Arthritis & Rheumatology

An Official Journal of the American College of Rheumatology
www.arthritisrheum.org and wileyonlinelibrary.com

Arthritis & Rheumatology

An Official Journal of the American College of Rheumatology
www.arthritisrheum.org and wileyonlinelibrary.com

VOLUME 73 • January 2021 • NO. 1

Cover image: The figure on the cover is an image of the Lyme disease spirochete (*Borrelia burgdorferi*) in mouse ear skin. Mice expressing tdTomato (red) in all tissues were inoculated intradermally at 3 weeks of age with green fluorescent protein–expressing *B. burgdorferi* (green) and imaged by two-photon intravital microscopy at 10 days of infection. Spirochetes (green) are easily visualized in the collagen matrix of skin (blue due to second harmonic generation) and around hair follicles. This issue of *Arthritis & Rheumatology* features clinical practice guidelines for the prevention, diagnosis, and treatment of Lyme disease. Image courtesy of Linda K. Bockenstedt, MD.

In this Issue

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

Anakinra Well Tolerated and Effective for Intravenous Immunoglobulin–Resistant Kawasaki Disease

p.151

Previous studies have demonstrated that the interleukin-1 (IL-1) signaling blockade anakinra is successful in preventing and treating cardiovascular disease lesions in experimental murine models of Kawasaki disease (KD) as well as intravenous immunoglobulin (IVIG)– and steroid-resistant patients with KD. In this issue, Kone-Paut et al (p. 151) report the results of the first investigative trial of IL-1 signaling blockade in KD. Results from their phase II open-label study indicate that anakinra is well tolerated in patients with IVIG-resistant KD. Moreover, anakinra appears to have some efficacy in reducing fever, markers of systemic inflammation, and coronary artery dilatation.

The primary outcome measure of this study was reduction in fever within 48 hours of anakinra treatment. The investigators found that 75% of patients in the intent-to-treat group and 87.5% of patients in the per-protocol group became afebrile within 48 hours of the last escalation dose of anakinra. When the researchers looked at reduction of disease activity by 50%, they found that this was achieved in 93.3% of physician evaluations and 100% of parent evaluations. They also found that patient C-reactive protein values normalized by day 30.

One of the secondary outcomes of the study was coronary artery Z score. At the initial screening, 12 of 16 patients had a maximum coronary artery Z score of >2, and 10 of 16 patients had a maximum Z score of >2.5. At day 45, 5 of 10 patients had achieved coronary artery Z scores of <2.5, and 6 of 12 patients had achieved coronary artery Z scores of <2. While the investigators observed 5 serious adverse events in 3 patients, no serious infections or deaths occurred, and the authors concluded that the safety and tolerability of anakinra in the study was very good.

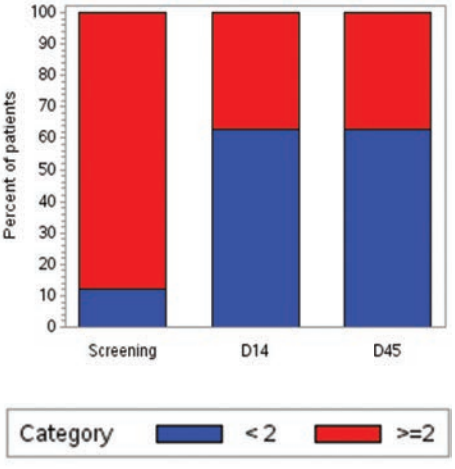


Figure 1. Distribution of coronary artery Z scores before and after treatment with anakinra in the per-protocol group (n = 8).

Mediterranean Diet May Reduce the Risk of Rheumatoid Arthritis in Ever-Smoking Women

p.69

While the Mediterranean diet has been associated with a significant reduction in risk of noncommunicable diseases, to date, only 3 studies have investigated the association between the Mediterranean diet and risk of rheumatoid arthritis (RA). One of these, a case–control study from the Swedish Epidemiological Investigation of RA, found an inverse association between the Mediterranean diet score and RA risk. In this issue, Nguyen et al (p. 69) report the results of a large, population-based prospective cohort study of French women that reinforces these findings, suggesting that adherence to the Mediterranean diet could reduce the high risk of RA among ever-smoking women. The investigators did not, however, find an inverse association between adherence to the Mediterranean diet and RA risk among never-smokers.

The study included 62,629 women and 480 incident cases of RA from the E3N-EPIC study (Etude Epidémiologique Auprès des Femmes de la Mutuelle Générale de l'Education Nationale). The French prospective cohort began in 1990 and includes 98,995 women. The study collected dietary data since 1993 via a validated food frequency questionnaire. The investigators used this data to calculate adherence to the Mediterranean diet using a 9-point dietary score evaluating consumption of vegetables, legumes, cereal products, fish, meat, dairy products, olive oil, and alcohol. When analyzing their data, the researchers adjusted for age and the main potential confounders, including smoking. The investigators found that, among ever-smokers, a higher Mediterranean diet adherence score was associated with a decreased risk of RA (hazard ratio 0.91 for a 1-point increase in score). Additionally, in ever-smokers, the absolute risks of RA in those with high scores and those with low scores were 38.3 and 51.5 per 100,000 person-years, respectively, compared with 35.8 per 100,000 person-years in never-smokers with high Mediterranean diet scores.

Novel RNase Fc Fusion Protein Eases Fatigue in Patients with Primary Sjögren's Syndrome

An earlier phase III clinical trial involving the treatment of patients with SLE with the anti–interferon (IFN) receptor anifrolumab documented improvements in the British Isles Lupus Assessment Group–based Composite Lupus Assessment score at week 52, relative to placebo, a finding consistent with the understanding that increased expression of IFN-inducible genes is associated with higher disease activity. In this issue, Posada et al (p. 143) report the results of a phase II randomized, double-blind, placebo-controlled clinical trial of the RNase Fc fusion protein RSLV-132 in patients with primary Sjögren's syndrome (SS). RSLV-132 contains a catalytically active RNase enzyme moiety, which they hypothesized would digest RNA associated with the immune complexes that induce immune cell IFN in these patients.

p. 143

The investigators measured efficacy using 4 independent patient-reported measures of fatigue: European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index, EULAR Sjögren's Syndrome Patient Reported Index, Functional Assessment of Chronic Illness Therapy–Fatigue, Profile of Fatigue, and the Digit Symbol Substitution Test. They randomized 30 patients with primary SS to receive treatment with RSLV-132 or placebo intravenously once per week for 2 weeks, and then every 2 weeks for 12 weeks. A total of 8 patients received placebo, and 20 patients received RSLV-132 at a dose of 10 mg/kg.

The researchers found that patients who received RSLV-132 experienced clinically meaningful improvements in all fatigue scores from baseline to day 99, whereas patients who received placebo demonstrated no changes in any of the clinical efficacy measures. The improvements correlated with increased expression of the selected IFN-inducible genes. Thus, not only did RSLV-132 improve severe fatigue in patients with primary SS, but it also unexpectedly induced up-regulation of selected IFN-inducible genes.

Journal Club

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

Mediterranean Diet and Risk of Rheumatoid Arthritis: Findings from the French E3N-EPIC Cohort Study

Nguyen et al *Arthritis Rheumatol* 2020;84:69–77

The Mediterranean diet, widely used in Southern European countries, mainly consists of olive oil, cereals, fresh or dried fruit and vegetables, legumes, cereal products, fish, and a moderate amount of dairy, meat, and wine. It has been reported to be associated with significant reduction of noncommunicable diseases, including cardiovascular events, cancers, and overall mortality. A few studies have investigated potential beneficial effects on rheumatoid arthritis (RA) activity, and even fewer have prospectively investigated a potential beneficial effect on the risk of RA occurrence. Nguyen et al aimed to assess the relationship between adherence to the Mediterranean diet and the risk of RA. To this end, they used the French prospective E3N-EPIC (Etude Epidémiologique Auprès des Femmes de la Mutuelle Générale de l'Education Nationale) general population cohort, which has followed 98,995 women since 1990, and for whom data on lifestyle, diet, socioeconomic status, health, and medications were available.

Adherence to the Mediterranean diet was assessed using a 9-point dietary score evaluating consumption of each dietary component, which was recorded in a dietary questionnaire. The authors used Mediterranean diet adherence score categories (i.e., low 0–3, medium 4–5, and high 6–9) and a continuous score (1-point increments in score) as the exposure of interest. Only incident cases of RA occurring after the dietary questionnaire were considered. The researchers observed that a strong adherence to the Mediterranean diet was associated with a decreased risk of RA, only among ever-smokers. This risk reduction was not found among never-smokers.

Questions

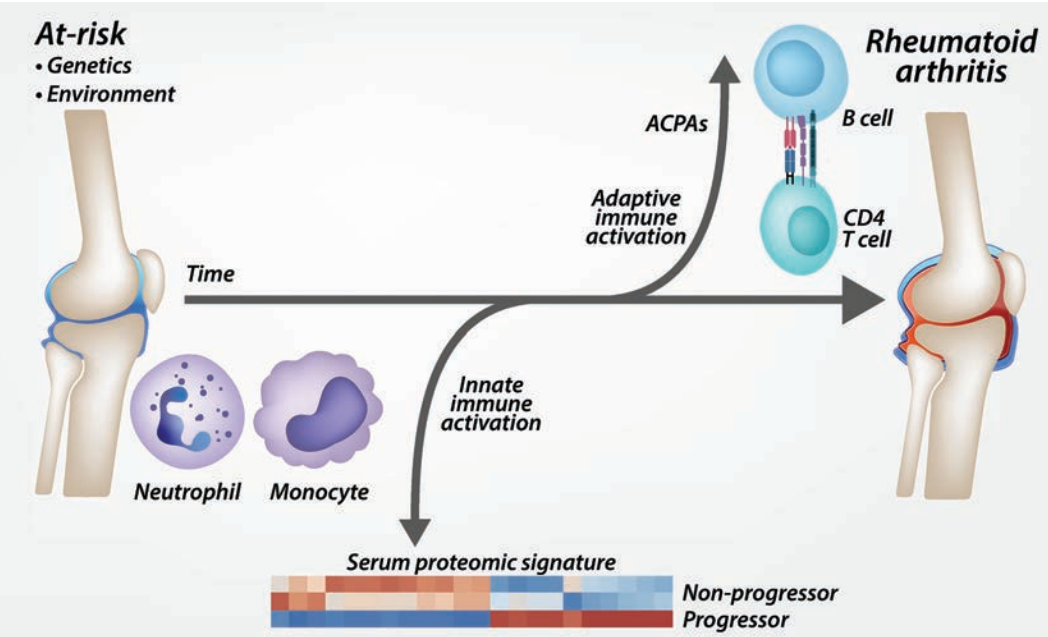
1. **What is currently known about the association between diet and the risk of RA?**
2. **Why does the Mediterranean diet adherence score depend on participants' median consumption of each of the 9 components?**
3. **Why did the authors exclude prevalent cases of RA that occurred before the dietary questionnaire?**
4. **Why did the authors use age as the time scale for their analyses?**
5. **Why did the authors stratify their analyses on smoking status (never or ever smokers)?**

Clinical Connections

Association of a Serum Protein Signature With Rheumatoid Arthritis Development

O'Neil et al, *Arthritis Rheumatol* 2021;84:78–88

CORRESPONDENCE
Liam J. O'Neil, MD: liam.oneil@umanitoba.ca



KEY POINTS

- Unsupervised serum proteomics provides a rich data set for understanding the biologic events that occur in preclinical RA.
- Serum proteomic changes occur years before the onset of clinical disease.
- Innate immune activity may be an important indicator of risk for future RA development.

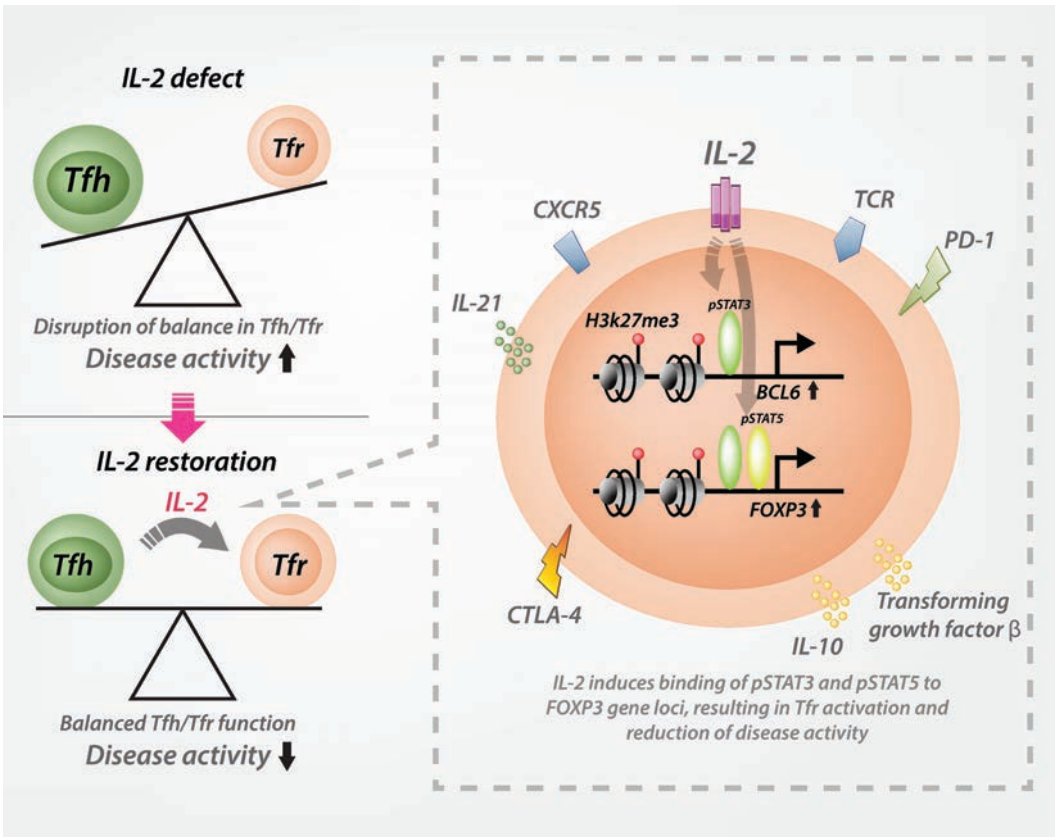
SUMMARY

Rheumatoid arthritis (RA) is an autoimmune disease that targets the synovial joints, tends to be persistent, and leads to progressive joint damage. Prior to the onset of clinically detectable joint inflammation, RA-associated autoantibodies directed toward anti–citrullinated proteins (ACPAs) are biomarkers for future disease development. However, many individuals with detectable levels of these autoantibodies do not develop disease. To enhance the capacity to identify those with the highest risk of developing future RA, O'Neil et al applied a broad-based proteomic technique that interrogates 1,300 proteins to study longitudinal serum samples from a group of first-degree relatives of RA patients. Some of these individuals developed RA, while most did not. The researchers demonstrate changes in the serum proteome that are highly predictive of RA onset, independent of a patient's baseline ACPA status. These proteomic changes were detectable not only in the period immediately preceding RA onset, but also in serum samples obtained an average of 3 years prior to onset. Proteins most predictive of RA included those linked to innate immune activation, such as Toll-like receptor pathways. These findings are consistent with the hypothesis that early activation of innate immunity plays a key role in the progression toward disease onset. Complex interactions between innate and adaptive immunity, the latter represented by autoantibody development and expansion, ultimately result in the onset of immune-mediated joint inflammation that characterizes RA. Thus, a combination of innate and adaptive immune biomarkers might optimize the capacity to identify individuals at the highest risk of developing seropositive RA.

Conversion of T Follicular Helper Cells to T Follicular Regulatory Cells by Interleukin-2 Through Transcriptional Regulation in Systemic Lupus Erythematosus

Hao et al, *Arthritis Rheumatol* 2021;84:132–142

CORRESPONDENCE
Yoshiya Tanaka, MD, PhD: tanaka@med.uoeh-u.ac.jp



KEY POINTS

- Imbalance of Tfr and Tfh activation is associated with disease activity in SLE patients.
- Impaired function of Tfr cells is due to defective IL-2 production.
- Exogenous IL-2 restores function of Tfr cells.
- Fine-tuning the balance between Tfh and Tfr cells provides therapeutic approaches in SLE.

SUMMARY

Follicular helper T (Tfh) cells promote autoantibody production, whereas follicular regulatory T (Tfr) cells suppress Tfh-mediated antibody responses. In this issue, Hao et al report that peripheral blood programmed death 1 (PD-1)^{high} Tfh cells increase, but activated Tfr cells decrease in patients with systemic lupus erythematosus (SLE). Exogenous interleukin-2 (IL-2) restores the balance between Tfh and Tfr cells by converting memory Tfh cells to functional Tfr cells, characterized by CXCR5+Bcl-6+FoxP3^{high}STAT3+pSTAT5+ cells. During this process, IL-2–activated STAT3 and STAT5 selectively bound to *FOXP3* and *BCL6* gene loci accompanied by suppression of H3K27me3. These data support the use of potential therapeutic approaches to targeting the IL-2–Tfr axis in SLE.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 1–4
DOI 10.1002/art.41479

American College of Rheumatology
Empowering Rheumatology Professionals

EDITORIAL

Challenges and Opportunities: Using Omics to Generate Testable Insights Into Pathogenic Mechanisms in Preclinical Seropositive Rheumatoid Arthritis

V. Michael Holers

Current understanding of the natural history of rheumatoid arthritis

Our knowledge of the phenotypic and immunologic features present during the preclinical period of seropositive rheumatoid arthritis (RA) has rapidly evolved since the beginning of the 21st Century. In the early and mid-2000s, an important series of reports (for review, see ref. 1) reexamined the question, originally posed in the 1960s, of when during the development of RA do autoantibodies appear, and specifically, do these biomarkers precede the development of clinically apparent arthritis? Subsequently, we now know that IgG and IgA isotypes of the RA-associated autoantibodies designated anti–citrullinated protein antibodies (ACPAs), which are typically measured as anti–cyclic citrullinated peptide (anti-CCP) antibodies, as well as rheumatoid factors (RFs) are found in the peripheral blood on average 3–5 years prior to the onset of clinically apparent arthritis (1). Indeed, a recent study demonstrated that separation of eventual cases from controls based on the levels of IgG ACPA autoantibodies, even those within the "normal" range, can occur ~18 years prior to diagnosis (2). In addition, increased levels of multiple cytokines and chemokines, including the pathogenic interleukin-1 (IL-1), tumor necrosis factor (TNF), and IL-6, as well as epitope spreading of the ACPA response are found in the peripheral blood as the onset of clinically detectable arthritis nears (3). Importantly, although studies have demonstrated subtle changes in the synovium in some RA-related autoantibody–positive individuals (4), a general consensus is that the prolonged disease process is not simply a joint-centered "asymptomatic arthritis."

With these now widely replicated findings, ongoing questions relevant to our contemporary understanding of the natural history of RA currently include those addressing what immune processes drive the initial break in tolerance to citrullinated and other self antigens during this asymptomatic preclinical period, and how the disease process then transitions to involve the joints, with the potential for eventual destruction. These are also questions of increasing relevance across the field of autoimmune disease research, as many other such diseases, including type 1 diabetes and systemic lupus erythematosus, clearly evolve through the same type of autoantibody-positive preclinical period and exhibit similar forms of immune dysregulation during that time (5), including involvement of autoantibodies that can also cross these disease boundaries throughout the disease course (6). In this issue of *Arthritis & Rheumatology*, O'Neil and colleagues (7) add important information to our understanding of these concepts in the preclinical RA period.

What immune processes are at-risk individuals undergoing during the preclinical RA period?

A fundamental question inherent in any discussion of the natural history of RA is what immune processes, if not those associated with overt arthritis, are ongoing in individuals during this preclinical period. Some important clues have come from studies of individuals who are identified as being at-risk for future RA. In these studies, an at-risk individual can be defined as either a first-degree relative of a patient with RA, an individual who exhibits a shared epitope (SE) genotype, or an individual who, through either screening or clinical care pathways, is found to exhibit anti-CCP antibodies or multiple RFs in the absence of RA or inflammatory arthritis.

Insights into the pathogenic processes emerging during this preclinical period have come from studying peripheral blood cell phenotypes in at-risk anti-CCP+ individuals. These studies have demonstrated alterations in circulating CD4+ and CD8+ T cell compartments, as well as an imbalance in effector T cell subsets with Treg cells, accompanied by dysfunctional inhibitory pathways including the programmed death 1 pathway (for review, see

V. Michael Holers, MD: University of Colorado School of Medicine, Aurora.
Dr. Holers has received consulting fees, speaking fees, and/or honoraria from Celgene, Janssen, and Bristol Myers Squibb (less than $10,000 each).

Address correspondence to V. Michael Holers, MD, Box B115, 1775 Aurora Court, Aurora, CO 80045. Email: michael.holers@UCDenver.edu.
Submitted for publication June 22, 2020; accepted in revised form August 4, 2020.

ref. 5). In addition, B cell abnormalities, including an elevated percentage of highly mutated IgA plasmablasts, reduced frequencies of peripheral memory B cells, relative decreases in CD80+ B cells, and higher numbers of dominant B cell receptor clones (≥5), are predictive of the onset of arthritis (5).

In addition to peripheral blood studies, findings from analyses of mucosal sites in at-risk individuals, including the lung (8) and periodontum (9) among others, have strongly suggested that chronic inflammation accompanied by dysbiosis may be playing an important role in this initial break in self tolerance to citrullinated target proteins. In aggregate, results of these studies suggest a "mucosal origins" hypothesis, in that initial localized mucosal ACPA production, typically predominantly, but not exclusively, of the IgA isotype, reflects either a hard-wired local protective response meant to recognize and clear products of inflammation gone awry, an immune response to specific microbial antigen exposure, and/or chronic inflammation due to multiple types of injury or damage (for review, see ref. 10). Indeed, Figure 1 provides one model of the stages of RA development that incorporates these initial concepts of mucosal origins, which is followed by the development of systemic autoantibodies and eventual arthritis. How these mucosal studies intersect with studies of peripheral blood cells remains unknown, as correlations have not yet been drawn. Notably, support for the notion that different types of mucosal mechanisms, and perhaps other pathways, are operative during preclinical RA is provided by the finding of at least 2 distinct RA-related autoantibody endotypes in the preclinical period (2).

Contributions of studies of indigenous populations to the current understanding of the problem

Starting with investigations of Pima Indians in the United States (11), studies of uniquely informative populations with a high risk of developing RA have been undertaken to understand the preclinical evolution of RA. The advantages of using Indigenous North American populations, as in the study by O'Neil et al (7), are many (for review, see ref. 12). This cohort study by O'Neil et al has been under the long-term direction of Prof. Hani El-Gabalawy

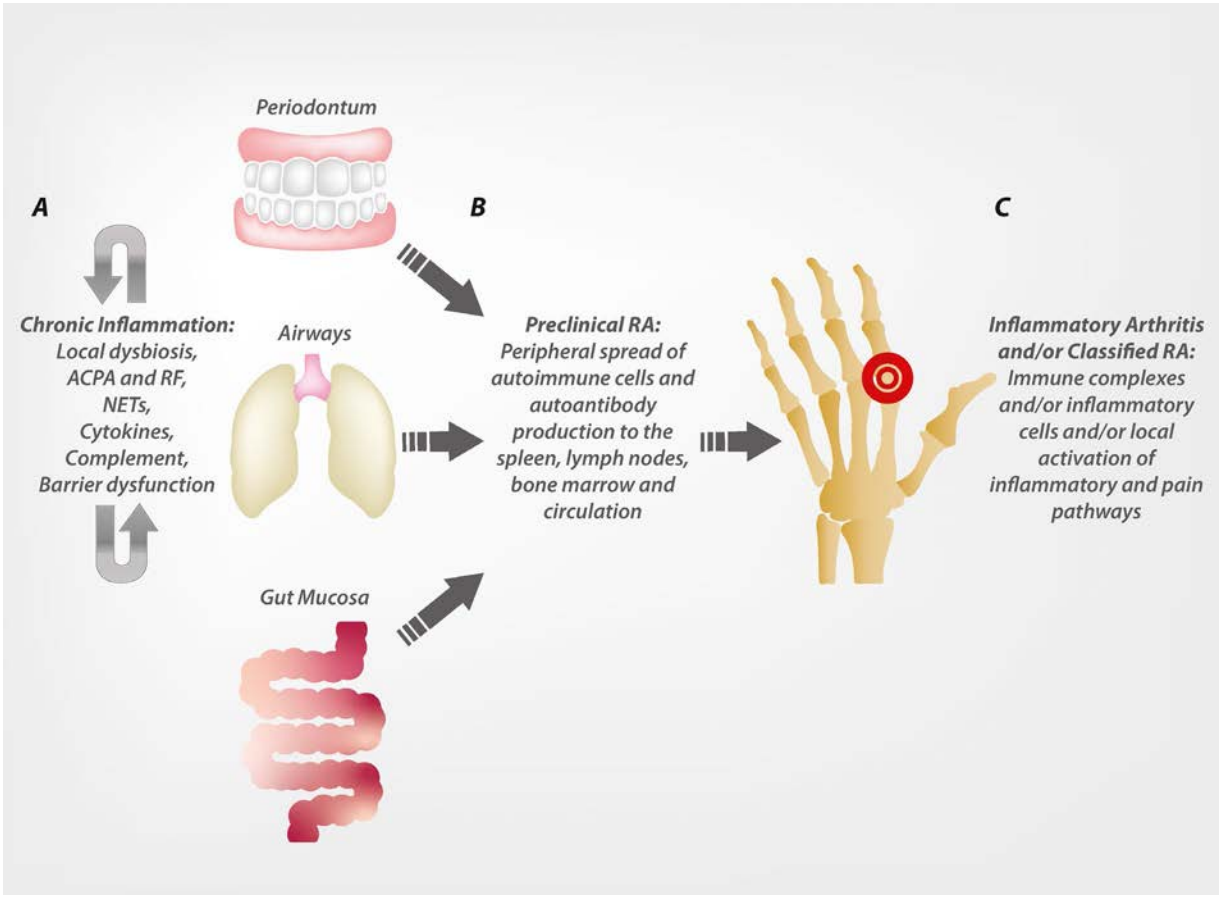


Figure 1. Mechanisms and steps in the proposed "mucosal origins" hypothesis for the preclinical initiation of rheumatoid arthritis (RA). These include local mucosal processes that promote and maintain inflammation without appropriate resolution thereof (**A**), processes that extend this mucosal process to the peripheral blood and lymphoid tissue, thereby maintaining the expression and expansion of autoantibodies (**B**), and transition to the joints through distinct mechanisms promoted by local articular and periarticular factors (**C**). ACPA = anti–citrullinated protein antibody; RF = rheumatoid factor; NETs = neutrophil extracellular traps.

at the University of Manitoba in Canada. Benefits of studying this population include the high prevalence of RA (0.9–2.4%) and its association with the SE, familial aggregation, and a more severe disease course, and the presence of a carefully performed clinical and biomarker phenotyping and follow-up effort lasting more than 15 years. In addition, this research group has consistently applied cutting-edge methodologies and investigators have participated in collaborative studies with other leaders in the field. This approach has resulted in an understanding, within this population, of the evolution of different types of autoantibodies, cytokine/chemokine elevations, and glycosylation status over time, among other factors. There is also a substantial opportunity to develop beneficial treatment strategies for this population, if safe preventive approaches can be identified.

One limitation to the study of this Indigenous North American population, however, is whether the findings are applicable across other ethnic groups, and whether the high rate of autoantibody reversion (~30% reversion to a negative status) will turn out to be similar to other populations or will limit the ability to translate findings into other populations. Unfortunately, directly comparative studies of ethnic and population differences in the preclinical evolution of RA have not yet been performed in depth across any groups.

Hypothesis-generating omics versus hypothesis-testing studies

Tools of the Omics revolution, i.e. methods with a suffix of "omics," such as proteomics, lipidomics, genomics, microbiomics, and metabolomics (for review, see ref. 13), are increasingly being applied to population studies, in which the primary goal is to generate extensive data in an unbiased hypothesis-generating exercise. Although they require replication using more focused biomarker studies and validated methods (14), these approaches have provided potential clues with regard to the causal pathways of RA.

The specific approach utilized by O'Neil and colleagues takes advantage of the generation of an array method based on pairs of highly stable RNA-derived aptamers that bind with very high affinity to 2 unique and physically dispersed protein epitopes on each analyte in a "sandwich"-like configuration, resulting in the ability to measure the levels of thousands of proteins at once in a small volume of serum across large sample sets (15). The method can be applied to separated blood products, such as serum or plasma, as well as other liquid-containing biologic samples. The primary findings of the study include the identification of a large number of analytes that were either increased or decreased at >3 years prior to the onset of inflammatory arthritis in individuals, termed progressors, who developed inflammatory arthritis within the current time course of the study. Notably, further statistical analyses allowed the identification of a smaller proteome signature, and network analyses implicated innate immune processes through Toll-like receptor 2 (TLR-2) and the cytokines TNF and IL-1 as key drivers of eventual transition.

There are limitations to this aptamer-based method, which have been recognized by the authors, and there is an obvious need to replicate the findings using independent assays and to expand the study into non-indigenous populations. Nevertheless, the results are intriguing and continue to point us in the direction of ongoing non-articular immune dysregulation as a preclinical driver of eventual arthritis.

Implications for identifying the right therapeutic approach for each period of RA development

Of course, a major goal of these types of preclinical RA studies is to identify actionable therapeutic targets that are on the critical pathway to disease prevention or amelioration. Although therapeutic approaches to date have focused on administration of "RA drugs," including hydroxychloroquine, methotrexate, glucocorticoids, rituximab, and CTLA4-Ig, the hope going forward is to identify targeted interventions, including promotion of antigen-specific tolerance, that address the specific ongoing non-articular immune alterations that occur in the preclinical period. A major focus across the many groups now working in this area is on the microbiome and dysbiosis, mucosal immune processes including barrier function, and how systemic autoimmunity develops and progresses or resolves. Moreover, studies are seeking to identify the transition mechanisms that then lead, in a subset of individuals, to the initial development and propagation of arthritis. This research area is greatly facilitated by study findings such as those reported by O'Neil and colleagues (7), especially those pointing to a TLR-2–mediated pathway as a novel therapeutic target, as well as other investigations yet to come.

ACKNOWLEDGMENTS

The author appreciates the many ongoing contributions to the concepts included in this editorial provided by the principal investigators, collaborators, and staff members working on the pathogenesis of preclinical RA through in-depth analyses of several preclinical and at-risk populations under the SERA (Studies of the Etiology of Rheumatoid Arthritis) research umbrella.

AUTHOR CONTRIBUTIONS

Dr. Holers drafted the article, revised it critically for important intellectual content, and approved the final version to be published.

REFERENCES

1. Malmström V, Catrina AI, Klareskog L. The immunopathogenesis of seropositive rheumatoid arthritis: from triggering to targeting [review]. Nat Rev Immunol 2017;17:60–75.
2. Kelmenson LB, Wagner BD, McNair BK, Frazer-Abel A, Demoruelle MK, Bergstedt DT, et al. Timing of elevations of autoantibody isotypes

in rheumatoid arthritis prior to diagnosis of rheumatoid arthritis. Arthritis Rheumatol 2020;72:251–61.

3. Sokolove J, Bromberg R, Deane KD, Lahey LJ, Derber LA, Chandra PE, et al. Autoantibody epitope spreading in the preclinical phase predicts progression to rheumatoid arthritis. PLoS One 2012;7:e35296.
4. De Hair MJ, van de Sande MG, Ramwadhdoebe TH, Hansson M, Landewé R, van der Leij C, et al. Features of the synovium of individuals at risk of developing rheumatoid arthritis: implications for understanding preclinical rheumatoid arthritis. Arthritis Rheumatol 2014;66:513–22.
5. Slight-Webb S, Bourn RL, Holers VM, James JA. Shared and unique immune alterations in preclinical autoimmunity. Curr Opin Immunol 2019;61:60–8.
6. James JA, Chen H, Young KA, Bemis EA, Seifert J, Bourn RL, et al. Latent autoimmunity across disease-specific boundaries in at-risk first-degree relatives of SLE and RA patients. EBioMedicine 2019;42:76–85.
7. O'Neil LJ, Spicer V, Smolik I, Meng X, Goel RG, Anaparti V, et al. Association of a serum protein signature with rheumatoid arthritis development. Arthritis Rheumatol 2021;73:78–88.
8. Demoruelle MK, Harrall KK, Ho L, Purmalek M, Seto NL, Rothfuss HM, et al. Anti–citrullinated protein antibodies are associated with neutrophil extracellular traps in the sputum in relatives of rheumatoid arthritis patients. Arthritis Rheumatol 2017;69:1165–75.
9. Mankia K, Cheng Z, Do T, Hunt L, Meade J, Kang J, et al. Prevalence of periodontal disease and periodontopathic bacteria in anti–cyclic citrullinated protein antibody-positive at-risk adults without arthritis. JAMA Netw Open 2019;5:e195394.
10. Holers VM, Demoruelle MK, Kuhn KA, Buckner JH, Robinson WH, Okamoto Y, et al. Rheumatoid arthritis and the mucosal origins hypothesis: protection turns to destruction [review]. Nat Rev Rheumatol 2018;14:542–57.
11. Del Puente A, Knowler WC, Pettitt DJ, Bennett PH. The incidence of rheumatoid arthritis is predicted by rheumatoid factor titer in a longitudinal population study. Arthritis Rheum 1988;31:1239–44.
12. Tanner S, Dufault B, Smolik I, Meng X, Anaparti V, Hitchon C, et al. A prospective study of the development of inflammatory arthritis in the family members of Indigenous North American people with rheumatoid arthritis. Arthritis Rheumatol 2019;71:1494–503.
13. Hasin Y, Seldin M, Lusis A. Multi-omics approaches to disease. Genome Biol 2017;18:1186.
14. Heller R, Bogomolov M, Benjamini Y. Deciding whether follow-up studies have replicated findings in a preliminary large-scale omics study. Proc Natl Acad Sci U S A 2014;111:16262–7.
15. Williams SA, Kivimaki M, Langenberg C, Hingorani AD, Casas JP, Bouchard C, et al. Plasma protein patterns as comprehensive indicators of health. Nat Med 2019;25:1851–7.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 5–8
DOI 10.1002/art.41498

American College of Rheumatology
Empowering Rheumatology Professionals

EDITORIAL

Would a 'Rosendo' by Another Name Smell as Sweet? Gender Disparity in Academic Rank and Publications in Rheumatology

Janet E. Pope

We are all familiar with Shakespeare's quote from *Romeo and Juliet*: "A rose by any other name would smell as sweet." The answer with regard to promotion in rheumatology, if there is gender equality, would be that Rose or Rosendo (the male version of Rose) would have equal opportunity to be promoted in academic rheumatology in the US. However, in this issue of *Arthritis & Rheumatology*, Jorge et al (1) demonstrate that there is a gender imbalance in faculty promotions, after adjustment for important variables related to promotion, such as age; years in practice; numbers of publications, National Institutes of Health (NIH) grants received, and registered trials; and an appointment at a top-ranked medical school. They found that 15% of 6,125 rheumatologists practiced in academic centers in 2014. After adjustment for women being younger and having fewer publications and grants (to compare on an equal playing field), women were found to be less likely to hold the rank of associate or full professor compared to men, with an adjusted odds ratio that was (barely) statistically significant. Interestingly, there was not a gender difference for registered rheumatology clinical trials, for which equal numbers of men and women were principal/site investigators.

Similarly, Bagga et al, whose article also appears in this issue of *Arthritis & Rheumatology* (2), studied the probability of women being first or senior authors in highly ranked rheumatology journals (2). Of course, the distribution would be dependent on the proportion of male and female academic faculty, since most papers are published by academics. Although there was approximately equal representation overall of women as first authors, they were only one-third as likely to be senior authors. The largest discrepancy was with respect to randomized controlled trials, especially industry-funded studies, for which women had an ~1 in 5 odds of being first authors and ~1 in 4 odds of being senior authors. This finding seems to demonstrate bias, especially within the pharmaceutical industry.

The methods used by Jorge et al are novel (1). The authors used data from Doximity (https://www.doximity.com), the largest network of US health care professionals, which registers all US licensed physicians even if they do not apply for an account, and includes age, gender, year of graduation from medical school and residency, certification, NIH grants, and publications; this eliminates bias if, for instance, there were gender differences in enrolling or entering data (1). Jorge et al validated the academic rank of 25 randomly selected rheumatologists in order to verify the data. In addition, the authors reviewed all 117 US academic rheumatology programs for division and program directors.

Women within academic rheumatology divisions were more likely than men to be assistant professors (55.5% versus 31.5%), but were less likely to be associate or full professors (17.5% versus 28% and 12.6% versus 36.8%, respectively) (1). Other studies have demonstrated that in academic medicine, women are less likely than men to achieve promotion to full professor or associate professor at US academic medical centers, which holds true after adjustment for experience, age, productivity, and specialty (3).

One could ask why this finding is important. There are many reasons. Women are enrolling in medical school in increasing numbers, accounting for at least half of the enrollment (4). There is also the feminization of rheumatology and other non-procedural subspecialties of medicine (5). In fact, two-thirds of rheumatology trainees are women (5).

If women are the majority in rheumatology and their numbers are increasing, then leadership should reflect their values and opinions, so balance in leadership allows for many opinions to be represented. Additionally, role models are important for young faculty, including diversity in ethnicity and gender in leadership roles. The way we think cannot easily be separated from these variables. The American College of Physicians (ACP) has published a position paper about the relatively low number of female doctors who are leaders and/or achieve equal academic advancement (6).

Janet E. Pope, MD, MPH, FRCPC: University of Western Ontario and St. Joseph's Health Care London, London, Ontario, Canada.

No potential conflicts of interest relevant to this article were reported.

Address correspondence to Janet E. Pope, MD, MPH, FRCPC, St. Joseph's Health Care London, 268 Grosvenor Street, London, Ontario N6A 4V2, Canada. Email: Janet.Pope@sjhc.london.on.ca.

Submitted for publication June 1, 2020; accepted in revised form August 18, 2020.

The ACP states that women in medicine are lacking mentors and face challenges such as discrimination, gender bias, a work culture that may be unfair to women, and a lack of work–life integration. Due to these experiences, their full potential may not be reached. The ACP also acknowledged a lack of pay equity. Women are at a higher risk of developing imposter syndrome, in which a woman may doubt her own accomplishments and have a fear of being exposed as a fraud (6).

If we look at the distribution of executive members and committee chairs of the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR), there is an imbalance of men to women. In 2017, the organizations' websites showed that 41% of EULAR Executive Committee members (13 of 32) were women, and 38% of the members of the ACR Board of Directors (8 of 21, including 2 ex officio members) were women. A review of the websites this year indicated that 10 of 21 members of the ACR Board of Directors (48%) and 10 of 34 EULAR Executive Committee members (29%) were women (7,8).

The findings presented by Jorge el al (1) are also important because they show a lack of equality with respect to academic rank promotions, which may result from a series of prior biases. Women seemed to have received fewer grants and have fewer publications on which they were first or senior author (perhaps there is a gender bias in peer review of grants and journal articles), which can result in a longer time to academic promotion for women, but even when these variables are adjusted for, the disparity remains. There are also biases within institutions where young or mid-level faculty are groomed to pursue leadership roles and men are often selected by male leaders. This may be an unconscious bias that leads to passing over an equally qualified woman due to perceptions that she may not have adequate leadership qualities (too emotional, too busy at home, too lenient, etc.). In general, leaders should reflect the population that they serve, and leadership committees may be more creative and proactive if several ideas and backgrounds are reflected.

The differences in academic promotion between men and women are inherently unfair and challenge rheumatologists to examine other potential biases within academia, such as those based on race/ethnicity. These biases may go beyond how colleagues are perceived to biases toward patients, who may be treated differently due to unconscious or conscious perceptions about the individual. A basic principle within medicine is to treat patients fairly, providing proper care irrespective of gender or ethnicity. It is difficult to change attitudes when the bias is subtle, unspoken, and even unconscious. These beliefs may be held by both men and women, so a solution needs to encompass attitudinal shifts among both male and female physicians and academic leaders.

The generalizability of the articles by Bagga et al and Jorge et al is broad. Although endocrinology will become the most female-predominant internal medicine subspecialty, the specialty faces similar issues (a pay gap between women and men, fewer women in academic leadership roles, and fewer publications authored by women than by men) (9,10).

The gap in academic rank in rheumatology has not changed from 2002 to 2014 (1,11). Eighty percent of graduating rheumatologists over the last 20 years have been women, but only 25% of full professors were women in 2002, with a 3:1 ratio of men to women as full professors in rheumatology (11). More rheumatologists may be entering private practice, but only 13% of female rheumatologists in 2014 were full professors, and the ratio of 3:1 has remained constant over 12 years (1). See Table 1 for a comparison of the data extracted from two articles on this topic (1,11).

In general, academic promotion is associated with prestige and a pay raise. Thus, if gender bias plays a role in promotions in rheumatology, then there will continue to be pay gaps. Men are paid more than women in medicine. This seems counterintuitive, as many terms of employment are equal at academic institutions. However, women tend to negotiate less when signing a contract and are less apt to ask for a better deal than the standard contract (12). Also, female physicians often spend more time with each patient, so when paid according to the number patients seen, they will make less money (such as in a fee-for-service model). It appears that women are discriminated against throughout their career, not just in terms of promotion (13). Self-promotion by men is perceived better by others than self-promotion by women. However, similar to corporate structures, there may be an advantage in academia for those who self-promote. Many members of large academic medical faculties are unaware of the achievements of most of their colleagues. Awareness facilitates nominations for awards and other recognition. The Association of Women in Rheumatology allows membership of both women and men (12) and provides education, advocacy, and training in skills such as

Table 1. A comparison of academics in rheumatology in the US in 2002 and 2014*

	All academic professor ranks	Professor		Associate professor		Assistant professor	
		Women	Men	Women	Men	Women	Men
2002							
M:F ratio	3:1	–	–	–	–	–	–
%	–	25	75	–	–	–	–
2014 (n = 941)							
M:F ratio	3:1	–	–	–	–	–	–
No.	–	49	200	68	152	216	171
% of total	–	12.6	36.8	17.5	28.0	55.5	31.5

* Data for 2002 were obtained from ref. 11; data for 2014 were obtained from ref. 1.

contract negotiations, especially for rheumatologists beginning their practices.

There are unspoken factors in the workplace that enhance gender inequity. Some ideas are from the business world (14). Both men and women have preconceived ideas of how women should behave. Care and nurturing are thought to be less useful as leadership skills than being dominant or assertive (the former traits are more associated with women and the latter with men). In fact, in the workplace, women who act assertive are liked less than men who have similar traits. Leadership styles differ between the sexes—men tend to be transactional, while women tend to be transformative (13). There is resistance to quotas to decrease gender gaps. No one wants to be perceived as being promoted if not deserving. Gender targets are not set in many institutions, since there is a belief that individuals are promoted based on merit, and therefore targets aren't necessary.

Often a person in a leadership role promotes someone with a style similar to their own, which can lead to the gender gap in academia. It is difficult to be promoted if, due to your gender, you are passed over for a series of several roles that will eventually lead to academic promotion. Women seem to be evaluated less highly for equal performance. Women tend to do more of the unpaid work at home, and when it comes to academic promotion, the clock doesn't always stop when a mother is on maternity leave. Many universities don't count the time taken for maternity leave when determining the usual number of years over which promotion from assistant to associate professor should occur. Motherhood is a time-out for many women, requiring more hours for child-rearing and domestic responsibilities and leaving less time to build professional networks and socializing, which translates into a lower chance of attaining a leadership role or promotion (13). There are many prejudices (held by both men and women) that penalize women yet benefit men with respect to leadership (13).

How can this be addressed? Institutions should make their faculty aware of the prejudice against female leaders and what drives these perceptions and try to dispel them (13). Performance evaluations should be based on criteria and be less subjective. Work hours should not be really long, as this is not conducive to productivity and may lead to negative opinions of those who go home before others, and a family-friendly work environment for both sexes should be cultivated. Leadership opportunities should be advertised with clear expectations instead of using word-of-mouth within certain networks. Institutions can avoid having committees that include women as "tokens" only by implementing rules regarding minimum representation, and should avoid having large teams that include only one woman. In addition, institutions should educate their faculty on why social capital and networking are important and provide strong mentors who can help women early in their career navigate their way to future leadership success.

Bates et al have outlined that networking for women, improving the gender pay gap, updating work policies for women, and improving grant awards to women are steps to improve gender equity in medicine (15). There have been programs developed for early-career female faculty to improve assertive communication, career management, and other skills (16).

There will be increased human resources required for providing academic and clinical services with the increasing feminization of the workforce in rheumatology. It has been found that with higher numbers of rheumatologists, the same numbers of patients are being seen over 15 years in Ontario, Canada (which has a population of >11 million adults) (17). Leaders will be required to understand the changing demographics of the workforce in rheumatology and develop a plan to sustain patient care, which may include training larger numbers of rheumatology residents and/or developing other models of care. Innovative models such as team care with allied health professionals may improve job satisfaction and decrease burnout for academic and community rheumatologists.

Legislating change such as pay equity is only part of the solution, since women have been found not to negotiate as frequently as their male counterparts, and this gap in pay early on is maintained throughout their career since each pay change is based on the previous pay level. Advocacy and formal training in leadership skills in the medical school curriculum, during residency, and for faculty could facilitate more women and men considering leadership in their careers.

In summary, we should all be aware that we see the world with many biases and that gender inequities do exist within academic rheumatology. Incremental change may be enacted by changing attitudes, providing opportunities to help young faculty reach their career potential, and specifically considering the needs of junior faculty, which may vary by gender. Specific courses for female faculty may be warranted, as well as other methods to improve the odds of being promoted for all faculty.

REFERENCES

1. Jorge A, Bolster M, Fu X, Blumenthal DM, Gross N, Blumenthal KG, et al. The association between physician gender and career advancement among academic rheumatologists in the United States. Arthritis Rheumatol doi: https://onlinelibrary.wiley.com/doi/abs/10.1002/art.41492. E-pub ahead of print.
2. Bagga E, Stewart S, Gamble GD, Hill J, Grey A, Dalbeth N. Representation of women as authors of rheumatology research articles. Arthritis Rheumatol doi: https://onlinelibrary.wiley.com/doi/abs/10.1002/art.41490. E-pub ahead of print.
3. Ruzycki SM, Freeman G, Bharwani A, Brown A. Association of physician characteristics with perceptions and experiences of gender equity in an academic internal medicine department. JAMA Netw Open 2019;2:e1915165.
4. Association of American Medical Colleges. Applicants, matriculants, enrollment, graduates, MD-PhD, and residency applicants data. 2019. URL: https://www.aamc.org/data-reports/students-residents/report/facts.
5. Battafarano DF, Ditmyer M, Bolster MB, Fitzgerald JD, Deal C, Bass AR, et al. 2015 American College of Rheumatology Workforce Study: supply and demand projections of adult rheumatology workforce, 2015–2030. Arthritis Care Res (Hoboken) 2018;70: 617–26.

6. Butkus R, Serchen J, Moyer DV, Bornstein SS, Hingle ST, on behalf of the Health and Public Policy Committee of the American College of Physicians. Achieving gender equity in physician compensation and career advancement: a position paper of the American College of Physicians. Ann Intern Med 2018;168:721–3.
7. EULAR. The structure of EULAR. URL: https://www.eular.org/eular_structure.cfm.
8. American College of Rheumatology. Board of Directors: ACR Executive Committee. URL: https://www.rheumatology.org/About-Us/Leadership/Board-of-Directors.
9. Elhakimi W, al Othman A, el Yahia M, al Dawood A, al Sadiq S, Mosli M, et al. Female authorship in major endocrinology journals: a 25-year progression. J Endocrinol, Metabolism, and Diabetes of South Africa 2018;23:76–9.
10. Pelley E, Danoff A, Cooper DS, Becker C. Female physicians and the future of endocrinology. J Clin Endocrinol Metab 2016;101:16–22.
11. Lundberg IE, Ozen S, Gunes-Ayata A, Kaplan MJ. Women in academic rheumatology. Arthritis Rheum 2005;52:697–706.
12. Association of Women in Rheumatology (AWIR). URL: https://awirgroup.org.
13. Eagly A, Carli LL. Women and the labyrinth of leadership. Harv Bus Rev 2007;85:62–71.
14. Ward M. Five unspoken truths getting in the way of gender equality. Mumbrella. November 2015. URL: https://mumbrella.com.au/five-unspoken-truths-getting-in-the-way-gender-equlaity-228328.
15. Bates C, Gordon L, Travis E, Chatterjee A, Chaudron L, Fivush B, et al. Striving for gender equity in academic medicine careers: a call to action. Acad Med 2016;91:1050–2.
16. Danhauer SC, Tooze JA, Barrett NA, Blalock JS, Shively CA, Voytko ML, et al. Development of an innovative career development program for early-career women faculty. Glob Adv Health Med 2019;8:2164956119862986.
17. Widdifield J, Bernatsky S, Pope JE, Ahluwalia V, Barber CE, Eder L, et al. Encounters with rheumatologists in a publicly-funded Canadian healthcare system: a population-based study. J Rheumatol 2020;47:468–76.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 9–11
DOI 10.1002/art.41461

American College of Rheumatology
Empowering Rheumatology Professionals

NOTES FROM THE FIELD

Treat-to-Target From the Patient Perspective Is Bowling for a Perfect Strike

Casper G. Schoemaker[1] and Maarten P. T. de Wit[2]

A treat-to-target approach is gaining ground as an effective and efficient strategy for a range of rheumatic diseases (1–4). It is assumed that a treatment continuously aimed at a single target—abrogation of inflammation, leading to remission—will have "domino effects" on all other treatment goals as well (1). Since the first recommendations were published there have been new insights, and there is a need to revisit the discussion. In this commentary we will reflect on treat-to-target in rheumatic diseases from the patient perspective, based on our experiences as patient representatives in research on rheumatoid arthritis (RA), psoriatic arthritis (PsA), and juvenile idiopathic arthritis (JIA).

Overarching principles

All treat-to-target recommendations start by formulating a set of overarching principles, including the ultimate goals. For treat-to-target in RA, the primary goal is to maximize long-term health-related quality of life through control of symptoms (e.g., pain, inflammation, stiffness, and fatigue), prevention of structural damage, normalization of function, and improved/restored ability to participate in social and work-related activities (2). For JIA, the ultimate treatment goals have been described as follows: "to control signs and symptoms; to prevent structural damage; to avoid comorbid conditions and drug toxicities; and to optimise function, growth and development, quality of life, and social participation" (3). From a patient perspective, the acknowledgment of all goals, including those related to pain, fatigue, activities of daily living, and social participation, is highly valued (5,6).

In the next overarching principle, abrogation of inflammation is assumed to be essential to reach these goals (2–4). In the final overarching principle, it is assumed that treatment to target by regularly assessing disease activity and adapting therapy accordingly is important to achieve these goals. The treat-to-target recommendations are derived from this last overarching principle.

Reaching all goals

In these treat-to-target recommendations, abrogation of inflammation, leading to remission, is implicitly assumed to be necessary and sufficient for reaching all treatment goals. This assumption is justified for some of the outcomes directly associated with inflammation, such as number of swollen joints, C-reactive protein level, and erythrocyte sedimentation rate (7). However, for several of the main symptoms of JIA and RA (pain, fatigue, functional limitations, morning stiffness, and comorbidities), there is compelling evidence that in a substantial proportion of patients, a treat-to-target strategy is not enough (6,8,9).

Carpenter et al conducted a large-scale longitudinal meta-analysis of 46 cohorts of patients with early RA, with sufficient data from 18,046 patients (8). They concluded that "the introduction of more aggressive, treat-to-target–based therapies coincided with improvements in disease activity and physical function over the last few decades during the first 60-months of the disease. However, these large-scale improvements in disease activity did not translate into equally large improvements in patient-reported outcomes, namely pain, functional disability and mental well-being." Furthermore, in a Cochrane review, it was concluded that treatment of RA with biologic agents has only a small-to-moderate effect on fatigue (9). As a result, some patients whose RA is considered to be in remission still experience fatigue. Walter et al, for example, reported that at 12 months, despite a strict treat-to-target strategy and decreased disease activity, nearly half of their studied patients with early RA (43%) still experienced fatigue (10). Finally, there is some indirect evidence of the effects of treat-to-target on some of the activities of daily living and social participation goals in RA patients (7,11). Findings of studies on treat-to-target in JIA (6) have been consistent with the findings of these studies in RA. Shiff et al, for example, found that a majority of children with JIA continued to report frequent pain and its

[1]Casper G. Schoemaker, PhD: Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands, and Netherlands JIA Patient and Parent Organisation, European Network for Children with Arthritis and Autoinflammatory Diseases, Rijssen, The Netherlands; [2]Maarten P. T. de Wit, PhD, European League Against Rheumatism patient research partner: VU University Medical Centre, Amsterdam, The Netherlands.

No potential conflicts of interest relevant to this article were reported.

Address correspondence to Casper G. Schoemaker, PhD, University Medical Centre Utrecht, Department of Paediatric Rheumatology, Room KC.03.063.0, PO Box 85090, 3508 AB Utrecht, The Netherlands. Email: C.G.Schoemaker-4@umcutrecht.nl.

Submitted for publication May 20, 2020; accepted in revised form July 23, 2020.

debilitating consequences, in spite of effective disease control with biologic therapies (12).

We do believe that a treat-to-target strategy is a promising approach. At this point, however, despite the progress that has been made by introducing principles of tight control (6–8), we find it premature to speak of "dramatic effectiveness" of treat-to-target in RA (1), or to state that transferring treat-to-target recommendations into clinical practice "will significantly improve the outcomes in JIA" (3). The holy grail has not yet been found.

A bowling analogy may be helpful to clarify this issue. A bowling ball will never directly knock down all 10 pins at once. Therefore, bowlers aim at the so-called "head pin" in the front, which is knocked down directly by ball impact and then starts a process of "pin action" by which pins interact and knock each other down. The ultimate goal of bowling is a strike: all 10 pins knocked down on the first roll (Figure 1). The success of a bowler is not measured by the impact on the head pin, or the adjacent pins in the middle, but on all pins. Similarly, to measure the success of a treat-to-target strategy, a disease activity score will not suffice. A proper "pin count" must be conducted.

Unfortunately, in the recommendations for treat-to-target in JIA or RA, no such pin count is included (2,3). Decisions regarding disease management are based almost solely on disease activity scores, and on use of pharmaceutical treatments to affect these scores. The recommendations do not take into account other patient-relevant outcomes, e.g., pain, fatigue, morning stiffness, and daily functioning, some of which may require other interventions, e.g., exercise or physical therapy, specialized surgery, or psychosocial support, rather than a change in the pharmaceutical treatment (5,6,8).

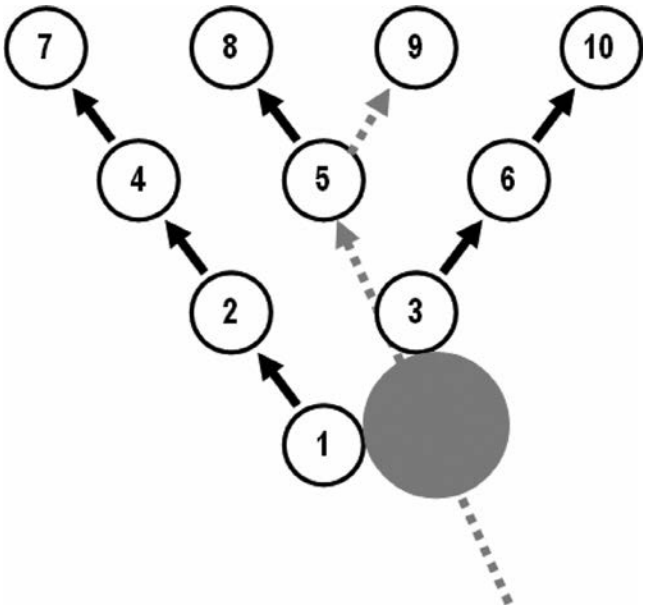


Figure 1. The angle of a bowling ball impacting the head pin and the subsequent pin action leading to a perfect strike.

Shared decision-making

The above illustrates the mismatch between the recommended treatment target (remission) and the emphasis on personal goal setting as the result of shared decision-making, another important stated overarching principle of treat-to-target (2,3). How can treatment decisions genuinely be shared, when the most relevant outcomes are not discussed?

In our experience, conversations between rheumatologists and patients on treatment success often resemble the confusion of tongues in Babel. For most patients, treatment success is about the whole spectrum of goals in the aforementioned overarching principles. For most rheumatologists, treatment success is a synonym for achieving remission, or—more precisely—what Ferreira et al have coined "*biological* remission" (5). When lay patients and their caregivers discuss treatment outcomes with the doctor, they often assume that the term remission includes the entire impact of their disease: not only physical signs and symptoms, but also the social and psychological impact. It has been suggested that patients should be educated about the "true" meaning of remission. From a patient perspective, it is instead time for a more widely encompassing definition of remission, including inflammation as well as disease impact, to cover all treatment goals in the overarching principles of treat-to-target (5).

Numerous composite indices have been developed to measure disease impact in rheumatic diseases. These measures can be very helpful, as long as they allow assessment of each component separately (in bowling terms, a pin count). This is specifically the case for the Rheumatoid Arthritis Impact of Disease and the Psoriatic Arthritis Impact of Disease, 2 patient-reported outcomes that were also developed for clinical practice with the explicit purpose of the individual domains being visible to both patient and physician at all times (13). This visibility to the patient and the provider promotes personal goal setting and monitoring in the context of routine clinical care.

Some people may argue that the patient perspective in all its diversity is captured by the patient global assessment. This single-item question is part of almost all composite indices that are recommended in treat-to-target strategies to measure disease impact. However, the patient global assessment has many flaws, as shown in recent studies (5). Furthermore, it provides no insight into which specific goals have been reached.

Future research

We agree with the treat-to-target task forces that there is an urgent need for more research to elucidate the causal relationships between the currently designated target and the other goals (2–4). Trajectory analyses are clearly needed in order to understand the complex domino effects between the various outcomes (12). Using the bowling analogy, an approach aimed at

2 targets may be more effective to start the pin action (5) (see Figure 1). Ultimately, well-conducted strategic trials will be needed to demonstrate the presumed superiority of the treat-to-target strategies with regard to all relevant goals. Unfortunately, thus far in most treat-to-target-trials a measure of disease activity or "biological remission" has been the main, or even the single, end point (5–12,14). From the patient perspective, this is clearly insufficient to judge success.

We believe the bowling metaphor helps to clarify the discussions on treat-to-target and remission. It demonstrates the importance of focusing on the entire spectrum of patients' quality of life. However, some limitations are worth noting. The outcomes in bowling are binary: pins can either stand or fall. Most outcomes in rheumatic diseases are continuous variables, although they are often dichotomized using cutoffs. Not all patients with rheumatic disease have the same symptoms: the "pins" for each rheumatic disease, disease stage, and even for each individual patient, may differ. In PsA, for instance, skin and nail disease are essential outcome measures (4,15). Patients may even add their own individual treatment goals, with reference to their daily life (6). Finally, while in bowling every pin counts for 1, an individual patient will have personal preferences for reaching some of the goals over others. In general, an open discussion of the goals of therapy should be the start of every treatment strategy.

AUTHOR CONTRIBUTIONS

Drs. Schoemaker and de Wit drafted the article, revised it critically for important intellectual content, and approved the final version to be published.

REFERENCES

1. Smolen JS. Treat-to-target as an approach in inflammatory arthritis [review]. Curr Opin Rheumatol 2016;28:297–302.
2. Smolen JS, Breedveld FC, Burmester GR, Bykerk V, Dougados M, Emery P, et al. Treating rheumatoid arthritis to target: 2014 update of the recommendations of an international task force. Ann Rheum Dis 2016;75:3–15.
3. Ravelli A, Consolaro A, Horneff G, Laxer RM, Lovell DJ, Wulffraat NM, et al. Treating juvenile idiopathic arthritis to target: recommendations of an international task force. Ann Rheum Dis 2018;77:819–28.
4. Smolen JS, Schöls M, Braun J, Dougados M, FitzGerald O, Gladman DD, et al. Treating axial spondyloarthritis and peripheral spondyloarthritis, especially psoriatic arthritis, to target: 2017 update of recommendations by an international task force. Ann Rheumc Dis 2018;77:3–17.
5. Ferreira RJ, Duarte C, Ndosi M, de Wit M, Gossec L, da Silva JA. Suppressing inflammation in rheumatoid arthritis: does patient global assessment blur the target? A practice-based call for a paradigm change. Arthritis Care Res (Hoboken) 2018;70:369–78.
6. Schoemaker CG, Swart JF, Wulffraat NM. Treating juvenile idiopathic arthritis to target: what is the optimal target definition to reach all goals? Pediatr Rheumatol Online 2020;18:34.
7. Stoffer MA, Schoels MM, Smolen JS, Aletaha D, Breedveld FC, Burmester G, et al. Evidence for treating rheumatoid arthritis to target: results of a systematic literature search update. Ann Rheum Dis 2016;75:16–22.
8. Carpenter L, Barnett R, Mahendran P, Nikiphorou E, Gwinnutt J, Verstappen S, et al. Secular changes in functional disability, pain, fatigue and mental well-being in early rheumatoid arthritis: a longitudinal meta-analysis. Semin Arthritis Rheum 2020;50:209–19.
9. Almeida C, Choy EH, Hewlett S, Kirwan JR, Cramp F, Chadler T, et al. Biologic interventions for fatigue in rheumatoid arthritis. Cochrane Database Syst Rev 2016:Cd008334.
10. Walter MJ, Kuijper TM, Hazes JM, Weel AE, Luime JJ. Fatigue in early, intensively treated and tight-controlled rheumatoid arthritis patients is frequent and persistent: a prospective study. Rheumatol Int 2018;38:1643–50.
11. Wechalekar MD, Quinn S, Lester S, Metcalf RG, Shanahan E, Walker JG, et al. A treat-to-target strategy preserves work capacity in a rheumatoid arthritis inception cohort treated with combination conventional DMARD therapy. J Clin Rheumatol 2017;233:131–7.
12. Shiff NJ, Tupper S, Oen K, Guzman J, Lim H, Lee CH, et al. Trajectories of pain severity in juvenile idiopathic arthritis: results from the Research in Arthritis in Canadian Children Emphasizing Outcomes cohort. Pain 2018;159:57–66.
13. Mistry J, Sharif M, Prideaux A, Smith C, Sumbwanyambe M, Sibley M, et al. Use of rheumatoid arthritis impact of disease (RAID) in routine care; identification of DAS28 remission and unmet patient reported outcomes. Rheumatol Adv Pract 2020;4:rkaa013.
14. Muller PH, Brinkman DM, Schonenberg-Meinema D, van den Bosch WB, Koopman-Keemink Y, Brederije IC, et al. Treat to target (drug-free) inactive disease in DMARD-naive juvenile idiopathic arthritis: 24-month clinical outcomes of a three-armed randomised trial. Ann Rheum Dis 2019;78:51–9.
15. Singh JA, Guyatt G, Ogdie A, Gladman DD, Deal C, Deodhar A, et al. 2018 American College of Rheumatology/National Psoriasis Foundation guideline for the treatment of psoriatic arthritis. Arthritis Rheumatol 2019;71:5–32.

American College of Rheumatology
Empowering Rheumatology Professionals

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 12–20
DOI 10.1002/art.41562

Clinical Practice Guidelines by the Infectious Diseases Society of America (IDSA), American Academy of Neurology (AAN), and American College of Rheumatology (ACR): 2020 Guidelines for the Prevention, Diagnosis, and Treatment of Lyme Disease

Paul M. Lantos,[1] Jeffrey Rumbaugh,[2] Linda K. Bockenstedt,[3] Yngve T. Falck-Ytter,[4] Maria E. Aguero-Rosenfeld,[5] Paul G. Auwaerter,[6] Kelly Baldwin,[7] Raveendhara R. Bannuru,[8] Kiran K. Belani,[9] William R. Bowie,[10] John A. Branda,[11] David B. Clifford,[12] Francis J. DiMario Jr.,[13] John J. Halperin,[14] Peter J. Krause,[15] Valery Lavergne,[16] Matthew H. Liang,[17] H. Cody Meissner,[8] Lise E. Nigrovic,[18] James (Jay) J. Nocton,[19] Mikala C. Osani,[8] Amy A. Pruitt,[20] Jane Rips,[21] Lynda E. Rosenfeld,[3] Margot L. Savoy,[22] Sunil K. Sood,[23] Allen C. Steere,[11] Franc Strle,[24] Robert Sundel,[18] Jean Tsao,[25] Elizaveta E. Vaysbrot,[8] Gary P. Wormser,[26] and Lawrence S. Zemel[13]

It is important to realize that guidelines cannot always account for individual variation among patients. They are assessments of current scientific and clinical information provided as an educational service; are not continually updated and may not reflect the most recent evidence (new evidence may emerge between the time information is developed and when it is published or read); should not be considered inclusive of all proper treatments methods of care, or as a statement of the standard of care; do not mandate any particular course of medical care; and are not intended to supplant physician judgment with respect to particular patients or special clinical situations. Whether and the extent to which to follow guidelines is voluntary, with the ultimate determination regarding their application to be made by the physician in the light of each patient's individual circumstances. Although IDSA, AAN, and ACR make every effort to present accurate, complete, and reliable information, these guidelines are presented "as is" without any warranty, either express or implied. IDSA, AAN, and ACR (and their officers, directors, members, employees, and agents) assume no responsibility for any loss, damage, or claim with respect to any liabilities, including direct, special, indirect, or consequential damages, incurred in connection with these guidelines or reliance on the information presented.

This evidence-based clinical practice guideline for the prevention, diagnosis, and treatment of Lyme disease was developed by a multidisciplinary panel representing the Infectious Diseases Society of North American (IDSA), the American Academy of Neurology (AAN), and the American College of Rheumatology (ACR). The scope of this guideline includes prevention of Lyme disease,

This guideline was jointly developed by the Infectious Diseases Society of America, the American Academy of Neurology Institute, and the American College of Rheumatology. The article was peer reviewed by *Arthritis & Rheumatology* and simultaneously published by *Clinical Infectious Diseases*, *Neurology*, *Arthritis Care & Research*, and *Arthritis & Rheumatology*. Each editor of the 4 journals appointed 1 reviewer for peer review. The articles

and the diagnosis and treatment of Lyme disease presenting as erythema migrans, Lyme disease complicated by neurologic, cardiac, and rheumatologic manifestations, Eurasian manifestations of Lyme disease, and Lyme disease complicated by coinfection with other tick-borne pathogens. This guideline does not include comprehensive recommendations for babesiosis and tick-borne rickettsial infections, which are published in separate guidelines. The target audience for this guideline includes primary care physicians and specialists caring for this condition such as infectious diseases specialists, emergency physicians, internists, pediatricians, family physicians, neurologists, rheumatologists, cardiologists, and dermatologists in North America.

Summarized below are the 2020 recommendations for the prevention, diagnosis, and treatment of Lyme disease. The panel followed a systematic process used in the development of other Infectious Diseases Society of America (IDSA), American Academy of Neurology (AAN), and American College of Rheumatology (ACR) clinical practice guidelines, which included a standardized methodology for rating the certainty of the evidence and strength of recommendation using the GRADE approach (Grading of Recommendations Assessment, Development, and Evaluation) (see Figure 1). A detailed description of background, methods, evidence summary and rationale that support each recommendation, and knowledge gaps can be found online in the full text (http://onlinelibrary.wiley.com/doi/10.1002/art.41562/abstract).

I. Which measures should be used to prevent tick bites and tick-borne infections?

A. Personal protective measures

Recommendation:

1. Individuals at risk of exposure should implement personal protective measures to reduce the risk of tick exposure and infection with tick-borne pathogens *(good practice statement).*

B. Repellents to prevent tick bites

Recommendation:

1. For the prevention of tick bites, we recommend N, N-Diethyl-meta-toluamide (DEET), picaridin, ethyl-3-(N-n-butyl-N- acetyl) aminopropionate (IR3535), oil of lemon eucalyptus (OLE), p-methane-3,8-diol (PMD), 2-undecanone, or permethrin *(strong recommendation, moderate-quality evidence)*.

C. Removal of attached ticks

Recommendations:

1. We recommend promptly removing attached ticks by mechanical means using a clean fine-tipped tweezer (or a comparable device) inserted between the tick body and the skin *(good practice statement)*.
2. We recommend against burning an attached tick (with a match or other heat device) or applying noxious chemicals or petroleum products to coax its detachment *(good practice statement)*.

II. Which diagnostic tests should be used following a tick bite?

A. Diagnostic tick testing

Recommendations:

1. We recommend submitting the removed tick for species identification *(good practice statement)*.

---

are identical except for minor stylistic and spelling differences in keeping with each journal's style. The full guideline is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41562/abstract.

Support for this guideline was provided by the Infectious Diseases Society of America, the American Academy of Neurology, and the American College of Rheumatology.

[1]Paul M. Lantos, MS, MD: Duke University School of Medicine, Durham, North Carolina; [2]Jeffrey Rumbaugh, MD, PhD: Pathway Neurology, Tampa, Florida; [3]Linda K. Bockenstedt, MD, Lynda E. Rosenfeld, MD: Yale University, New Haven, Connecticut; [4]Yngve T. Falck-Ytter, MD: Case Western Reserve University, VA Northeast Ohio Healthcare System, Cleveland, Ohio; [5]Maria E. Aguero-Rosenfeld, MD: New York University School of Medicine, New York, New York; [6]Paul G. Auwaerter, MBA, MD: Johns Hopkins University School of Medicine, Baltimore, Maryland; [7]Kelly Baldwin, MD: Geisinger Medical Center, Danville, Pennsylvania; [8]Raveendhara R. Bannuru, MD, H. Cody Meissner, MD, Mikala C. Osani, MPH, Elizaveta E. Vaysbrot, MD: Tufts Medical Center, Boston, Massachusetts; [9]Kiran K. Belani, MD: Children's Hospital and Clinics of Minnesota, Minneapolis; [10]William R. Bowie, MD: University of British Columbia, Vancouver, British Columbia, Canada; [11]John A. Branda, MD, Allen C. Steere, MD: Massachusetts General Hospital, Boston; [12]David B. Clifford, MD: Washington University School of Medicine, St. Louis, Missouri; [13]Francis J. DiMario Jr., MD, Lawrence S. Zemel, MD: Connecticut Children's Medical Center, Hartford; [14]John J. Halperin, MD: Atlantic Health System, Summit, New Jersey; [15]Peter J. Krause, MD: Yale School of Public Health, New Haven, Connecticut; [16]Valery Lavergne, MSc, MD: University of Montreal, Montreal, Quebec, Canada; [17]Matthew H. Liang, MPH, MD: Brigham and Women's Hospital, Boston, Massachusetts; [18]Lise E. Nigrovic, MPH, MD, Robert Sundel, MD: Boston Children's Hospital, Boston, Massachusetts; [19]James (Jay) J. Nocton, MD: Medical College of Wisconsin, Waowatosa; [20]Amy A. Pruitt, MD: University of Pennsylvania, Philadelphia; [21]Jane Rips: Consumer Representative, Omaha, Nebraska; [22]Margot L. Savoy, MPH, MD: Temple University, Philadelphia, Pennsylvania; [23]Sunil K. Sood, MD: Northwell Health, New York, New York; [24]Franc Strle, PhD: University Medical Centre Ljubljana, Ljubljana, Slovenia; [25]Jean Tsao, PhD: Michigan State University, East Lansing; [26]Gary P. Wormser, MD: New York Medical College, Valhalla, New York.

Conflict of interest information appears at the end of the text.

Address correspondence to Paul M. Lantos, MD, MS, Duke University School of Medicine, 2424 Erwin Road, Hock Plaza, Suite 405, Durham, NC. Email: paul.lantos@duke.edu.

Submitted for publication August 21, 2019; accepted in revised form June 5, 2020.

[Correction added on 11 December, 2020 after first online publication: a paragraph was inserted on page 1, prior to the existing first paragraph.]

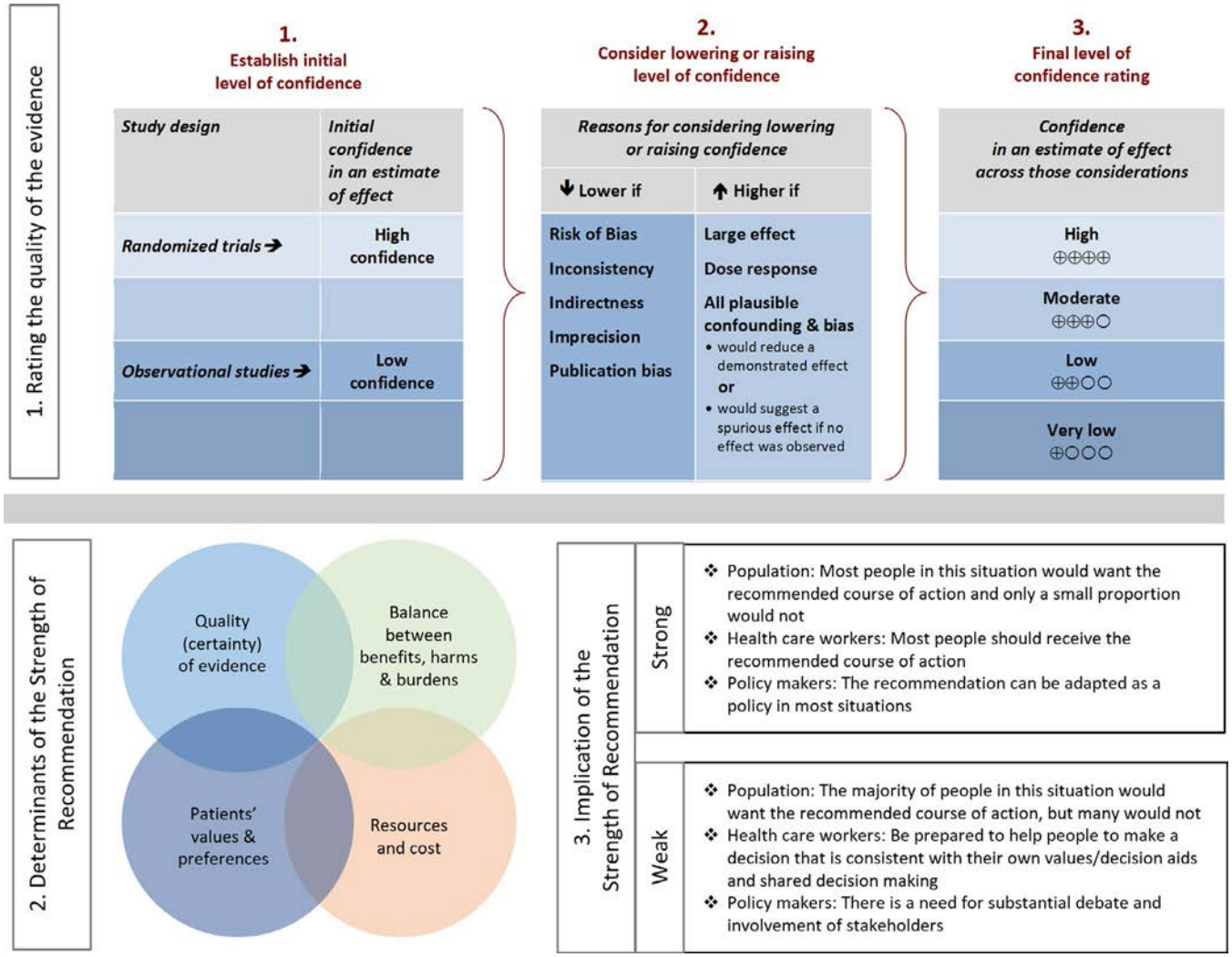


Figure 1. Approach and implications to rating the quality of evidence and strength of recommendations using the GRADE (Grading of Recommendations Assessment, Development, and Evaluation) methodology (unrestricted use of the figure granted by the US GRADE Network) (1,2).

2. We recommend against testing a removed *Ixodes* tick for *B. burgdorferi (strong recommendation, moderate-quality evidence)*. **Comment:** The presence or absence of *B. burgdorferi* in an *Ixodes* tick removed from a person does not reliably predict the likelihood of clinical infection.

B. Diagnostic testing of asymptomatic patients following tick bites

Recommendation:

1. We recommend against testing asymptomatic patients for exposure to *B. burgdorferi* following an *Ixodes* spp. tick bite *(strong recommendation, moderate-quality evidence).*

III. Who should receive antibiotic prophylaxis to prevent Lyme disease following presentation with a tick bite?

Recommendation:

1. We recommend that prophylactic antibiotic therapy be given only to adults and children within 72 hours of removal of an identified high-risk tick bite, but not for bites that are equivocal risk or low risk *(strong recommendation, high-quality evidence)*. **Comment**: If a tick bite cannot be classified with a high level of certainty as a high-risk bite, a wait-and-watch approach is recommended. A tick bite is considered to be high-risk only if it meets the following 3 criteria: the tick bite was from (a) an identified *Ixodes* spp. vector species, (b) it occurred in a highly endemic area, and (c) the tick was attached for ≥36 hours.

IV. What is the preferred antibiotic regimen for the chemoprophylaxis of Lyme disease following a high-risk tick bite?

Recommendation:

1. For high-risk *Ixodes* spp. bites in all age groups, we recommend the administration of a single dose of oral doxycycline within 72 hours of tick removal over observation *(strong recommendation, moderate-quality evidence)*. **Comment:** Doxycycline is given as a single oral dose, 200 mg for adults and 4.4 mg/kg (up to a maximum dose of 200 mg) for children.

V. What is the preferred diagnostic testing strategy for erythema migrans?

Recommendations:

1. In patients with potential tick exposure in a Lyme disease endemic area who have 1 or more skin lesions compatible with erythema migrans, we recommend clinical diagnosis rather than laboratory testing *(strong recommendation, moderate-quality evidence)*.
2. In patients with 1 or more skin lesions suggestive of, but atypical for erythema migrans, we suggest antibody testing performed on an acute-phase serum sample (followed by a convalescent-phase serum sample if the initial result is negative) rather than currently available direct detection methods such as polymerase chain reaction (PCR) or culture performed on blood or skin samples *(weak recommendation, low-quality evidence)*. **Comment:** If needed, the convalescent-phase serum sample should be collected at least 2–3 weeks after collection of the acute-phase serum sample.

VI. What are the preferred antibiotic regimens for the treatment of erythema migrans?

Recommendation:

1. For patients with erythema migrans, we recommend using oral antibiotic therapy with doxycycline, amoxicillin, or cefuroxime axetil *(strong recommendation; moderate-quality evidence)*. **Comment:** For patients unable to take both doxycycline and beta-lactam antibiotics, the preferred second-line agent is azithromycin.

VII. How long should a patient with erythema migrans be treated?

Recommendation:

1. We recommend that patients with erythema migrans be treated with either a 10-day course of doxycycline or a 14-day course of amoxicillin or cefuroxime axetil rather than longer treatment courses *(strong recommendation, moderate-quality evidence)*. **Comment:** If azithromycin is used, the indicated duration is 5–10 days, with a 7-day course preferred in the United States, as this duration of therapy was used in the largest clinical trial performed in the United States (3).

VIII. Should patients with the southern tick–associated rash illness (STARI) be treated with antibiotics?

Recommendation:

1. In patients who develop an erythema migrans–like skin lesion following the bite of the lone star tick (*Amblyomma americanum*), an illness referred to as STARI, we make no recommendation for or against the use of antibiotics *(no recommendation; knowledge gap)*. **Comment:** In certain geographic regions both STARI and Lyme disease are endemic (4). Distinguishing single erythema migrans due to Lyme disease from STARI may not be possible clinically unless the responsible tick has been identified (5). When STARI cannot be distinguished from Lyme disease–associated erythema migrans in areas endemic for both conditions, antibiotic therapy directed toward Lyme disease is indicated.

IX. What is the preferred diagnostic testing strategy for Lyme neuroborreliosis?

Recommendations:

1. When assessing patients for possible Lyme neuroborreliosis involving either the peripheral nervous system (PNS) or central nervous system (CNS), we recommend serum antibody testing rather than PCR or culture of either cerebrospinal fluid (CSF) or serum *(strong recommendation, moderate-quality evidence)*.

2. If CSF testing is performed in patients with suspected Lyme neuroborreliosis involving the CNS, we (a) recommend obtaining simultaneous samples of CSF and serum for determination of the CSF:serum antibody index, carried out by a laboratory using validated methodology, (b) recommend against CSF serology without measurement of the CSF:serum antibody index, and (c) recommend against routine PCR or culture of CSF or serum *(strong recommendation, moderate-quality evidence)*.

X. For which neurologic presentations should patients be tested for Lyme disease?

Recommendations:

1. In patients presenting with 1 or more of the following acute disorders: meningitis, painful radiculoneuritis, mononeuropathy multiplex including confluent mononeuropathy multiplex, acute cranial neuropathies (particularly VII, VIII, less commonly III, V, VI, and others), or in patients with evidence of spinal cord (or rarely brain) inflammation, the former particularly in association with painful radiculitis involving related spinal cord segments, and with epidemiologically plausible exposure to ticks infected with *B. burgdorferi*, we recommend testing for Lyme disease *(strong recommendation, moderate-quality evidence)*.
2. In patients with typical amyotrophic lateral sclerosis, relapsing-remitting multiple sclerosis, Parkinson's disease, dementia or cognitive decline, or new-onset seizures, we recommend against routine testing for Lyme disease *(strong recommendation, low-quality evidence)*.
3. In patients with neurologic syndromes other than those listed in [1] or [2], in the absence of a history of other clinical or epidemiologic support for the diagnosis of Lyme disease, we recommend against screening for Lyme disease *(strong recommendation, low-quality evidence)*.
4. In patients presenting with nonspecific magnetic resonance imaging white matter abnormalities confined to the brain in the absence of a history of other clinical or epidemiologic support for the diagnosis of Lyme disease, we suggest against testing for Lyme disease *(weak recommendation, low-quality evidence)*.

XI. Should adult patients with psychiatric illnesses be tested for Lyme disease?

Recommendation:

1. In patients with psychiatric illness, we recommend against routine testing for Lyme disease *(strong recommendation, low-quality evidence)*.

XII. Should children with developmental, behavioral, or psychiatric disorders be tested for Lyme disease?

Recommendation:

1. In children presenting with developmental, behavioral, or psychiatric disorders, we suggest against routinely testing for Lyme disease *(weak recommendation, low-quality evidence)*.

XIII. What are the preferred antibiotic regimens for the treatment of acute neurologic manifestations of Lyme disease without parenchymal involvement of the brain or spinal cord?

Recommendation:

1. In patients with Lyme disease–associated meningitis, cranial neuropathy, radiculoneuropathy, or with other PNS manifestations, we recommend using intravenous (IV) ceftriaxone, cefotaxime, penicillin G, or oral doxycycline over other antimicrobials *(strong recommendation, moderate-quality evidence)*. **Comment:** Decisions about the choice of antibiotic among these, including the route of administration, should primarily be made based on individual factors such as side effect profile, ease of administration, ability to tolerate oral medication, concerns about compliance unrelated to effectiveness. Treatment route may be changed from IV to oral during treatment. The preferred antibiotic duration is 14–21 days.

XIV. Should patients with Lyme disease–related parenchymal involvement of the brain or spinal cord be treated with oral or intravenous antibiotics?

Recommendation:

1. In patients with Lyme disease–associated parenchymal involvement of the brain or spinal cord, we recommend using IV over oral antibiotics *(strong recommendation, moderate-quality evidence)*.

XV. Should patients with Lyme disease and facial nerve palsy receive corticosteroids in addition to antimicrobial therapy?

Recommendation:

1. In patients with Lyme disease–associated facial nerve palsy, we make no recommendation on the use of corti-

costeroids in addition to antibiotics (no recommendation; knowledge gap). **Comment:** In patients age 16 or older presenting with acute facial nerve palsy but without other objective clinical or serologic evidence of Lyme disease, corticosteroid treatment should be administered within 72 hours in accordance with current facial nerve palsy guideline recommendations (6).

XVI. Should all patients with early Lyme disease receive an electrocardiogram (ECG) to screen for Lyme carditis?

Recommendation:

1. We suggest performing an ECG only in patients with signs or symptoms consistent with Lyme carditis *(weak recommendation, low-quality evidence)*. **Comment:** Symptoms and signs of cardiac involvement in Lyme disease include dyspnea, edema, palpitations, lightheadedness, chest pain, and syncope.

XVII. Which patients with Lyme carditis require hospitalization?

Recommendation:

1. In patients with or at risk for severe cardiac complications of Lyme disease including those with significant PR prolongation (PR >300 milliseconds), other arrhythmias, or clinical manifestations of myopericarditis, we recommend hospital admission with continuous ECG monitoring *(strong recommendation, very low-quality evidence).* **Comment:** Clinical manifestations of Lyme carditis include exercise intolerance, palpitations, presyncope, syncope, pericarditic pain, evidence of pericardial effusion, elevated biomarkers (such as troponin), edema, and shortness of breath.

XVIII. What pacing modality should be used if needed for the management of Lyme carditis?

Recommendation:

1. For patients with symptomatic bradycardia due to Lyme carditis that cannot be managed medically, we recommend temporary pacing modalities rather than implanting a permanent pacemaker *(strong recommendation, moderate-quality evidence)*.

XIX. What are the preferred antibiotic regimens for the treatment of Lyme carditis?

Recommendations:

1. In outpatients with Lyme carditis, we suggest oral antibiotics over IV antibiotics *(weak recommendation, very low-quality evidence)*.
2. In the hospitalized patient with Lyme carditis, we suggest initially using IV ceftriaxone over oral antibiotics until there is evidence of clinical improvement, then switching to oral antibiotics to complete treatment *(weak recommendation, very low-quality evidence)*.
3. For the treatment of Lyme carditis, we suggest 14–21 days of total antibiotic therapy over longer durations of treatment *(weak recommendation, very low-quality evidence)*. **Comment:** Oral antibiotic choices for Lyme carditis are doxycycline, amoxicillin, cefuroxime axetil, and azithromycin.

XX. Should patients being evaluated for acute myocarditis/pericarditis or chronic cardiomyopathy of unknown cause be tested for Lyme disease?

Recommendations:

1. In patients with acute myocarditis/pericarditis of unknown cause in an appropriate epidemiologic setting, we recommend testing for Lyme disease *(strong recommendation, low-quality evidence).*
2. In patients with chronic cardiomyopathy of unknown cause, we suggest against routine testing for Lyme disease *(weak recommendation, low-quality evidence)*.

XXI. What is the preferred diagnostic testing strategy for Lyme arthritis?

Recommendations:

1. When assessing possible Lyme arthritis, we recommend serum antibody testing over PCR or culture of blood or synovial fluid/ tissue *(strong recommendation, moderate-quality evidence)*.
2. In seropositive patients for whom the diagnosis of Lyme arthritis is being considered but treatment decisions require more definitive information, we recommend PCR applied to synovial fluid or tissue rather than *Borrelia* culture of those samples *(strong recommendation, moderate-quality evidence).*

XXII. What are the preferred antibiotic regimens for the initial treatment of Lyme arthritis?

Recommendation:

1. For patients with Lyme arthritis, we recommend using oral antibiotic therapy for 28 days *(strong recommendation, moderate-quality evidence)*.

XXIII. What are the approaches to patients in whom Lyme arthritis has not completely resolved?

Recommendations:

1. In patients with Lyme arthritis with partial response (mild residual joint swelling) after a first course of oral antibiotic, we make no recommendation for a second course of antibiotic versus observation *(no recommendation, knowledge gap)*. **Comment:** Consideration should be given to exclusion of other causes of joint swelling than Lyme arthritis, medication adherence, duration of arthritis prior to initial treatment, degree of synovial proliferation versus joint swelling, patient preferences, and cost. A second course of oral antibiotics for up to 1 month may be a reasonable alternative for patients in whom synovial proliferation is modest compared to joint swelling and for those who prefer repeating a course of oral antibiotics before considering IV therapy.
2. In patients with Lyme arthritis with no or minimal response (moderate to severe joint swelling with minimal reduction of the joint effusion) to an initial course of oral antibiotic, we suggest a 2–4-week course of IV ceftriaxone over a second course of oral antibiotics *(weak recommendation, low-quality evidence)*.

XXIV. How should post-antibiotic (previously termed antibiotic-refractory) Lyme arthritis be treated?

Recommendation:

1. In patients who have failed 1 course of oral antibiotics and 1 course of IV antibiotics, we suggest a referral to a rheumatologist or other trained specialist for consideration of the use of disease-modifying antirheumatic drugs, biologic agents, intraarticular steroids, or arthroscopic synovectomy *(weak recommendation, very low-quality evidence)*. **Comment:** Antibiotic therapy for longer than 8 weeks is not expected to provide additional benefit to patients with persistent arthritis if that treatment has included 1 course of IV therapy.

XXV. Should patients with persistent symptoms following standard treatment of Lyme disease receive additional antibiotics?

Recommendation:

1. For patients who have persistent or recurring nonspecific symptoms such as fatigue, pain, or cognitive impairment following recommended treatment for Lyme disease, but who lack objective evidence of reinfection or treatment failure, we recommend against additional antibiotic therapy *(strong recommendation, moderate-quality evidence)*. **Comment:** Evidence of persistent infection or treatment failure would include objective signs of disease activity, such as arthritis, meningitis, or neuropathy.

XXVI. What is the preferred antibiotic regimen for the treatment of borrelial lymphocytoma?

Recommendation:

1. In patients with borrelial lymphocytoma, we suggest oral antibiotic therapy for 14 days *(weak recommendation, low-quality evidence)*.

XXVII. What is the preferred antibiotic regimen for the treatment of acrodermatitis chronica atrophicans?

Recommendation:

1. In patients with acrodermatitis chronica atrophicans, we suggest oral antibiotic therapy for 21–28 days over shorter durations *(weak recommendation, low-quality evidence)*.

XXVIII. Under what circumstances should a patient with Lyme disease be evaluated for coinfection with *A. phagocytophilum* or *B. microti*?

Recommendation:

1. In patients with Lyme disease who have a high-grade fever or characteristic laboratory abnormalities, clinicians should assess for possible coinfection with *Anaplasma phagocytophilum* and/or *B. microti* infection in geographic regions where these infections are endemic *(good practice statement)*. **Comment:** Coinfection should be investigated in patients who have a persistent fever for >1 day while on antibiotic treatment for Lyme disease. If fever persists despite treatment with doxycycline, *B. microti* infection is an important consideration.

Characteristic laboratory abnormalities found in both anaplasmosis and babesiosis include thrombocytopenia, leukopenia, neutropenia, and/or anemia. Evidence of hemolysis, such as elevated indirect bilirubin level, anemia, and elevated lactate dehydrogenase, is particularly suggestive of babesiosis.

Supplementary data. Supplementary materials (in addition to the full guideline) are available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41562/abstract. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Conflict of interest statement. See the Methodology section in the full guideline (on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41562/abstract) for approach to conflict of interest (COI) by the IDSA/AAN/ACR COI review group. The following list is a reflection of what has been reported to the IDSA/AAN/ACR COI review group. To provide thorough transparency, the IDSA/AAN/ACR requires full disclosure of all relationships, regardless of relevancy to the guideline topic. The assessment of disclosed relationships for possible COI is based on the relative weight of the financial relationship (i.e., monetary amount) and the relevance of the relationship (i.e., the degree to which an association might reasonably be interpreted by an independent observer as related to the topic or recommendation of consideration). The reader of these guidelines should be mindful of this when the list of disclosures is reviewed. Dr. Lantos has received research funding from the National Cytomegalovirus Foundation and from the NIH and educational funding from Duke University; and has served as a consultant and reviewed trial protocol for Frederick O'Connor Medical Consultants, LLC. Dr. Bockenstedt has received research funding from the NIH and the Gordon and Llura Gund Foundation; has received remuneration from L2 Diagnostics for investigator-initiated NIH-sponsored research; and was awarded an endowed professorship as the Harold W. Jockers Professor of Medicine at Yale University. Dr. Falck-Ytter serves as director of the Evidence Foundation and the GRADE Network; conducts GRADE workshops with the Evidence Foundation; has served as the chair of the Guidelines Committee for the American Gastroenterological Association; and has received research funding from the Cleveland VA Medical Research and Education Foundation. Dr. Aguero-Rosenfeld serves as a council member for the New York City chapter of the American Society of Microbiology (ASM) and as a Board member of the American Lyme Disease Foundation; has provided legal testimony and consultation regarding Lyme disease and tick-borne diseases; and has received research grants from the NIH, BioFire, New York State Department of Health, and ViraMed. Dr. Auwaerter receives research funding from the Fisher Center for Environmental Infectious Diseases and the NIH; serves on the Board of Directors of the American Lyme Disease Foundation and as the Vice Chair of the Infectious Diseases Society of America (IDSA) Foundation; serves as a scientific advisor for DiaSorin, Adaptive Technologies, and Shionogi; provides legal expert opinion testimony regarding Lyme disease; had stock in Johnson & Johnson; has served as an editor for Johns Hopkins POC-IT ABX Guide, an advisor for the Food and Drug Administration (FDA), Genentech, Dynavax, Aradigm, Cempra, BioMérieux, Cerexa, and Medscape; has received research funding from Cerexa; has served on the FDA Advisory Board, the Medscape Advisory Board, and the IDSA Board of Directors; and his spouse has equity interest in venture capital–funded Capricor. Dr. Belani reviews non-continuing medical education (CME) lectures for and received honoraria and travel reimbursement from Horizon Therapeutics; and has received research funding from the NIH and the Children's Hospitals and Clinics of Minnesota. Dr. Bowie has provided expert testimony to the Canadian Senate Subcommittee on Bill C-442: An Act Respecting a National Lyme Disease Strategy on behalf of the Association of Medical Microbiology and Infectious Disease Canada; and has received research funding from GlaxoSmithKline, Pfizer Canada, the Canadian Institutes of Health Research, and Vancouver Coastal Health Research Institute. Dr. Branda receives research funding from the Lyme Disease Biobank Foundation and Zeus Scientific; serves as a scientific advisor and consultant to DiaSorin, Inc.; has served as a scientific advisor and consultant for T2 Biosystems; has served on the scientific advisory board of Roche Diagnostics and AdvanDx; has received research funding from Karius, Inc., Alere, Inc., T2 Biosystems, BioMérieux, TBS Technologies, Immunetics, Inc., DiaSorin, Inc., Kephera Diagnostics, Inc., and the Bay Area Lyme Foundation; has participated in unfunded research collaborations with Karius Inc. and Kephera Diagnostics; was a member of the editorial board of the *Journal of Clinical Microbiology*; was a co-inventor on an application for a patent to protect intellectual property; and his spouse is an employee of Informed DNA. Dr. Clifford receives research funding from the NIH and the Alzheimer's Association; serves as scientific consultant to Inhibikase and Excision BioTherapeutics; serves on Data and Safety Monitoring Boards (DSMB) for Biogen, Genzyme/Sanofi, Genentech, EMD Serono, Shire, Wave Life Sciences, Pfizer, Atara, Mitsubishi Tanabe, and IQVIA (formerly Quintiles); serves on Progressive Multifocal Leukoencephalopathy (PML) adjudication committees for Amgen, GlaxoSmithKline, EMD Serono, Bristol Myers Squibb, Roche, and the Takeda Oncology (formerly Millennium) Adjudication Committee–FDA, as well as Dr. Reddy's Laboratories; has previously received research funding from the NIH; and his spouse formerly held stock in Johnson & Johnson. Dr. DiMario has received research funding from Novartis. Dr. Halperin serves as an Editorial Board Member of *Neurology*, and Vice Chair of the American Academy of Neurology (AAN) Guideline Subcommittee; has stock in Abbott Labs, AbbVie, Merck, and Johnson & Johnson; provides and has previously provided legal expert testimony defending physicians in medical malpractice cases on various neurologic issues, including Lyme disease; has received research funding from NIH, the Centers for Disease Control and Prevention (CDC); and has served as a section editor of neuroinfectious diseases in *Neurology & Neuroscience Reports*. Dr. Krause receives research funding from the Yale Emerging Infections Program; receives remuneration from Gold Standard Diagnostics for a collaborative research project; has stock in Gilead Sciences and First Trust NASDAQ Pharmaceuticals ETF; has received research funding from the NIH, the Centers for Disease Control and Prevention (CDC), the Gordon and Llura Gund Foundation, and L2 Diagnostics for NIH-sponsored research; has served as a scientific consultant and provided medical education and training for Oxford Immunotec, Inc.; has a patent pending (Enhanced Chemiluminescent enzyme-linked immunosorbent assay for detection of antibodies against *Babesia microti*), for which US Provisional Patent Application No. 62/580,588, was filed on November 2, 2017; serves on the Board of Directors for the American Lyme Disease Foundation and the Editorial Boards of Pathogens and *Plos* Neglected Tropical Diseases and the Editorial Advisory Board of *Clinical Infectious Diseases*; was on the Editorial Board of *Journal of Clinical Microbiology*, and will be on the Editorial Board of *Clinical Microbiology Reviews* starting January 2021. Dr. Liang has stock in Johnson & Johnson; received research funding from the Veterans Health Administration, the Arthritis Foundation, and the NIH; has served on the FDA Advisory Panel, Institute of Medicine panels; served as a scientific reviewer for the Research Grant Council of Hong Kong and the NIH; served on the Board of the Lupus Clinical Trials Consortium, Beacon Hill Villages, and Rx Foundation and advised the Institute for Clinical and Economic Review and the China Medical Board; previously had stock in Sequenom; and his spouse has stock in Johnson & Johnson. Dr. Meissner is a current member of the CDC Workgroups and serves as a volunteer consultant on the American Academy of Pediatrics Committee on Infectious Diseases and the NIH DSMB. Dr. Nigrovic receives research funding from the NIH, Department of Defense, and the NIH Center for Research Resources and for Advancing Translational Sciences (NCATS), Global Lyme Alliance, and Peabody Foundation; serves on the Editorial Board for *Annals of Emergency Medicine*; has served as scientific consultant for Adaptive Technologies; has received research funding from the NIH, Provider and Payer Quality Initiative (PPQI) Research Foundation, Harvard Catalyst, Hood Foundation, Bay Area Lyme Foundation, CDC, Emergency Medical Services for Children (EMSC), the National Patient-Centered Clinical Research Network (PCORNet), Milton Foundation, and Boston Children's Hospital. Dr. Nocton receives research funding from Bristol Myers Squibb; serves as

a member of the Subboard of Pediatric Rheumatology of the American Board of Pediatrics; and has received research funding from AbbVie, NIH, and the Arthritis Foundation. Dr. Pruitt has received research funding from Teva Pharmaceuticals and has served on the AAN Editorial Board of *Neurology Clinical Practice*. Ms Rips has received research funding from the Center for AIDS Research, Biogen Idec, Hoffmann-LaRoche, Sun Pharmaceutical Industries Ltd., Genzyme, the Alzheimer's Association, and the American College of Radiology; and has served as a speaker for Teva Pharmaceuticals. Dr. Rosenfeld serves as a Council Member of the American College of Cardiology; has stock in Abbott, Proctor & Gamble, and General Electric; has received Fellowship Support from Boston Scientific, Medtronic, and Abbott Laboratories (formerly St. Jude Medical); has received research funding from Boehringer Ingelheim Pharmaceuticals, Inc.; and has served on the Program Committee and the Patient and Caregivers Committee of the Heart Rhythm Society. Dr. Savoy serves on the American Academy of Family Physicians (AAFP) Board of Directors, as an ex-officio Board member of Delaware Academy of Family Physicians (DAFP), as the Chair of the Centers for Medicare and Medicaid Services (CMS) Advisory Panel on Outreach and Education, and as Secretary of the Board of Directors of the Association of Departments of Family Medicine; receives honoraria from AAFP, DAFP, CMS, and Merck; has served on an Advisory Council for Highmark Health and as an advisor to the AAFP Adolescent Immunization Project; has received honoraria from AAFP; has served as the President of DAFP, as Editor of *DelFamDoc*, and as a member of AAFP Commissions. Dr. Sood has received research funding from the NIH; and has provided expert testimony for Danaher Lagnese, PC. Dr. Steere receives research funding from the NIH and the Mathers Foundation; has received research funding from the NIH, the American College of Rheumatology, the Mathers Foundation, the English-Bonter-Mitchell Foundation, Immunetics, Inc., Zeus Diagnostics, and the Ounsworth-Fitzgerald Foundation; and has served as a scientific advisor for Baxter Bioscience Institute of Systems Biology, Immunetics, Inc., Roche Diagnostics, and Viramed. Dr. Strle receives research funding from the Slovenian Research Agency; serves as the Head of Health Counsel of the Ministry of Health of the Republic of Slovenia and as a member of the Steering Committee for the European Society of Clinical Microbiology and Infectious Diseases Study Group for Lyme Borreliosis; serves on the Roche Diagnostics Advisory Board on Lyme Disease Diagnostics; and has received honoraria from Roche Diagnostics. Dr. Sundel receives research funding from the NIH and AbbVie, Inc.; serves as a content author and editor for UpToDate; provides expert testimony to Chin-Caplan, PC; has provided expert testimony for Conway Homer, PC; has served as an advisor for Paul Hastings, LLC; has served as a content editor for SimulConsult and as a Medical Education Resources lecturer for CME-granting educational courses; has received remuneration from SimulConsult as a co-investigator for an NIH-sponsored grant; and has received research funding from the NIH. Dr. Tsao receives research funding from the National Science Foundation, NIH, CDC, the Michigan Lyme Disease Association, and the Michigan Department of Health and Human Services; serves as a Scientific Council Advisor Member for the Canadian Lyme Disease Research Network and as a scientific advisor for the American Lyme Disease Association; has received research funding from Michigan State University; has served as an Associate Editor for *Ticks and Tick-Borne Diseases* and on the Tick Vectors, Surveillance, and Prevention Subcommittee of the US Department of Health and Human Services Tick-Borne Disease Working Group; and has received remuneration for providing educational seminars for Boehringer Ingelheim (formerly Merial). Dr. Wormser receives research funding from Immunetics, Inc., Rarecyte, Inc., Institute for Systems Biology, and Quidel Corporation; serves on the Board of the American Lyme Disease Foundation; provides and has previously provided expert testimony in malpractice cases; has stock in AbbVie, Inc. and Abbott Laboratories; has received research funding from the CDC, NIH, BioMérieux, Bio-Rad Laboratories, and DiaSorin, Inc; has served as a scientific research advisor for Baxter International and as a Lyme disease advisor and expert for the Missouri Board of Registration for the Healing Arts; has a patent approved (US patent no. 10,669,567 B2) for High Sensitivity Method for Early Lyme Disease Detection; filed 2 patent applications related to early Lyme disease detection (application no: 62/277,252) and Lyme arthritis and post-treatment Lyme disease syndrome (application no: 62/725,745); and has served on the Editorial Boards for *Clinical Infectious Diseases*, *Vector-Borne and Zoonotic Diseases*, and *Ticks and Tick-Borne Diseases*. Dr. Zemel has served as an advisor for Novartis Promotional Speakers Bureau. No other disclosures relevant to this article were reported. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

ACKNOWLEDGMENTS

The expert panel expresses its gratitude for thoughtful reviews of an earlier version to the external reviewers. The panel thanks the IDSA, AAN, and ACR for supporting the guideline development process.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Lantos had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Lantos, Rumbaugh, Bockenstedt, Falck-Ytter, Aguero-Rosenfeld, Auwaerter, Baldwin, Bannuru, Belani, Bowie, Branda, Clifford, DiMario, Halperin, Krause, Lavergne, Liang, Meissner, Nigrovic, Nocton, Osani, Pruitt, Rips, Rosenfeld, Savoy, Sood, Steere, Strle, Sundel, Tsao, Vaysbrot, Wormser, Zemel.

Acquisition of data. Lantos, Rumbaugh, Bockenstedt, Falck-Ytter, Aguero-Rosenfeld, Auwaerter, Baldwin, Bannuru, Belani, Bowie, Branda, Clifford, DiMario, Halperin, Krause, Lavergne, Liang, Meissner, Nigrovic, Nocton, Osani, Pruitt, Rosenfeld, Savoy, Sood, Steere, Strle, Sundel, Tsao, Vaysbrot, Wormser, Zemel.

Analysis and interpretation of data. Lantos, Rumbaugh, Bockenstedt, Falck-Ytter, Aguero-Rosenfeld, Auwaerter, Baldwin, Bannuru, Belani, Bowie, Branda, Clifford, DiMario, Halperin, Krause, Lavergne, Liang, Meissner, Nigrovic, Nocton, Osani, Pruitt, Rips, Rosenfeld, Savoy, Sood, Steere, Strle, Sundel, Tsao, Vaysbrot, Wormser, Zemel.

REFERENCES

1. Guyatt GH, Oxman AD, Vist GE, Kunz R, Falck-Ytter Y, Alonson-Coello P, et al, on behalf of the GRADE Working Group. GRADE: an emerging consensus on rating quality of evidence and strength of recommendations. BMJ 2008;336:924–6.
2. Schunemann H, Brozek J, Guyatt G, Oxman AG. Handbook for grading the quality of evidence and the strength of recommendations using the GRADEapproach. Hamilton (Ontario): GRADEpro; 2015. URL: https://gdt.gradepro.org/app/handbook/handbook.html.
3. Luft BJ, Dattwyler RJ, Johnson RC, Luger SW, Bosler EM, Rahn DW, et al. Azithromycin compared with amoxicillin in the treatment of erythema migrans: a double-blind, randomized, controlled trial. Ann Intern Med 1996;124:785–91.
4. Feder Jr HM, Hoss DM, Zemel L, Telford SR III, Dias F, Wormser GP. Southern tick-associated rash illness (STARI) in the North: STARI following a tick bite in Long Island, New York. Clin Infect Dis 2011;53:e142–6.
5. Wormser GP, Masters E, Nowakowski J, McKenna D, Holmgren D, Ma K, et al. Prospective clinical evaluation of patients from Missouri and New York with erythema migrans-like skin lesions. Clin Infect Dis 2005;41:958–65.
6. Baugh RF, Basura GJ, Ishii LE, Schwartz SR, Drumheller CM, Burkholder R, et al. Clinical practice guideline: Bell's palsy executive summary. Otolaryngol Head Neck Surg 2013;149:656–63.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 21–22
DOI 10.1002/art.41556

American College of Rheumatology
Empowering Rheumatology Professionals

SPECIAL ARTICLE

Winners of the 2020 American College of Rheumatology Annual Image Competition

American College of Rheumatology Image Library Subcommittee

The mission of the American College of Rheumatology Image Library Subcommittee is to provide ACR members, as well as the entire medical community, access to a wide variety of clinical images to help educators effectively present the manifestations of rheumatic diseases. Additionally, the images have been widely used in peer-reviewed publications and textbooks. Since its inception, the ACR's Rheumatology Image Library has become the preeminent collection devoted to rheumatic diseases. The collection is a dynamic one, changing yearly because of submissions from the medical community. The Image Library Subcommittee meets annually to review these new images and awards prizes based on the panel's consensus. Additionally, many nonwinning images are introduced into the Image Library, greatly enhancing the collection. Winners, as well as those images selected for inclusion in the Image Library, are chosen based on image quality and educational value. For the 2020 competition, 23 entries were received, and the subcommittee carefully evaluated each entry.

The 2020 grand prize winner was a series of images showing a lesion on the left cheek of a 32-year-old patient with *Blastomyces dermatitidis* and its improvement after treatment with itraconazole (Figures 1 and 2). The winning submissions, as well as several other outstanding images, will be added to the Image Library.

The Rheumatology Image Library provides the medical community with 24/7 online access to the world's foremost collection of rheumatology images. It features contributions from all over the world and is an invaluable resource for countless physicians and other health care professionals, researchers, and journalists. To view the winning images and many others, visit the Rheumatology Image Library at http://images.rheumatology.org.

The ACR encourages the continued submission of images to its annual Image Competition. Submissions of high-quality images that illustrate rheumatic conditions or are relevant to the practice of rheumatology are welcomed. Visit https://www.rheumatology.org/Annual-Meeting/Program/Image-Compeition for competition

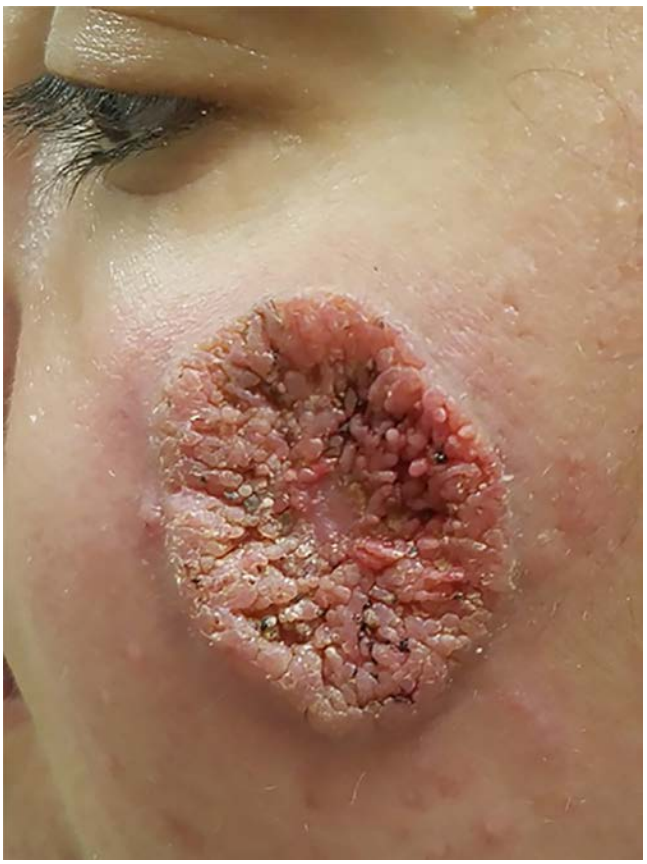


Figure 1. Cutaneus blastomycosis. The patient, a 32-year old woman, presented with a new skin lesion on her left cheek. Four months previously, she had been diagnosed as having Behçet's disease based on pulmonary embolism, inflammatory arthritis, oral ulcers, and a positive pathergy test. She was treated with adalimumab, with full resolution of the arthritis and aphthous ulcers. However, 1 month after therapy was initiated, the skin lesion appeared. The lesion was scraped for fungal culture, which was positive for *Blastomyces dermatitidis*, as confirmed by DNA probe.

Members of the Image Library Subcommittee of the American College of Rheumatology Committee on Education: Christopher E. Collins, MD, Washington, DC (Chair); Senada Arabelovic, DO, Boston, Massachusetts; Sharon Banks, DO, Hershey, Pennsylvania; Elana Bernstein, MD, New York, New York; Michael Jennings, RT, CBDT, New Lebanon, New York; Abhishek Nandan, MD, Richmond, Virginia; Rochella Ostrowski, MD, MS, Maywood, Illinois; Lesley Ann Saketkoo, MD, New Orleans, Louisiana.

Submitted for publication October 2, 2020; accepted in revised form October 6, 2020.

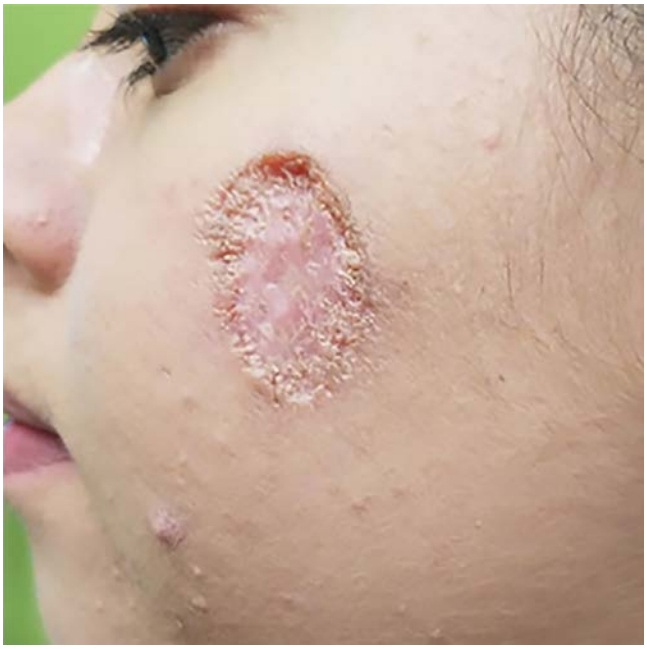


Figure 2. Improvement of the *Blastomyces dermatitidis* lesion in the patient shown in Figure 1, after 4 months of treatment with itraconazole. Submitted by Aleksandra Bukiej, MD, Chicago, IL.

rules and entry/deadline dates. Details about the 2021 Image Competition will be available in spring 2021. If you have any questions regarding the Image Competition, please contact education@rheumatology.org.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 23–35
DOI 10.1002/art.41526

American College of Rheumatology
Empowering Rheumatology Professionals

REVIEW

The Longitudinal Immune Response to Coronavirus Disease 2019: Chasing the Cytokine Storm

Alice S. Chau,[1] Andrew G. Weber,[2] Naomi I. Maria,[3] Sonali Narain,[4] Audrey Liu,[2] Negin Hajizadeh,[4] Prashant Malhotra,[4] Ona Bloom,[5] Galina Marder,[4] and Blanka Kaplan[4]

The clinical progression of the severe acute respiratory syndrome coronavirus 2 infection, coronavirus 2019 (COVID-19), to critical illness is associated with an exaggerated immune response, leading to magnified inflammation termed the "cytokine storm." This response is thought to contribute to the pathogenicity of severe COVID-19. There is an initial weak interferon response and macrophage activation that results in delayed neutrophil recruitment leading to impeded viral clearance. This causes prolonged immune stimulation and the release of proinflammatory cytokines. Elevated levels of inflammatory markers in COVID-19 (e.g., D-dimer, C-reactive protein, lactate dehydrogenase, ferritin, and interleukin-6) are reminiscent of the cytokine storm seen in severe hyperinflammatory macrophage disorders. The dysfunctional immune response in COVID-19 also includes lymphopenia, reduced T cells, reduced natural killer cell maturation, and unmitigated plasmablast proliferation causing aberrant IgG levels. The progression to severe disease is accompanied by endotheliopathy, immunothrombosis, and hypercoagulability. Thus, both parts of the immune system—innate and adaptive—play a significant role in the cytokine storm, multiorgan dysfunction, and coagulopathy. This review highlights the importance of understanding the immunologic mechanisms of COVID-19 as they inform the clinical presentation and suggest potential therapeutic targets.

INTRODUCTION

The first cases of coronavirus disease 2019 (COVID-19) were encountered in December 2019 in Wuhan, China, and the causative agent was identified as severe acute respiratory syndrome coronavirus 2 (SARS–CoV-2) shortly thereafter. With wider testing, it appears that >80% of individuals with COVID-19 may be asymptomatic or have mild disease that does not require hospitalization (1). However, 5–15% of patients progress from mild disease to severe viral pneumonia and hypoxemic respiratory failure, followed by a hyperinflammatory response associated with coagulopathy and multiorgan damage (2) (Figure 1). Of the hospitalized patients, up to 32% require intensive care (3,4), with mortality rates varying between 20% and 26% in the critically ill and between 88% and 97% among those receiving mechanical ventilation (5,6). It has been suggested that mild, moderate, and severe illness represent distinct phenotypes characterized by variable inflammatory marker expression. Clinical progression to critical illness is associated with an unbalanced immune response, which leads to exuberant inflammation termed the "cytokine storm" (7). Understanding the loss of homeostasis in the immunologic response to SARS–CoV-2 will allow for better comprehension of the mechanisms of autoimmune and inflammatory conditions. In

The opinions and assertions contained herein are those of the authors and do not necessarily represent those of the United States Department of Defense.

Dr. Chau's work was supported by The Primary Immune Deficiency Treatment Consortium (grant U54-AI-082973), jointly funded by the National Center for Advancing Translational Sciences and the National Institute of Allergy and Infectious Diseases, NIH. Dr. Maria's work was supported by The Lupus Research Alliance (grant LRA-540716). Dr. Bloom's work was supported by grants from the United States Department of Defense (grant W81XWH-15-1-0614) and from the New York State Spinal Cord Injury Research Board (grant DOH01-ISSCI6-2016-00018).

[1]Alice S. Chau, MD, MSE: University of Washington Center for Immunity and Immunotherapies and Seattle Children's Research Institute, Seattle, Washington; [2]Andrew G. Weber, MD, Audrey Liu, MD: Northwell Health, Manhasset, New York; [3]Naomi I. Maria, PhD: Feinstein Institutes for Medical Research, Manhasset, New York, and Red Cross Blood Bank Foundation Curaçao, Willemstad, Curaçao; [4]Sonali Narain, MD, MPH, Negin Hajizadeh, MD, MPH, Prashant Malhotra, MD, Galina Marder, MD, Blanka Kaplan, MD: Northwell Health, Manhasset, New York, and Donald and Barbara Zucker School of Medicine at Hofstra/Northwell, Hempstead, New York; [5]Ona Bloom, PhD, Northwell Health and Feinstein Institutes for Medical Research, Manhasset, New York, and Donald and Barbara Zucker School of Medicine at Hofstra/Northwell, Hempstead, New York.

No potential conflicts of interest relevant to this article were reported.

Address correspondence to Alice S. Chau, MD, MSE, Seattle Children's Research Institute, Jack MacDonald Building, 6th Floor, 1900 9th Avenue, Seattle, WA 98101. Email: achau22@uw.edu.

Submitted for publication June 9, 2020; accepted in revised form September 10, 2020.

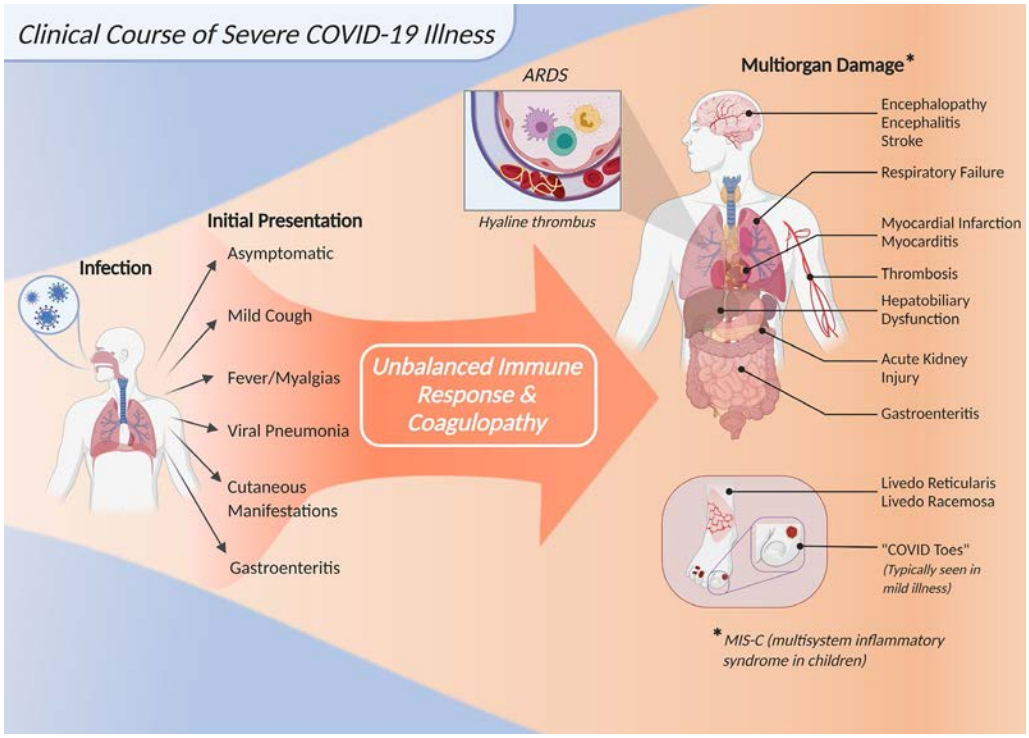


Figure 1. Clinical course of severe coronavirus disease 2019 (COVID-19). Clinical presentations range from asymptomatic to severe COVID-19 illness, which includes major organ damage as the end product of an unbalanced immune response. Dysfunctional immune responses lead to uncontrolled inflammation, which in turn leads to hypercoagulability, thrombosis, and organ damage. Multiple skin lesions and vascular manifestations of COVID-19 have been observed, such as livedo reticularis and racemosa, targetoid erythema, and urticarial, vesicular, morbilliform, and dengue-like rashes. Some patients present with ischemic digits (e.g., "COVID toe"). More recently, multisystem inflammatory syndrome in children has been described. ARDS = acute respiratory distress syndrome.

this review, we focus on immunologic and clinical changes in COVID-19 and describe potential therapeutic targets.

Viral immune response

Viral sensing: extracellular and intracellular. A variety of cellular receptors are involved in sensing viruses. Membrane-associated receptors, such as Toll-like receptors (TLRs), respond to extracellular pathogen components. Intracellular pathogen components are sensed by cytosolic pattern-recognition receptors (PRRs), such as nucleotide-binding oligomerization domain–like receptors, retinoic acid–inducible gene 1–like receptors, and endosomal TLRs. Damage-associated molecular patterns (DAMPs) are breakdown products of injured or dying cells. Pattern-associated molecular patterns (PAMPs) include viral components, such as single-stranded RNA (ssRNA) or viral capsid elements. DAMPs and PAMPs activate the innate immune system by binding to PRRs (8).

Innate immune response. *Macrophage and plasmacytoid dendritic cell response: innate immune response.* Resident tissue macrophages are the early antiviral responders of innate immunity and are activated by PRRs. Plasmacytoid dendritic cells (pDCs) then migrate from nearby lymphoid tissues to the site of infection to activate cells involved in innate and adaptive immunity. Macrophages attract and activate lymphocytes (Figure 2A). Pathogen recognition via PRRs activates intracellular cascades within pDCs that produce cytokines called type I interferons (IFNs) (e.g., IFNα, IFNβ), while macrophages produce type I and type III IFNs (e.g., IFNλ) (8).

Natural killer cells and IFNs. IFNs are integral to the antiviral effect of innate immune cells and nearby epithelial cells. IFNs mediate the recruitment of neutrophils, natural killer (NK) cells, and the adaptive immune cells, naive CD8+ T cells. NK cells recognize infected host tissue and induce necroptosis, playing a central role in host viral defense. Their arrival attenuates viral replication while awaiting the slower arrival and formation of CD8+ cytotoxic T lymphocytes (CTLs), which provide a more targeted and effective attack (8). In severe viral infections, interleukin-6 (IL-6) has been shown to inhibit NK cell cytotoxicity (9,10) (Figure 2A).

IFNs induce the transcription of >100 IFN-stimulated genes. These genes enhance the antiviral state in the host and contribute to a positive feedback loop. IFN pathways facilitate macrophages' secretion of additional proinflammatory (IL-1β, IL-6, IL-8, IL-12, and tumor necrosis factor [TNF]) and antiinflammatory (IL-10) cytokines and chemokines—attracting and activating neutrophils,

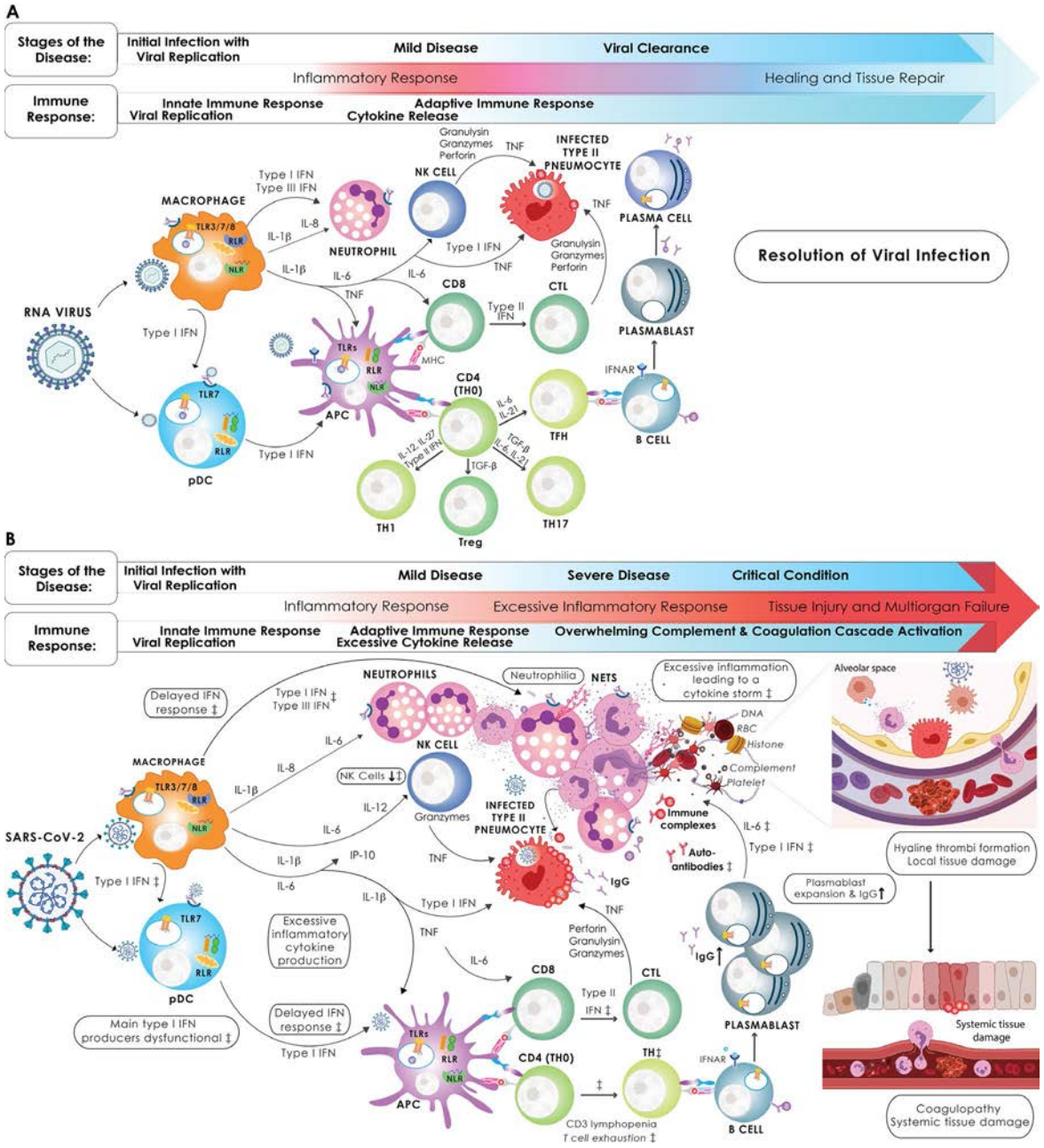


Figure 2. Longitudinal immune response to viruses and to severe acute respiratory syndrome coronavirus 2 (SARS–CoV-2). **A**, Typical viral immune response. The innate immune cells are activated, releasing cytokines. Nonprogrammed cell death of infected tissue activates antigen-presenting cells (APCs), which trigger T cells to differentiate from naive CD8+ and CD4+ T cells into mature, active forms. Follicular helper T (Tfh) cells interact with B cells to become antibody-secreting cells. Together, these coordinated responses typically result in resolution of viral infections. **B**, Unbalanced and disrupted viral immune response in coronavirus disease 2019. A delayed interferon (IFN) release results in an increased peak of cytokines. Natural killer (NK) cell numbers and function are decreased. Activated T cells lead to T cell derangements. Tfh cells activate naive B cells. Neutrophil extracellular traps (NETs) interact with platelets and complement. Immune complexes form, and hyaline thrombi collect in microvasculature. ‡ Limited data and under investigation. TLR = Toll-like receptor; RLR = retinoic acid–inducible gene 1–like receptor; NLR = nucleotide-binding oligomerization domain–like receptor; IL-1β = interleukin-1β; pDC = plasmacytoid dendritic cell; CTL = cytotoxic T lymphocyte; TGFβ = transforming growth factor β; TNF = tumor necrosis factor; MHC = major histocompatibility complex; IFNAR = IFN α/β/ω receptor; IP-10 = IFNγ-induced 10-kd protein; RBC = red blood cell. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41526/abstract.

DCs, and lymphocytes. IL-1β contributes to neutrophil antimicrobial activity (11), while IL-6 induces T cell differentiation, fibroblast activation, angiogenesis, collagen production, and B cell activation and maturation (12). The balance between pro- and antiinflammatory cytokines regulates the inflammatory cascade and maintains immunologic homeostasis (8).

The proinflammatory response and biomarkers. In inflammatory conditions, including sepsis and other autoinflammatory conditions, levels of IL-1β, IL-6, and TNF increase, inducing a dominant proinflammatory response and immune aberrancy (3,13). Acute-phase proteins, such as C-reactive protein (CRP, a type of PRR), ferritin, and fibrinogen, are surrogate markers of up-regulated inflammatory cytokines. In response, macrophages, mesenchymal cells, and hepatocytes secrete ferritin, which decreases the availability of iron (8). Lactate dehydrogenase (LDH) is released by infected cells via IL-1β– and TNF-induced nonprogrammed cell death (14). D-dimer is a product of degraded crosslinked fibrin and is a marker of coagulopathy and inflammation (15).

Adaptive immune response. *CTLs: CD8+ recruitment and differentiation.* Initial steps in the adaptive immune system's antiviral response include the recruitment of naive CD8+ T cells. These cells are attracted by IL-6 (12) and are activated to become CTLs both by viral infection and via cross-presentation by major histocompatibility complex (MHC) class I on DCs (Figure 2A). CTLs release TNF, cytolytic enzymes, and proteins to induce apoptosis in infected cells. CTLs also release IFNγ (a type II IFN) to augment the antiviral response. Simultaneously, naive CD4+ T cells become activated via interaction with MHC class II–antigen complexes presented by DCs. These helper CD4+ T cells interact with naive CD8+ T cells to activate additional CD8+ T cells and to support CTLs and DCs (8).

CD4+ T lymphocytes: maintaining immunologic homeostasis. T helper cells/CD4+ T cells can increase or decrease inflammation depending on the lineage, maintaining immunologic balance. JAKs and the STAT family of transcriptional activators play a role in cytokine signaling and determine T cell differentiation into T helper cells. The predominant form of T helper cells in viral infections is Th1, which produces additional IFNγ. Other CD4+ T cells and their signature cytokines, such as Treg cells (IL-10), Th17 cells (IL-17), and Th2 cells (IL-4), counteract Th1 cells, thereby maintaining homeostasis (Figure 2A). IL-10, an anti-inflammatory cytokine, inhibits IL-12–dependent IFNγ production and MHC class II expression, thereby decreasing T cell activation. IL-17 increases the production of antimicrobial substances, induces neutrophil inflammation, and inhibits Th1 differentiation. IL-4 decreases Th1 and macrophage differentiation, interferes with the effects of IFNγ, and increases B cell activation and differentiation into plasma cells (8).

Antibody formation and establishing immunologic memory. B cells mature into long-lived plasma cells within lymphoid tissue called germinal centers. There, naive memory B cells are activated with the assistance of follicular DCs, macrophages, and special CD4+ T-cells, called follicular helper T (Tfh) cells (Figure 2A). B cells, activated by antigens, interact with Tfh cells and mature into IgM-expressing plasmablasts, eventually becoming class-switched IgG plasmablasts. Plasmablasts proliferate rapidly and some become long-lived plasma cells, allowing for the durability of humoral memory. Other plasmablasts become effector cells that secrete antibodies that opsonize and neutralize pathogens (8).

Indeed, the viral immune response requires a fastidious balance to maintain homeostasis between the adaptive and innate immune systems, as well as between pro- and antiinflammatory effectors. Loss of such harmony may result in the hyperinflammatory state that is seen with COVID-19.

Hyperinflammation in SARS–CoV-2

Epidemiology. Coronaviridae (CoV) are a large family of positive single-stranded RNA viruses notable for 4 human strains (229E, NL63, OC43, and HKU1) that cause upper respiratory tract infections. However, SARS-CoV, Middle East respiratory syndrome (MERS)–CoV, and the newly identified SARS–CoV-2 also cause lower respiratory tract infections (16,17). The latter 3 CoV viruses are zoonoses, likely originating from bats with intermediary hosts of various species, such as the camel with MERS-CoV. The intermediary host of SARS–CoV-2 is unknown. There is high homology among CoV genomes, with SARS–CoV-2 sharing ~79% of its genomic sequence with SARS-CoV (18). All 3 viruses that cause lower respiratory tract infections incite an exuberant immune response, leading to systemic inflammation and resultant end-organ damage (7,19,20).

SARS–CoV-2 is transmitted between persons via droplets or aerosolization, entering epithelial cells via the pathognomonic spike ("corona") protein interacting with the angiotensin-converting enzyme 2 (ACE2) receptor (21). Upon cell entry, the time from infection to symptoms is 1–3 days for typical respiratory viruses (22). However, in SARS–CoV-2, the median incubation is 5–7 days; the majority of patients become symptomatic within 13 days of infection (23). Following symptom onset, up to 32% of patients develop severe or critical disease ~9 days later (3), which leads to hypoxemic or mixed respiratory failure due to severe lung injury (Figure 1). Higher viral loads and prolonged infection correlate with increased severity; however, the ultimate cause of severe COVID-19 is unclear (24).

The cytokine storm. Markedly elevated levels of inflammatory markers, including D-dimer, CRP, LDH, ferritin, and IL-6, correspond with severe illness and mortality risk (3,4). Some studies have shown that high IL-1β levels correlate with increased morbidity and mortality, with a peak prior to respiratory decompensation (3,13,25) (Figure 2B). This constellation of markers has come to represent the cytokine storm (7). A similar phenomenon has been observed in severe hyperinflammatory macrophage disorders, such as familial hemophagocytic lymphohistiocytosis, macrophage activation syndrome, and cytokine release syndrome caused by chimeric antigen receptor T cell therapy.

Delayed release of proinflammatory cytokines. In SARS-CoV and SARS–CoV-2 infections, the delayed but pronounced release of proinflammatory cytokines causing systemic inflammation is poorly understood. In SARS-CoV, the type I IFN peak is delayed and associated with elevated lung cytokine and chemokine levels, vascular leakage, and impaired T cell response that are linked to poor outcomes (26,27). When mice with SARS-CoV were administered type I IFN early in the disease course, there was no immunopathology, while later administration promoted lung pathology, underscoring the importance of timing and immune homeostasis in viral infections (27). Similarly, in SARS–CoV-2, peak IFN levels are also delayed but reach a lower peak than in SARS-CoV, especially in severely affected patients (Figure 2B). Correspondingly, viral clearance is delayed and the expression of proinflammatory cytokines is increased (28).

Delayed neutrophil recruitment and activation. With a weaker initial IFN response, neutrophil recruitment may be delayed. Neutrophils release leukotrienes, reactive oxygen species, and neutrophil extracellular traps (NETs) to fight infections. NETs are web-like structures composed of DNA, associated proteins, and microbial enzymes, and have been implicated in the initiation and propagation of inflammation and thrombosis in COVID-19 (29) (Figure 2B). However, these substances also damage tissue and likely contribute to the pathogenesis of lung injury and multiorgan dysfunction syndrome. The timing of neutrophil recruitment may play a role in the severe COVID-19 phenotypes. Lower IFN levels lead to slower macrophage activation and reduced initial neutrophil recruitment. This diminished initial response results in higher viral loads, leading to prolonged immune stimulation. Potentially, delayed neutrophilic inflammation may contribute to the pathogenesis of the cytokine storm.

Fewer NK cells. Peripheral NK cell numbers and function are detrimentally affected in SARS–CoV-2 infection (Figure 2B). Similar to other viral infections, NK cells in patients with severe COVID-19 have been found to have decreased functional markers (e.g., CD107a) and cytokine expression (e.g., TNF) (30). Fewer mature NK cells have been found in patients with COVID-19. Their ability to communicate with monocytes and exert their cytolytic functions upon SARS–CoV-2–infected cells is reduced, likely from lower granzyme and perforin expression that is in part due to inhibition by IL-6 (10). However, NK cells in the lung, unlike in other tissues, do not express the ACE2 receptor, so it is unclear how SARS–CoV-2 affects lung NK cell function (31).

T cell activation delay. A poorly controlled viral infection, evidenced by the high viral load and delayed cytokine signatures seen in patients with severe COVID-19, implies that T cell activation is also likely delayed. It typically takes ~7–15 days for T cells to respond to a novel antigen (8). In patients with COVID-19, SARS–CoV-2–specific T cells appear in peripheral blood within 2 weeks of symptom onset (31). Collectively, given an ~1-week delay between infection and symptom onset, T cell engagement takes ~3 weeks in COVID-19, which is delayed compared to typical viruses. This delay can be deleterious, considering the integral role of T cells in the positive feedback machinery of the immune response. Together, the decreased type I IFN signature and delayed T cell effector mechanisms may account for the timing of the cytokine storm.

Aberrant Th17 response. Th17 cells may be the primary CD4+ T cell perpetrator in the cytokine storm. Th17 cells predominantly express the proinflammatory cytokine IL-17, which promotes granulopoiesis and neutrophil recruitment, as well as the expression of IL-1β, IL-6, TNF, chemokines, and matrix metalloproteinases, which contributes to tissue damage (32). Aberrant Th17 cell response is also implicated in cytokine dysregulation in autoimmune diseases.

Neutrophil:lymphocyte ratio and lymphopenia. Lymphopenia and relative neutrophilia have been observed early in the clinical course of disease, with an increased neutrophil:lymphocyte ratio in individuals with severe COVID-19. A high neutrophil:lymphocyte ratio is an independent risk factor for mortality (33). Lymphopenia is largely due to decreased CD3+ T cells (Figure 2B). Increased mortality has been identified in patients with CD3+ T cell counts of <800 cells/μl, CD4+ T cell counts of <300 cells/μl, and CD8+ T cell counts of <400 cells/μl (34). Initial autopsies of lung tissue from patients who died shortly after the onset of the cytokine storm and acute respiratory distress syndrome (ARDS) demonstrated neutrophil-predominant infiltrates. Atrophy and necrosis of lymphoid organs have also been noted, suggesting that tissue sequestration of T cells was not the etiology of lymphopenia (13). However, a recent case series comparing pulmonary histopathology in patients who died due to either COVID-19 or influenza H1N1 demonstrated lymphocyte-predominant infiltration (35). Therefore, T cell lymphopenia may be the result of tissue sequestration, direct viral infection of this cell type (36), delayed IFN response, or T cell exhaustion due to the intensity and duration of the disease (34). The inability of CD4+ and CD8+ T cells to sustain long-term activation results in their exhaustion, which is akin to that seen in high-grade chronic viral infections (37).

Exaggerated humoral response. Antibodies generated against SARS–CoV-2 have been found to be specific for the internal nucleoprotein (NP) and surface protein receptor binding domain (RBD). In general, patients develop antibodies against RBD prior to those against NP (38). Seroconversion occurs between 4 and 40 days following symptom onset. In another study, most patients were seropositive by day 10 after clinical presentation, but some patients had an increase in IgG titer prior to IgM titer (38). This is surprising and atypical, as most infections are known to induce IgM prior to IgG. Furthermore, in the study by Tan et al, antibody levels were shown to be 10 times higher in patients with severe disease compared to patients with mild and moderate disease (39). None of the aforementioned studies showed that the severity of illness influenced the amount of time before patients became

Table 1. Therapies studied for COVID-19*

Therapy type, drug name	Mechanism of action	No. of clinical trials
rhACE2	Generates Ang 1–7 from Ang II, preventing Ang II–induced myocardial injury; forms fusion protein of rhACE2 with an IgG1 Fc fragment; potently neutralized SARS–CoV-2 in vitro	1
NETs/DNA accumulation		
Dornase alfa	Degrades extracellular DNA	7
Antivirals		
Chloroquine/HCQ	Interferes with the cellular receptor ACE2 binding with spike protein; structurally changes the gp120 envelope protein-reducing reactivity and pathogenicity of virions; inhibits TNF, IL-6, and IL-1 production, thereby promoting higher Th2:Th1 ratio	198
Liponivir/ritonavir	Protease inhibitor combination	30
IFNβ1a	Type I IFN, up-regulates macrophages; other activities (see Figure 2)	2
Ribavirin	Guanosine analog that halts viral RNA synthesis and viral mRNA capping (nucleoside inhibitor)	3
Remdesivir	Mutagenic nucleoside that targets the RNA-dependent RNA polymerase, preventing replication of the viral RNA genome	10
Immunosuppressants		
Glucocorticoids	Broad immunosuppressive	30
MTX	Dihydrofolate reductase inhibitor	1
B and T cell inhibitor		
Duvelisib	PI3Kγ and PI3Kδ inhibitor	1
Anti–IL-6R antibodies		
Tocilizumab	Anti–IL-6R antibody	36
Sarilumab	Anti–IL-6R antibody	11
IL-1R antagonists		
Anakinra	IL-1R antagonist	13
Canakinumab	Anti–IL-1 antibody	3
Cytokine modulators		
Colchicine	Microtubule inhibitor; decreases IL-1β and TNF release	15
Emapalumab	Anti-IFNγ antibody	1
Infliximab	Anti-TNF antibody	1
GM-CSF modulators		
Gimsilumab	Anti–GM-CSF antibody	1
Lenzilumab	Anti–GM-CSF antibody	
Mavrilimumab	Anti–GM-CSF receptor α antibody	3
Otilimab	Anti–GM-CSF antibody	1
TJM2	Anti–GM-CSF antibody	1
Complement C5		
Eculizumab	Anti-C5 antibody, disallowing its cleavage and preventing formation of MAC	3
IFX-1	Anti-C5a antibody that does not impact C5b function, allowing formation of MAC	1
Anti-CD20 antibodies		
Ocrelizumab	Anti-CD20 antibody	0
Rituximab	Anti-CD20 antibody	0
BTK inhibitors		
Acalabrutinib	Inhibitor of BTK	2
Ibrutinib	Irreversible inhibitor of BTK	2
Lymphocyte inhibitor		
Fingolimod	Activates lymphocyte S1P1 via high-affinity receptor binding with subsequent S1P1 down-regulation, preventing lymphocyte egress from lymphoid tissues	1
mTOR (T and B cell inhibitor)		
Sirolimus	Binds to an immunophilin, FKBP12, generating a complex that inhibits the activation of mTOR	5
T cell inhibitors		
Tacrolimus	Binds to an immunophilin, FKBP12, generating a complex that inhibits calcineurin phosphatase	2
Cyclosporine	Inhibitor of IL-2 production	3
Chemokine receptor antagonists		
Leronlimab	Anti–CCR5 receptor antibody	2
Maraviroc	CCR5 receptor antagonist	3

(Continued)

Table 1. (*Cont'd*)

Therapy type, drug name	Mechanism of action	No. of clinical trials
JAK inhibitors		
Fedratinib	JAK2-selective inhibitor	0
Baricitinib	JAK1/2 inhibitor	12
Ruxolitinib	JAK1/2 inhibitor	15

* From https://clinicaltrials.gov. COVID-19 = coronavirus disease 2019; rhACE2 = recombinant human angiotensin-converting enzyme 2; Ang 1–7 = angiotensin 1–7; SARS–CoV-2 = severe acute respiratory syndrome coronavirus 2; NETs = neutrophil extracellular traps; HCQ = hydroxychloroquine; TNF = tumor necrosis factor; IL-6 = interleukin-6; IFNβ1a = interferon-β1a; MTX = methotrexate; PI3Kγ = phosphatidylinositol 3-kinase γ; anti–IL-6R = anti–IL-6 receptor; GM-CSF = granulocyte–macrophage colony-stimulating factor; MAC = membrane attack complex; BTK = Bruton's tyrosine kinase; S1P1 = sphingosine 1-phosphate receptor 1; mTOR = mechanistic target of rapamycin; FKBP12 = FK506 binding protein 12.

seropositive. Taken together, these findings suggest that an exaggerated humoral response is associated with a hyperinflammatory response (Figure 2B).

Critical illness in COVID-19: acute respiratory failure and immunothrombosis. As disease progresses in critically ill patients, their respiratory injury follows the natural history of ARDS, moving through 3 phases: exudative, proliferative, and fibrotic. The exudative phase of ARDS has been noted to be NET-rich in patients with COVID-19 (29). Platelet aggregates with NETs have been well documented in sepsis and ARDS and play a role in immunothrombosis in COVID-19 (29) (Figure 2B). Complement factors induce neutrophils to form NETs, which in turn activate the coagulation cascade and recruit platelets. Complement anaphylatoxin (C5a) and other molecules produced during activation of the complement cascade are major contributors to hypercoagulability (40). Disordered interactions between monocytes and macrophages, activated endothelial cells expressing tissue factor, and impaired fibrinolysis also contribute to coagulopathy (35,41).

Thrombotic events occur with greater frequency in critically ill patients with COVID-19. Studies have shown a cumulative incidence of both venous and arterial thrombosis of up to 31% (41). Thrombosis results in multiorgan damage and contributes to mortality. Evidence of thrombotic microangiopathy on autopsies of some patients who had COVID-19 suggests that immunothrombosis plays a critical role in this disease (35). Interestingly, preliminary reports demonstrate the presence of newly identified antiphospholipid antibodies and lupus anticoagulant in some patients (13,42).

Multisystem inflammatory syndrome in children. There are reports of multisystem inflammatory syndrome in children (MIS-C), manifesting as a late complication of SARS–CoV-2 infection (Figure 1). In contrast to adults, children present with increased frequency of gastrointestinal symptoms (43) and features of Kawasaki disease, including cardiogenic shock and dilated coronary arteries. Laboratory evaluations revealed significantly elevated levels of inflammatory markers with positive SARS–CoV-2 serology and, typically, a negative polymerase chain reaction result. Reports indicate that the onset of MIS-C may be delayed, potentially by months, following COVID-19 infection (44). There have also been reports of myocarditis in adult patients with severe COVID-19 that occur up to 26 days following symptom onset (45). It is unclear whether these instances of myocarditis occur due to viral infection and the direct damage of myocardial tissue, host response, or a combination of both.

In summary, every part of the immune system plays a significant role in the cytokine storm, tissue damage, and ensuing coagulopathy of severe COVID-19.

Demographics and comorbidities

Groups that have a high risk of severe COVID-19 have been well described and include older Black men and patients with hypertension, coronary artery disease, congestive heart failure, diabetes mellitus, chronic pulmonary diseases (e.g., chronic obstructive pulmonary disease, smoking history), and obesity (3,4,6,46). However, the etiology that explains why some comorbid states are associated with high risk is unclear. Hypothetically, cytokine storm will have more severe consequences in people with unopposed proinflammatory conditions, such as in obesity, which is associated with increased IL-6 and CRP levels (47). Paradoxically, some populations that canonically have been at high risk for other types of severe infections have not been documented to have a high incidence of overwhelming host response to SARS–CoV-2. Here, we discuss other possible risk factors that may assist in the understanding of the pathophysiology of COVID-19 and may influence the strategic implementation of potential therapeutics.

Atopic asthma. Despite initial concerns, patients with asthma have not clearly been documented as having a higher risk of severe disease. This is surprising, as viral respiratory infections are the most frequent cause of asthma exacerbation. In an effort to determine a molecular mechanism, Jackson et al analyzed the brush samples of nasal and lower respiratory tract epithelium and identified low *ACE2* expression in patients with respiratory allergies and atopic asthma. The authors posited that the reduced *ACE2* expression may be responsible for the decreased COVID-19 severity in this population (48). Recombinant human ACE2 is being examined as a potential therapeutic (Table 1).

Immunosuppressive states. It is not yet clear which individuals with primary or acquired immunodeficiency are more susceptible to SARS–CoV-2 infection. Early reports have demonstrated that some forms of immunodeficiency or immunosuppression may protect against the cytokine storm and progression to severe disease (49). Three retrospective reports from China documented an increased risk for COVID-19 critical illness and case fatality rates in patients with a history of malignancy (1,50,51). Additional risk factors included older age (50) and having received antitumor therapies within 14 days of hospital admission (51). Similarly, the solid organ transplant literature has shown poor outcomes in kidney, liver, and heart transplant patients. Despite decreased doses of immunosuppressive medications, posttransplant patients have pronounced T cell lymphopenia and higher case fatality rates earlier in the COVID-19 course (52–54).

Patients with autoimmune disease who are receiving immunosuppressive therapy, including steroids, nonbiologic and biologic therapies, and disease-modifying antirheumatic modalities, appear not to have an increased risk of SARS–CoV-2 infection compared to the general population (55). Likewise, patients with inflammatory bowel disease who are being treated with immunosuppressants may not have an increased risk of severe infection (56). However, systemic lupus erythematosus patients treated with hydroxychloroquine (HCQ) monotherapy appeared to have increased severity in a small case series (57). This underscores the notion that the type of immunosuppression in the setting of underlying immune system dysregulation may affect disease course in COVID-19. It is yet unknown whether people with untreated rheumatic or other autoimmune diseases are at higher risk for developing a cytokine storm.

Therapies

Antiviral therapies. Based on in vitro data demonstrating the efficacy of chloroquine and HCQ to interfere with coronavirus entry, replication, and antigen processing, these aminoquinolines were widely used in the early days of the pandemic, despite minimal evidence-based data from large clinical trials (Table 1 and Figure 3). As of July 2020, results from 6 randomized controlled trials and 26 nonrandomized studies evaluating >29,000 patients in order to assess the role that HCQ may play in the treatment and prevention of COVID-19 have been published (58). The findings have been conflicting in nature. In most studies, there has been no difference between all-cause mortality, invasive mechanical ventilation requirement, disease progression, symptom resolution, or upper airway viral clearance with HCQ, compared to conventional therapy. The reports of potential cardiovascular adverse effects of HCQ have also been conflicting (59). There is currently no conclusive evidence to recommend or discourage the use of HCQ as prophylaxis (59,60). There are multiple ongoing trials evaluating the use of HCQ for both the treatment and the prevention of COVID-19.

Other antivirals have been repurposed, such as lopinavir/ritonavir, with lopinavir demonstrating in vitro activity against

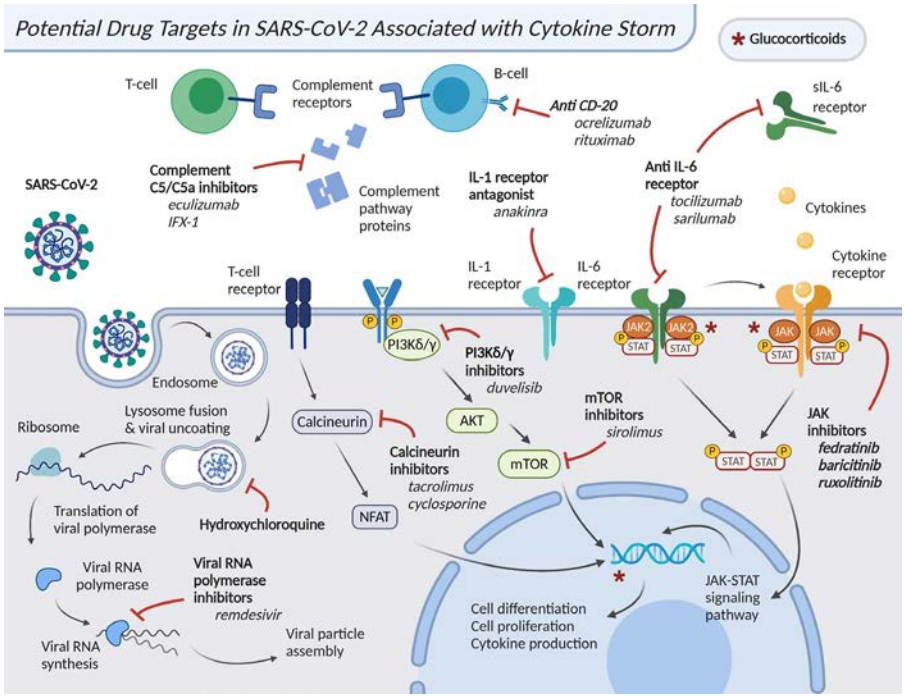


Figure 3. Drug targets in severe acute respiratory syndrome coronavirus 2 (SARS–CoV-2) associated with cytokine storm. Drugs that target both intracellular and extracellular events, being assessed around the world in efforts to prevent or reduce the cytokine storm, are depicted. IL-1 = interleukin-1; PI3Kδ/γ = phosphatidylinositol 3-kinase δ/γ; mTOR = mechanistic target of sirolimus; sIL-6 = soluble IL-6. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41526/abstract.

SARS-CoV but poor efficacy against SARS–CoV-2 (61). One study demonstrated a decrease in days in which SARS–CoV-2 was still detectable in patients with mild-to-moderate COVID-19 when lopinavir/ritonavir was used in combination with ribavirin, a guanosine analog, and IFNβ1a (62). This beneficial outcome is suspected to be due to IFNβ1a, with possible positive influence from ribavirin. Thus far, the most successful antiviral therapy has been remdesivir, an adenosine analog, which was shown in a preliminary report to decrease recovery time by 31% in patients with COVID-19 (63).

Targeting NETs. In the absence of robust SARS–CoV-2 agents, the therapeutic focus has been on mitigating the hyperinflammatory response in an attempt to decrease disease severity using clinical features, laboratory findings, and correlations with other diseases. NETs are an increasing area of focus in COVID-19 due to the neutrophil-predominant sputum observed in severe disease as well as the prominent role they play in inflammation and coagulopathy. The cystic fibrosis medication dornase alfa is a recombinant human DNase that has been delivered via nebulization as a mucolytic and to target NETs in patients with COVID-19 receiving invasive mechanical ventilation, with encouraging results (64).

Broad immunosuppressive therapies. Glucocorticoids have been used in moderate doses in attempts to temper the hyperinflammatory state, especially in severe lung injury, with mixed results (4,65). More recently, glucocorticoids and intravenous IgG have been used to treat myocarditis (45) and MIS-C (44). Studies have suggested that the use of steroids to treat patients with COVID-19 may result in improved mortality, reduced intubation, and decreased inflammatory marker levels (66,67). The RECOVERY Collaborative Group demonstrated that oral or intravenous dexamethasone (6 mg), administered once daily for up to 10 days, reduced 28-day mortality in patients with COVID-19 who required either invasive mechanical ventilation (29.3% versus 41.4%; rate ratio (RR) 0.64 [95% confidence interval (95% CI) 0.51–0.81]) or supplemental oxygen alone (23.3% versus 26.2%; RR 0.82 [95% CI 0.72–0.94]). There was no mortality benefit in patients with COVID-19 who did not require oxygen therapy, with a trend toward harm (17.8% versus 14.0%; RR 1.19 [95% CI 0.91–1.55]) (68).

Methotrexate also acts broadly upon immune cells by inhibiting enzymes required for nucleotide synthesis and is being investigated in severe COVID-19. Duvelisib inhibits phosphatidylinositol 3-kinase δ/γ, which mediates extracellular signals that govern development, activation, and mobilization in many innate and adaptive cells.

Cytokines. Given the high IL-6 signature in the cytokine storm, anti–IL-6 receptor therapy with tocilizumab has been utilized with promising results (69), and sarilumab is currently undergoing randomized clinical trials. IL-1 receptor antagonism has been used to treat hyperinflammation in hemophagocytic lymphohistiocytosis (70) and sepsis with hyperferritinemia (71). A small retrospective study of high-dose anakinra, an IL-1 receptor antagonist, demonstrated improved outcomes and survival benefits for patients with COVID-19 (72). Subsequently, Huet et al reported findings of a retrospective analysis of 52 patients who received low-dose anakinra, with some patients also receiving methylprednisolone, with improved survival (73).

Ucciferri et al performed a retrospective analysis of 10 patients with COVID-19 who had hyperinflammation and respiratory failure and who received canakinumab, a human monoclonal antibody against IL-1β. Following administration of the treatment, there was a reduction in CRP levels and oxygen requirements. At 45 days after hospitalization, all patients were alive, none were still receiving oxygen supplementation, and none had developed neutropenia or bacterial sepsis (74). Given the strong role of IFNγ in the cytokine storm, emapalumab is being studied in comparison to anakinra in preventing the need for invasive mechanical ventilation and extracorporeal membrane oxygenation.

TNF is now being discussed as a potential target, given its prominent inflammatory role in COVID-19 cytokine storm (3,34) and the finding that patients receiving anti-TNF therapies have not demonstrated increased incidence of severe COVID-19 (56). Colchicine is a microtubule inhibitor that decreases IL-1β and TNF release and is used to treat familial Mediterranean fever and gout flares (75).

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is implicated in myelopoiesis, hyperactive inflammation, and maintenance of pulmonary protective mechanisms. Thus, there are biologically plausible reasons to use bidirectional modulation therapy. Currently available anti–GM-CSF therapeutics include otilimab, gimsilumab, lenzilumab, TJM2, mavrilimumab, and namilumab (76). Mavrilimumab, an anti–GM-CSF receptor α monoclonal antibody, has shown improved clinical outcomes in a prospective study of non–mechanically ventilated patients with severe COVID-19 and systemic hyperinflammation (77). Further studies are needed to confirm this finding.

Complement C5. The complement anaphylatoxin C5a plays a role not only in coagulation but also as a neutrophil chemoattractant and activator, enhancing neutrophil longevity and increasing vascular permeability (40). C5a and C5b are cleavage products of C5, with the latter being integral to the establishment of the membrane attack complex that allows pathogen cell lysis. Eculizumab is a humanized monoclonal antibody targeted against complement C5, disallowing its cleavage. In murine models of MERS-CoV, C5a levels are increased, and treatment of mice with a C5 inhibitor reduced lung damage. Eculizumab has been suggested to have potential in treating the endotheliopathy associated with COVID-19. Promising early data from China also support the use of the novel IFX-1, a specific anti-C5a monoclonal antibody that does not impact C5b function.

B cell and T cell modulation. In a case report, a multiple sclerosis patient with COVID-19 who was treated with B cell–depleting ocrelizumab (an anti-CD20 monoclonal antibody) had mild disease without IL-6 elevation, raising the question of whether peripheral B cells might release IL-6 (78). Moreover, rituximab is widely used for the treatment of rheumatoid arthritis, antineutrophil cytoplasmic autoantibody vasculitis, refractory lupus, and other rheumatic conditions, but its effect on the severity of COVID-19 is unclear at this time (79). Preliminary observations suggest that B cell depletion following rituximab treatment may have the ability to mitigate the severity of the cytokine storm and need for mechanical ventilation in patients with COVID-19 (79).

Two case series describing patients with X-linked agammaglobulinemia (XLA) further support the idea that B cells contribute to the cytokine storm. XLA is caused by the loss of function of Bruton's tyrosine kinase (BTK), an enzyme essential to B cell maturation and activation. Patients with XLA appear to be at a lower risk of developing a cytokine storm compared to patients with common variable immune deficiency who have residual B cell function (80). Moreover, the BTK deficiency was recently found to impair T cell activation (81). Patients who were treated for a hematologic malignancy with the BTK inhibitor ibrutinib and then contracted COVID-19 demonstrated milder disease (82). Likewise, 19 patients with severe COVID-19 who were given another BTK inhibitor, acalabrutinib, had favorable results (83).

By broadly targeting both B cells and T cells, fingolimod prevents lymphocyte egress from the bone marrow. Likewise, sirolimus is being studied, as it blocks signal transduction from mechanistic target of rapamycin, downstream of cytokine receptors to disallow cellular activation. Furthermore, sirolimus has been suggested to reduce the serum viral load of MERS-CoV in mice by preventing the virus from usurping host cell machinery for replication—making this drug enticing for treatment of patients with COVID-19 (84). With the goal of preventing or ameliorating the cytokine storm, tacrolimus and cyclosporine are being considered as therapies due to their focused inhibition of an enzyme, the cytoplasmic protein calcineurin, which is required for T cell activity.

Therapies against the chemokine receptor CCR5 have been administered for compassionate use in critically ill patients. After administration, observations included receptor occupancy on T cell and macrophages and reductions in IL-6 levels, SARS–CoV-2 viremia, and CD4:CD8 ratios (85). In addition, due to the potential role of Th17 cells in the cytokine storm, JAK2, an intracellular regulator of T cell signaling, has been suggested as a therapeutic target. JAK inhibitors have been proposed as agents to decrease Th17 and other CD3+ T cell activity (32). It must be noted, however, that JAK inhibitors carry black box warnings for increased risk of infection and thrombosis, both of which are potentially problematic in COVID-19.

Reviewing antiviral immune mechanisms in COVID-19 accentuates the importance of therapeutic timing. Patients with severe COVID-19 lack a balanced immune response and require treatment prior to becoming critically ill. Thus far, glucocorticoid therapy in low-to-moderate doses presents the best evidence for curtailing the SARS–CoV-2–associated hyperinflammation. As multiple clinical trials are ongoing, the search continues for a definitive therapy to prevent or calm the cytokine storm.

Conclusions

Our understanding of the mechanism of the immunologic response and cytokine storm incited by SARS–CoV-2 remains incomplete. There are very few studies that have examined how the clinical symptoms and laboratory findings evolve over time, which is especially relevant given the dynamic nature of the immune response and the importance of timely, targeted interventions. Small sample sizes and heterogeneity in cohort studies, disease phenotype, and immune evaluation methodologies complicate the comparisons between analyses and lead to inconclusive deductions. It is not understood why some populations are at lower or higher risk for a cytokine storm than others. There are studies underway to address the contribution of genetic and epigenetic risk factors in conferring protection to some populations and increasing risk in others. Additionally, understanding mechanisms at play in triggering hyperinflammation in COVID-19 may provide critical insights into the progression of inflammatory and autoimmune diseases and improve our ability to repurpose and develop effective therapies.

ACKNOWLEDGMENTS

The authors thank Kathleen Luskin for her commentary, scientific editor Wendy Spitzer for her insights, and the Northwell COVID-19 Research Consortium for facilitating the manuscript. Illustrations were created with Biorender.com and Adobe Illustrator CS6. We apologize to all authors whose work we could not cite due to space limitations.

AUTHOR CONTRIBUTIONS

All authors drafted the article, revised it critically for important intellectual content, and approved the final version to be published.

REFERENCES

1. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72,314 cases from the Chinese Center for Disease Control and Prevention. JAMA 2020;323:1239–42.
2. Siddiqi HK, Mehra MR. COVID-19 illness in native and immunosuppressed states: a clinical-therapeutic staging proposal [editorial]. J Heart Lung Transplant 2020;39:405–7.
3. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet 2020;395:497–506.
4. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. JAMA Intern Med 2020;180:934–43.

5. Grasselli G, Zangrillo A, Zanella A, Antonelli M, Cabrini L, Castelli A, et al. Baseline characteristics and outcomes of 1591 patients infected with SARS-CoV-2 admitted to ICUs of the Lombardy Region, Italy. JAMA 2020;323:1574–81.
6. Richardson S, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW, et al. Presenting characteristics, comorbidities, and outcomes among 5700 patients hospitalized with COVID-19 in the New York City area. JAMA 2020;323:2052–9.
7. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ. COVID-19: consider cytokine storm syndromes and immunosuppression [letter]. Lancet 2020;395:1033–4.
8. Murphy K, Weaver C. Janeway's immunobiology. 9th ed. New York: Garland Science; 2016.
9. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods 2004;294:15–22.
10. Cifaldi L, Prencipe G, Caiello I, Bracaglia C, Locatelli F, de Benedetti F, et al. Inhibition of natural killer cell cytotoxicity by interleukin-6: implications for the pathogenesis of macrophage activation syndrome. Arthritis Rheumatol 2015;67:3037–46.
11. Dinarello CA. Overview of the IL-1 family in innate inflammation and acquired immunity. Immunol Rev 2018;281:8–27.
12. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol 2014;6:a016295.
13. Zhang W, Zhao Y, Zhang F, Wang Q, Li T, Liu Z, et al. The use of antiinflammatory drugs in the treatment of people with severe coronavirus disease 2019 (COVID-19): the perspectives of clinical immunologists from China. Clin Immunol 2020;214:108393.
14. Rayamajhi M, Zhang Y, Miao EA. Detection of pyroptosis by measuring released lactate dehydrogenase activity. In: de Nardo CM, Latz E, editors. The inflammasome: methods and protocols. Totowa (NJ): Humana Press; 2013. p. 85–90.
15. Schutte T, Thijs A, Smulders YM. Never ignore extremely elevated D-dimer levels: they are specific for serious illness. Neth J Med 2016;74:443–8.
16. Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med 2003;348: 1967–76.
17. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med 2012;367:1814–20.
18. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. Lancet 2020;395:565–74.
19. Wong CK, Lam CW, Wu AK, Ip WK, Lee NL, Chan IH, et al. Plasma inflammatory cytokines and chemokines in severe acute respiratory syndrome. Clin Exp Immunol 2004;136:95–103.
20. Mahallawi WH, Khabour OF, Zhang Q, Makhdoum HM, Suliman BA. MERS–CoV infection in humans is associated with a proinflammatory Th1 and Th17 cytokine profile. Cytokine 2018;104:8–13.
21. Letko M, Marzi A, Munster V. Functional assessment of cell entry and receptor usage for SARS–CoV-2 and other lineage B betacoronaviruses. Nat Microbiol 2020;5:562–9.
22. Flint SJ, Racaniello VR, Rall GF, Skalka AM, Enquist LW. Principles of virology. 4th ed. Washington, DC: ASM Press; 2015.
23. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus–infected pneumonia. N Engl J Med 2020;382:1199–207.
24. Liu Y, Yan LM, Wan L, Xiang TX, Le A, Liu JM, et al. Viral dynamics in mild and severe cases of COVID-19 [letter]. Lancet Infect Dis 2020;20:656–7.
25. Ong EZ, Chan YF, Leong WY, Lee NM, Kalimuddin S, Mohideen SM, et al. A dynamic immune response shapes COVID-19 progression. Cell Host Microbe 2020;27:879–82.e2.
26. Cameron MJ, Ran L, Xu L, Danesh A, Bermejo-Martin JF, Cameron CM, et al. Interferon-mediated immunopathological events are associated with atypical innate and adaptive immune responses in patients with severe acute respiratory syndrome. J Virol 2007;81:8692–706.
27. Channappanavar R, Fehr AR, Vijay R, Mack M, Zhao J, Meyerholz DK, et al. Dysregulated type I interferon and inflammatory monocyte-macrophage responses cause lethal pneumonia in SARS–CoV-infected mice. Cell Host Microbe 2016;19:181–93.
28. Blanco-Melo D, Nilsson-Payant BE, Liu WC, Uhl S, Hoagland D, Møller R, et al. Imbalanced host response to SARS–CoV-2 drives development of COVID-19. Cell 2020;181:1036–45.e9.
29. Zuo Y, Yalavarthi S, Shi H, Gockman K, Zuo M, Madison JA, et al. Neutrophil extracellular traps in COVID-19. JCI Insight 2020;5:e138999.
30. Salomé B, Mahmood Z. Modulation of immune crosstalk in COVID-19 [review]. Nat Rev Immunol 2020;20:406.
31. Vabret N, Britton GJ, Gruber C, Hegde S, Kim J, Kuksin M, et al. Immunology of COVID-19: current state of the science [review]. Immunity 2020;52:910–41.
32. Wu D, Yang XO. TH17 responses in cytokine storm of COVID-19: an emerging target of JAK2 inhibitor Fedratinib. J Microbiol Immunol Infect 2020;53:368–70.
33. Lagunas-Rangel FA. Neutrophil-to-lymphocyte ratio and lymphocyte-to-C-reactive protein ratio in patients with severe coronavirus disease 2019 (COVID-19): a meta-analysis. J Med Virol 2020. E-pub ahead of print.
34. Diao B, Wang C, Tan Y, Chen X, Liu Y, Ning L, et al. Reduction and functional exhaustion of T cells in patients with coronavirus disease 2019 (COVID-19). Front Immunol 2020;11:827.
35. Ackermann M, Verleden SE, Kuehnel M, Haverich A, Welte T, Laenger F, et al. Pulmonary vascular endothelialitis, thrombosis, and angiogenesis in COVID-19. N Engl J Med 2020;383:120–8.
36. Wang X, Xu W, Hu G, Xia S, Sun Z, Liu Z, et al. SARS–CoV-2 infects T lymphocytes through its spike protein-mediated membrane fusion [article retracted in Cell Mol Immunol 2020;17:894]. Cell Mol Immunol 2020. E-pub ahead of print.
37. Chiappelli F, Khakshooy A, Greenberg G. COVID-19 immunopathology and immunotherapy. Bioinformation 2020;16:219–22.
38. To KK, Tsang OT, Leung WS, Tam AR, Wu TC, Lung DC, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS–CoV-2: an observational cohort study. Lancet Infect Dis 2020;20:565–74.
39. Tan W, Lu Y, Zhang J, Wang J, Dan Y, Tan Z, et al. Viral kinetics and antibody responses in patients with COVID-19. medRxiv 2020:2020.03.24.20042382.
40. De Bont CM, Boelens WC, Pruijn GJ. NETosis, complement, and coagulation: a triangular relationship. Cell Mol Immunol 2019;16:19–27.
41. Klok FA, Kruip M, van der Meer NJ, Arbous MS, Gommers D, Kant KM, et al. Incidence of thrombotic complications in critically ill ICU patients with COVID-19. Thromb Res 2020;191:145–7.
42. Harzallah I, Debliquis A, Drénou B. Lupus anticoagulant is frequent in patients with COVID-19 [letter]. J Thromb Haemost 2020;18:2064–5.
43. Dong Y, Mo X, Hu Y, Qi X, Jiang F, Jiang Z, et al. Epidemiology of COVID-19 among children in China. Pediatrics 2020;145:e20200702.
44. Verdoni L, Mazza A, Gervasoni A, Martelli L, Ruggeri M, Ciuffreda M, et al. An outbreak of severe Kawasaki-like disease at the Italian epicentre of the SARS–CoV-2 epidemic: an observational cohort study. Lancet 2020;395:1771–8.

45. Zeng JH, Liu YX, Yuan J, Wang FX, Wu WB, Li JX, et al. First case of COVID-19 complicated with fulminant myocarditis: a case report and insights. Infection 2020. E-pub ahead of print.
46. Yancy CW. COVID-19 and African Americans [letter]. JAMA 2020; 323:1891–2.
47. Chiappetta S, Sharma AM, Bottino V, Stier C. COVID-19 and the role of chronic inflammation in patients with obesity. Int J Obes (Lond) 2020;44:1790–2.
48. Jackson DJ, Busse WW, Bacharier LB, Kattan M, O'Connor GT, Wood RA, et al. Association of respiratory allergy, asthma, and expression of the SARS–CoV-2 receptor ACE2 [letter]. J Allergy Clin Immunol 2020;146:203–6.e3.
49. Minotti C, Tirelli F, Barbieri E, Giaquinto C, Dona D. How is immunosuppressive status affecting children and adults in SARS–CoV-2 infection? A systematic review. J Infect 2020;81:e61–6.
50. Liang W, Guan W, Chen R, Wang W, Li J, Xu K, et al. Cancer patients in SARS–CoV-2 infection: a nationwide analysis in China [letter]. Lancet Oncol 2020;21:335–7.
51. Zhang L, Zhu F, Xie L, Wang C, Wang J, Chen R, et al. Clinical characteristics of COVID-19-infected cancer patients: a retrospective case study in three hospitals within Wuhan, China. Ann Oncol 2020;31:894–901.
52. Bhoori S, Rossi RE, Citterio D, Mazzaferro V. COVID-19 in long-term liver transplant patients: preliminary experience from an Italian transplant centre in Lombardy [letter]. Lancet Gastroenterol Hepatol 2020;5:532–3.
53. Akalin E, Azzi Y, Bartash R, Seethamraju H, Parides M, Hemmige V, et al. COVID-19 and kidney transplantation [letter]. N Engl J Med 2020;382:2475–7.
54. Fernández-Ruiz M, Andrés A, Loinaz C, Delgado JF, López-Medrano F, San Juan R, et al. COVID-19 in solid organ transplant recipients: a single-center case series from Spain. Am J Transplant 2020;20:1849–58.
55. Emmi G, Bettiol A, Mattioli I, Silvestri E, Scala GD, Urban ML, et al. SARS–CoV-2 infection among patients with systemic autoimmune diseases [review]. Autoimmun Rev 2020;102575.
56. Bezzio C, Saibeni S, Variola A, Allocca M, Massari A, Gerardi V, et al. Outcomes of COVID-19 in 79 patients with IBD in Italy: an IG-IBD study. Gut 2020;69:1213–7.
57. Mathian A, Mahevas M, Rohmer J, Roumier M, Cohen-Aubart F, Amador-Borrero B, et al. Clinical course of coronavirus disease 2019 (COVID-19) in a series of 17 patients with systemic lupus erythematosus under long-term treatment with hydroxychloroquine [letter]. Ann Rheum Dis 2020;79:837–9.
58. Cortegiani A, Ingoglia G, Ippolito M, Giarratano A, Einav S. A systematic review on the efficacy and safety of chloroquine for the treatment of COVID-19. J Crit Care 2020;57:279–83.
59. Hernandez AV, Roman YM, Pasupuleti V, Barboza JJ, White CM. Hydroxychloroquine or chloroquine for treatment or prophylaxis of COVID-19: a living systematic review. Ann Intern Med 2020;173:287–96.
60. Kim AH, Sparks JA, Liew JW, Putman MS, Berenbaum F, Duarte-García A, et al. A rush to judgment? Rapid reporting and dissemination of results and its consequences regarding the use of hydroxychloroquine for COVID-19. Ann Intern Med 2020;172:819–21.
61. Cao B, Wang Y, Wen D, Liu W, Wang J, Fan G, et al. A trial of lopinavir-ritonavir in adults hospitalized with severe COVID-19. N Engl J Med 2020;382:1787–99.
62. Hung IF, Lung KC, Tso EY, Liu R, Chung TW, Chu MY, et al. Triple combination of interferon β-1b, lopinavir-ritonavir, and ribavirin in the treatment of patients admitted to hospital with COVID-19: an open-label, randomised, phase 2 trial. Lancet 2020;395:1695–704.
63. Beigel JH, Tomashek KM, Dodd LE, Mehta AK, Zingman BS, Kalil AC, et al. Remdesivir for the treatment of COVID-19: preliminary report [letter]. N Engl J Med 2020;383:993–4.
64. Weber AG, Chau AS, Egeblad M, Barnes BJ, Janowitz T. Nebulized in-line endotracheal dornase alfa and albuterol administered to mechanically ventilated COVID-19 patients: a case series. medRxiv 2020:2020.05.13.20087734.
65. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA 2020;323:1061–9.
66. Rubio JL, del Castillo JD, de la Hera Fernández J, Arrabal EG, Ruiz MC, Centeno NO. Eficacia de los pulsos de corticoides en pacientes con síndrome de liberación de citocinas inducido por infección por SARS–CoV-2. Med Clin (Barc) 2020;155:159–61.
67. Fadel R, Morrison AR, Vahia A, Smith ZR, Chaudhry Z, Bhargava P, et al. Early short course corticosteroids in hospitalized patients with COVID-19. Clin Infect Dis 2020. E-pub ahead of print.
68. Horby P, Lim WS, Emberson JR, Mafham M, Bell JL, Linsell L, et al, on behalf of the RECOVERY Collaborative Group. Dexamethasone in hospitalized patients with COVID-19: preliminary report. N Engl J Med 2020. E-pub ahead of print.
69. Toniati P, Piva S, Cattalini M, Garrafa E, Regola F, Castelli F, et al. Tocilizumab for the treatment of severe COVID-19 pneumonia with hyperinflammatory syndrome and acute respiratory failure: a single center study of 100 patients in Brescia, Italy [review]. Autoimmun Rev 2020;19:102568.
70. Mehta P, Cron RQ, Hartwell J, Manson JJ, Tattersall RS. Silencing the cytokine storm: the use of intravenous anakinra in haemophagocytic lymphohistiocytosis or macrophage activation syndrome. Lancet Rheumatol 2020;2:e358–67.
71. Shakoory B, Carcillo JA, Chatham WW, Amdur RL, Zhao H, Dinarello CA, et al. Interleukin-1 receptor blockade is associated with reduced mortality in sepsis patients with features of macrophage activation syndrome: reanalysis of a prior phase III trial. Crit Care Med 2016;44:275–81.
72. Cavalli G, de Luca G, Campochiaro C, Della-Torre E, Ripa M, Canetti D, et al. Interleukin-1 blockade with high-dose anakinra in patients with COVID-19, acute respiratory distress syndrome, and hyperinflammation: a retrospective cohort study. Lancet Rheumatol 2020;2:e325–31.
73. Huet T, Beaussier H, Voisin O, Jouveshomme S, Dauriat G, Lazareth I, et al. Anakinra for severe forms of COVID-19: a cohort study. Lancet Rheumatol 2020;2:e393–400.
74. Ucciferri C, Auricchio A, di Nicola M, Potere N, Abbate A, Cipollone F, et al. Canakinumab in a subgroup of patients with COVID-19. Lancet Rheumatol 2020;2:e457–8.
75. Jamilloux Y, Henry T, Belot A, Viel S, Fauter M, el Jammal T, et al. Should we stimulate or suppress immune responses in COVID-19? Cytokine and anticytokine interventions [review]. Autoimmun Rev 2020;19:102567.
76. Lang FM, Lee KM, Teijaro JR, Becher B, Hamilton JA. GM-CSF-based treatments in COVID-19: reconciling opposing therapeutic approaches [review]. Nat Rev Immunol 2020;20:507–14.
77. De Luca G, Cavalli G, Campochiaro C, Della-Torre E, Angelillo P, Tomelleri A, et al. GM-CSF blockade with mavrilimumab in severe COVID-19 pneumonia and systemic hyperinflammation: a single-centre, prospective cohort study. Lancet Rheumatol 2020;2:e465–73.
78. Novi G, Mikulska M, Briano F, Toscanini F, Tazza F, Uccelli A, et al. COVID-19 in a MS patient treated with ocrelizumab: does immunosuppression have a protective role? Mult Scler Relat Disord 2020;42:102120.
79. Guilpain P, le Bihan C, Foulongne V, Taourel P, Pansu N, Maria AT, et al. Rituximab for granulomatosis with polyangiitis in the pandemic of COVID-19: lessons from a case with severe pneumonia. Ann Rheum Dis 2020. E-pub ahead of print.
80. Soresina A, Moratto D, Chiarini M, Paolillo C, Baresi G, Foca E, et al. Two X-linked agammaglobulinemia patients develop pneumonia as COVID-19 manifestation but recover. Pediatr Allergy Immunol 2020. E-pub ahead of print.

81. Xia S, Liu X, Cao X, Xu S. T-cell expression of Bruton's tyrosine kinase promotes autoreactive T-cell activation and exacerbates aplastic anemia. Cell Mol Immunol 2019. E-pub ahead of print.
82. Chong EA, Roeker LE, Shadman M, Davids MS, Schuster SJ, Mato AR. BTK inhibitors in cancer patients with COVID-19: "the winner will be the one who controls that chaos" (Napoleon Bonaparte). Clin Cancer Res 2020;26:3514–6.
83. Roschewski M, Lionakis MS, Sharman JP, Roswarski J, Goy A, Monticelli MA, et al. Inhibition of Bruton tyrosine kinase in patients with severe COVID-19. Sci Immunol 2020;5:eabd0110.
84. Kindrachuk J, Ork B, Hart BJ, Mazur S, Holbrook MR, Frieman MB, et al. Antiviral potential of ERK/MAPK and PI3K/AKT/mTOR signaling modulation for Middle East respiratory syndrome coronavirus infection as identified by temporal kinome analysis. Antimicrob Agents Chemother 2015;59:1088–99.
85. Patterson BK, Seethamraju H, Dhody K, Corley MJ, Kazempour K, Lalezari JP, et al. Disruption of the CCL5/RANTES-CCR5 pathway restores immune homeostasis and reduces plasma viral load in critical COVID-19. medRxiv 2020:2020.05.02.20084673.

American College of Rheumatology
Empowering Rheumatology Professionals

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 36–47
DOI 10.1002/art.41469

Antirheumatic Disease Therapies for the Treatment of COVID-19: A Systematic Review and Meta-Analysis

Michael Putman,[1] Yu Pei Eugenia Chock,[2] Herman Tam,[3] Alfred H. J. Kim,[4] Sebastian E. Sattui,[5] Francis Berenbaum,[6] Maria I. Danila,[7] Peter Korsten,[8] Catalina Sanchez-Alvarez,[9] Jeffrey A. Sparks,[10] Laura C. Coates,[11] Candace Palmerlee,[12] Andrea Peirce,[13] Arundathi Jayatilleke,[14] Sindhu R. Johnson,[15] Adam Kilian,[16] Jean Liew,[17] Larry J. Prokop,[18] M. Hassan Murad,[18] Rebecca Grainger,[19] Zachary S. Wallace,[20] and Alí Duarte-García,[18] on behalf of the COVID-19 Global Rheumatology Alliance

Objective. Antirheumatic disease therapies have been used to treat coronavirus disease 2019 (COVID-19) and its complications. We conducted a systematic review and meta-analysis to describe the current evidence.

Methods. A search of published and preprint databases in all languages was performed. Included studies described ≥1 relevant clinical outcome for ≥5 patients who were infected with severe acute respiratory syndrome coronavirus 2 and were treated with antirheumatic disease therapy between January 1, 2019 and May 29, 2020. Pairs of reviewers screened articles, extracted data, and assessed risk of bias. A meta-analysis of effect sizes using random-effects models was performed when possible.

Results. The search identified 3,935 articles, of which 45 were included (4 randomized controlled trials, 29 cohort studies, and 12 case series). All studies evaluated hospitalized patients, and 29 of the 45 studies had been published in a peer-reviewed journal. In a meta-analysis of 3 cohort studies with a low risk of bias, hydroxychloroquine use was not significantly associated with mortality (pooled hazard ratio [HR] 1.41 [95% confidence interval (95% CI) 0.83, 2.42]). In a meta-analysis of 2 cohort studies with some concerns/higher risk of bias, anakinra use was associated with lower mortality (pooled HR 0.25 [95% CI 0.12, 0.52]). Evidence was inconclusive with regard to other antirheumatic disease therapies, and the majority of other studies had a high risk of bias.

Conclusion. In this systematic review and meta-analysis, hydroxychloroquine use was not associated with benefit or harm regarding COVID-19 mortality. The evidence supporting the effect of other antirheumatic disease therapies in COVID-19 is currently inconclusive.

INTRODUCTION

Several antirheumatic disease therapies have emerged as potential treatments for coronavirus disease 2019 (COVID-19), the disease caused by severe acute respiratory syndrome coronavirus 2 (SARS–CoV-2). There has been particular interest in the antimalarial agents hydroxychloroquine (HCQ) and chloroquine (1), which may inhibit SARS–CoV-2 replication by elevating endosomal pH or altering the glycosylation of the angiotensin-converting enzyme 2 (ACE2) receptor (2). After preliminary

The views expressed are those of the authors and not necessarily those of the NHS, the NIHR, or the Department of Health.

The COVID-19 Global Rheumatology Alliance receives financial support from Amgen and Johnson & Johnson. This project was planned and completed prior to receipt of any funding. Dr. Putman's work was supported in part by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH (grant T32-AR-007611-13). Dr. Sattui's work was supported by the Vasculitis Clinical Research Consortium/Vasculitis Foundation Fellowship. The Vasculitis Clinical Research Consortium is part of the Rare Diseases Clinical Research Network, an initiative of the Office of Rare Diseases Research, National Center for Advancing Translational Science. The Vasculitis Clinical Research Consortium is funded through collaboration between the National Center for Advancing Translational Science and the National Institute of Arthritis and Musculoskeletal and Skin Diseases (U54-AR-057319). Dr. Sparks' work was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH (grants K23-AR-069688, R03-AR-075886, L30-AR-066953, P30-AR-070253, and P30-AR-072577), a Rheumatology Research Foundation K Supplement Award, the Brigham Research Institute, and the R. Bruce and Joan M. Mickey Research Scholar Fund. Dr. Coates' work was supported by the NIHR Oxford Biomedical Research Centre and a National Institute for Health Research Clinician Scientist award. Dr. Johnson's work was supported by a Canadian Institutes of Health Research New Investigator award. Dr. Liew's work was supported in part by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH (grant T32-AR-007108). Dr. Wallace's work was supported by the National Institute of Arthritis and Musculoskeletal and Skin Disease, NIH (grants K23-AR-073334 and L30-AR-070520). Dr. Duarte-García's work was supported by the Robert D. and Patricia E. Kern Center for the Science of Health Care Delivery and the Eaton Family Career Development Award.

[1]Michael Putman, MD, MSci: Northwestern Medicine, Chicago, Illinois; [2]Yu Pei Eugenia Chock, MD, MPH: Yale School of Medicine, New Haven, Connecticut; [3]Herman Tam, MBBS, MSc: Hospital for Sick Children, Toronto, Ontario, Canada; [4]Alfred H. J. Kim, MD, PhD: Washington University School of Medicine, St. Louis, Missouri; [5]Sebastian E. Sattui, MD, MS: Hospital for Special Surgery,

evidence also suggested a clinical benefit of HCQ (3), public acquisition resulted in shortages (4,5). More recently, a now-retracted study by Mehra et al demonstrated an association between HCQ use and increased mortality (6,7). Both concern for this potential risk and the aforementioned HCQ shortages have negatively impacted patients who take HCQ for rheumatic diseases.

Antirheumatic disease therapies may also mitigate the hyperinflammatory state caused by SARS–CoV-2 infection, which has been associated with elevated levels of inflammatory cytokines (8,9). Therapies that directly target the inflammatory cascade, including interleukin-6 (IL-6) inhibitors, IL-1 inhibitors, and glucocorticoids, have been widely adopted in clinical practice prior to the publication of ongoing randomized controlled trials (RCTs). Similar considerations have led to speculation that tumor necrosis factor (TNF) inhibitors and the JAK inhibitor baricitinib may be beneficial (10–12).

Recent systematic reviews have primarily focused on antimalarial therapy (13,14), and no reviews to date have included a meta-analysis of recently published large observational studies of antirheumatic disease therapies. In this systematic review and meta-analysis, we have identified and summarized published and preprint original scientific articles that describe the use of antirheumatic disease therapies for the treatment of COVID-19.

METHODS

This systematic review was performed according to the Cochrane Handbook for Systematic Reviews of Interventions (15) and was reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (16) and the Synthesis Without Meta-Analysis guidelines (17). The protocol was registered on the International Prospective Register of Systematic Reviews (no. CRD42020176896) (18).

Data sources and literature search. A comprehensive search in any language was performed on March 17, 2020 and included all articles published between January 1, 2019 and April 1, 2020. The search was refreshed on May 7, 2020. The following databases were included: Ovid Medline and E-pub Ahead of Print, In-Process & Other Non-Indexed Citations, and Daily, Ovid Embase, Ovid Cochrane Central Register of Controlled Trials, Ovid Cochrane Database of Systematic Reviews, Scopus, Web of Science, and ClinicalTrials.Gov. The search strategy was designed and conducted by an experienced librarian (LJP) with input from the study investigators. Controlled vocabulary supplemented with keywords was used to search for drug therapy for COVID-19.

Given the rapid development of new evidence, all articles available on the preprint servers medRxiv, bioRxiv, and ChinaXiv were also included. Coronavirus resource centers of *The Lancet, Journal of the American Medical Association*, and *New England Journal of Medicine* were manually searched until May 29, 2020. The studies that were identified as preprints were replaced by peer-reviewed published versions if available and identified by May 23, 2020. A detailed description of the search strategy is available in the Supplementary Materials (available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41469/abstract).

Study selection eligibility criteria. Original eligibility criteria were refined after review of the initial search (18). The final eligibility criteria were as follows: 1) included ≥5 people infected with SARS–CoV-2; 2) focused on antirheumatic disease therapy (Supplementary Materials, http://onlinelibrary.wiley.com/doi/10.1002/art.41469/abstract); 3) was published after January 1, 2019; 4) was original research; 5) had one of the following outcomes: death, ventilator-free days, escalation of care (intensive care unit [ICU] transfer), length of hospital stay, symptom resolution, viral

New York, New York; [6]Francis Berenbaum, MD, PhD: Sorbonne Université, INSERM CRSA, AP-HP, Hôpital Saint Antoine, Paris, France; [7]Maria I. Danila, MD, MSc, MSPH: University of Alabama at Birmingham; [8]Peter Korsten, MD: University Medical Center Göttingen, Göttingen, Germany; [9]Catalina Sanchez-Alvarez, MD: University of Florida, Gainesville; [10]Jeffrey A. Sparks, MD, MMSc: Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts; [11]Laura C. Coates, MBChB, MRCP, PhD: University of Oxford, Oxford, UK; [12]Candace Palmerlee, BA, CMT: Berkeley, California; [13]Andrea Peirce, BA: New York, New York; [14]Arundathi Jayatilleke, MD, MS: Lewis Katz School of Medicine, Temple University, Philadelphia, Pennsylvania; [15]Sindhu R. Johnson, MD, PhD: Toronto Western Hospital, Mount Sinai Hospital, and University of Toronto, Toronto, Ontario, Canada; [16]Adam Kilian, MD: George Washington University School of Medicine and Health Sciences, Washington, DC; [17]Jean Liew, MD, MS: University of Washington, Seattle; [18]Larry J. Prokop, MLS, M. Hassan Murad, MD, MPH, Alí Duarte-García, MD, MSc: Mayo Clinic, Rochester, Minnesota; [19]Rebecca Grainger, MBChB, PhD: University of Otago, Wellington, New Zealand; [20]Zachary S. Wallace, MD, MSc: Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts.

Dr. Kim has received consulting fees, speaking fees, and/or honoraria from Exagen Diagnostics and GlaxoSmithKline (less than $10,000 each) and research support from GlaxoSmithKline. Dr. Berenbaum has received consulting fees, speaking fees, and/or honoraria from MSD, Nordic, Novartis, Pfizer, Roche, Sandoz, Sanofi, and UCB (less than $10,000 each). Dr. Danila has received consulting fees from Amgen, Novartis, and Sanofi Regeneron (less than $10,000 each) and research support from Genentech, Pfizer, Boehringer, and Horizon. Dr. Korsten has received consulting fees, speaking fees, and/or honoraria from AbbVie, Bristol Myers Squibb, Chugai, Gilead, GlaxoSmithKline, Eli Lilly, Novartis, Pfizer, and Sanofi-Aventis (less than $10,000 each). Dr. Sparks has received consulting fees, speaking fees, and/or honoraria from Bristol Myers Squibb, Gilead, Inova, Janssen, and Optum (less than $10,000 each) and research support from Bristol Myers Squibb and Amgen. Dr. Coates has received consulting fees, speaking fees, and/or honoraria from Celgene, Eli Lilly, Pfizer, Amgen, Galápagos, Gilead, Janssen, UCB, and Biogen (less than $10,000 each) and from AbbVie and Novartis (more than $10,000 each) and research support from Novartis, Celgene, Eli Lilly, and Pfizer. Dr. Johnson has received consulting fees and/or honoraria from Boehringer Ingelheim and Ikaria (less than $10,000 each) and research support from GlaxoSmithKline, Corbus, Roche, Merck, Boehringer Ingelheim, and Bayer. Dr. Grainger has received consulting fees, speaking fees, and/or honoraria from Pfizer Australia, Cornerstones, Janssen New Zealand, and Novartis (less than $10,000 each). Dr. Wallace has received consulting fees, speaking fees, and/or honoraria from Viela Bio and research support from Bristol Meyers Squibb. No other potential conflicts of interest relevant to this article were reported.

Address correspondence to Michael Putman, MD, Northwestern University, 251 East Huron, Suite 1400, Chicago, IL 60611 (email: msputman@gmail.com); or to Alí Duarte-García, MD, MSc, Mayo Clinic, 200 First Street SW, Rochester, MN 55905 (email: duarte.ali@mayo.edu).

Submitted for publication June 12, 2020; accepted in revised form July 26, 2020.

Table 1. Studies investigating antimalarial therapies and COVID-19 (n = 14 for HCQ and n = 5 for chloroquine)*

Medication, outcome measure, author (ref.)	Study design	n	Outcome and inference	Bias assessment†	Direction of effect‡
HCQ					
Mortality					
Rosenberg et al (26)	Cohort	1,438	No significant difference in mortality (adjusted HR 1.08 [95% CI 0.63, 1.85])	Low	QS
Magagnoli et al (27)	Cohort	368	Increased mortality in HCQ group (adjusted HR 2.6 [95% CI 1.1, 6.21])	Low	QS
Mahévas et al (28)	Cohort	173	No difference in overall survival at 21 days (weighted HR 1.2 [95% CI 0.4, 3.3]) or survival without transfer to ICU (weighted HR 0.9 [95% CI 0.4, 2.1])	Low	QS
Yu et al (66)	Cohort	568	Lower mortality in HCQ group among those critically ill (adjusted HR 0.33 [95% CI 0.17, 0.64])	High	+
Ashraf et al (67)	Case series	100	Higher rate of survival in HCQ group (OR 61.9 [95% CI 9.0, 424.7])	High	NA
Mathian et al (68)	Case series	17	2 of 14 hospitalized patients taking HCQ died	High	NA
Composite of intubation and death					
Mahévas et al (28)	Cohort	173	No difference in the combined outcome of ICU care or death (HR 0.9 [95% CI 0.4, 2.1])	Low	QS
Geleris et al (29)	Cohort	1,376	No difference in the combined outcome of IMV or death (HR 1.04 [95% CI 0.82, 1.32])	Low	QS
Escalation of care					
Magagnoli et al (27)	Cohort	368	No difference in IMV (adjusted HR 1.43 [95% CI 0.53, 3.79])	Low	–
Mathian et al (68)	Case series	17	Of 17 patients taking HCQ, 14 were admitted to hospital and 7 to ICU	High	NA
Hospital/ICU discharge					
Mahévas et al (28)	Cohort	173	No difference in discharge at 21 days (RR 1.0 [95% CI 0.9, 1.3])	Low	NA
Clinical improvement					
Tang et al (30)	RCT	150	No difference in symptom resolution at 28 days (60% vs. 67% SoC; $P = 0.97$)	High	+
Chen et al (31)	RCT	62	Shorter recovery for fever (2.2 days vs. 3.2 days; $P < 0.001$) and cough (2.0 days vs. 3.1 days; $P = 0.002$)	High	+
Mahévas et al (28)	Cohort	173	No difference in oxygen weaning at 21 days (RR 1.1 [95% CI 0.9, 1.3])	Low	+
Gautret et al (69)	Case series	80	81% with "favorable outcome" and only 15% required oxygen	High	NA
SARS–Cov-2 clearance					
Tang et al (30)	RCT	150	No difference in viral clearance at 28 days (85% vs. 81% SoC; $P = 0.34$)	High	+
Mallat et al (32)	Cohort	34	Longer duration of SARS–CoV-2 test positivity in HCQ (17 days vs. 10 days SoC; $P = 0.023$)	Some	–
Gautret et al (3)	Cohort	42	Higher rate of viral clearance at 6 days (70% vs. 13% SoC at other hospitals; $P = 0.001$)	High	+
Molina et al (70)	Case series	11	Viral load persistent 6 days after treatment in 8 of 10 patients	High	NA
Million et al (71)	Case series	1,061	Persistent SARS–CoV-2 test positivity at 10 days in 47 patients	High	NA
Gautret et al (69)	Case series	80	Viral clearance in 74 of 80 patients at 8 days	High	NA
Chloroquine					
Mortality					
Borba et al (33)	RCT	81	Higher mortality in high-dose group vs. low-dose group (log rank −2.183; $P = 0.03$)	High	–
Composite of intubation and death					
Million et al (71)	Case series	1,061	10 patients transferred to ICU and 8 patients died	High	NA
Hospital/ICU discharge					
Huang et al (34)	RCT	22	Increased likelihood of discharge in chloroquine group vs. lopinavir/ritonavir group (RR 1 [95% CI 1.33, 4])	High	+
Clinical improvement					
Huang et al (35)	Cohort	373	Shorter fever duration in the chloroquine group (1.2 days vs. 1.9 days; $P = 0.003$)	High	+

(Continued)

Table 1. (*Cont'd*)

Medication, outcome measure, author (ref.)	Study design	n	Outcome and inference	Bias assessment†	Direction of effect‡
SARS–CoV-2 clearance					
Huang et al (34)	RCT	22	Increased likelihood of negative RT-PCR on chloroquine vs. lopinavir/ritonavir (RR 1.09 [95% CI 1, 1.33])	High	+
Chen et al (36)	Cohort	284	No significant change in viral clearance with chloroquine (OR 0.7 [95% CI 0.2, 2.0])	High	+
Huang et al (35)	Cohort	373	Shorter time to viral clearance (median difference −5.4 [95% CI −6.0, −4.0]; $P < 0.001$)	High	+

* Escalation of care included intensive care unit (ICU) transfer, intubation, and mechanical ventilation. COVID-19 = coronavirus disease 2019; HCQ = hydroxychloroquine; HR = hazard ratio; 95% CI = 95% confidence interval; QS = quantitative synthesis; OR = odds ratio; NA = not applicable; IMV = invasive mechanical ventilation; RR = risk ratio; RCT = randomized controlled trial; SoC = standard of care; SARS–CoV-2 = severe acute respiratory syndrome coronavirus 2; RT-PCR = reverse transcriptase–polymerase chain reaction.
† Bias assessed using the Newcastle-Ottawa Scale for cohort studies and the Risk of Bias 2.0 tool for randomized controlled trials; case series assumed to be high risk by default.
‡ Quantified using the Cochrane vote counting method for data synthesis. Studies eligible for quantitative synthesis and case series were excluded.

clearance. Studies that did not present primary data (i.e., editorials, opinions, meta-analysis, and reviews) were excluded.

Patient research partners. Four patient research partners who have had COVID-19 (2 patients with an autoimmune disease and 2 rheumatologists) were involved throughout the project. Patient research partners participated in the selection of outcomes and the drafting of the manuscript.

Data collection process. Pairs of reviewers working independently (MP, YPEC, HT, SES, FB, MID, PK, CS-A, JS, AK, and AD-G) evaluated eligibility based on review of abstracts and titles. Records with disagreements on inclusion/exclusion were included in full-text review. Pairs of the same reviewers working independently evaluated full-text articles. Disagreements were resolved by consensus discussion and, if necessary, by involving a third reviewer. Abstract, title, and full-text review were conducted using DistillerSR software (Evidence Partners). A standardized extraction tool was developed by consensus and refined after preliminary testing on a subset of the full-text articles. The extraction tool included a full description of study characteristics, the medications patients received (dose, frequency, route), and the inferences made in each study. Pairs of reviewers extracted data independently, and differences were reconciled by the corresponding authors (MP and AD-G).

Risk of bias in individual studies. Two reviewers working independently (MP and AD-G) assessed the risk of bias. RCTs were assessed using the Risk of Bias 2.0 tool (19) and were reported using the recommended 3-item ordinal scale ("high risk of bias," "some concerns," or "low risk of bias"). Cohort studies were assessed using the Newcastle-Ottawa Scale (20). The comparability domain of the Newcastle-Ottawa Scale was the primary differentiation point for a study's risk of bias in this context and was used to determine global risk of bias (0 = high risk, 1 = some concerns, and 2 = low risk) (21). Disagreements were resolved by consensus discussion. Studies were defined as case series if they did not include an unexposed group and were deemed to have a high risk of bias by default (22,23).

Data analysis. When ≥1 study demonstrated the same outcome for the same antirheumatic therapy and showed an estimate of effect size, we performed a meta-analysis. Adjusted effect size estimates were used if available. Otherwise, unadjusted effect size estimates were used. Each study was weighted based on its log-transformed inverse variance. The meta-analysis was conducted using random-effects models due to expected clinical and methodologic heterogeneity (24). The I^2 statistic was calculated to describe heterogeneity. All analyses were conducted using RevMan 5.3 software.

We grouped the studies according to antirheumatic disease therapy and outcomes. The data were synthesized narratively and in tables. For reporting purposes and due to the methodologic diversity of the studies, we prioritized results for summary and synthesis based on study design (RCT > cohort studies > case series), risk of bias assessment (low risk > some concerns > high risk), and relevance of the outcome (e.g., mortality > viral clearance). Given the substantial heterogeneity of study design and reporting, we used the vote counting method, as described in the Cochrane handbook, to summarize the direction of the effect for a given outcome (25).

RESULTS

Study selection. The initial search was performed on March 17, 2020 and identified 1,315 studies, including 290 studies in the peer-reviewed published literature and 1,025 in preprint archives. An updated search was performed on May 7, 2020 and identified an additional 2,614 studies, including 634 studies in the published literature and 1,980 in the preprint archives. Six additional studies were identified prior to May 29, 2020 by manual search and were

included in the second extraction. After title and abstract screening, 3,660 studies were excluded. Of the 275 articles included for full-text review, 230 were excluded and 45 were included in qualitative review. One study identified by manual count was subsequently retracted (6,7) and therefore removed. Six of these studies were also eligible for meta-analysis (Supplementary Figure 1, http://onlinelibrary.wiley.com/doi/10.1002/art.41469/abstract).

Overall study characteristics. We included 4 RCTs, 29 cohort studies, and 12 case series. Sixteen studies had been posted to a preprint archive only, and 29 had been published in a peer-reviewed journal. Studies were conducted in China (n = 22), France (n = 10), Italy (n = 5), the US (n = 4), Brazil (n = 1), the United Arab Emirates (n = 1), Iran (n = 1), and Qatar (n = 1). All studies evaluated hospitalized patients with COVID-19 (Supplementary Table 1, http://onlinelibrary.wiley.com/doi/10.1002/art.41469/abstract). Of the 4 RCTs included, all had a high risk of bias. Of the 29 cohort studies, 6 had a low risk of bias, 5 had some concerns related to risk of bias, and 18 had a high risk of bias (Supplementary Tables 2 and 3, http://onlinelibrary.wiley.com/doi/10.1002/art.41469/abstract).

Antimalarial therapy. *HCQ.* Fourteen studies assessed HCQ, including 2 RCTs, 7 cohort studies, and 5 case series (Table 1). Three cohort studies (pooled n = 932) evaluated mortality and were included in quantitative synthesis (26–28). In the meta-analysis, HCQ use was not associated with a significant risk of death (pooled HR 1.41 [95% CI 0.83, 2.42]) (Figure 1A). Two cohort studies (pooled n = 1,549) were conducted to evaluate a composite risk of invasive mechanical ventilation and mortality and were included in quantitative synthesis (28,29). HCQ use was not associated with the pooled composite outcome (HR 1.03 [95% CI 0.82, 1.29]) (Figure 1B). All studies included in the quantitative synthesis had a low risk of bias.

Escalation of care and rate of discharge were each evaluated in 1 cohort study. Neither the study by Magagnoli et al assessing the risk of mechanical ventilation (27) nor one by Mahévas and colleagues evaluating discharge at 21 days (28) showed differences among patients with COVID-19 who received HCQ compared to those who did not. Both studies were considered to have a low risk of bias.

Two RCTs and 1 cohort study assessed clinical improvement. An RCT by Tang et al demonstrated no significant difference with regard to symptom alleviation at 28 days (30), while a smaller RCT by Chen et al showed a shorter recovery time with regard to both fever and cough (31). Based on vote counting, the direction of effect in both studies was toward a faster resolution of symptoms. In the aforementioned cohort study by Mahévas et al, researchers also evaluated the proportion of patients who were successfully weaned from oxygen after 21 days and found no significant difference. Both RCTs had a high risk of bias.

With regard to SARS–CoV-2 clearance, the RCT by Tang et al demonstrated no improvement in the proportion of people who had negative SARS–CoV-2 results at 28 days after treatment commenced. In a cohort study, Mallat et al found a longer duration of SARS–CoV-2 test positivity (32), while a cohort study by Gautret et al showed a higher rate of viral clearance (3). According to vote counting, there was no clear effect of HCQ on the time to viral clearance. The study by Mallat et al had some concerns about risk of bias, and the study by Gautret et al had a high risk of bias.

Chloroquine. Five studies assessed chloroquine, including 2 RCTs, 2 cohort studies, and 1 case series (Table 1). In an RCT by Borba et al, researchers assessed mortality (33), and the study was stopped early due to a safety signal that suggested a higher rate of mortality with a higher dose of chloroquine. It had a high risk of bias and did not include a placebo group as a comparator.

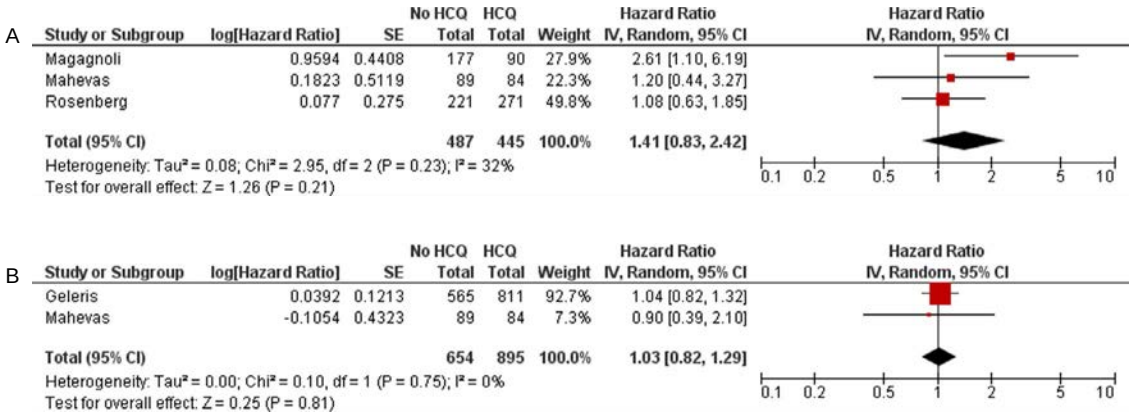


Figure 1. **A**, Meta-analysis of 3 observational studies investigating hydroxychloroquine (HCQ) and mortality among patients hospitalized with coronavirus disease 2019 (COVID-19). **B**, Meta-analysis of 2 observational studies investigating HCQ and the composite outcome of death or intubation among patients hospitalized with COVID-19. IV = inverse variance; 95% CI = 95% confidence interval.

Table 2. Studies investigating IL-6 inhibitors and COVID-19 (n = 7 for TCZ and n = 1 for siltuximab)*

Outcome measure, author (ref.)	Study design	n	Outcome and inference	Bias assessment†	Direction of effect‡
Mortality					
Roumier et al (37)	Cohort	59	No difference in mortality in TCZ group (17.2% vs. 18.7% SoC; *P* = 0.837)	Some	+
Quartuccio et al (39)	Cohort	111	Higher mortality in TCZ group (9.5% vs. 0% SoC)	High	–
Klopfenstein et al (38)	Cohort	45	Numerically lower mortality in TCZ group (25% vs. 48% historical SoC; *P* = 0.07)	High	+
Sciascia et al (72)	Case series	63	Mortality of 11% at day 14; increased survival with early TCZ (HR 2.2 [95% CI 1.3, 6.7])	High	NA
Luo et al (73)	Case series	15	Death in 3 of 15 patients (20%) treated with TCZ at 1 week of follow-up	High	NA
Alattar et al (74)	Case series	25	Death in 3 of 25 patients (12%) treated with TCZ at day 14	High	NA
Gritti et al (75)	Case series	21	IMV or death in 5 of 21 patients (24%) treated with siltuximab	High	NA
Composite of intubation and death					
Klopfenstein et al (38)	Cohort	45	Lower death/ICU admission in TCZ group (25% vs. 72% historical SoC; *P* = 0.002)	High	+
Escalation of care					
Roumier et al (37)	Cohort	59	Lower rate of IMV in TCZ group (adjusted OR 0.42 [95% CI 0.2, 0.9])	Some	+
Klopfenstein et al (38)	Cohort	45	Lower rate of IMV in TCZ group (0% vs. 32% historical SoC; *P* = 0.006)	High	+
Hospital/ICU discharge					
Klopfenstein et al (38)	Cohort	45	No difference in hospital discharge rate with TCZ (55% vs. 44% historical SoC; *P* = 0.453)	High	+
Alattar et al (74)	Case series	25	Discharge after improvement from ICU at day 14 in 9 of 25 patients (36%) treated with TCZ	High	NA
Clinical improvement					
Quartuccio et al (39)	Cohort	111	Lower rate of "complete" recovery in TCZ group (21% vs. 100% SoC)	High	–
Sciascia et al (72)	Case series	63	Pao_2:Fio_2 improved (152 ± 53 day 0; 284 ± 116 day 7; 302 ± 126 day 14; *P* < 0.05)	High	NA
Gritti et al (75)	Case series	21	Improvement in 7 of 21 patients (33%) treated with siltuximab	High	NA
Xu et al (76)	Case series	21	Improved oxygenation in 15 of 20 patients (75%) and discharge in 21 of 21 patients (100%) treated with TCZ	High	NA

* Escalation of care included ICU transfer, intubation, and mechanical ventilation. IL-6 = interleukin-6; TCZ = tocilizumab; Pao_2:Fio_2 = arterial partial pressure oxygen to fractional inspired oxygen ratio (see Table 1 for other definitions).
† Bias assessed using the Newcastle-Ottawa Scale; case series assumed to be high-risk by default.
‡ Quantified using the Cochrane vote counting method for data synthesis. Studies eligible for quantitative synthesis and case series were excluded.

An RCT by Huang et al that compared chloroquine to lopinavir/ritonavir demonstrated that participants receiving chloroquine were twice as likely to be discharged (34), and a cohort study by Huang et al showed a significantly shorter duration of fever in the chloroquine group (35). The same 2 studies also addressed SARS–CoV-2 clearance. The RCT showed a higher likelihood of clearance with chloroquine compared to ritonavir/lopinavir, while the cohort study showed a shorter time for viral clearance. In another cohort study, Chen et al found no significant change in viral clearance at 14 days (36). All studies assessing viral clearance had a high risk of bias and, according to vote counting, had the same direction of effect toward a shorter time for viral clearance.

IL-6 inhibitors. Seven studies assessed tocilizumab, an IL-6 receptor inhibitor, including 3 cohort studies and 4 case series; 1 case series assessed the IL-6 inhibitor siltuximab (Table 2). Three cohort studies assessed mortality. Roumier et al found no difference after adjustment (37), Klopfenstein et al found a numerically lower mortality rate (38), and Quartuccio et al found a numerically higher mortality rate with tocilizumab (39). The cohort studies by Roumier et al and Klopfenstein et al showed a significantly lower rate of escalation of care to mechanical ventilation, while the cohort study by Quartuccio et al described a lower rate of "complete" recovery among tocilizumab users. In the study by Roumier et al, there were some concerns regarding risk of bias, and the studies by Quartuccio et al and Klopfenstein et al both had a high risk of bias.

Table 3. Studies investigating GCs and COVID-19 (n = 14)*

Outcome measure, author (ref.)	Study design	n	Outcome and inference	Bias assessment†	Direction of effect‡
Mortality					
Fadel et al (42)	Cohort	213	Lower mortality with early GC protocol (14% vs. 26%; *P* = 0.024; OR 0.5 [95% CI 0.2, 0.9])	Some	+
Lu et al (77)	Cohort	244	No difference in mortality (adjusted HR 1.1 [95% CI 0.2, 7.4])	Some	–
Wu et al (78)	Cohort	201	Reduced mortality in patients with ARDS (HR 0.38 [95% CI 0.2, 0.7])	Some	+
Shi et al (79)	Cohort	101	No difference in mortality at 3 days (51% survived vs. 35% died; *P* = 0.12)	High	+
Liu et al (49)	Cohort	109	No difference in survival (*P* = 0.56; effect not available)	High	–
Qi et al (51)	Cohort	21	In people with cirrhosis, lower rate of GC use in survivors (3 of 16 [19%]) vs. nonsurvivors (5 of 5 [100%])	High	–
Wang et al (41)	Cohort	46	No difference in mortality with methylprednisolone (7.7% vs. 5.0% SoC; *P* = 0.71)	High	–
Jacobs et al (80)	Cohort	221	No association with GCs and ICU mortality (9.5 days vs. 11.0 days discharge; *P* = 0.21)	High	–
Cao et al (50)	Cohort	102	No difference in GCs among survivors (47%) and nonsurvivors (65%) (*P* = 0.18)	High	–
Composite of intubation and death					
Wang et al (40)	Cohort	115	No difference in ICU admission or mortality (OR 2.2 [95% CI 0.5, 9.4])	High	–
Escalation of care					
Fadel et al (42)	Cohort	213	Lower progression to IMV with early GC protocol (22% vs. 37%; *P* = 0.025)	Some	+
Wang et al (41)	Cohort	46	Lower rate of ventilation in methylprednisolone group (12% vs. 35% SoC; *P* = 0.05)	High	+
Hospital/ICU discharge					
Fadel et al (42)	Cohort	213	No difference in hospital discharge (67% vs. 62%; *P* = 0.58)	Some	–
Wang et al (41)	Cohort	46	Shorter hospitalization in methylprednisolone group (14 days [IQR 11–6] vs. 22 days [IQR 18–26]; *P* < 0.001)	High	+
SARS–CoV-2 clearance					
Chen et al (44)	Cohort	25	No difference in viral clearance (43% clearance vs. 73% no clearance; *P* = 0.23)	High	–
Fang et al (45)	Cohort	78	No change in time to viral clearance (17.6 ± 4.9 days vs. 18.7 ± 7.7 days with no GCs)	High	+
Ling et al (43)	Cohort	66	Longer time to viral clearance (15 days vs. 8 days; *P* = 0.01)	High	–
Chen et al (81)	Case series	97	No difference in time to negative conversion (10.0 days vs 10.0 days; *P* > 0.05)	High	NA

* Escalation of care included ICU transfer, intubation, and mechanical ventilation. GCs = glucocorticoids; ARDS = acute respiratory distress syndrome; IQR = interquartile range (see Table 1 for other definitions).
† Bias assessed using the Newcastle-Ottawa Scale; case series assumed to be high-risk by default.
‡ Quantified using the Cochrane vote counting method for data synthesis. Studies eligible for quantitative synthesis and case series were excluded.

Glucocorticoids. Fourteen studies assessed glucocorticoid use, including 13 cohort studies and 1 case series (Table 3). Nine cohort studies evaluated mortality and glucocorticoids. There was variability regarding timing of glucocorticoid use and COVID-19 disease severity. Based on vote counting, the direction of effect was positive in one-third of the studies and negative in the remaining two-thirds. One cohort study by Wang et al showed no difference in a composite outcome of ICU admission or mortality (40). Two cohort studies both demonstrated a lower rate of escalation of care (41,42). The study by Wang et al (41) showed a shorter hospitalization time with methylprednisolone, but the cohort study by Fadel (42) et al did not. Three cohort studies evaluated SARS–CoV-2 clearance with glucocorticoids. One study showed a significantly increased time to viral clearance (43), and 2 studies showed no significant difference (44,45). Eleven of the 14 studies had a high risk of bias.

Anakinra. Three studies assessed the IL-1 inhibitor anakinra, including 2 cohort studies and 1 case series (Table 4). The 2 cohort studies (pooled n = 141) evaluated mortality and were included in the quantitative analysis (46,47). Anakinra was associated with a significantly lower risk of mortality (pooled HR 0.25 [95% CI 0.12, 0.52]), compared to the standard of care (Figure 2). Huet et al (46) also

Table 4. Studies investigating other antirheumatic therapies and COVID-19 (n = 3 for anakinra, n = 4 for IVIG, and n = 1 for baricitinib)*

Medication, outcome measure, author (ref.)	Study design	n	Outcome and inference	Bias assessment†	Direction of effect‡
Anakinra					
Mortality					
Huet et al (46)	Cohort	96	Anakinra associated with lower rate of death (HR 0.3 [95% CI 0.1, 0.7])	Some	QS
Cavalli et al (47)	Cohort	52	High-dose anakinra (5 mg/kg BID) associated with lower mortality at 21 days (HR 0.2 [95% CI 0.04, 0.63])	High	QS
Composite of intubation and death					
Huet et al (46)	Cohort	96	Anakinra associated with lower rate of composite IMV/death (HR 0.2 [95% CI 0.1, 0.5])	Some	+
Escalation of care					
Huet et al (46)	Cohort	96	Anakinra associated with lower rate of invasive mechanical ventilation (HR 0.2 [95% CI 0.1, 0.6])	Some	+
Cavalli et al (47)	Cohort	52	No difference in high-dose anakinra and IMV-free survival at 21 days (HR 0.5 [95% CI 0.2, 1.3])	High	+
Clinical improvement					
Aouba et al (82)	Case series	9	9 of 9 patients treated with anakinra improved	High	NA
IVIG					
Mortality					
Shao et al (48)	Cohort	325	Lower 60-day mortality with IVIG (HR 0.3 [95% CI 0.1, 0.6])	Some	+
Liu et al (49)	Cohort	109	No difference in survival with IVIG (P = 0.51; effect not available)	High	–
Qi et al (51)	Cohort	21	No difference in survival with IVIG (P = 0.063)	High	–
Cao et al (50)	Cohort	102	No difference in IVIG among survivors (6%) and nonsurvivors (0%) (P = 0.68)	High	+
Baricitinib					
Escalation of care					
Cantini et al (52)	Cohort	24	No difference in ICU transfer at week 2 with baricitinib (0% vs. 33% SoC; P = 0.09)	High	+
Hospital/ICU discharge					
Cantini et al (52)	Cohort	24	Higher rate of discharge at week 2 with baricitinib (58% vs. 8% SoC; P = 0.03)	High	+

* Escalation of care included ICU transfer, intubation, and mechanical ventilation. IVIG = intravenous immunoglobulin; BID = twice daily (see Table 1 for other definitions).
† Bias assessed using the Newcastle-Ottawa Scale; case series assumed to be high-risk by default.
‡ Quantified using the Cochrane vote counting method for data synthesis. Studies eligible for quantitative synthesis and case series were excluded.

found a lower rate of a composite end point of mechanical ventilation or death, but Cavalli and colleagues (47) did not find a difference with regard to ventilator-free survival at 21 days. The study by Cavalli et al had a high risk of bias, while there were some concerns related to the risk of bias in the study by Huet et al.

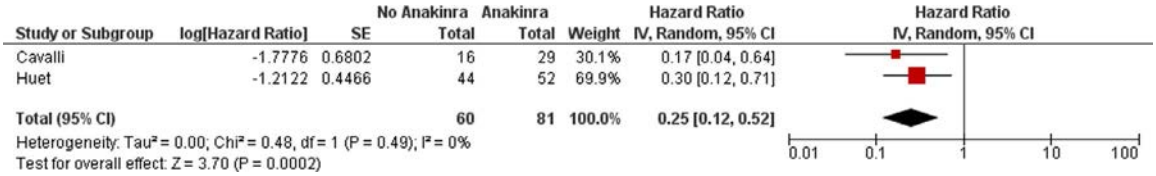


Figure 2. Meta-analysis of 2 observational studies investigating anakinra and mortality among patients hospitalized with COVID-19. See Figure 1 for definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41481/abstract.

Intravenous immunoglobulin (IVIG). Four cohort studies evaluated mortality and the use of IVIG (Table 4). One study demonstrated a lower risk of mortality at 60 days with IVIG, while 2 other cohorts demonstrated no difference in survival (48–50). In a study of patients with cirrhosis and COVID-19, there was no difference in mortality between patients receiving and those not receiving IVIG (51). The direction of effect was split evenly according to vote counting. There were some concerns pertaining to the risk of bias in the cohort study by Shao et al, and the other 3 studies had a high risk of bias.

Baricitinib. One cohort study with a high risk of bias showed no significant difference in ICU transfer at 2 weeks, but there was higher rate of discharge at week 2 among patients who received baricitinib (52) (Table 4).

DISCUSSION

In this systematic review and meta-analysis of antirheumatic disease therapies for the treatment of COVID-19, the use of HCQ was not associated with mortality. The effects of other antirheumatic disease therapies were frequently contradictory with respect to mortality, escalation of care, discharge, clinical improvement, and SARS–CoV-2 clearance. This may reflect important limitations of the included studies, the majority of which had small sample sizes and inadequate or absent comparator groups. Many also relied upon viral clearance as their primary outcome measure, a surrogate measure that may not be clinically relevant. These results extend recent systematic reviews of HCQ (13,14) to a broader range of antirheumatic disease therapies and complement guidance from the American College of Rheumatology that focused on patients with rheumatic diseases (53).

Despite limitations of the available evidence, patterns have begun to emerge. Contrary to early enthusiasm for HCQ (1,4), in this meta-analysis, HCQ use was not associated with a mortality benefit in people with COVID-19. These findings are consistent with general observations from another systematic review (13) and from a recently published RCT that assessed postexposure prophylaxis (54). In contrast to reported findings from a now-retracted study by Mehra et al (6,7), HCQ use was not associated with increased mortality. This may reassure patients with rheumatic diseases, who were understandably concerned about taking HCQ after these apparently unverifiable data were published. Definitive data from large randomized trials are expected to be published soon, including the National Institutes of Health–sponsored ORCHID trial, the RECOVERY trial from the UK, and the World Health Organization Solidarity trial. All 3 trials recently halted enrollment and have shown a lack of benefit as reported in press releases (55–57). Overall, our findings and other data support a growing consensus that antimalarial therapies for COVID-19 should be limited to use in ongoing clinical trials (58,59).

Therapies that target the hyperinflammatory state of COVID-19, including IL-1 and IL-6 inhibitors, have been widely used despite a relative paucity of data. Results from our meta-analysis of 2 studies showed an association between anakinra and lower mortality, but this should be interpreted with caution. One study did not adequately control for confounders, and the other study used a historical cohort as a comparator group (46,47). Neither study provided adequate evidence to support widespread use of drugs inhibiting IL-1 for treatment of COVID-19, which must await high-quality evidence from ongoing RCTs. The available data for IL-6 inhibition were similarly limited. Few studies of IL-6 inhibitors used an adequate comparator, and the results of IL-6 inhibitor studies were frequently conflicting. It should be noted that both IL-1 and IL-6 inhibitors were typically used for patients with moderate-to-severe acute respiratory distress syndrome. Selection bias, publication bias, and confounding by indication may have influenced purported associations. Press releases from ongoing RCTs have been encouraging, but peer-reviewed data will be essential in determining the role of these therapies.

Glucocorticoids have also been widely used in hospitalized patients with COVID-19. As with IL-1 and IL-6 inhibitors, they typically have been reserved for patients with moderate-to-severe disease, likely biasing risk estimates. Overall, no definitive conclusions could be drawn from our data synthesis. Small studies with inadequate or absent comparator groups generally suggested no difference with regard to mortality. Those that included a comparator had conflicting findings, and none were assessed as having a low risk of bias. After the final date of our search, preliminary findings from the adaptive RECOVERY trial, which assessed dexamethasone in hospitalized patients with COVID-19, were published (60). The RECOVERY trial was well designed and showed a significant reduction in mortality at 28 days in patients randomized to receive open-label dexamethasone as opposed to usual care (age-adjusted rate ratio 0.83 [95% CI 0.74, 0.92]). These data support current recommendations for prescribing glucocorticoids in a select group of patients with COVID-19 (58,61,62).

IVIG and baricitinib have also been studied. One study with an inadequate comparator showed an association between IVIG use and lower mortality at 60 days. Only 1 small cohort study with a high risk of bias evaluated baricitinib. It demonstrated no difference with respect to escalation of care, but patients who received baricitinib were more likely to be discharged at 2 weeks. Although it did not meet inclusion criteria, we identified 1 case series of eculizumab use in 4 patients (63), all of whom recovered.

Our search did not identify any studies as of May 29, 2020 that evaluated other antirheumatic disease therapies, such as colchicine or TNF inhibitors. Clinical trials are underway to further assess IVIG, baricitinib, and eculizumab, among others (63–65).

Strengths of this review were a rigorous application of systematic review methodology and a comprehensive search of the literature, which included published and preprint archives

in all languages. Another strength was the inclusion of patients with rheumatic diseases and patients with COVID-19 in the review process. In fact, several members of the review team contracted COVID-19 during the execution of this review.

Our study also had a number of limitations. First, the COVID-19 literature has rapidly expanded and indexing may be delayed, which makes performing a systematic review difficult. At the time of this writing (June 10, 2020), we are not aware of any consequential publications that have been missed. Second, although we used validated risk of bias assessments with 2 reviewers working in parallel, such judgments may be open to interpretation, and use of other validated tools may have led to different conclusions. Third, all of the observational data came from hospitalized patients and may not be generalizable to a broader population. This highlights an important limitation of the literature itself, as we found no studies of outpatients infected with COVID-19 who received antirheumatic disease therapies. Finally, the degree to which publication bias has influenced the current literature was not assessed, but preprint archives were included to mitigate such biases.

These limitations notwithstanding, this comprehensive systematic review and meta-analysis suggests that HCQ use is not associated with benefit or harm with regard to COVID-19 mortality. Antirheumatic disease therapies should be investigated further in RCTs. In the interim, physicians should be cautious in offering off-label antirheumatic disease therapies to patients with COVID-19 based on the currently available literature.

ACKNOWLEDGMENTS

The authors would like to acknowledge the American College of Rheumatology, Amy Turner, and Regina Parker for their logistical support.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Putman had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Putman, Chock, Tam, Kim, Sattui, Berenbaum, Danila, Korsten, Sanchez-Alvarez, Sparks, Coates, Palmerlee, Peirce, Jayatilleke, Johnson, Kilian, Liew, Prokop, Grainger, Wallace, Duarte-García.

Acquisition of data. Putman, Chock, Tam, Kim, Sattui, Berenbaum, Danila, Korsten, Sanchez-Alvarez, Sparks, Duarte-García.

Analysis and interpretation of data. Putman, Chock, Tam, Kim, Sattui, Berenbaum, Danila, Korsten, Sanchez-Alvarez, Sparks, Jayatilleke, Johnson, Kilian, Liew, Murad, Grainger, Wallace, Duarte-García.

REFERENCES

1. Kim AH, Sparks JA, Liew JW, Putman MS, Berenbaum F, Duarte-García A, et al. A rush to judgment? Rapid reporting and dissemination of results and its consequences regarding the use of hydroxychloroquine for COVID-19 [editorial]. Ann Intern Med 2020;172:819–21.
2. Liu J, Cao R, Xu M, Wang X, Zhang H, Hu H, et al. Hydroxychloroquine, a less toxic derivative of chloroquine, is effective in inhibiting SARS-CoV-2 infection in vitro [letter]. Cell Discov 2020;6:16.
3. Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Mailhe M, et al. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. Int J Antimicrob Agents 2020;56:105949.
4. Graef ER, Liew JW, Putman MS, Simard JF, Sirotich E, Berenbaum F, et al. Festina lente: hydroxychloroquine, COVID-19 and the role of the rheumatologist. Ann Rheum Dis 2020;79:734–6.
5. Mendel A, Bernatsky S, Thorne JC, Lacaille D, Johnson SR, Vinet É. Hydroxychloroquine shortages during the COVID-19 pandemic. Ann Rheum Dis 2020. E-pub ahead of print.
6. Mehra MR, Desai SS, Ruschitzka F, Patel AN. Hydroxychloroquine or chloroquine with or without a macrolide for treatment of COVID-19: a multinational registry analysis [article retracted in Lancet 2020;395:1820]. Lancet 2020. E-pub ahead of print.
7. Mehra MR, Ruschitzka F, Patel AN. Retraction—Hydroxychloroquine or chloroquine with or without a macrolide for treatment of COVID-19: a multinational registry analysis. Lancet 2020;395:1820.
8. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ, et al. COVID-19: consider cytokine storm syndromes and immunosuppression [letter]. Lancet 2020;395:1033–4.
9. Moore JB, June CH. Cytokine release syndrome in severe COVID-19. Science 2020;368:473–4.
10. Feldmann M, Maini RN, Woody JN, Holgate ST, Winter G, Rowland M, et al. Trials of anti–tumour necrosis factor therapy for COVID-19 are urgently needed. Lancet 2020;395:1407–9.
11. Richardson P, Griffin I, Tucker C, Smith D, Oechsle O, Phelan A, et al. Baricitinib as potential treatment for 2019-nCoV acute respiratory disease [letter]. Lancet 2020;395:e30–1.
12. Tay MZ, Poh CM, Rénia L, MacAry PA, Ng LF. The trinity of COVID-19: immunity, inflammation and intervention [review]. Nat Rev Immunol 2020;20:363–74.
13. Hernandez AV, Roman YM, Pasupuleti V, Barboza JJ, White CM. Hydroxychloroquine or chloroquine for treatment or prophylaxis of COVID-19: a living systematic review. Ann Intern Med 2020. E-pub ahead of print.
14. Shah S, Das S, Jain A, Misra DP, Negi VS. A systematic review of the prophylactic role of chloroquine and hydroxychloroquine in coronavirus disease-19 (COVID-19). Int J Rheum Dis 2020;23:613–9.
15. Higgins JP, Thomas J, Chandler J, Cumpston M, Li T, Page M, et al, editors. Cochrane handbook for systematic reviews of interventions: version 6.0, 2019. July 2019. URL: www.training.cochrane.org/handbook.
16. Moher D, Liberati A, Tetzlaff J, Altman DG, for the PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. J Clin Epidemiol 2009;62:1006–12.
17. Campbell M, McKenzie JE, Sowden A, Katikireddi SV, Brennan SE, Ellis S, et al. Synthesis without meta-analysis (SWiM) in systematic reviews: reporting guideline. BMJ 2020;368:l6890.
18. NIHR. PROSPERO international prospective register of systematic reviews. Preclinical and clinical outcomes of rheumatic disease therapy in patients with COVID-19 infection: a systematic review. April 2020. URL: https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=176896.
19. Sterne JA, Savović J, Page MJ, Elbers RG, Blencowe NS, Boutron I, et al. RoB 2: a revised tool for assessing risk of bias in randomised trials. BMJ 2019;366:l4898.
20. Wells G, Shea B, O'Connell D, Peterson J, Welch V, Losos M, et al. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses. URL: http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp.
21. Viswanathan M, Patnode CD, Berkman ND, Bass EB, Chang S, Hartling L, et al. Recommendations for assessing the risk of bias in systematic reviews of health-care interventions. J Clin Epidemiol 2018;97:26–34.

22. Dekkers OM, Egger M, Altman DG, Vandenbroucke JP. Distinguishing case series from cohort studies. Ann Intern Med 2012;156:37–40.
23. Mathes T, Pieper D. Clarifying the distinction between case series and cohort studies in systematic reviews of comparative studies: potential impact on body of evidence and workload. BMC Med Res Methodol 2017;17:107.
24. DerSimonian R, Laird N. Meta-analysis in clinical trials. Control Clin Trials 1986;7:177–88.
25. McKenzie JE, Brennan S. Synthesizing and presenting findings using other methods. In: Higgins JP, Thomas J, Chandler J, Cumpston M, Li T, Page MJ, et al, editors. Cochrane handbook for systematic reviews of interventions version 6.0 2019. URL: https://training.cochrane.org/handbook/current/chapter-12.
26. Rosenberg ES, Dufort EM, Udo T, Wilberschied LA, Kumar J, Tesoriero J, et al. Association of treatment with hydroxychloroquine or azithromycin with in-hospital mortality in patients with COVID-19 in New York State. JAMA 2020;323:2493–502.
27. Magagnoli J, Narendran S, Pereira F, Cummings T, Hardin JW, Sutton SS, et al. Outcomes of hydroxychloroquine usage in United States veterans hospitalized with COVID-19. medRxiv 2020. E-pub ahead of print.
28. Mahévas M, Tran VT, Roumier M, Chabrol A, Paule R, Guillaud C, et al. Clinical efficacy of hydroxychloroquine in patients with COVID-19 pneumonia who require oxygen: observational comparative study using routine care data. BMJ 2020;369:m1844.
29. Geleris J, Sun Y, Platt J, Zucker J, Baldwin M, Hripcsak G, et al. Observational study of hydroxychloroquine in hospitalized patients with COVID-19. N Engl J Med 2020;382:2411–8.
30. Tang W, Cao Z, Han M, Wang Z, Chen J, Sun W, et al. Hydroxychloroquine in patients with mainly mild to moderate coronavirus disease 2019: open label, randomised controlled trial. BMJ 2020;369:m1849.
31. Chen Z, Hu J, Zhang Z, Jiang S, Han S, Yan D, et al. Efficacy of hydroxychloroquine in patients with COVID-19: results of a randomized clinical trial. medRxiv 2020. E-pub ahead of print.
32. Mallat J, Hamed F, Balkis M, Mohamed MA, Mooty M, Malik A, et al. Hydroxychloroquine is associated with slower viral clearance in clinical COVID-19 patients with mild to moderate disease: a retrospective study. medRxiv 2020. E-pub ahead of print.
33. Borba MGS, Val FFA, Sampaio VS, Alexandre MAA, Melo GC, Brito M, et al. Effect of high vs low doses of chloroquine diphosphate as adjunctive therapy for patients hospitalized with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection: a randomized clinical trial. JAMA Netw Open 2020;3:e208857.
34. Huang M, Tang T, Pang P, Li M, Ma R, Lu J, et al. Treating COVID-19 with chloroquine. J Mol Cell Biol 2020;12:322–5.
35. Huang M, Li M, Xiao F, Pang P, Liang J, Tang T, et al. Preliminary evidence from a multicenter prospective observational study of the safety and efficacy of chloroquine for the treatment of COVID-19 [review]. Nat Sci Rev 2020. E-pub ahead of print.
36. Chen X, Zhang Y, Zhu B, Zeng J, Hong W, He X, et al. Associations of clinical characteristics and antiviral drugs with viral RNA clearance in patients with COVID-19 in Guangzhou, China: a retrospective cohort study. April 2020. URL: http://medrxiv.org/lookup/doi/10.1101/2020.04.09.20058941.
37. Roumier M, Paule R, Groh M, Vallee A, Ackermann F. Interleukin-6 blockade for severe COVID-19. April 2020. URL: http://medrxiv.org/lookup/doi/10.1101/2020.04.20.20061861.
38. Klopfenstein T, Zayet S, Lohse A, Balblanc JC, Badie J, Royer PY, et al. Tocilizumab therapy reduced intensive care unit admissions and/or mortality in COVID-19 patients. Med Mals Infect 2020;50:397–400.
39. Quartuccio L, Sonaglia A, McGonagle D, Fabris M, Peghin M, Pecori D, et al. Profiling COVID-19 pneumonia progressing into the cytokine storm syndrome: results from a single Italian Centre study on tocilizumab versus standard of care. J Clin Virol 2020. E-pub ahead of print.
40. Wang D, Wang J, Jiang Q, Yang J, Li J, Gao C, et al. No clear benefit to the use of corticosteroid as treatment in adult patients with coronavirus disease 2019: a retrospective cohort study. April 2020. URL: http://medrxiv.org/lookup/doi/10.1101/2020.04.21.20066258.
41. Wang Y, Jiang W, He Q, Wang C, Wang B, Zhou P, et al. A retrospective cohort study of methylprednisolone therapy in severe patients with COVID-19 pneumonia [letter]. Sig Transduct Target Ther 2020;5:57.
42. Fadel R, Morrison A, Vahia A, Smith ZR, Chaudhry Z, Bhargava P, et al. Early short course corticosteroids in hospitalized patients with COVID-19. May 2020. URL: https://www.medrxiv.org/content/10.1101/2020.05.04.20074609v1.
43. Ling Y, Xu SB, Lin YX, Tian D, Zhu ZQ, Dai FH, et al. Persistence and clearance of viral RNA in 2019 novel coronavirus disease rehabilitation patients. Chin Med J (Engl) 2020;133:1039–43.
44. Chen X, Ling J, Mo P, Zhang Y, Jiang Q, Ma Z, et al. Restoration of leukomonocyte counts is associated with viral clearance in COVID-19 hospitalized patients. March 2020. URL: http://medrxiv.org/lookup/doi/10.1101/2020.03.03.20030437.
45. Fang X, Mei Q, Yang T, Li L, Wang Y, Tong F, et al. Low-dose corticosteroid therapy does not delay viral clearance in patients with COVID-19 [letter]. J Infect 2020;81:147–78.
46. Huet T, Beaussier H, Voisin O, Jouveshomme S, Dauriat G, Lazareth I, et al. Anakinra for severe forms of COVID-19: a cohort study. Lancet Rheumatol 2020. E-pub ahead of print.
47. Cavalli G, de Luca G, Campochiaro C, Della-Torre E, Ripa M, Canetti D, et al. Interleukin-1 blockade with high-dose anakinra in patients with COVID-19, acute respiratory distress syndrome, and hyperinflammation: a retrospective cohort study. Lancet Rheumatol 2020;2:e325–31.
48. Shao Z, Feng Y, Zhong L, Xie Q, Lei M, Liu Z, et al. Clinical efficacy of intravenous immunoglobulin therapy in critical patients with COVID-19: a multicenter retrospective cohort study. April 2020. URL: medRxiv 2020:2020.04.11.20061739.
49. Liu Y, Sun W, Li J, Chen L, Wang Y, Zhang L, et al. Clinical features and progression of acute respiratory distress syndrome in coronavirus disease 2019. February 2020. URL: http://medrxiv.org/lookup/doi/10.1101/2020.02.17.20024166.
50. Cao J, Tu WJ, Cheng W, Yu L, Liu YK, Hu X, et al. Clinical features and short-term outcomes of 102 patients with coronavirus disease 2019 in Wuhan, China. Clin Infect Dis 2020;71:748–55.
51. Qi X, Liu Y, Wang J, Fallowfield JA, Wang J, Li X, et al. Clinical course and risk factors for mortality of COVID-19 patients with pre-existing cirrhosis: a multicentre cohort study. Gut 2020. E-pub ahead of print.
52. Cantini F, Niccoli L, Matarrese D, Nicastri E, Stobbione P, Goletti D. Baricitinib therapy in COVID-19: a pilot study on safety and clinical impact [letter]. J Infect 2020;81:318–56.
53. Mikuls TR, Johnson SR, Fraenkel L, Arasaratnam RJ, Baden LR, Bermas BL, et al. American College of Rheumatology guidance for the management of rheumatic diseases in adult patients during the COVID-19 pandemic: version 1. Arthritis Rheumatol 2020;72:1241–51.
54. Boulware DR, Pullen MF, Bangdiwala AS, Pastick KA, Lofgren SM, Okafor EC, et al. A randomized trial of hydroxychloroquine as postexposure prophylaxis for COVID-19. N Engl J Med 2020. E-pub ahead of print.
55. RECOVERY. Randomised evaluation of COVID-19 therapy. No clinical benefit from use of hydroxychloroquine in hospitalised patients with COVID-19. June 2020. URL: https://www.recoverytr

ial.net/news/statement-from-the-chief-investigators-of-the-randomised-evaluation-of-covid-19-therapy-recovery-trial-on-hydroxychloroquine-5-june-2020-no-clinical-benefit-from-use-of-hydroxychloroquine-in-hospitalised-patients-with-covid-19.

56. World Health Organization. WHO discontinues hydroxychloroquine and lopinavir/ritonavir treatment arms for COVID-19. July 2020. URL: https://www.who.int/news-room/detail/04-07-2020-who-discontinues-hydroxychloroquine-and-lopinavir-ritonavir-treatment-arms-for-covid-19.
57. National Institutes of Health. News releases. NIH halts clinical trial of hydroxychloroquine. June 2020. URL: https://www.nih.gov/news-events/news-releases/nih-halts-clinical-trial-hydroxychloroquine.
58. Bhimraj A, Morgan RL, Shumaker AH, Lavergne V, Baden L, Cheng VC, et al. Infectious Diseases Society of America guidelines on the treatment and management of patients with COVID-19. Clin Infect Dis 2020. E-pub ahead of print.
59. RECOVERY. Randomised evaluation of COVID-19 therapy. No clinical benefit from use of hydroxychloroquine in hospitalised patients with COVID-19. June 2020. URL: https://www.recoverytrial.net/news/statement-from-the-chief-investigators-of-the-randomised-evaluation-of-covid-19-therapy-recovery-trial-on-hydroxychloroquine-5-june-2020-no-clinical-benefit-from-use-of-hydroxychloroquine-in-hospitalised-patients-with-covid-19.
60. Horby P, Lim WS, Emberson J, Mafham M, Bell J, Linsell L, et al, for the RECOVERY Collaborative Group. Dexamethasone in hospitalized patients with COVID-19: preliminary report. N Engl J Med 2020. E-pub ahead of print.
61. Alhazzani W, Møller MH, Arabi YM, Loeb M, Gong MN, Fan E, et al. Surviving sepsis campaign: guidelines on the management of critically ill adults with coronavirus disease 2019 (COVID-19). Crit Care Med 2020;48:e440–69.
62. Poston JT, Patel BK, Davis AM. Management of critically ill adults with COVID-19. JAMA 2020. E-pub ahead of print.
63. Diurno F, Numis FG, Porta G, Cirillo F, Maddaluno S, Ragozzino A, et al. Eculizumab treatment in patients with COVID-19: preliminary results from real life ASL Napoli 2 Nord experience. Eur Rev Med Pharmacol Sci 2020;24:4040–7.
64. ClinicalTrials.gov. National Library of Medicine. Eculizumab (Soliris) in COVID-19 infected patients (SOLID-C19). March 2020. URL: https://clinicaltrials.gov/ct2/show/NCT04288713.
65. ClinicalTrials.gov. National Library of Medicine. Octagam 10% therapy in COVID-19 patients with severe disease progression. August 2020. URL: https://clinicaltrials.gov/ct2/show/NCT04400058.
66. Yu B, Wang DW, Li C. Hydroxychloroquine application is associated with a decreased mortality in critically ill patients with COVID-19. May 2020. URL: http://medrxiv.org/lookup/doi/10.1101/2020.04.27.20073379.
67. Ashraf MA, Shokouhi N, Memar O, Sanginabadi M, Ghaderkhani S. COVID-19 in Iran, a comprehensive investigation from exposure to treatment outcomes. April 2020. URL: https://www.medrxiv.org/content/10.1101/2020.04.20.20072421v1.
68. Mathian A, Mahévas M, Rohmer J, Roumier M, Cohen-Aubert F, Amador-Borrero B, et al. Clinical course of coronavirus disease 2019 (COVID-19) in a series of 17 patients with systemic lupus erythematosus under long-term treatment with hydroxychloroquine [letter]. Ann Rheum Dis 2020;79:837–9.
69. Molina JM, Delaugerre C, le Goff J, Mela-Lima B, Ponscarme D, Goldwirt L, et al. No evidence of rapid antiviral clearance or clinical benefit with the combination of hydroxychloroquine and azithromycin in patients with severe COVID-19 infection [letter]. Med Mal Infect 2020;50:384.
70. Million M, Lagier JC, Gautret P, Colson P, Fournier PE, Amrane S, et al. Early treatment of COVID-19 patients with hydroxychloroquine and azithromycin: a retrospective analysis of 1061 cases in Marseille, France. Travel Med Infect Dis 2020:101738.
71. Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Sevestre J, et al. Clinical and microbiological effect of a combination of hydroxychloroquine and azithromycin in 80 COVID-19 patients with at least a six-day follow up: a pilot observational study. Travel Med Infect Dis 2020;34:101663.
72. Sciascia S, Aprà F, Baffa A, Baldovino S, Boaro D, Boero R, et al. Pilot prospective open, single-arm multicentre study on off-label use of tocilizumab in patients with severe COVID-19. Clin Exp Rheumatol 2020;38:529–32.
73. Luo P, Liu Y, Qiu L, Liu X, Liu D, Li J. Tocilizumab treatment in COVID-19: a single center experience. J Med Virol 2020;92:814–8.
74. Alattar R, Ibrahim TB, Shaar SH, Abdalla S, Shukri K, Daghfal JN, et al. Tocilizumab for the treatment of severe coronavirus disease 2019. J Med Virol 2020. E-pub ahead of print.
75. Gritti G, Raimondi F, Ripamonti D, Riva I, Landi F, Alborghetti L, et al. Use of siltuximab in patients with COVID-19 pneumonia requiring ventilatory support. June 2020. URL: http://medrxiv.org/lookup/doi/10.1101/2020.04.01.20048561.
76. Xu X, Han M, Li T, Sun W, Wang D, Fu B, et al. Effective treatment of severe COVID-19 patients with tocilizumab. Proc Natl Acad Sci U S A 2020;117:10970–5.
77. Lu X, Chen T, Wang Y, Wang J, Yan F. Adjuvant corticosteroid therapy for critically ill patients with COVID-19 [letter]. Crit Care 2020;24:241.
78. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. JAMA Intern Med 2020. E-pub ahead of print.
89. Shi Q, Zhao K, Yu J, Jiang F, Feng J, Zhao K, et al. Clinical characteristics of 101 COVID-19 nonsurvivors in Wuhan, China: a retrospective study. May 2020. URL: http://medrxiv.org/lookup/doi/10.1101/2020.03.04.20031039.
80. Jacobs JP, Stammers AH, St. Louis J, Hayanga JW, Firstenberg MS, Mongero LB, et al. Extracorporeal membrane oxygenation in the treatment of severe pulmonary and cardiac compromise in COVID-19: experience with 32 patients. ASAIO J 2020;66:722–30.
81. Chen M, Tu C, Tan C, Zheng X, Wang X, Wu J, et al. Key to successful treatment of COVID-19: accurate identification of severe risks and early intervention of disease progression. April 2020. URL: http://medrxiv.org/lookup/doi/10.1101/2020.04.06.20054890.
82. Aouba A, Baldolli A, Geffray L, Verdon R, Bergot E, Martin-Silva N, et al. Targeting the inflammatory cascade with anakinra in moderate to severe COVID-19 pneumonia: case series. Ann Rheum Dis 2020. E-pub ahead of print.

American College of Rheumatology
Empowering Rheumatology Professionals

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 48–52
DOI 10.1002/art.41475

BRIEF REPORT

Susceptibility to COVID-19 in Patients Treated With Antimalarials: A Population-Based Study in Emilia-Romagna, Northern Italy

Carlo Salvarani,[1] Pamela Mancuso,[2] Federica Gradellini,[2] Nilla Viani,[3] Paolo Pandolfi,[4] Massimo Reta,[4] Giuliano Carrozzi,[5] Gilda Sandri,[6] Gianluigi Bajocchi,[2] Elena Galli,[6] Francesco Muratore,[2] Luigi Boiardi,[2] Nicolò Pipitone,[2] Giulia Cassone,[6] Stefania Croci,[2] Anna Maria Marata,[7] Massimo Costantini,[2] and Paolo Giorgi Rossi[2]

Objective. To evaluate the susceptibility to coronavirus disease 2019 (COVID-19) in patients with autoimmune conditions treated with antimalarials in a population-based study.

Methods. All residents treated with chloroquine (CQ)/hydroxychloroquine (HCQ) from July through December 2019 and living in 3 provinces of Regione Emilia-Romagna were identified by drug prescription registries and matched with the registry containing all residents living in the same areas who have had swabs and tested positive for severe acute respiratory syndrome coronavirus 2 (SARS–CoV-2). Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated.

Results. A total of 4,408 patients were identified. The prevalence of patients receiving antimalarials was 0.85 per 1,000 men and 3.3 per 1,000 women. The cumulative incidence of testing during the study period was 2.7% in the general population and 3.8% among those receiving CQ or HCQ, while the cumulative incidence of testing positive was 0.55% in the general population and 0.70% among those receiving CQ/HCQ. Multivariate models showed that those receiving CQ/HCQ had a slightly higher probability of being tested compared to the general population (OR 1.09 [95% CI 0.94–1.28]), the same probability of being diagnosed as having COVID-19 (OR 0.94 [95% CI 0.66–1.34]), and a slightly lower probability of being positive once tested (OR 0.83 [95% CI 0.56–1.23]). None of the differences were significant.

Conclusion. Our findings do not support the use of antimalarials as a prophylactic treatment of COVID-19.

INTRODUCTION

Given the increasingly widespread use of the antimalarial drugs chloroquine (CQ) and hydroxychloroquine (HCQ), not only as therapy but also as prophylaxis for coronavirus disease 2019 (COVID-19) (1–4), there is an immediate unmet need to obtain insights into their efficacy, particularly because of their potential toxicity (5).

Antimalarial drugs are well-known, disease-modifying antirheumatic drugs (DMARDs) used in the treatment of several autoimmune conditions such as rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), systemic lupus erythematosus (SLE), discoid lupus erythematosus (DLE), and other off-label uses including antiphospholipid syndrome and primary Sjögren's syndrome. In addition to their immunomodulatory capacity, these drugs protect patients with inflammatory rheumatic diseases against infection. For example, in SLE, the duration of antimalarial treatment is a protective factor against infections (6). Antimalarials have also been reported to inhibit severe acute respiratory syndrome

[1]Carlo Salvarani, MD: Azienda USL-IRCCS di Reggio Emilia and Università di Modena e Reggio Emilia, Reggio Emilia, Italy; [2]Pamela Mancuso, BS, Federica Gradellini, MD, Gianluigi Bajocchi, MD, Francesco Muratore, MD, Luigi Boiardi, MD, Nicolò Pipitone, MD, Stefania Croci, MB, Massimo Costantini, MD, Paolo Giorgi Rossi, MD: Azienda USL-IRCCS di Reggio Emilia, Reggio Emilia, Italy; [3]Nilla Viani, MD: Azienda USL di Modena and Azienda OU di Modena, Modena, Italy; [4]Paolo Pandolfi, MD, Massimo Reta, MD: Azienda USL di Bologna, Bologna, Italy; [5]Giuliano Carrozzi, MD: Azienda USL di Modena, Modena, Italy; [6]Gilda Sandri, MD, Elena Galli, MD, Giulia Cassone, MD: Università di Modena e Reggio Emilia, Modena, Italy; [7]Anna Maria Marata, MD: Regione Emilia Romagna, Bologna, Italy.

No potential conflicts of interest relevant to this article were reported.

Address correspondence to Carlo Salvarani, MD, Unità Operativa di Reumatologia, Azienda Ospedaliera-IRCCS di Reggio Emilia, Viale Risorgimento 80, 42100 Reggio Emilia, Italy. Email: salvarani.carlo@ausl.re.it.

Submitted for publication June 2, 2020; accepted in revised form July 30, 2020.

coronavirus 2 (SARS–CoV-2) in vitro (7,8). Therefore, because of their immunomodulatory and antiviral effects, these drugs have been proposed to be repurposed not only for the treatment of COVID-19, but also for the primary prophylaxis in healthy subjects living in highest risk areas.

Patients with autoimmune conditions who received long-term treatment with antimalarials before the onset of SARS–CoV-2 infection, potentially represent the best candidates to test the efficacy of these drugs in preventing symptomatic COVID-19 (9,10). In these patients, CQ and HCQ accumulate at the cell and tissue level, including in the lungs, where they may exert an antiviral effect, although it is unclear whether such antiviral action may be achieved using the standard therapeutic doses of antimalarials (7,8,11). We decided to evaluate, in a population-based study, the risk of COVID-19 in patients treated with antimalarials before the start of the infection in a large geographic area (3 provinces of Emilia-Romagna) with a high rate of spread of COVID-19.

PATIENTS AND METHODS

Study population. The 3 provinces included in the catchment areas (Bologna, Modena, and Reggio Emilia) have 2,251,903 residents. We identified all resident populations who had been prescribed CQ or HCQ during the period from July 1 through December 31, 2019, via the local drug prescription registries. The database is updated every 3 months. Those receiving CQ or HCQ were cross-referenced with the archive of residents who had oral nasopharyngeal swabs for SARS–CoV-2 reverse transcriptase–polymerase chain reaction (RT-PCR) testing and with the COVID-19 registry. All residents in the study areas who have had oral nasopharyngeal swabs since February 21, 2020, the date of diagnosis of the first COVID-19 case in Italy, are registered in a local registry. Those who tested positive were included in the COVID-19 registry, with data collected at the local level and gathered at the national level (12,13).

With a few exceptions, swabs were performed only in symptomatic subjects. Therefore, all patients included in the COVID-19 registry are considered to be COVID-19 patients. Initially, only patients who had contact with other SARS–Cov-2 patients were tested, but after the second week of the outbreak, all patients with symptoms compatible with COVID-19 were tested with RT-PCR on oral nasopharyngeal samples.

A fiscal code (a government-issued identification number used in Italy) was used to identify and match patients treated with antimalarial agents and those with COVID-19 infection. We used data updated on May 13, 2020. In Emilia-Romagna, the epidemic curve peaked in the last third of March and then decreased. At the end of the study period, the cumulative incidence of COVID-19 in the general population was 0.48%, 0.54%, and 0.9%, in Bologna, Modena, and Reggio Emilia, respectively.

The study was approved by the Reggio Emilia Provincial Ethics Committee, and all participants and their relatives provided informed consent. Approval was obtained on July 4, 2020 (no. 2020/0045199).

Statistical analysis. We identified age- and sex-specific cumulative rates of being tested and of testing positive in the general population and in patients who received CQ or HCQ during the second half of 2019, with odds ratios (ORs) and 95% confidence intervals (95% CIs) calculated. Multivariate logistic regression models were used to evaluate whether treatment, age classes, and sex increased the odds of being tested or having a positive test. We also identified the probability of being positive once tested.

RESULTS

The drug prescription databases indicated that 4,408 patients had at least 1 prescription for CQ or HCQ during the second half of 2019. Their mean ± SD age was 62.4 ± 18.2 years, and 80.2% were women. The median number of packs per patient (each pack containing 30 tablets) was 6 (interquartile range 4–9). Only 3.6% of the patients were prescribed only 1 pack of tablets during the period. CQ and HCQ were mainly prescribed for their approved indications, i.e., RA, JIA, SLE, and DLE.

The prevalence of individuals receiving CQ or HCQ was 0.85 per 1,000 men and 3.3 per 1,000 women, with no differences between provinces. Prevalence increased with age until 80–89 years, when it reached 2.7 per 1,000 men and 6.1 per 1,000 women. After age 90 years, the prevalence of receiving CQ or HCQ decreased, at least among women, to 3.8 per 1,000.

The cumulative incidence of being tested during the study period was 2.7% in the general population and 3.8% among those receiving CQ or HCQ. Age- and sex-specific rates did not differ between those who were receiving CQ or HCQ and those who were not (Table 1). The cumulative incidence of testing positive was 0.55% in the general population and 0.70% among those receiving CQ or HCQ.

Multivariate models confirmed that women were more frequently tested, while individuals younger than 40 years were less frequently tested. Among individuals ages 40–79 years, the probability of being tested was quite homogenous; it increased among older individuals (Table 2). Those receiving CQ or HCQ had a slightly higher probability (nonsignificant) of being tested compared to the general population (OR 1.09 [95% CI 0.94–1.28]).

The cumulative incidence of COVID-19 increased exponentially with age, with women showing a slightly higher incidence. Those receiving CQ or HCQ had almost the same probability of being diagnosed as having COVID-19 as the general population (OR 0.94 [95% CI 0.66–1.34]). The probability of being positive once tested was slightly, albeit nonsignificantly, lower among those receiving CQ or HCQ than in the general population (OR 0.83 [95% CI 0.56–1.23]).

Table 1. Cumulative incidence of testing for severe acute respiratory syndrome coronavirus 2 and of testing positive, by age, sex, and use of hydroxychloroquine or chloroquine

	Population, no.		Tested, no (%)		Tested positive, no. (%)	
	Men	Women	Men	Women	Men	Women
Individuals taking antimalarials						
Age, years						
<40	47	318	1 (2.1)	9 (2.8)	0 (0.0)	1 (0.3)
40–49	84	483	2 (2.4)	19 (3.9)	1 (1.2)	4 (0.8)
50–59	152	671	2 (1.3)	29 (4.3)	1 (0.7)	6 (0.9)
60–69	162	707	6 (3.7)	9 (1.3)	2 (1.2)	0 (0.0)
70–79	254	781	14 (5.5)	33 (4.2)	0 (0.0)	7 (0.9)
80–89	151	500	7 (4.6)	30 (6.0)	1 (0.7)	6 (1.2)
≥90	24	74	3 (12.5)	4 (5.4)	0 (0.0)	2 (2.7)
Overall	874	3,534	35 (4.0)	133 (3.8)	5 (0.6)	26 (0.7)
General population						
Age, years						
<40	413,462	395,505	5,620 (1.4)	7,448 (1.9)	912 (0.2)	1,036 (0.3)
40–49	164,156	164,407	3,513 (2.1)	6,051 (3.7)	738 (0.4)	992 (0.6)
50–59	162,369	167,056	3,959 (2.4)	6,030 (3.6)	937 (0.6)	1,198 (0.7)
60–69	119,176	132,315	3,286 (2.8)	3,142 (2.4)	852 (0.7)	698 (0.5)
70–79	96,687	113,531	3,200 (3.3)	2,909 (2.6)	787 (0.8)	667 (0.6)
80–89	56,948	82,140	3,072 (5.4)	4,541 (5.5)	722 (1.3)	1,081 (1.3)
≥90	10,332	26,235	1,015 (9.8)	3,278 (12.5)	235 (2.3)	708 (2.7)
Overall	1,023,130	1,081,189	23,665 (2.3)	33,399 (3.1)	5,183 (0.5)	6,380 (0.6)

DISCUSSION

In a recent observational study involving a large sample of consecutive patients who had been hospitalized in New York City with COVID-19, HCQ use was not associated with a significantly higher or lower risk of intubation or death (14). Although these results may be affected by prescription bias, with patients with severe disease receiving the drug, they do not support the use of HCQ at present, outside of randomized clinical trials testing its efficacy. Furthermore, a randomized trial did not demonstrate a significant benefit of HCQ as postexposure prophylaxis for COVID-19 (15). Accordingly, the Italian Medicines Agency (AIFA), in addition to other regulatory national agencies, has recently stopped the use of HCQ both for treatment of and prophylaxis for COVID-19, outside of clinical trials.

Our study is the first population-based study in a geographic area with a high level of spread of COVID-19 to evaluate if antimalarials might be effective in preventing symptomatic COVID-19 in a large number of patients (n = 4,408) treated with long-term CQ or HCQ for autoimmune conditions. These drugs have been reported to have antiviral activity in vitro against SARS–CoV-2; in particular, they seem able to block or decrease viral replication in a time- and concentration-dependent manner, as well as to inhibit the fusion of the virus to the cell membrane (7,8). Taken together, these effects have prompted suggestions for the use of antimalarials as prophylactic treatment of COVID-19. However, in our study, those individuals receiving antimalarials had the same probability of being diagnosed as having COVID-19 as the general population; therefore, our study does not support a role for CQ or

Table 2. Adjusted odds ratios of being tested for severe acute respiratory syndrome coronavirus 2, testing positive, and testing positive if tested in Emilia-Romagna, Italy between March 2020 and May 2020*

	Cumulative incidence of being tested	Cumulative incidence of testing positive	Probability of being positive, if tested
Individuals taking antimalarials	1.09 (0.94–1.28)	0.94 (0.66–1.34)	0.83 (0.56–1.23)
Men	1 (referent)	1 (referent)	1 (referent)
Women	1.24 (1.22–1.26)	1.05 (1.01–1.09)	0.85 (0.81–0.89)
Age, years			
<40	1 (referent)	1 (referent)	1 (referent)
40–49	1.82 (1.77–1.87)	2.19 (2.05–2.34)	1.27 (1.19–1.37)
50–59	1.90 (1.85–1.95)	2.70 (2.54–2.87)	1.56 (1.46–1.67)
60–69	1.58 (1.54–1.63)	2.56 (2.4–2.74)	1.79 (1.66–1.93)
70–79	1.80 (1.75–1.86)	2.88 (2.69–3.08)	1.76 (1.63–1.90)
80–89	3.45 (3.35–3.55)	5.42 (5.08–5.78)	1.78 (1.66–1.91)
≥90	7.72 (7.45–8.01)	10.84 (10.02–11.74)	1.66 (1.52–1.81)

* Values are the adjusted odds ratio (95% confidence interval).

HCQ in preventing symptomatic COVID-19 at the dosage used to treat autoimmune conditions. The maximum prescribed dosage of HCQ, the most commonly used antimalarial, is 400 mg daily. Safety is a major concern at higher doses.

The probability of those receiving CQ or HCQ being tested for SARS–CoV-2 was slightly increased, while the probability of those who were taking CQ or HCQ receiving a positive swab once tested was slightly lower. These differences are compatible with an increased propensity to test patients with autoimmune conditions who are considered at higher risk of infection, including patients with less typical symptoms or at lower risk of COVID-19. However, the differences were minimal and not significant and cannot have impeded the observation of an important prophylactic effect of antimalarials. In particular, the 95% CI suggests that a reduction larger than one-third is extremely unlikely.

Among patients who were followed up for at least 4 weeks, we observed a high rate of fatality (18%) in the Emilia Romagna COVID-19 population, which outlined the severity of the disease among our patients (16). We cannot rule out the possibility that a group of patients with asymptomatic or mildly symptomatic COVID-19 may have been tested; however, such a high case fatality rate suggests that patients with asymptomatic disease did not represent a substantial part of our COVID-19 registry.

Only 3.6% of the patients were treated with a single pack of antimalarials, possibly prescribed as antimalarial prophylaxis in travelers, suggesting that most patients were treated long-term for autoimmune conditions and therefore, with regard to the accumulation of the drugs in the cells and tissues related to long-term treatment, our patients represented an ideal population for evaluating the prophylactic effectiveness of antimalarials.

This study has many limitations, but also some strengths. First, the number of patients with COVID-19 was too small to provide definitive conclusions; however, our study is the first population-based study on this topic, the case ascertainment was accurate using 2 reliable sources, and we examined a large population of patients (>4,000 patients) who received long-term antimalarials. However, we compared the incidence of COVID-19 in patients with autoimmune conditions with that of the general population, and we could adjust only for sex and age. The 2 populations are not comparable with regard to health conditions and possibly also for their probability of being infected by SARS–CoV-2 and developing COVID-19. In fact, the underlying autoimmune condition and immunosuppressive treatment could have influenced the susceptibility or the course of the infection. It is worth noting that, at least for susceptibility, we did not observe any impact of prolonged use of biologic DMARDs or targeted synthetic DMARDs (17). Finally, we cannot exclude the possibility that higher dosages of CQ or HCQ than those used in autoimmune diseases could be effective in treating COVID-19. Balevic et al showed that patients receiving HCQ treatment for rheumatic diseases are unlikely to achieve total serum or plasma concentrations shown to inhibit SARS–CoV-2 in vitro; however, patients receiving HCQ long term may have tissue concentrations far exceeding serum/plasma levels (18).

In conclusion, our study did not show a prophylactic effect of antimalarial for symptomatic COVID-19 in a large population of patients with autoimmune conditions. If confirmed in larger observational studies, these results do not support the rationale for conducting large trials.

ACKNOWLEDGMENTS

We would like to thank Roberto Grilli, Massimiliano Marino, Giulio Formoso, Debora Formisano, Manuela Bedeschi, Cinzia Perilli, Elisabetta Larosa, Eufemia Bisaccia, Ivano Venturi, Massimo Vicentini, Cinzia Campari, Francesco Gioia, Serena Broccoli, Marta Ottone, Pierpaolo Pattacini, Giulia Besutti, Valentina Iotti, Lucia Spaggiari, Pamela Mancuso, Chiara Seidenari, Licia Veronesi, Paola Affanni, Maria Eugenia Colucci, Andrea Nitrosi, Marco Foracchia, Rossana Colla, Alessandro Zerbini, Marco Massari, Anna Maria Ferrari, Mirco Pinotti, Nicola Facciolongo, Ivana Lattuada, Laura Trabucco, Stefano De Pietri, Giorgio Francesco Danelli, Laura Albertazzi, Enrica Bellesia, Simone Canovi, Mattia Corradini, Tommaso Fasano, Elena Magnani, Annalisa Pilia, Alessandra Polese, Silvia Storchi Incerti, Piera Zaldini, Efrem Bonelli, Bonanno Orsola, Matteo Revelli, Carmine Pinto, and Francesco Venturelli from the Reggio Emilia COVID-19 working group.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Salvarani had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Salvarani, Sandri, Bajocchi, Galli, Muratore, Boiardi, Pipitone, Cassone, Croci, Marata, Costantini.

Acquisition of data. Salvarani, Gradellini, Viani, Pandolfi, Reta, Carrozzi, Rossi.

Analysis and interpretation of data. Mancuso.

REFERENCES

1. Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Mailhe M, et al. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. Int J Antimicrob Agents 2020;56:105949.
2. Million M, Lagier JC, Gautret P, Colson P, Fournier PE, Amrane S, et al. Early treatment of COVID-19 patients with hydroxychloroquine and azithromycin: a retrospective analysis of 1061 cases in Marseille, France. Travel Med Infect Dis 2020;35:101738.
3. Hernandez AV, Roman YM, Pasupuleti V, Barboza JJ, White CM. Hydroxychloroquine or chloroquine for treatment or prophylaxis of COVID-19: a living systematic review. Ann Intern Med 2020;173:287–96.
4. Spinelli FR, Ceccarelli F, di Franco M, Conti F. To consider or not antimalarials as a prophylactic intervention in the SARS-CoV-2 (COVID-19) pandemic [letter]. Ann Rheum Dis 2020;79:666–7.
5. Schrezenmeier E, Dörner T. Mechanisms of action of hydroxychloroquine and chloroquine: implications for rheumatology [review]. Nat Rev Rheumatol 2020;16:155–66.
6. González-Echavarri C, Capdevila O, Espinosa G, Suárez S, Marín-Ballvé A, González-León R, et al, on behalf of RELES, Autoimmune Diseases Study Group GEAS. Infections in newly diagnosed

Spanish patients with systemic lupus erythematosus: data from the RELES cohort. Lupus 2018;27:2253–61.
7. Yao X, Ye F, Zhang M, Cui C, Huang B, Niu P, et al. In vitro antiviral activity and projection of optimized dosing design of hydroxychloroquine for the treatment of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Clin Infect Dis 2020;71:732–9.
8. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro [letter]. Cell Res 2020;30:269–71.
9. Mathian A, Mahevas M, Rohmer J, Roumier M, Cohen-Aubart F, Amador-Borrero B, et al. Clinical course of coronavirus disease 2019 (COVID-19) in a series of 17 patients with systemic lupus erythematosus under long-term treatment with hydroxychloroquine [letter]. Ann Rheum Dis 2020;79:837–9.
10. Cassione EB, Zanframundo G, Biglia A, Codullo V, Montecucco C, Cavagna L. COVID-19 infection in a northern-Italian cohort of systemic lupus erythematosus assessed by telemedicine. Ann Rheum Dis 2020;79:1382–3.
11. Kang CK, Seong MW, Choi SJ, Kim TS, Choe PG, Song SH, et al. In vitro activity of lopinavir/ritonavir and hydroxychloroquine against severe acute respiratory syndrome coronavirus 2 at concentrations achievable by usual doses. Korean J Intern Med 2020;35:782–7.
12. Onder G, Rezza G, Brusaferro S. Case-fatality rate and characteristics of patients dying in relation to COVID-19 in Italy. JAMA 2020;323:1775–6.
13. Riccardo F, Ajelli M, Andrianou XD, Bella A, del Manso M, Fabiani M, et al. Epidemiological characteristics of COVID-19 cases in Italy and estimates of the reproductive numbers one month into the epidemic. medRxiv. April 2020. URL: https://doi.org/10.1101/2020.04.08.20056861.
14. Geleris J, Sun Y, Platt J, Zucker J, Baldwin M, Hripcsak G, et al. Observational study of hydroxychloroquine in hospitalized patients with COVID-19. N Engl J Med 2020;382:2411–8.
15. Boulware DR, Pullen MF, Bangdiwala AS, Pastick KA, Lofgren SM, Okafor EC, et al. A randomized trial of hydroxychloroquine as postexposure prophylaxis for COVID-19. N Engl J Med 2020;383:517–25.
16. Rossi PG, Broccoli S, Angelini P, for the Emilia-Romagna COVID-19 working group. Case fatality rate in patients with COVID-19 infection and its relationship with length of follow up [letter]. J Clin Virol 2020;128:104415.
17. Salvarani C, Bajocchi G, Mancuso P, Galli E, Muratore F, Boiardi L, et al. Susceptibility and severity of COVID-19 in patients treated with bDMARDS and tsDMARDs: a population-based study. Ann Rheum Dis 2020;79:986–8.
18. Balevic SJ, Hornik CP, Green TP, Clowse ME, Gonzalez D, Maharaj AR, et al. Hydroxychloroquine in patients with rheumatic disease complicated by COVID-19: clarifying target exposures and the need for clinical trials. J Rheumatol 2020. E-pub ahead of print.

Errata

DOI 10.1002/art.41617

In the article by Xu et al in the August 2020 issue of *Arthritis & Rheumatology* (Interleukin-17A Is Produced by CD4+ but Not CD8+ T Cells in Synovial Fluid Following T Cell Receptor Activation and Regulates Different Inflammatory Mediators Compared to Tumor Necrosis Factor in a Model of Psoriatic Arthritis Synovitis [pages 1303–1313]), a second institutional affiliation of one of the authors was inadvertently omitted from the title page footnotes. Dr. Dominique Baeten's information should have read "Academic Medical Center and UCB Pharma, Amsterdam, The Netherlands." Dr. Baeten was not, however, employed by UCB Pharma at the time of his work on the study reported in the August 2020 issue.

DOI 10.1002/art.41609

In the letter by Bertin et al in the November 2020 issue of *Arthritis & Rheumatology* (Anticardiolipin IgG Autoantibody Level Is an Independent Risk Factor for COVID-19 Severity [pages 1953–1955]), two errors were inadvertently introduced in copyediting. The sentence "To this end, levels of IgG and IgM anticardiolipin antibodies (aCLs) and anti–β_2-glycoprotein I (anti-β_2GPI) autoantibodies were measured using real-time polymerase chain reaction in serum samples from 56 COVID-19 patients with severe acute respiratory syndrome coronavirus 2 (SARS–CoV-2)" (page 1953, right column) should have read "To this end, levels of IgG and IgM anti–β_2-glycoprotein I (anti-β_2GPI) and anticardiolipin (aCL) autoantibodies were measured by enzyme-linked immunosorbent assay in serum samples from 56 COVID-19 patients who were positive for severe acute respiratory syndrome coronavirus 2 (SARS–CoV-2) by reverse transcriptase–polymerase chain reaction." The sentence "Except for 1 patient who presented with a history of stroke, no other IgG aCL–positive patient with a severe manifestation of COVID-19 presented with a history of thrombosis, which suggests that positivity for aCL could be attributed to infection with SARS–CoV-2" (page 1954, right column) should have read "Except for 1 patient who presented with a history of stroke, no other IgG aCL–positive patient with a severe manifestation of COVID-19 presented with a history of thrombosis, which suggests that positivity for aCL could be attributed to severe infection with SARS–CoV-2."

We regret the errors.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 53–60
DOI 10.1002/art.41499

American College of Rheumatology
Empowering Rheumatology Professionals

Improvements in Fatigue Lag Behind Disease Remission in Early Rheumatoid Arthritis: Results From the Canadian Early Arthritis Cohort

Melissa Holdren,[1] Orit Schieir,[2] Susan J. Bartlett,[3] Louis Bessette,[4] Gilles Boire,[5] Glen Hazlewood,[6] Carol A. Hitchon,[7] Edward Keystone,[8] Diane Tin,[9] Carter Thorne,[9] Vivian P. Bykerk,[10] and Janet E. Pope,[11] on behalf of the Canadian Early Arthritis Cohort Investigators

Objective. To examine the relationship between disease activity and fatigue over time in early rheumatoid arthritis (RA).

Methods. Data were from patients with early RA (duration of symptoms ≤12 months) enrolled in the Canadian Early Arthritis Cohort (CATCH). Patients rated the level of their fatigue over the past week using an 11-point numerical rating scale for up to 5 years of follow-up. Fatigue severity was classified as low (≤2), moderate (>2 but <5), or high (≥5). Fatigue severity ratings in patients who achieved a low disease activity state (Disease Activity Score in 28 joints [DAS28] ≤3.2) were compared to those in patients who did not achieve a low disease activity state within 3 months of cohort entry.

Results. Of 1,864 patients included, 88% met RA criteria, and 72% were women. The mean ± SD baseline DAS28 was 4.9 ± 1.5. Nineteen percent of the patients reported moderate baseline fatigue, and 59% reported severe baseline fatigue. Fatigue was correlated with pain and patient global disease activity ratings (r = 0.56–0.67, $P < 0.001$), and was weakly correlated with the DAS28, tender joint count, swollen joint count, physician global assessment of disease activity, erythrocyte sedimentation rate, and C-reactive protein level. Patients who reported low fatigue by 3 months had significantly lower fatigue throughout follow-up compared to those who had moderate or high fatigue at 3 months ($P < 0.001$). Patients who achieved a DAS28 ≤3.2 within 3 months had significantly lower fatigue ratings than those with a DAS28 >3.2 (mean ± SD fatigue severity score 2.7 ± 2.6 versus 4.6 ± 3.0; $P < 0.001$), with improvements in fatigue that persisted through 5 years of follow-up. Maximal improvements in fatigue lagged behind remission by 6 months.

Conclusion. Fatigue is common in early RA, and improvements may occur after remission. Early treatment response within 3 months after treatment initiation was associated with short-term and long-term improvements in fatigue over time.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease with accompanying pain, fatigue, disability, and poor quality of life. Despite advances in the treatment of RA, fatigue continues to be common, with 80% of RA patients reporting clinically relevant fatigue (determined as having a fatigue severity score of ≥2 on a visual analog scale [VAS]), and 50% of patients reporting high fatigue or a fatigue severity score of ≥5 on a VAS (1). Patients with severe fatigue reported the experience as frustrating

The Canadian Early Arthritis Cohort (CATCH) study was supported by Amgen, Pfizer, AbbVie, Medexus, Eli Lilly, Merck Frosst Canada, Sandoz, Canada Biopharmaceuticals, Hoffmann-La Roche, Janssen Biotech, UCB, Bristol Myers Squibb, and Sanofi Genzyme.

[1]Melissa Holdren, MD: University of Western Ontario, London, Ontario, Canada; [2]Orit Schieir, PhD: University of Toronto, Toronto, Ontario, Canada; [3]Susan J. Bartlett, PhD: McGill University, Montreal, Quebec, Canada; [4]Louis Bessette, MD: CHU de Québec-Université Laval, Laval, Quebec, Canada; [5]Gilles Boire, MD, MSc: Centre Intégré Universitaire de Santé et de Services Sociaux de l'Estrie–Centre Hospitalier Universitaire de Sherbrooke and Université de Sherbrooke, Sherbrooke, Quebec, Canada; [6]Glen Hazlewood, MD, PhD: University of Calgary, Calgary, Alberta, Canada; [7]Carol A. Hitchon, MD, MSc: University of Manitoba, Winnipeg, Manitoba, Canada; [8]Edward Keystone, MD: Mount Sinai Hospital, Toronto, Ontario, Canada; [9]Diane Tin, RN, Carter Thorne, MD: Southlake Regional Health Centre, Newmarket, Ontario, Canada; [10]Vivian P. Bykerk, MD: Mount Sinai Hospital, Toronto, Ontario, Canada, and Hospital for Special Surgery and Weill Cornell Medicine, New York, New York; [11]Janet E. Pope, MD: University of Western Ontario and St. Joseph's Health Care London, London, Ontario, Canada.

Dr. Bartlett has received consulting fees from Pfizer, UCB, Eli Lilly, Novartis, Merck, Janssen, and AbbVie (less than $10,000 each). Dr. Bessette has received consulting fees, speaking fees, and/or honoraria from Amgen, Bristol Myers Squibb, Janssen, Roche, UCB, AbbVie, Pfizer, Merck, Celgene, Sanofi, Eli Lilly, and Novartis (less than $10,000 each). Dr. Boire has received consulting fees, speaking fees, and/or honoraria from Amgen, Bristol Myers Squibb, Celgene, Eli Lilly, Pfizer, Merck, AbbVie, and Novartis (less than $10,000 each). Dr. Hitchon has received research support from Pfizer and UCB. Dr. Keystone has received consulting fees, speaking fees, and/or honoraria from AbbVie, Amgen, AstraZeneca, Biotest, Bristol Myers Squibb, Celltrion, Crescendo Bioscience, F. Hoffmann-La Roche, Genentech, Gilead, Janssen, Eli Lilly, Merck, Pfizer, Sandoz, UCB, and Sanofi Genzyme (less than $10,000 each) and research support from AbbVie, Amgen, Bristol

and exhausting, and patients often feel it is dismissed by health care professionals (2,3). Possible consequences of fatigue include reduced physical and mental health–related quality of life, depression, reduced work ability, and increased morning stiffness (4). It is likely, for these reasons, that fatigue is becoming a focus for research. Patients with RA have identified fatigue as an important outcome, and the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) recommend that it be reported in trials with RA patients (5).

However, fatigue research has provided inconsistent answers to many questions. A prior systematic review of the causes and consequences of fatigue found that none of the variables studied was consistently and strongly related to fatigue across all studies (4). It was also found that some studies reported a variable that statistically predicted fatigue, while others found the variable to be statistically predicted by fatigue (4). Variables studied included pain, inflammatory activity, disease duration, disability, sleep, depression, age, and sex (4). Inconsistent results have also been found when investigating whether fatigue is correlated with disease activity in RA (3,4,6,7). Fatigue is a complex process with multiple causes, which may vary between individuals (4). For instance, fatigue may be the result of chronic pain and poor sleep, which initially could be due to disease activity, but later it can become less dependent on RA activity. This may lead to some of the inconsistencies that have been seen between previous related studies.

Studying patients with early RA provides a unique opportunity to look at how early treatment can affect future fatigue. A previous study of an early RA cohort using a treat-to-target strategy found that the mean fatigue score only decreased by a small amount at 1 year, and that of those who reported having fatigue at baseline, 77% remained fatigued despite decreased disease activity (8). Another early RA study using a fatigue score on a VAS found that 24%, 34%, and 42% of patients showed worsened, stable, or improved fatigue, respectively, at 1 year (9). Neither of those studies looked at fatigue past 1 year in patients with early RA.

Many studies have analyzed disease activity measures and fatigue levels at baseline as potential predictors of future outcomes. We believe that the 3-month time point after starting disease-modifying antirheumatic drug (DMARD) treatment may provide a unique perspective, as patients may have shown a clinical response to treatment within 3 months, allowing future predictions to be made.

The Canadian Early Arthritis Cohort (CATCH) allows us to extract high-quality data since patients are followed up regularly over time, with multiple disease variables being collected. The performance of the CATCH cohort has recently been assessed, and the cohort has shown high rates of yearly follow-up, DMARD use, and treatment with DMARDs within 2 weeks of diagnosis (10). Using this incident early arthritis cohort, we aimed to determine if the level of fatigue at baseline and at 3 months could be predictive of patient outcomes at 1 year, if early control of disease activity would result in sustained reductions in fatigue well beyond 1 year, and if improvement in fatigue could be attained in those who achieved sustained disease remission within the first year, and if so, whether the maximum improvement in fatigue lagged behind the iniital achievement of remission.

PATIENTS AND METHODS

Study design and patients. Patient data were from the CATCH cohort, a multicenter, prospective, observational cohort of patients with incident early RA or suspected RA in Canada. CATCH includes patients age >18 years who have had fixed joint symptoms for ≥6 weeks and ≤12 months, have ≥2 swollen joints or 1 swollen joint at the metacarpophalangeal or proximal interphalangeal joint, and 1 of the following: rheumatoid factor ≥20 IU, anti–citrullinated protein antibody positivity, response to nonsteroidal antiinflammatory drugs, painful joints determined by the metatarsophalangeal squeeze test, or morning stiffness lasting ≥45 minutes (11). Patients are excluded from the cohort or withdrawn if they have an alternate diagnosis, such as psoriatic arthritis, crystal-induced arthritis, infection-induced arthritis, or connective tissue disease (11). Patients are followed up with standardized assessments at baseline, every 3 months for the first year, every 6 months for the second year, and annually thereafter. Patients who were enrolled in CATCH between its inception in January 2007 and March 2017, and had at least 1 follow-up visit, were included in this study. Those who were missing data on fatigue at baseline or at 12 months of follow-up were excluded. The study was conducted according to the Declaration of Helsinki. All sites received institutional review board approval, and all patients provided written informed consent.

Data collection. In addition to demographic data, information on comorbidities such as fibromyalgia, osteoarthritis, anxiety, and depression was collected from the patients at baseline. The primary outcome of fatigue was assessed using a standardized question addressing the severity of fatigue at every follow-up visit, with the outcome being expressed as a score on a numerical rating scale (NRS) ranging from 0 (no fatigue) to 10 (extreme fatigue).

Myers Squibb, F. Hoffmann-La Roche, Gilead, Janssen, Eli Lilly, Pfizer, and Sanofi-Aventis. Dr. Thorne has received consulting fees, speaking fees, and/or honoraria from AbbVie, Amgen, Celgene, Eli Lilly, Medexus/Medac, Merck, Novartis, Pfizer, Sanofi, Centocor/Janssen, and CaREBiodam (less than $10,000 each). Dr. Bykerk has received consulting fees from Amgen, Pfizer, UCB, Scipher Medicine, and Sanofi/Genzyme/Regeneron (less than $10,000 each). Dr. Pope has received consulting fees, speaking fees, and/or honoraria from AbbVie, Actelion, Amgen, Bayer, Bristol Myers Squibb, Emerald, Genzyme, Janssen, Eli Lilly, Merck, Novartis, Pfizer, Roche, Sandoz, Sanofi, UCB, and Seattle Genetics (less than than $10,000 each). No other disclosures relevant to this article were reported.

Address correspondence to Janet E. Pope, MD, St. Joseph's Health Care London, 268 Grosvenor Street, London, Ontario N6A,4V2, Canada. Email: Janet.Pope@sjhc.london.on.ca.

Submitted for publication January 28, 2020; accepted in revised form August 18, 2020.

Previous studies have defined fatigue as low if scores were ≤2, moderate if scores were >2 but <5, and severe if scores were ≥5 (12). Other covariates that were measured at follow-up visits included the Disease Activity Score in 28 joints (DAS28) (13), C-reactive protein (CRP) level, erythrocyte sedimentation rate (ESR), physician global assessment of disease activity and patient global assessment of disease activity scores, Health Assessment Questionnaire (HAQ) scores (14), and pain NRS scores. Disease activity states were defined using the DAS28, in which a DAS28 score <2.6 indicates remission, ≥2.6 and ≤3.2 indicates low disease activity, >3.2 and ≤5.1 indicates moderate disease activity, and >5.1 indicates high disease activity (15).

Statistical analysis. Univariate analyses were conducted to assess baseline characteristics, and data for continuous variables are presented as the mean ± SD. Categorical data are presented as the frequency (expressed as a percentage). All baseline data were also assessed according to each fatigue level (low, moderate, and severe).

Bivariate analysis was used to determine the correlation between fatigue severity scores and disease activity measures over the first year of follow-up after cohort enrollment. Bivariate analyses were performed to study the correlation between fatigue severity scores at baseline or 3 months with disease activity measures at 1 year. To compare differences in fatigue in patients who achieved a low disease activity state (DAS28 ≤3.2) versus those who did not achieve a low disease activity state within 3 months of cohort entry, *t*-tests were used. Repeated-measures analysis of variance (ANOVA) was used to describe the change in fatigue and DAS28 over time, and one-way ANOVA was used to analyze the differences between fatigue and DAS28 categories. Paired *t*-tests were used to compare fatigue at different time points in patients who achieved remission (DAS28 <2.6) at ≥3 consecutive visits within the first

Table 1. Baseline demographic and disease characteristics in patients with rheumatoid arthritis, by fatigue severity score*

	All (n = 1,864)	Fatigue score ≤2 (n = 420)	Fatigue score >2 but <5 (n = 349)	Fatigue score ≥5 (n = 1,095)	*P*†
Patients, %	100	22.5	18.7	58.7	–
Age, mean ± SD years	54.5 ± 14.7	56.7 ± 14.5	54.7 ± 15.1	53.6 ± 14.4	0.001
Sex, female	1,339 (71.8)	264 (62.9)	250 (71.6)	825 (75.3)	<0.001
High school education	1,664 (89.2)	378 (90.0)	308 (88.2)	978 (89.3)	0.279
Married	1,115 (59.8)	265 (63.1)	203 (58.2)	647 (59.1)	0.140
Smoking status					0.114
Never	811 (43.5)	185 (44.0)	142 (40.7)	484 (44.2)	>0.05
Previous smoker	727 (39)	167 (39.8)	152 (43.6)	408 (37.3)	>0.05
Current smoker	323 (17.3)	68 (16.2)	53 (15.2)	202 (18.4)	>0.05
SJC28, median (25th, 75th percentiles)	6.0 (2.0, 11.0)	4.0 (2.0, 8.0)	6.0 (2.0, 11.0)	7.0 (3.0, 12.0)	<0.001
TJC28, median (25th, 75th percentiles)	6.0 (3.0, 12.0)	4.0 (2.0, 9.0)	5.0 (2.0, 10.0)	8.0 (4.0, 13.0)	<0.001
HAQ score (scale 0–3), mean ± SD	1.01 ± 0.71	0.56 ± 0.61	0.78 ± 0.58	1.26 ± 0.67	<0.001
DAS28, mean ± SD	4.86 ± 1.46	4.13 ± 1.38	4.49 ± 1.38	5.25 ± 1.38	<0.001
ESR, median (25th, 75th percentiles)	20.0 (10.0, 38.0)	18.0 (9.5, 33.0)	20.0 (10.0, 34.5)	21.0 (10.0, 41.0)	0.003
CRP, median (25th, 75th percentiles)	6.7 (2.2, 19.0)	5.0 (1.8, 14.6)	5.6 (2.0, 16.0)	8.0 (2.8, 23.0)	<0.001
Erosions	368 (19.7)	82 (19.5)	96 (27.5)	190 (17.4)	0.001
RF positive	1,042 (55.9)	244 (58.1)	202 (57.9)	596 (54.4)	0.394
ACPA positive	741 (39.8)	166 (39.5)	155 (44.4)	420 (38.4)	0.320
PhGA, mean ± SD‡	4.7 ± 2.5	3.9 ± 2.4	4.25 ± 2.5	5.1 ± 2.4	<0.001
PtGA, mean ± SD‡	5.7 ± 2.9	3.5 ± 2.9	4.4 ± 2.3	6.9 ± 2.3	<0.001
Pain VAS, mean ± SD‡	5.4 ± 2.8	3.1 ± 2.6	4.3 ± 2.3	6.6 ± 2.3	<0.001
Fatigue NRS, mean ± SD‡	5.1 ± 3.0	–	–	–	–
Comorbid conditions					
Fibromyalgia	38 (2.0)	3 (0.7)	7 (2.0)	28 (2.6)	0.043
Osteoarthritis	227 (12.2)	41 (9.8)	51 (14.6)	135 (12.3)	0.060
Depression	184 (9.9)	21 (5.0)	46 (13.2)	117 (10.7)	<0.001
MCS, mean ± SD	45.8 ± 11.4	52.8 ± 9.1	48.7 ± 9.6	42.3 ± 11.2	<0.001
Medication					
Nonbiologic DMARD	1,644 (88.2)	368 (87.7)	310 (88.8)	966 (88.2)	>0.05
Biologic DMARD	39 (2.1)	5 (1.2)	7 (2.0)	27 (2.5)	>0.05
Oral steroids	563 (30.2)	121 (28.8)	96 (27.5)	346 (31.6)	0.272
Parenteral steroids	572 (30.7)	88 (21.0)	89 (25.6)	395 (36.1)	<0.001

* Except where indicated otherwise, values are the number (%). SJC28 = swollen joint count in 28 joints; TJC28 = tender joint count in 28 joints; HAQ = Health Assessment Questionnaire; DAS28 = Disease Activity Score in 28 joints; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; RF = rheumatoid factor; ACPA = anti–citrullinated protein antibody; PhGA = physician global assessment of disease activity; PtGA = patient global assessment of disease activity; VAS = visual analog scale; NRS = numerical rating scale; MCS = mental component summary score on the Short Form 36 health survey; DMARD = disease-modifying antirheumatic drug.
† Difference between fatigue groups.
‡ Score scale 0–10.

Table 2. Correlations between fatigue severity scores and disease activity measures at various time points over the first year of follow-up after cohort entry in patients with rheumatoid arthritis*

	Pearson's r				
	Baseline	3 months	6 months	9 months	12 months
ESR	0.126	0.096†	0.107	0.096†	0.099
CRP	0.139	0.138	0.105	0.124	0.103
TJC28	0.229	0.314	0.368	0.377	0.360
SJC28	0.154	0.161	0.239	0.252	0.223
PhGA‡	0.231	0.270	0.337	0.351	0.295
PtGA‡	0.562	0.589	0.653	0.671	0.643
Pain VAS score‡	0.591	0.628	0.646	0.656	0.635
DAS28 score	0.363	0.401	0.470	0.488	0.464

* $P < 0.001$ for all correlations, except where indicated. ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; TJC28 = tender joint count in 28 joints; SJC28 = swollen joint count in 28 joints; PhGA = physician global assessment of disease activity; PtGA = patient global assessment of disease activity; VAS = visual analog scale; DAS28 = Disease Activity Score in 28 joints.
† $P < 0.01$.
‡ Score scale 0–10.

year of follow-up. All analyses were performed using IBM SPSS Statistics, version 25.

RESULTS

Baseline characteristics. A total of 1,864 patients with early RA met inclusion criteria (Supplementary Figure S1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41499/abstract). Demographic and baseline clinical characteristics of the overall sample as well as the sample broken down by baseline fatigue severity scores are shown in Table 1. Fatigue was common, with 23%, 19%, and 59% of patients reporting low, moderate, or severe fatigue at baseline, respectively. The mean ± SD age of the study cohort was 54.5 ± 14.7 years, and 71.8% of the patients were women. At baseline, the mean ± SD fatigue NRS score was 5.1 ± 3.0 and the mean ± SD pain NRS score was 5.4 ± 2.8, with mean pain scores being significantly different among the 3 fatigue levels. The mean ± SD DAS28 at baseline was 4.86 ± 1.46, with all fatigue groups having a mean DAS28 consistent with moderate disease activity, but still significantly different between the 3 fatigue levels. The median ESR at baseline was 20.0 (25th, 75th percentiles 10.0, 38.0) and the median CRP level at baseline was 6.7 (25th, 75th percentiles 2.2, 19.0). At baseline, 38 patients (2%) reported having fibromyalgia, 227 (12%) reported having osteoarthritis, and 184 (10%) reported having depression. Short Form 36 mental component summary scores at baseline differed significantly between the fatigue groups. A total of 1,644 patients (88%) were prescribed or already receiving a conventional synthetic DMARD, and 39 patients (2%) were prescribed or already receiving a biologic DMARD at the baseline visit. The flow diagram of eligible patients is shown in Supplementary Figure S1 (available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41499/abstract).

Table 3. Correlations between fatigue severity scores at baseline and 3 months and disease activity measures at 1 year of follow-up after cohort entry in patients with rheumatoid arthritis

		Correlation with baseline fatigue severity scores		Correlation with 3-month fatigue severity scores	
	Measure at 1 year*	Pearson's r	Spearman's correlation	Pearson's r	Spearman's correlation
ESR	10.0 (5.0, 20.0)	0.007	0.028	0.006	0.008
CRP	2.7 (1.0, 6.0)	0.005	0.019	0.005	0.041
TJC28	1.0 (0.0, 3.0)	0.131†	0.137†	0.227†	0.241†
SJC28	0.0 (0.0, 2.0)	0.050‡	0.043	0.107†	0.120†
PhGA	1.38 ± 1.89	0.102†	0.120†	0.195†	0.207†
PtGA	2.9 ± 2.6	0.219†	0.224†	0.370†	0.386†
Pain VAS score	2.79 ± 2.59	0.233†	0.233†	0.367†	0.381†
Fatigue severity score	3.17 ± 2.87	0.353†	0.355†	0.494†	0.507†
DAS28 score	2.77 ± 1.37	0.156†	0.148†	0.267†	0.257†

* The 1-year values for the erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) level, tender joint count in 28 joints (TJC28), and swollen joint count in 28 joints (SJC28) are the median (25th, 75th percentiles), while the 1-year values for the physician global assessment (PhGA) and patient global assessment (PtGA) of disease activity (scale 0–10), pain visual analog scale (VAS) score (scale 0–10), fatigue severity score (scale 0–10), and Disease Activity Score in 28 joints (DAS28) are the mean ± SD.
† $P < 0.001$.
‡ $P < 0.05$.

Relationship between fatigue and disease activity. Throughout the first year of follow-up, fatigue severity scores at each time point were significantly correlated with all disease activity measures (Table 2). Fatigue severity scores were positively and moderately correlated with pain and patient global disease activity ratings (r = 0.56–0.67, $P < 0.001$), positively and weakly correlated with the DAS28 (r = 0.36–0.49, $P < 0.001$), and positively but very weakly correlated with the tender joint count (TJC) and swollen joint count (SJC), physician global disease activity assessment, ESR, and CRP levels (r = 0.10–0.38, $P < 0.01$). Baseline fatigue severity scores were significantly correlated with the TJC, physician global disease activity assessment, patient global disease activity assessment, pain VAS scores, fatigue severity scores, and DAS28 at 1 year (Table 3). Fatigue severity scores at 3 months were significantly correlated with the TJC, SJC, physician global disease activity assessment and patient global disease activity assessment, pain VAS scores, DAS28, and fatigue severity scores at 1 year (Table 3). Neither the baseline fatigue score nor the fatigue score at 3 months was significantly correlated with the ESR or CRP level at 1 year.

Fatigue trends over time. Fatigue scores decreased over time, with the fatigue score at baseline being significantly higher than the fatigue score at any follow-up visit (Supplementary Figure S2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41499/abstract). The largest decrease in fatigue score was seen at the 3-month follow-up visit. Patients reporting low fatigue by 3 months had significantly lower fatigue throughout the follow-up period compared to those with moderate or severe fatigue at 3 months ($P < 0.001$) (Figure 1). Patients who achieved remission or low disease activity according to the DAS28 within 3 months had significantly lower fatigue scores throughout the follow-up period compared to those who still had moderate or high disease activity ($P < 0.05$) (Figure 2).

Fatigue lags behind early sustained remission. In patients who achieved sustained remission (DAS28 <2.6) from months 3–9, there was a significant decrease in fatigue score from baseline to time of first remission ($P < 0.001$). Further, there was a significant decrease in fatigue score from the time of first remission at the 3-month visit to the 9-month visit, with the fatigue score decreasing by 0.39, resulting in a 6-month lag in further improvement in fatigue score ($P < 0.05$) (Figure 3). There was no difference in fatigue score 3 months after the time of first remission, but further improvement in fatigue was seen 6 months after the time of initial remission.

In patients who achieved sustained remission from 6–12 months, there was a significant decrease in fatigue at each visit, leading to time of first remission ($P < 0.001$). There was a further significant decrease in fatigue score by 0.36 from the 6-month visit to the 12-month visit, when remission first occurred at the 6-month visit ($P < 0.05$), resulting in a 6-month lag in final improvement in fatigue score (Supplementary Figure S3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41499/abstract). Again, there was no difference in fatigue 3 months after the time of first remission. It appears that sustained remission, defined as a sustained DAS28 score of <2.6 for 6 months, in the first year was predictive of a further small improvement in fatigue 6 months after the first achievement of remission.

DISCUSSION

This longitudinal study of fatigue in early RA supports the notion that three-fourths of patients have at least moderate fatigue

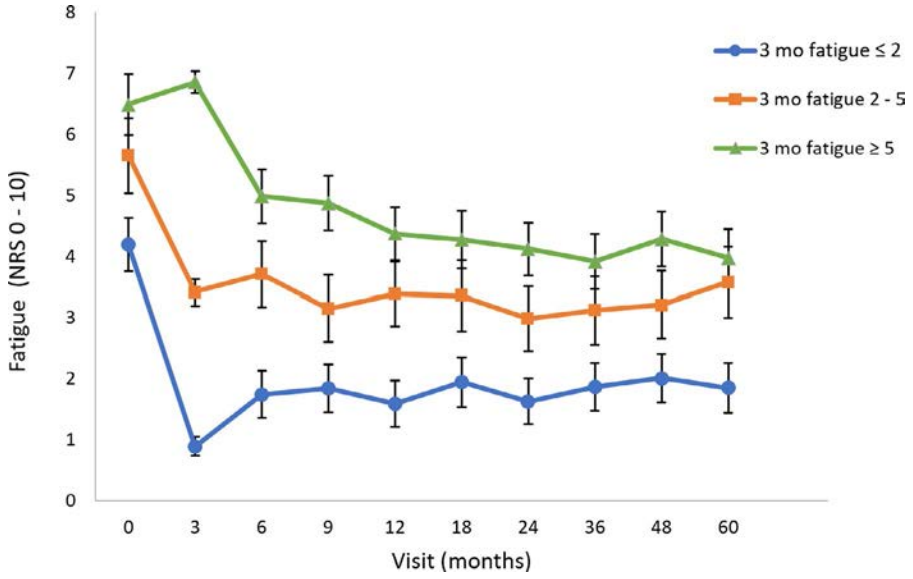


Figure 1. Mean fatigue scores over time in patients with early rheumatoid arthritis who reported low fatigue (≤2 on a numerical rating scale [NRS]), moderate fatigue (2–5 on an NRS), and high fatigue (≥5 on an NRS) at the 3-month visit. Values are the mean ± SD.

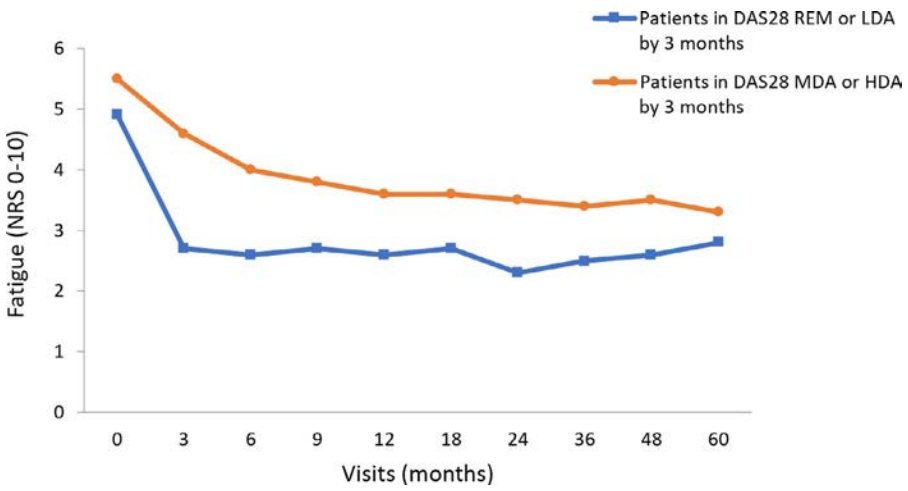


Figure 2. Mean fatigue scores over time in patients with early rheumatoid arthritis whose disease was in remission (REM) or who had low disease activity (LDA) according to the Disease Activity Score in 28 joints (DAS28) versus those who had moderate disease activity (MDA) or high disease activity (HDA) according to the DAS28 at 3 months. Values are the mean. NRS = numerical rating scale. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41499/abstract.

in early RA. This finding is consistent with previously published RA studies on fatigue (1). Fatigue improves significantly over time, with the greatest improvement seen 3 months after cohort entry. This may be due to DMARDs taking effect typically within the first 3 months after initiation, leading to significant improvements in fatigue by the first follow-up time point.

Fatigue continued to be significantly correlated with each disease activity measure at each follow-up time point within the first year. Fatigue was most strongly correlated with pain and patient global disease activity ratings over the first year. Previous studies have also shown associations between pain and fatigue, but did not find pain to be a predictor of future fatigue (16). Controlling disease activity and also pain may have the greatest benefits to reduce fatigue in patients with RA. It was found that fatigue is more strongly correlated with pain and patient global disease activity ratings while only very weakly correlated with the physician global disease activity assessment, SJC, ESR, and CRP levels over the first year. This discrepancy may be due to physicians focusing more on SJCs and markers of inflammation than on patient-reported pain and fatigue.

Early fatigue (at baseline and 3 months) was significantly correlated with future fatigue severity scores, pain VAS scores, patient and physician global disease activity ratings, DAS28 scores, and TJCs at 1 year but was not significantly correlated with the ESR or CRP level at 1 year, suggesting that fatigue may be starting to unlink from inflammation. A previous cohort study of early RA found that the strongest predictor of fatigue at 12 months was baseline fatigue (8). One study found that fatigue severity was not associated with inflammation but rather with pain severity and psychosocial factors (3). Another study found that fatigue was correlated more strongly with pain than with disease activity (6). Other studies have found inconsistent results between fatigue and disease activity and ESR over time (4).

Patients who reported low fatigue levels or who had low disease activity according to the DAS28 by 3 months continued to have lower fatigue levels throughout the 5-year follow-up

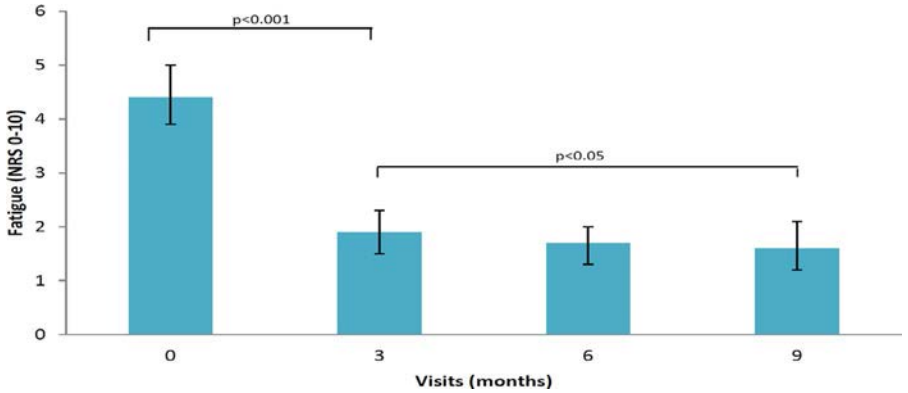


Figure 3. Change in fatigue score in patients who achieved sustained remission (Disease Activity Score in 28 joints <2.6) from month 3 to month 9. Values are the mean ± SD. NRS = numerical rating scale. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41499/abstract.

period. This further shows that an early treatment response within 3 months provides short-term and long-term benefits for fatigue. This may reflect a lower likelihood of chronic fatigue in those who have rapid response to treatment. This finding may also help to better identify patients who will have future ongoing fatigue due to difficulty in controlling disease activity quickly.

Future research considerations should include studying patients with early RA whose disease is not well controlled but who eventually develop better control of disease activity to determine whether their fatigue levels eventually improve to match that in patients with rapid remission/low disease activity. However, we observed that early rapid control of disease was predictive of the level of fatigue even 5 years later.

In patients who achieved sustained remission for ≥3 consecutive visits in the first year there was a significant decrease in fatigue at the time of first remission along with a subsequent further decrease in fatigue 6 months after the first visit in remission. Therefore, the lowest fatigue level seemed to lag behind disease remission by 6 months. Improvements in fatigue that occurred after the time of first remission were small and may not be clinically meaningful when compared to the larger improvement in fatigue that occurred at time of first remission. This finding may have implications when counseling patients who have ongoing fatigue despite achieving disease remission for the first time.

CATCH is a multicenter cohort, including sites with results that can be generalized to reflect typical patients with early RA receiving care from a rheumatologist in Canada. The generalizability to other countries is uncertain, as many of the patients start initial treatment with combination DMARDs, including high doses of methotrexate. There are several limitations to our study. The effect of initial treatment with oral, intraarticular, and intramuscular glucocorticoids was not determined in these analyses. Similarly, some patients develop fatigue, nausea, and a sense of feeling unwell over time after treatment with methotrexate, which we did not analyze in our study. Other limitations of this study include follow-up time points being 3 months apart, making it difficult to capture the level of fatigue between appointments. We were unable to study fatigue lag in patients who developed sustained remission past 1 year, as follow-up visits were spaced further apart. Due to missing data on fatigue at baseline or at the 12-month follow-up, 958 patients were excluded, which may have created a reporting bias. A more comprehensive fatigue instrument could have provided other insights. In addition, while the results of this study suggest a relationship between early disease control and lower fatigue over time, analyses were unadjusted; therefore, other factors could possibly contribute to fatigue over time. Fatigue is multifactorial and is never fully explained by disease activity. We have previously demonstrated that fibromyalgia incidence is increased in early RA, especially in the first 2 years, which could explain the residual fatigue present in some patients (17).

Fatigue is common in early RA and is most strongly correlated with pain. Early treatment response within 3 months was associated with short-term and long-term improvements in fatigue. Fatigue was significantly decreased at the time of first remission in those with sustained remission in the first year of diagnosis. Achievement of the lowest level of fatigue lagged behind the initial occurrence of remission in patients with early RA, with maximal improvement in fatigue reported at 6 months after the achievement of first early, sustained remission.

ACKNOWLEDGMENTS

We would like to thank the CATCH investigators Murray Baron, Ines Colmegna, Sabrina Fallavollita, Derek Haaland, Paul Haraoui, Shahin Jamal, Raman Joshi, Bindu Nair, Peter Panopoulos, Laurence Rubin, Edith Villeneuve, and Michel Zummer.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Holdren had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Holdren, Schieir, Bartlett, Bykerk, Pope.
Acquisition of data. Schieir, Bartlett, Bessette, Boire, Hazlewood, Hitchon, Keystone, Tin, Thorne, Bykerk, Pope.
Analysis and interpretation of data. Holdren, Schieir, Bykerk, Pope.

REFERENCES

1. Pollard LC, Choy HE, Gonzalez J, Khoshaba B, Scott DL. Fatigue in rheumatoid arthritis reflects pain, not disease activity. Rheumatology (Oxford) 2006;45:885–9.
2. Hewlett S, Cockshott Z, Byron M, Kitchen K, Tipler S, Pope D, et al. Patients' perception of fatigue in rheumatoid arthritis: overwhelming, uncontrollable, ignored. Arthritis Rheum 2005;53:697–702.
3. Van Hoogmoed D, Fransen J, Bleijenberg G, van Riel P. Physical and psychosocial correlates of severe fatigue in rheumatoid arthritis. Rheumatology (Oxford) 2010;49:1294–302.
4. Nikolaus S, Bode C, Taal E, van de Laar MA. Fatigue and factors relating to fatigue in rheumatoid arthritis: a systematic review. Arthritis Care Res (Hoboken) 2013;65:1128–46.
5. Aletaha D, Landewe R, Karonitsch T, Bathon J, Boers M, Bombardier C, et al. Reporting disease activity in clinical trials of patients with rheumatoid arthritis: EULAR/ACR collaborative recommendations. Arthritis Rheum 2008;59:1371–7.
6. Madsen SG, Danneskiold-Samsoe B, Stockmarr A, Bartels EM. Correlations between fatigue and disease duration, disease activity, and pain in patients with rheumatoid arthritis: a systematic review. Scand J Rheumatol 2016;45:255–61.
7. Bergman MJ, Shahouri SH, Shaver TS, Anderson JD, Weidensaul DN, Busch RE, et al. Is fatigue an inflammatory variable in rheumatoid arthritis (RA)? Analyses of fatigue in RA, osteoarthritis, and fibromyalgia. J Rheumatol 2009;36:2788–94.
8. Walter MJ, Kuijper TM, Hazes JM, Weel AE, Luime JJ. Fatigue in early, intensively treated and tight-controlled rheumatoid arthritis patients is frequent and persistent: a prospective study. Rheumatol Int 2018;38:1643–50.
9. Rat AC, Pouchot J, Fautrel B, Boumier P, Goupille P, Guillemin F. Factors associated with fatigue in early arthritis: results from a multicenter national French cohort study. Arthritis Care Res (Hoboken) 2012;64:1061–9.

10. Barber CE, Schieir O, Lacaille D, Marshall DA, Barnabe C, Hazlewood G, et al. High adherence to system-level performance measures for rheumatoid arthritis in a national early arthritis cohort over eight years. Arthritis Care Res (Hoboken) 2018;70:842–50.
11. Bykerk VP, Jamal S, Boire G, Hitchon CA, Haraoui B, Pope JE, et al. The Canadian Early Arthritis Cohort (CATCH): patients with new-onset synovitis meeting the 2010 ACR/EULAR classification criteria but not the 1987 ACR classification criteria present with less severe disease activity. J Rheumatol 2012;39:2071–80.
12. Tournadre A, Pereira B, Gossec L, Soubrier M, Dougados M. Impact of comorbidities on fatigue in rheumatoid arthritis patients: results from a nurse-led program for comorbidities management (COMEDRA). Joint Bone Spine 2018;86:55–60.
13. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight–joint counts: development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44–8.
14. Fries JF, Spitz PW, Kraines RG, Holman HR. Measurement of patient outcome in arthritis. Arthritis Rheum 1980;23:137.
15. Fransen J, Creemers MC, van Riel PL. Remission in rheumatoid arthritis: agreement of the disease activity score (DAS28) with the ARA preliminary remission criteria. Rheumatology (Oxford) 2004;43:1252–5.
16. Feldthusen C, Grimby-Ekman A, Forsblad-d'Elia H, Jacobsson L, Mannerkorpi K. Explanatory factors and predictors of fatigue in persons with rheumatoid arthritis: a longitudinal study. J Rehabil Med 2016;48:469–76.
17. Lee YC, Lu B, Boire G, Haraoui B, Hitchon CA, Pope JE, et al. Incidence and predictors of secondary fibromyalgia in an early arthritis cohort. Ann Rheum Dis 2013 Jun;72:949–54.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 61–68
DOI 10.1002/art.41491

American College of Rheumatology
Empowering Rheumatology Professionals

Respiratory Diseases as Risk Factors for Seropositive and Seronegative Rheumatoid Arthritis and in Relation to Smoking

Vanessa L. Kronzer,[1] Helga Westerlind,[2] Lars Alfredsson,[2] Cynthia S. Crowson,[1] Fredrik Nyberg,[3] Göran Tornling,[2] Lars Klareskog,[2] Marie Holmqvist,[2] and Johan Askling[2]

Objective. The link and interplay between different airway exposures and rheumatoid arthritis (RA) risk are unclear. This study was undertaken to determine whether respiratory disease is associated with development of RA, and specifically to examine this relationship by RA serostatus and smoking exposure.

Methods. Using data from the Epidemiological Investigation of Rheumatoid Arthritis study, this analysis included 1,631 incident RA cases and 3,283 matched controls recruited from 2006 to 2016. Linking these individuals to the National Patient Register provided information on past diagnoses of acute or chronic upper or lower respiratory disease. For each disease group, we estimated adjusted odds ratios (OR_{adj}) with 95% confidence intervals (95% CIs) for RA, using logistic regression models adjusted for age, sex, residential area, body mass index, and education level both overall and stratified by anti–citrullinated protein antibody (ACPA)/rheumatoid factor (RF) status and by smoking status.

Results. Respiratory disease diagnoses were associated with risk of RA, with an OR_{adj} of 1.2 (95% CI 0.8–1.7) for acute upper respiratory disease, 1.4 (95% CI 1.1–1.9) for chronic upper respiratory disease, 2.4 (95% CI 1.5–3.6) for acute lower respiratory disease, and 1.6 (95% CI 1.5–3.6) for chronic lower respiratory disease. These associations were present irrespective of RF or ACPA status, though the association was somewhat stronger for ACPA-positive or RF-positive RA than for ACPA-negative or RF-negative RA. The association between any respiratory disease and RA was stronger for nonsmokers (OR_{adj} 2.1 [95% CI 1.5–2.9]) than for smokers (OR_{adj} 1.2 [95% CI 0.9–1.5]).

Conclusion. Respiratory diseases increase the risk for both seropositive and seronegative RA, but only among nonsmokers. These findings raise the hypothesis that smoking and airway disease are associated with RA development through partly different mechanisms.

INTRODUCTION

Over the past decades, various airway exposures, including silica or coal (1–3) and cigarette smoking (4), have been linked both to airway disease and to rheumatoid arthritis (RA). Several studies have shown that smoking may generate anti–citrullinated peptide antibodies (ACPAs) (5,6) and rheumatoid factor (RF) (7,8), supporting smoking as an etiologic agent of seropositive RA. Some epidemiologic studies have identified associations between other types of airway disease and RA, such as asthma (9–15) and chronic obstructive pulmonary disease (COPD) (15–17), whereas other studies have not (10,18–20).

In newly diagnosed RA as well as RA prior to clinical diagnosis, smoking has been associated with citrullination and production of ACPAs in the lungs in the presence of subtle airway abnormalities, but in the absence of diagnosed airway disease (21–23). Other studies have demonstrated increased citrullination and/or ACPA production in patients with COPD (24), asthma (25), and interstitial lung disease (ILD) (26), also in the absence of smoking. These studies raise the question as to what extent the association between airway disease and RA may be attributable to smoking as a common risk factor and to what extent airway disease confers a risk of RA in the absence of smoking.

Drs. Kronzer and Holmqvist's work was supported by a Mayo Clinic–Karolinska Institutet Collaborative Travel Award. Dr. Kronzer's work was supported in part by a Rheumatology Research Foundation Resident Research Preceptorship. Dr. Askling's work was supported by the Swedish Research Council, the Swedish Heart Lung Foundation, the Region-Stockholm ALF, and The Nordic Research Council/Vinnova.

[1]Vanessa L. Kronzer, MD, MSCI, Cynthia S. Crowson, PhD: Mayo Clinic, Rochester, Minnesota; [2]Helga Westerlind, PhD, Lars Alfredsson, PhD, Göran Tornling, MD, PhD, Lars Klareskog, MD, PhD, Marie Holmqvist, MD, PhD, Johan Askling, MD, PhD: Karolinska Institutet, Stockholm, Sweden; [3]Fredrik Nyberg, MD, MPH, PhD: University of Gothenburg, Gothenburg, Sweden.

No potential conflicts of interest relevant to this article were reported.

Address correspondence to Vanessa L. Kronzer, MD, MSCI, 200 First Street SW, Rochester, MN 55905. Email: kronzer.vanessa@mayo.edu.

Submitted for publication May 4, 2020; accepted in revised form August 11, 2020.

Prior studies on this question are sparse. One recent study demonstrated an association between respiratory diseases and ACPA/RF–positive RA (16), though that study did not specifically investigate whether or not respiratory disease preceded RA, and did not take smoking status into account. Another recently published study from the Nurses' Health Study found an association of asthma and COPD with seronegative RA in women (15), but did not specifically address whether smoking and respiratory disease may confer risk for RA through similar or different mechanisms. Minimal data exist for other types of airway diseases. A few case reports and small studies have shown an association of RA with prior ILD (27,28), chronic tonsillitis (29), and influenza (30).

With this background, our objectives for the present study were two-fold. First, we aimed to define the relationship between a variety of preexisting respiratory disease groups and subsequent risk of RA using high-quality, population-based data. Second, we aimed to determine the effects of smoking on respiratory disease, and in particular, whether smoking conferred an added risk for RA in individuals with preexisting respiratory diseases. To accomplish these objectives, we leveraged the Epidemiological Investigation of Rheumatoid Arthritis (EIRA) case–control study, as well as administrative respiratory data from the Swedish National Patient Register.

PATIENTS AND METHODS

Study design. EIRA is a population-based, case–control study of incident RA in central and southern Sweden that began in 1995 (31). The participation rate from 1995 to 2005 was 93% for RA cases and 72% for controls, with reasons for nonparticipation outlined previously (32). At the time of enrollment, all participants completed a questionnaire, including smoking history. Index date was defined as the time of RA symptom onset as reported by the case. Controls were assigned the same index date as their corresponding case. For this particular analysis, we linked participants to the Swedish National Patient Register to obtain outpatient diagnosis codes, which became available in 2001. In Sweden, these codes are used primarily as part of the medical file with health care staff as the intended reader, rather than for billing. To achieve a minimum of 5 years of exposure assessment, we restricted analyses to EIRA participants with an index date of 2006 or after.

Participants. Inclusion criteria for EIRA included age ≥18 years, and a diagnosis of RA for the first time between 2006 and 2016. Exclusion criteria included inability to speak Swedish and age >70 years before 2009. For this analysis, we also excluded participants with >1 year since symptom onset and index date before 2006, resulting in 1,631 RA cases. All RA cases were examined and diagnosed by a rheumatologist at the time of enrollment and fulfilled either American College of Rheumatology (ACR) 1987 criteria or the ACR/European League Against Rheumatism 2010 criteria for RA (33,34). Controls were randomly selected from the general population and individually matched to each case 2:1 for age, sex, and residential area.

Exposures and covariates. The primary exposure was respiratory disease diagnosis codes before the index date, as assigned in outpatient specialist care (Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41491/abstract). Because respiratory disease diagnosis codes from outpatient specialized care became available in 2001 and participants were enrolled between 2006 and 2016, each participant had a total look-back period ranging from 5 years to 15 years. To ensure that respiratory diseases preceded RA, we required the respiratory disease diagnosis to be registered during a visit at least 1 calendar year prior to the index date. We classified respiratory diseases as acute or chronic and as upper or lower. Examples of acute upper respiratory diseases include sinusitis and pharyngitis, whereas chronic upper respiratory diseases include allergic and chronic rhinitis. Acute lower respiratory diseases include influenza and pneumonia, whereas chronic lower respiratory disease include asthma, COPD, and ILD. Analyses of respiratory diseases classified as "any" included all 4 of these groupings, whereas "any, no infection" excluded respiratory diseases considered to be infections—largely the acute respiratory diseases.

The positive predictive value (PPV) for influenza codes has been shown to range from 40% to 80% (35), and the PPV for pneumonia codes has been shown to be ~96% (36). However, the PPV for other respiratory diseases remains unknown, and for diseases such as sinusitis, the PPV may be very low. All diagnosis codes and their classification can be found in Supplementary Table 1 (available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41491/abstract). Respiratory disease duration before RA was approximated by subtracting the date of respiratory disease diagnosis registration from the index date.

Data on covariates including age, sex, residential area, body mass index (BMI), education level, and smoking status (never, non-regular, ex, and current) were self-reported by participants at the time of the EIRA enrollment questionnaire. We defined ever smokers as non-regular smokers, ex-smokers, and current smokers. ACPA status was determined using the CCP2 diagnostic kid for frozen sera from EIRA patients and controls. RF status was determined at the time of inclusion by the recruiting site.

Statistical analysis. Proportions were compared using chi-square tests, while Wilcoxon's rank sum tests were used to compare continuous variables. We performed separate, unconditional logistic regression models with each exposure (respiratory disease group, respiratory disease duration, or other covariate) as the main risk factor, adjusting for age, sex, residential area, BMI, education level, and smoking status as appropriate, to obtain

adjusted odds ratios (OR_{adj}) with 95% confidence intervals (95% CIs). A sensitivity analysis also adjusted each respiratory disease group for the others. The outcome measure was RA, both overall and separately for ACPA/RF–positive and ACPA/RF–negative RA. Selected analyses were also stratified by smoking status or cumulative exposure. For each disease, we also tested multiplicative interactions between respiratory disease and age, sex, and smoking status, and included them in the model if statistically significant ($P < 0.05$). We assessed interactions on the additive scale by quantifying the attributable proportion of deviation from additivity for interactions between respiratory disease and smoking and also by calculating the relative excess risk due to interaction (37). Finally, to determine whether smoking was associated with respiratory diseases in our study population, we performed analyses with smoking history (i.e., never, ever) as the exposure, and each respiratory disease group as the outcome, separately among RA cases and their controls.

Only 77 individuals (1.6%) in this analysis had missing data for ≥1 of the covariates: age, sex, residential area, BMI, education level, or smoking history. The models excluded participants with missing data. Analyses were performed using SAS version 9.4. This analysis received approval from the ethics committee (approval no. 2015/1844-31/2), followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for observational studies, and was conducted in accordance with the Declaration of Helsinki.

RESULTS

Demographic characteristics of the subjects. Of the 1,631 RA cases included in this analysis, the median age at enrollment was 57 years, and 71% were women. In addition, 1,088 (69%) of 1,573 cases were positive for ACPAs, and 1,059 (66%) of 1,615 cases were positive for RF. Table 1 summarizes the characteristics of the cases and their 3,283 matched controls. As expected, smoking was strongly associated with risk of RA (Table 1), especially ACPA-positive RA, as shown by prior EIRA data, and RF-positive RA (Supplementary Table 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41491/abstract). The characteristics of the 1.6% of the participants who were missing any of the covariates were similar to those with complete data, except that those with missing data had a slightly lower education level and were more likely to be nonsmokers (Supplementary Table 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41491/abstract).

Respiratory diseases before RA. Among the 4,914 participants, 522 (11%) had a history of at least 1 type of respiratory disease before RA onset. Of those, 409 participants (78%) had a history of only 1 type of respiratory disease. After adjusting for confounders, all respiratory disease groups were associated with an increased risk of developing RA later, except acute upper respiratory

Table 1. Association of risk of RA in cases and population controls at EIRA study enrollment*

Characteristic	All RA cases (n = 1,631)	Controls (n = 3,283)	OR_{adj} (95% CI), all RA (n = 1,631)†
Age, median (IQR) years	57 (46,64)	57 (46–65)	Matched
Female	1,152 (71)	2,315 (71)	Matched
BMI, kg/m^2			
<20	116 (7)	177 (5)	1.4 (1.1–1.8)
20–25	689 (42)	1540 (47)	Referent
25–30	564 (35)	1109 (34)	1.1 (1.0–1.3)
≥30	255 (16)	436 (13)	1.3 (1.0–1.5)
Education level			
Compulsory school only	345 (21)	532 (16)	Referent
Upper secondary school	868 (53)	1614 (49)	0.8 (0.7–1.0)
University degree	418 (25)	1137 (34)	0.6 (0.5–0.7)
Smoking status			
Never smoker	574 (36)	1612 (50)	Referent
Non-regular smoker	110 (7)	228 (7)	1.4 (1.1–1.8)
Ex-smoker	564 (35)	951 (30)	1.7 (1.4–1.9)
Current smoker	367 (23)	456 (14)	2.1 (1.8–2.5)
Cigarette smoking amount			
0 pack-years	575 (37)	1614 (51)	Referent
0.1–10 pack-years	371 (24)	723 (23)	1.4 (1.2–1.7)
10–20 pack-years	241 (15)	370 (12)	1.8 (1.5–2.2)
20–30 pack-years	167 (11)	216 (7)	2.1 (1.7–2.6)
≥30 pack-years	218 (14)	216 (7)	2.8 (2.3–3.5)

* Except where indicated otherwise, values are the number (%). RA = rheumatoid arthritis; EIRA = Epidemiological Investigation of Rheumatoid Arthritis; OR_{adj} = adjusted odds ratio; 95% CI = 95% confidence interval; IQR = interquartile range.
† Adjusted for age, sex, body mass index (BMI), education level, smoking status (never, non-regular, ex, current; not included in pack-years model).

Table 2. Association between respiratory disease before RA and developing RA in this EIRA analysis*

Respiratory disease†	RA cases, no. (%) (n = 1,631)	Controls, no. (%) (n = 3,283)	All RA, OR_{adj} (95% CI) (n = 1,631)‡	ACPA+ RA, OR_{adj} (95% CI) (n = 1,088)‡	ACPA− RA, OR_{adj} (95% CI) (n = 485)‡
Any	220 (13)	302 (9)	1.5 (1.2–1.8)	1.5 (1.2–1.9)	1.4 (1.0–1.9)
Any, no infection	160 (10)	203 (6)	1.6 (1.3–2.0)	1.7 (1.3–2.1)	1.3 (0.9–1.9)
Acute upper	60 (4)	102 (3)	1.2 (0.8–1.7)	1.0 (0.7–1.5)	1.6 (1.0–2.6)
Chronic upper	99 (6)	141 (4)	1.4 (1.1–1.8)	1.6 (1.2–2.1)	1.0 (0.6–1.6)
Acute lower	46 (3)	41 (1)	2.4 (1.5–3.6)	2.6 (1.6–4.2)	2.0 (1.0–3.9)
Chronic lower	66 (4)	77 (2)	1.6 (1.2–2.3)	1.6 (1.1–2.4)	1.4 (0.8–2.4)
Asthma	41 (3)	50 (2)	1.7 (1.1–2.6)	1.6 (0.9–2.6)	1.8 (1.0–3.4)
COPD	19 (1)	25 (0.8)	1.2 (0.7–2.2)	1.2 (0.6–2.5)	1.0 (0.4–2.5)
ILD	10 (0.6)	3 (0.1)	7.9 (2.1–29)	10.6 (2.7–40)	4.9 (0.8–30)

*ACPA = anti–citrullinated protein antibody; COPD = chronic obstructive pulmonary disease; ILD = interstitial lung disease (see Table 1 for other definitions).
† Of note, 113 participants had >1 respiratory disease type.
‡ Adjusted for age, sex, residential area, body mass index, education level, and smoking status (never, non-regular, ex, current).

diseases (Table 2). Crude models only adjusted for age, sex, and residential area had similar results. Taking precision into account, these associations were similar overall but slightly numerically stronger for ACPA-positive RA than for ACPA-negative RA (Table 2) and for RF-positive RA than for RF-negative RA (Supplementary Table 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41491/abstract). Respiratory diseases that occurred closer to the index date of RA onset tended to be more strongly associated with RA, though this finding was not statistically significant (OR_{adj} 0.6 per decade that any respiratory disease diagnosis code occurred before RA [95% CI 0.3–1.4]).

Stratification by smoking status. The association between respiratory disease and RA varied significantly depending on smoking status (Figure 1 and Supplementary Table 4, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41491/abstract). Except for acute lower respiratory disease, respiratory disease was not associated with RA in participants who were ever smokers (OR_{adj} 1.2 for any respiratory disease [95% CI 0.9–1.5]). However, in nonsmokers, respiratory diseases of all types were associated with RA (OR_{adj} 2.1 for any respiratory disease [95% CI 1.6–2.9]). Chronic lower respiratory disease was particularly strongly associated with RA among nonsmokers (OR_{adj} 3.1 [95% CI 1.8–5.5]). These results were nearly identical, even after adjustment for smoking pack-years (data not shown). A sensitivity analysis adjusting the association between each respiratory disease group and RA for each of the other respiratory disease types showed that the point estimates decreased slightly; yet chronic upper respiratory diseases, acute lower respiratory diseases, and chronic lower respiratory

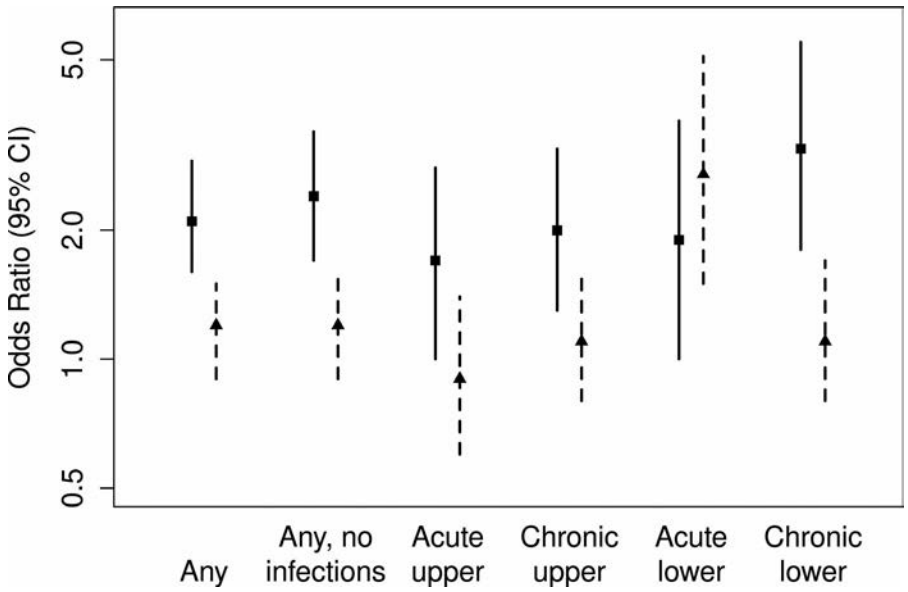


Figure 1. Association between history of respiratory disease and rheumatoid arthritis (RA), stratified by ever versus never smoking, among the 1,631 Epidemiological Investigation of Rheumatoid Arthritis cases with incident RA and their matched controls. Squares represent never smokers, and triangles represent ever smokers. 95% CI = 95% confidence interval.

Table 3. Association between respiratory diseases and RA in the EIRA cohort, stratified by pack-years of smoking*

Respiratory disease	Nonsmoker (n = 2,189)	0.1–10 pack-years (n = 1,094)	≥10 pack-years (n = 1,428)
Any	2.1 (1.6–2.9)	1.4 (0.9–2.0)	1.0 (0.8–1.4)
Any, no infection	2.4 (1.7–3.4)	1.6 (1.0–2.6)	1.1 (0.7–1.5)
Acute upper	1.7 (1.0–2.8)	1.0 (0.5–1.9)	0.9 (0.4–1.7)
Chronic upper	2.0 (1.3–3.1)	1.2 (0.6–2.1)	1.0 (0.7–1.6)
Acute lower	1.9 (1.0–3.7)	4.9 (1.5–16)	2.0 (1.0–4.1)
Chronic lower	3.1 (1.8–5.5)	2.4 (1.1–5.2)	0.9 (0.5–1.4)
Asthma	2.0 (1.1–3.7)	2.8 (1.2–6.7)	0.8 (0.4–1.8)
COPD	6.1 (1.1–34)	1.0 (0.1–11)	0.9 (0.4–1.8)
ILD	20.4 (2.5–167)	2.0 (0.1–32)	–†

* Values are the adjusted odds ratio (95% confidence interval), adjusted for age, sex, residential area, body mass index, and education level. COPD = chronic obstructive pulmonary disease; ILD = interstitial lung disease (see Table 1 for other definitions).
† Sample size too small to permit calculation.

diseases remained significantly associated with an increased risk of RA (Supplementary Table 4, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41491/abstract).

When stratifying participants who smoked by their pack-years of cigarette smoking instead of ever/never smoking status, the same phenomenon held true. Indeed, respiratory diseases were associated with RA more strongly among nonsmokers than among smokers who had smoked ≥10 pack-years (Table 3). When using a reference group of never smokers without a history of respiratory disease, the association remained significant with evidence of a negative additive interaction pattern (Supplementary Table 5, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41491/abstract). For example, the OR_{adj} of RA in nonsmokers with chronic lower respiratory disease was 3.1 (95% CI 1.8–5.5) compared to 1.8 (95% CI 1.6–2.0) for smokers without chronic lower respiratory disease and 2.0 (95% CI 1.3–3.2) for both exposures (Supplementary Table 5, http://onlinelibrary.wiley.com/doi/10.1002/art.41491/abstract).

Combining ACPAs and smoking status. Among both smokers and nonsmokers, the association between respiratory disease and RA did not vary appreciably by ACPA status (Table 4). The main exceptions were that in nonsmokers, acute upper respiratory diseases were only associated with ACPA-negative RA, whereas acute lower respiratory diseases were more associated with ACPA-positive RA, particularly in smokers (Table 4).

Association between smoking and respiratory disease. To delineate whether respiratory diseases are in the causal pathway between smoking and RA, we evaluated whether history of ever smoking was associated with respiratory disease in RA patients and in their EIRA controls. As expected, a history of smoking was associated with an increased risk of chronic upper and lower respiratory disease in controls (Table 5). However, smoking was not associated with an elevated risk of any respiratory disease group in RA cases (Table 5).

DISCUSSION

This analysis of a large, population-based, case–control study showed that a wide variety of respiratory diseases prior to RA onset, as assessed through health registry linkage, were associated with incident RA. This pattern was more evident for, but not confined to, ACPA-positive or RF-positive RA. Interestingly, the association between most of the respiratory disease groups

Table 4. Association between respiratory diseases and RA in the EIRA cohort of patients with incident RA, stratified by smoking and ACPA RA status*

	Never smokers		Ever smokers	
Respiratory disease	ACPA+ RA (n = 364)	ACPA− RA (n = 190)	ACPA+ RA (n = 712)	ACPA− RA (n = 292)
Any	2.0 (1.4–2.9)	2.3 (1.5–3.6)	1.3 (1.0–1.7)	0.9 (0.6–1.4)
Any, no infection	2.4 (1.6–3.6)	2.3 (1.4–3.9)	1.4 (1.0–1.9)	0.9 (0.6–1.5)
Acute upper	1.3 (0.7–2.5)	2.6 (1.4–5.2)	0.8 (0.5–1.4)	1.0 (0.5–2.1)
Chronic upper	2.1 (1.3–3.4)	1.7 (0.7–3.3)	1.3 (0.9–1.9)	0.7 (0.3–1.3)
Acute lower	2.1 (1.0–4.4)	1.8 (0.7–4.8)	2.9 (1.5–5.6)	2.2 (0.9–5.4)
Chronic lower	3.3 (1.7–6.2)	2.6 (1.1–6.1)	1.1 (0.7–1.8)	1.0 (0.5–2.0)

* Values are the adjusted odds ratio (95% confidence interval), adjusted for age, sex, residential area, body mass index, education level, and smoking status (never or ever). ACPA = anti–citrullinated protein antibody (see Table 1 for other definitions).

Table 5. Association between ever smoking (exposure) and developing respiratory disease (outcome), separately in EIRA cohort cases stratified by ACPA status and in controls*

Respiratory disease	ACPA+ RA cases (n = 1,088)	ACPA− RA cases (n = 485)	Controls (n = 3,283)
Any	0.9 (0.6–1.3)	0.6 (0.4–1.1)	1.5 (1.1–1.9)
Any, no infection	1.0 (0.6–1.5)	0.7 (0.4–1.3)	1.7 (1.2–2.2)
Acute upper	0.8 (0.4–1.7)	0.5 (0.2–1.3)	1.3 (0.8–1.9)
Chronic upper	0.9 (0.6–1.5)	0.5 (0.2–1.3)	1.4 (1.0–2.0)
Acute lower	0.9 (0.4–1.9)	1.0 (0.3–3.0)	0.7 (0.4–1.3)
Chronic lower	0.7 (0.4–1.4)	1.0 (0.4–2.6)	2.1 (1.3–3.5)

* Values are the adjusted odds ratio (95% confidence interval), adjusted for body mass index and education level. Cases and controls were matched for age, sex, and residential area. ACPA = anti–citrullinated protein antibody (see Table 1 for other definitions).

studied and RA was most prominent among nonsmokers; among ever smokers, the addition of a respiratory disease increased the risk of RA only in the context of acute lower airway exposure and for ACPA-positive RA.

The primary finding that a history of respiratory disease was associated with developing RA aligns with the growing body of evidence supporting a connection between the lungs and RA. The association between chronic lower respiratory diseases and overall RA risk is consistent with existing data for asthma (9–15,18,19,25), COPD (10,15–17,20), and ILD (27,28), which is mixed but mostly positive. Novel to our analysis were the associations between RA and acute upper respiratory diseases (e.g., sinusitis and pharyngitis), chronic upper respiratory diseases (e.g., rhinitis), and acute lower respiratory diseases (e.g., influenza and pneumonia), which have never been reported. One prior study identified no association of sinusitis, tonsillitis, or pneumonia with RA (38), but used self-reported data, which are easily prone to misclassification (e.g., by recall bias) and only studied diseases that presented 2 years before RA.

Another novel component of this analysis compared to prior studies was our separation of RA cases according to ACPA and RF status. A recent study showed an association between asthma/COPD and RA that was surprisingly similar for seropositive disease and seronegative disease, though that study did not separate patients according to ACPA and RF status specifically (15). The present study identified only a slightly higher association with ACPA/RF–positive RA than with ACPA/RF–negative RA. Acute upper respiratory diseases did not differ by serostatus at all, which is consistent with the understanding that ACPA and RF generation are lower respiratory and potentially chronic processes. As expected, smoking displayed a strong association with ACPA/RF–positive RA (5,6). Taken together, these results suggest that the associations with ACPA/RF differ by airway exposure, and that the association between smoking and RA may be much more ACPA/RF-dependent (as in our study) than the association between respiratory disease and RA. Future studies should explore whether the clinical phenotype of RA also differs in these 2 groups.

A third finding from this analysis that we initially found surprising was that most types of respiratory diseases were associated with an elevated risk of RA in nonsmokers, but not in smokers, and that there was no positive interaction between the 2 types of exposures. Other studies have found a similar pattern for asthma (15) and air pollution (39), supporting the validity of this result. These are notable findings, as smoking is associated with an increased risk of RA (40), and smoking also confers an increased risk of respiratory diseases as shown in a previous publication (41) and as replicated for individuals without RA in the present study.

One possible explanation for the lack of association between smoking and risk of RA in patients with respiratory disease is that lesions in the lungs that may confer risk for future RA (the mucosal hypothesis [5,6]) are present in individuals with certain respiratory diseases, and that smoking does not potentiate the RA-causative effects of such lung lesions. Another explanation may reside in the risk-factor paradox (42) arising from the conditioning on the presence of RA in cases, but no such conditioning among the controls. Among nonsmokers, our data suggest that respiratory disease may activate mucosal immunity to cause RA, not necessarily by ACPA/RF production. This finding is consistent with a pulmonary inflammation hypothesis of RA origination (24,43,44). Such inflammation might be produced by a wide variety of agents or types of stimuli, explaining why other pulmonary irritants like air pollution (39,45), organic dust (46), asbestos (47), and silica (47,48) also have been observed to be associated with an increased risk of RA, with only silica showing a strong association with seropositive RA alone (47,48). The mechanisms behind these associations may obviously differ by exposure, sometimes involving risk for only ACPA/RF–positive disease, and other times involving risk for both RA subtypes.

Our analysis benefited from precise incident RA classification, linkage to the Swedish National Patient Register for respiratory disease exposure assessment, and a large sample size. There are also several important limitations. First, the participants all came from central and southern Sweden, so the results may not be readily generalizable to other geographic areas, especially since

the burden and composition of respiratory diseases may vary worldwide (49). Second, any respiratory diseases that occurred prior to the start of the Swedish National Patient Register's outpatient data collection in 2001 were not captured, resulting in low power within analysis subsets such as smoking, serologic status, and chronic lower respiratory disease types.

Another limitation of our study is that since the exposure assessment stretched back to 2001, both RA cases and controls with respiratory exposure history occurring before that year were classified as unexposed. Thus, self-limited acute respiratory diseases or chronic respiratory diseases confined to childhood would not have been captured. Fourth, differential health care utilization could explain at least some of the observed associations if patients seeking care for respiratory diseases would be more likely to receive a diagnosis of RA, or vice versa. Similarly, diagnostic access bias would artificially increase the observed associations if RA cases had increased health care burden or other comorbidities before RA symptoms began. We mitigated this possibility by excluding EIRA cases with >1 year since symptom onset and requiring respiratory diseases to occur at least 1 year before RA symptom onset. Another important limitation is that because of the known risk factor paradox, the true association between respiratory diseases and RA might be higher than observed in smokers and/or lower than observed in nonsmokers (42). Finally, the timing between smoking and respiratory exposure in this study was uncertain. Considering that RA in some form (e.g., ACPA positivity) may actually begin years before RA symptoms (50), reverse causality cannot be excluded for any of these analyses.

In conclusion, respiratory disease is associated with an increased risk of both seropositive RA and seronegative RA. Our results raise the hypothesis of different pathogenic mechanisms underlying the association between various airway exposures and RA, exemplified by respiratory diseases and smoking. Future studies should assess whether a difference in clinical characteristics exists for RA generated from these 2 exposures. From an RA prevention point of view, taking precautions to prevent respiratory diseases may be just as important as smoking cessation in individuals at risk of RA and also in nonsmokers. From a research point of view, stratifying by smoking status may uncover additional risk factors and pathogenic mechanisms behind RA, which would otherwise be masked.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kronzer had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Kronzer, Crowson, Holmqvist, Askling.
Acquisition of data. Kronzer, Westerlind, Alfredsson, Klareskog, Holmqvist, Askling.
Analysis and interpretation of data. Kronzer, Westerlind, Alfredsson, Crowson, Nyberg, Tornling, Klareskog, Holmqvist, Askling.

REFERENCES

1. Caplan A. Certain unusual radiological appearances in the chest of coal-miners suffering from rheumatoid arthritis. Thorax 1953;8: 29–37.
2. Khuder SA, Peshimam AZ, Agraharam S. Environmental risk factors for rheumatoid arthritis. Rev Environ Health 2002;17:307–15.
3. Lippmann M, Eckert HL, Hahon N, Morgan WK. Circulating antinuclear and rheumatoid factors in coal miners: a prevalence study in Pennsylvania and West Virginia. Ann Intern Med 1973;79:807–11.
4. Vessey MP, Villard-Mackintosh L, Yeates D. Oral contraceptives, cigarette smoking and other factors in relation to arthritis. Contraception 1987;35:457–64.
5. Klareskog L, Stolt P, Lundberg K, Källberg H, Bengtsson C, Grunewald J, 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;54:38–46.
6. Makrygiannakis D, Hermansson M, Ulfgren AK, Nicholas AP, Zendman AJ, Eklund A, et al. Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells. Ann Rheum Dis 2008;67:1488–92.
7. Hedström AK, Rönnelid J, Klareskog L, Alfredsson L. Complex relationships of smoking, HLA–DRB1 genes, and serologic profiles in patients with early rheumatoid arthritis: update from a Swedish population-based case–control study. Arthritis Rheumatol 2019;71: 1504–11.
8. Jonsson T, Thorsteinsson J, Valdimarsson H. Does smoking stimulate rheumatoid factor production in non-rheumatic individuals? APMIS 1998;106:970–4.
9. Kronzer VL, Crowson CS, Sparks JA, Vassallo R, Davis JM III. Investigating asthma, allergic disease, passive smoke exposure, and risk of rheumatoid arthritis. Arthritis Rheumatol 2019;71:1217–24.
10. Sheen YH, Rolfes MC, Wi CI, Crowson CS, Pendegraft RS, King KS, et al. Association of asthma with rheumatoid arthritis: a population-based case–control study. J Allergy Clin Immunol Pract 2018;6: 219–26.
11. Hou YC, Hu HY, Liu IL, Chang YT, Wu CY. The risk of autoimmune connective tissue diseases in patients with atopy: a nationwide population-based cohort study. Allergy Asthma Proc 2017;38: 383–9.
12. Lai NS, Tsai TY, Koo M, Lu MC. Association of rheumatoid arthritis with allergic diseases: a nationwide population-based cohort study. Allergy Asthma Proc 2015;36:99–103.
13. Hemminki K, Li X, Sundquist J, Sundquist K. Subsequent autoimmune or related disease in asthma patients: clustering of diseases or medical care? Ann Epidemiol 2010;20:217–22.
14. De Roos AJ, Cooper GS, Alavanja MC, Sandler DP. Personal and family medical history correlates of rheumatoid arthritis. Ann Epidemiol 2008;18:433–9.
15. Ford JA, Liu X, Chu SH, Lu B, Cho MH, Silverman EK, et al. Asthma, chronic obstructive pulmonary disease, and subsequent risk for incident rheumatoid arthritis among women: a prospective cohort study. Arthritis Rheumatol 2020;72:704–13.
16. Doss J, Mo H, Carroll RJ, Crofford LJ, Denny JC. Phenome-wide association study of rheumatoid arthritis subgroups identifies association between seronegative disease and fibromyalgia. Arthritis Rheumatol 2017;69:291–300.
17. Bieber V, Cohen AD, Freud T, Agmon-Levin N, Gertel S, Amital H. Autoimmune smoke and fire: coexisting rheumatoid arthritis and chronic obstructive pulmonary disease: a cross-sectional analysis. Immunol Res 2013;56:261–6.
18. Yun HD, Knoebel E, Fenta Y, Gabriel SE, Leibson CL, Loftus EV Jr, et al. Asthma and proinflammatory conditions: a population-

based retrospective matched cohort study. Mayo Clin Proc 2012;87: 953–60.

19. Kronzer VL, Crowson CS, Sparks JA, Myasoedova E, Davis JM III. Comorbidities as risk factors for rheumatoid arthritis and their accrual after diagnosis. Mayo Clin Proc 2019;94:2488–98.
20. Bergstrom U, Jacobsson LT, Nilsson JA, Berglund G, Turesson C. Pulmonary dysfunction, smoking, socioeconomic status and the risk of developing rheumatoid arthritis. Rheumatology (Oxford) 2011;50:2005–13.
21. Demoruelle MK, Weisman MH, Simonian PL, Lynch DA, Sachs PB, Pedraza IF, et al. Airways abnormalities and rheumatoid arthritis–related autoantibodies in subjects without arthritis: early injury or initiating site of autoimmunity? Arthritis Rheum 2012;64:1756–61.
22. Sigari N, Moghimi N, Shahraki FS, Mohammadi S, Roshani D. Anti–cyclic citrullinated peptide (CCP) antibody in patients with wood-smoke-induced chronic obstructive pulmonary disease (COPD) without rheumatoid arthritis. Rheumatol Int 2015;35:85–91.
23. Reynisdottir G, Karimi R, Joshua V, Olsen H, Hensvold AH, Harju A, et al. Structural changes and antibody enrichment in the lungs are early features of anti–citrullinated protein antibody–positive rheumatoid arthritis. Arthritis Rheumatol 2014;66:31–9.
24. Lugli EB, Correia RE, Fischer R, Lundberg K, Bracke KR, Montgomery AB, et al. Expression of citrulline and homocitrulline residues in the lungs of non-smokers and smokers: implications for autoimmunity in rheumatoid arthritis. Arthritis Res Ther 2015;17:9.
25. Zaccardelli A, Liu X, Ford JA, Cui J, Lu B, Chu SH, et al. Asthma and elevation of anti–citrullinated protein antibodies prior to the onset of rheumatoid arthritis. Arthritis Res Ther 2019;21:246.
26. Yin Y, Liang D, Zhao L, Li Y, Liu W, Ren Y, et al. Anti–cyclic citrullinated peptide antibody is associated with interstitial lung disease in patients with rheumatoid arthritis. PLoS One 2014;9:e92449.
27. Fischer A, Solomon JJ, du Bois RM, Deane KD, Olson AL, Fernandez-Perez ER, et al. Lung disease with anti–CCP antibodies but not rheumatoid arthritis or connective tissue disease. Respir Med 2012;106:1040–7.
28. Kono M, Nakamura Y, Enomoto N, Hashimoto D, Fujisawa T, Inui N, et al. Usual interstitial pneumonia preceding collagen vascular disease: a retrospective case control study of patients initially diagnosed with idiopathic pulmonary fibrosis. PLoS One 2014;9:e94775.
29. Kawano M, Okada K, Muramoto H, Morishita H, Omura T, Inoue R, et al. Simultaneous, clonally identical T cell expansion in tonsil and synovium in a patient with rheumatoid arthritis and chronic tonsillitis. Arthritis Rheum 2003;48:2483–8.
30. Wruck K, Zoller B, Faust-Tinnefeldt G, Engel HJ. Demonstration of increased influenza-A-antibody levels in the serum of patients with chronic polyarthritis. Z Rheumatol 1985;44:259–62. In German.
31. Stolt P, Bengtsson C, Nordmark B, Lindblad S, Lundberg I, Klareskog L, et al. Quantification of the influence of cigarette smoking on rheumatoid arthritis: results from a population based case-control study, using incident cases. Ann Rheum Dis 2003;62:835–41.
32. Bengtsson C, Berglund A, Serra ML, Nise L, Nordmark B, Klareskog L, et al. Non-participation in EIRA: a population-based case-control study of rheumatoid arthritis [letter]. Scand J Rheumatol 2010;39:344–6.
33. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
34. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO III, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum 2010;62:2569–81.
35. Feemster KA, Leckerman KH, Middleton M, Zerr DM, Elward AM, Newland JG, et al. Use of administrative data for the identificationof laboratory-confirmed influenza infection: the validity of influenza-specific ICD-9 codes. J Pediatric Infect Dis Soc 2013;2:63–6.
36. Skull SA, Andrews RM, Byrnes GB, Campbell DA, Nolan TM, Brown GV, et al. ICD-10 codes are a valid tool for identification of pneumonia in hospitalized patients aged > or = 65 years. Epidemiol Infect 2008;136:232–40.
37. Andersson T, Alfredsson L, Kallberg H, Zdravkovic S, Ahlbom A. Calculating measures of biological interaction. Eur J Epidemiol 2005;20:575–9.
38. Sandberg ME, Bengtsson C, Klareskog L, Alfredsson L, Saevarsdottir S. Recent infections are associated with decreased risk of rheumatoid arthritis: a population-based case-control study. Ann Rheum Dis 2015;74:904–7.
39. Hart JE, Laden F, Puett RC, Costenbader KH, Karlson EW. Exposure to traffic pollution and increased risk of rheumatoid arthritis. Environ Health Perspect 2009;117:1065–9.
40. Pedersen M, Jacobsen S, Klarlund M, Pedersen BV, Wiik A, Wohlfahrt J, et al. Environmental risk factors differ between rheumatoid arthritis with and without autoantibodies against cyclic citrullinated peptides. Arthritis Res Ther 2006;8:R133.
41. National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health. The health consequences of smoking—50 years of progress: a report of the surgeon general. Atlanta (GA): Centers for Disease Control and Prevention (US); 2014.
42. Choi HK, Nguyen US, Niu J, Danaei G, Zhang Y. Selection bias in rheumatic disease research [review]. Nat Rev Rheumatol 2014;10: 403–12.
43. Makrygiannakis D, af Klint E, Lundberg IE, Lofberg R, Ulfgren AK, Klareskog L, et al. Citrullination is an inflammation-dependent process. Ann Rheum Dis 2006;65:1219–22.
44. Klareskog L, Ronnelid J, Saevarsdottir S, Padyukov L, Alfredsson L. The importance of differences: on environment and its interactions with genes, and immunity in the causation of rheumatoid arthritis. J Intern Med 2020;287:514–33.
45. De Roos AJ, Koehoorn M, Tamburic L, Davies HW, Brauer M. Proximity to traffic, ambient air pollution, and community noise in relation to incident rheumatoid arthritis. Environ Health Perspect 2014;122:1075–80.
46. Ilar A, Gustavsson P, Wiebert P, Alfredsson L. Occupational exposure to organic dusts and risk of developing rheumatoid arthritis: findings from a Swedish population-based case-control study. RMD Open 2019;5:e001049.
47. Miller FW, Alfredsson L, Costenbader KH, Kamen DL, Nelson LM, Norris JM, et al. Epidemiology of environmental exposures and human autoimmune diseases: findings from a National Institute of Environmental Health Sciences expert panel workshop. J Autoimmun 2012;39:259–71.
48. Zeng P, Chen Z, Klareskog L, Alfredsson L, Bengtsson C, Jiang X. Amount of smoking, duration of smoking cessation and their interaction with silica exposure in the risk of rheumatoid arthritis among males: results from the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA) study. Ann Rheum Dis 2018;77:1238–41.
49. Lim SS, Vos T, Flaxman AD, Danaei G, Shibuya K, Adair-Rohani H, et al. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 2012;380:2224–60.
50. Rantapää-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum 2003;48:2741–9.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 69–77
DOI 10.1002/art.41487

American College of Rheumatology
Empowering Rheumatology Professionals

Mediterranean Diet and Risk of Rheumatoid Arthritis: Findings From the French E3N-EPIC Cohort Study

Yann Nguyen,[1] Carine Salliot,[2] Amandine Gelot,[3] Juliette Gambaretti,[3] Xavier Mariette,[4] Marie-Christine Boutron-Ruault,[3] and Raphaèle Seror[4]

Objective. The Mediterranean diet has been reported to be associated with a significant reduction in risk of noncommunicable diseases. We undertook this study to assess the relationship between adherence to the Mediterranean diet and the risk of rheumatoid arthritis (RA), especially in high-risk individuals.

Methods. The E3N-EPIC study (Etude Epidémiologique Auprès des Femmes de la Mutuelle Générale de l'Education Nationale) is a French prospective cohort study that has included 98,995 women since 1990. Dietary data were collected via a validated food frequency questionnaire in 1993. Adherence to the Mediterranean diet was assessed using a 9-unit dietary score evaluating consumption of vegetables, legumes, cereal products, fish, meat, dairy products, olive oil, and alcohol. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for incident RA were estimated using Cox proportional hazards regression models adjusted for age and the main potential confounders, including smoking.

Results. Among 62,629 women, we identified 480 incident cases of RA. In the entire study population, the Mediterranean diet adherence score was not associated with RA risk (HR 0.86 [95% CI 0.67–1.09] for high score versus low score; *P* for trend = 0.09); however, among ever-smokers, a higher score was associated with a decreased risk of RA (HR 0.91 [95% CI 0.84–0.99] for 1-point increase in score; *P* = 0.03). In ever-smokers, the absolute risks of RA in those with high scores and those with low scores were 38.3 and 51.5 per 100,000 person-years, respectively, compared to 35.8 per 100,000 person-years in never-smokers with high Mediterranean diet scores.

Conclusion. Our results suggest that adherence to the Mediterranean diet could reduce the high risk of RA among ever-smoking women. Our results must be confirmed in future research.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease of complex and multifactorial etiology. RA preferentially affects women, who represent 70% of cases, and is often associated with antibodies (rheumatoid factor [RF] and/or anti–citrullinated protein antibodies [ACPAs]). Both environmental and genetic factors are thought to interact in its pathogenesis by triggering autoimmunity (1). To date, only smoking has been reproducibly reported as a risk factor for ACPA-positive RA, particularly in genetically predisposed patients who carry HLA–DRB1 shared epitope alleles (2–7). Prevalence of RA seems to be lower in Southern European countries compared to Northern European countries, following a north-to-south decreasing gradient (8). Environmental and lifestyle factors, including dietary habits, may partially explain this difference.

The Mediterranean diet, widely used in Southern European countries, mainly consists of olive oil, cereals, fresh or dried fruit and vegetables, fish, and a moderate amount of dairy, meat, and wine (9). This diet has been associated with a significant reduction of overall mortality, cardiovascular diseases, and neoplastic diseases (10). However, although the Mediterranean diet is rich in many bioactive components, especially antioxidants and ω-3

Supported by the Agence Nationale de la Recherche within the Investissement d'Avenir program (state grant ANR-10-COHO-0006), the FOREUM Foundation for Research in Rheumatology, and the Société Française de Rhumatologie.

[1]Yann Nguyen, MD, MSc: Centre de Recherche en Epidémiologie et Santé des Populations, INSERM U1018, Université Paris-Saclay, Université Paris-Sud, and AP-HP Nord, Hôpital Beaujon, Université de Paris, Paris, France; [2]Carine Salliot, MD, MSc: Centre de Recherche en Epidémiologie et Santé des Populations, INSERM U1018, Université Paris-Saclay, Université Paris-Sud, Paris, France, and Centre Hospitalier Régional d'Orléans, Orléans, France; [3]Amandine Gelot, MSc, Juliette Gambaretti, MSc, Marie-Christine Boutron-Ruault, MD, PhD: Centre de Recherche en Epidémiologie et Santé des Populations, INSERM U1018, Université Paris-Saclay, Université Paris-Sud, and Gustave Roussy Institute, Paris, France; [4]Xavier Mariette, MD, PhD, Raphaèle Seror, MD, PhD: Centre National de Référence pour les Maladies Auto-Immunes Systémiques Rares, AP-HP, Hôpital Bicêtre, Université Paris-Saclay, INSERM UMR1184, Paris, France.

Drs. Boutron-Ruault and Seror contributed equally to this work.

No potential conflicts of interest relevant to this article were reported.

Address correspondence to Marie-Christine Boutron-Ruault, MD, PhD, INSERM U1018, Health Across Generations Team, EMT, Institut Gustave Roussy, 114 Rue du Edouard Vaillant, 94708 Villejuif, France. Email: marie-christine.boutron@gustaveroussy.fr.

Submitted for publication May 13, 2020; accepted in revised form August 6, 2020.

fatty acids, the potential beneficial effect on autoimmune conditions, such as RA, has yet to be comprehensively studied. Some studies have investigated potential benefits on RA activity, but only a few have prospectively investigated a potential beneficial effect on RA occurrence.

Some studies have shown a role of olive oil, cooked vegetable, or fish consumption as protective against RA (11–13), but most of those focused on a single food or nutrient and were case–control studies, thus carrying the risk of recall bias. Moreover, pattern analysis is thought to be the most realistic approach to examine the relationship between diet and the risk of diseases, instead of focusing on individual food groups or nutrients (14). Evaluating an association between food pattern and RA could be used as a rationale for RA prevention strategies involving dietary interventions (15). The objective of this prospective study was to investigate the relationship between a Mediterranean dietary pattern and the risk of incident RA in a large prospective cohort of healthy French women.

SUBJECTS AND METHODS

E3N-EPIC cohort. The E3N-EPIC (Etude Epidémiologique Auprès des Femmes de la Mutuelle Générale de l'Education Nationale, the French component of the European Prospective Investigation into Cancer and Nutrition) is a large prospective cohort study conducted in France to investigate environmental factors associated with chronic diseases. It involves 98,995 healthy French women who are covered by a national health insurance primarily involving teachers, born between 1925 and 1950 (16). Participants were recruited in 1990 and completed and returned biennially mailed questionnaires (Q1–Q12) to update their health-related information, lifestyle characteristics, and newly diagnosed diseases. Since 2004, a linkage with a drug reimbursement claim database has been available from their medical insurance records (Mutuelle Générale de l'Éducation Nationale [MGEN]). The total proportion of patients lost to follow-up since 1990 is <3%, and the average response rate per follow-up questionnaire is 83%. All participants signed an informed consent form at study entry, and approval was obtained from the French National Commission for Data Protection and Individual Freedom (no. 327346-V14) and the French Advisory Committee on Information Processing in Material Research in the Field of Health (no. 13.794).

RA ascertainment. Identification and ascertainment of RA cases have been described previously (17). Briefly, potential RA subjects were first identified through the 2007, 2011, and 2014 follow-up questionnaires (Q9, Q10, and Q11, respectively), in which women were asked if they had RA or other inflammatory rheumatic diseases, or if they self-reported a hospital admission for RA in any follow-up questionnaire. A specific questionnaire for inflammatory rheumatic disease validation derived by Guillemin et al (18) was sent to all potential RA subjects in 2017. Women were considered RA subjects if they confirmed having RA in this specific questionnaire and fulfilled any of the following criteria: 1) RA was confirmed by a physician; 2) they self-reported receiving or having received any disease-modifying antirheumatic drugs or biologic therapies considered specific to RA (i.e., methotrexate, leflunomide, sulfasalazine, azathioprine, tumor necrosis factor inhibitors, rituximab, tocilizumab, rituximab, abatacept, anakinra); 3) they self-reported being positive for autoantibodies, such as RF or ACPA; or 4) they met the 1987 American College of Rheumatology criteria for RA (19). For those who did not answer the specific inflammatory rheumatic disease questionnaire, we used data from the MGEN database on medication reimbursements to ascertain RA subjects; women were considered RA subjects if they self-reported having RA in Q9, Q10, or Q11 and had received reimbursements for any medication considered specific to RA, as described in a previous study (20).

Study population. For the present study, we excluded subjects without available dietary data and those with extreme values for the ratio of energy intake to energy requirement (1% on both sides). Subjects were also excluded if they did not complete any of the 3 questionnaires that collected data on inflammatory rheumatic diseases, if they self-reported inflammatory rheumatic diseases other than RA, or if an RA diagnosis was ruled out after assessment of medical records (false-positive cases). We also excluded subjects with prevalent RA occurring prior to the dietary questionnaire and RA subjects for whom the data on diagnosis were unavailable. Follow-up began on the date the dietary questionnaire was returned (baseline), and person-time was calculated from baseline until the date of RA diagnosis, the date of the last completed questionnaire, the date of death, or the date of loss to follow-up, whichever occurred first.

Data collection. *Dietary assessment.* The dietary questionnaire was sent between 1993 and 1995 at the same time as the third questionnaire (Q3). It was developed and validated for the purpose of the cohort study (21). The questionnaire included quantitative questions on the intake and frequency of food group consumption with the help of a booklet that included pictures of portion sizes, as well as qualitative questions on food groups. With this questionnaire, the consumption of 208 food items could be assessed. The questionnaire had been previously validated in a dedicated study using 24-hour recalls (n = 12) carried out monthly as the referent (21). After 1 year, its reproducibility was shown to be satisfactory (the percentage of subjects categorized in the same or adjacent quintile according to the questionnaire and according to 24-hour recall was on average 76% for food).

Mediterranean diet score calculation. The traditional Mediterranean diet score included 9 components, as follows: 7 components that were positively associated with the score (legumes, vegetables, fruits and nuts, cereal products, fish, olive oil,

and moderate alcohol consumption [5–25 gm/day for women]) and 2 components that were negatively associated (meat and dairy products). However, the score has mostly been used in small cohorts with elderly participants or in the Greek population (22,23). To allow the score to be applied to non-Mediterranean populations, in which intake of olive oil is minimal, a variant of the score has been proposed, in which olive oil consumption was replaced by the ratio of unsaturated acids (the sum of monounsaturated and polyunsaturated fatty acids) to saturated fatty acids (24). Values of 0 or 1 were assigned to each of the 9 components, using as cutoff values the sex-specific medians among the participants. Subjects whose consumption of presumed beneficial components (vegetables, legumes, fruits, cereals, fish, unsaturated fat) was below the median consumption were assigned a value of 0, and a value of 1 otherwise. Subjects whose consumption of presumed detrimental components (meat and dairy products) was below the median consumption were assigned a value of 1, and a value of 0 otherwise. A value of 1 was given to women consuming a moderate amount of alcohol (i.e., 5 to 25 gm/day), and a value of 0 otherwise. Thus, the Mediterranean diet score ranged from 0 to 9. It was then further stratified into approximate tertiles to reflect low, medium, or high adherence to the diet (scores of 0–3, 4–5, and 6–9, respectively).

Other factors. Data on demographic characteristics (education level) and passive smoking status in childhood were available at inclusion. Smoking status (nonsmoker, former smoker, current smoker) at baseline were used. Baseline physical activity was assessed in metabolic equivalents of task (hours/week). Body mass index (BMI; kg/m^2) was calculated at baseline. Gastrointestinal disorders (normal transit, diarrhea, constipation, or alternating diarrhea/constipation), which have recently been shown to be associated with the risk of RA (25), were also assessed at baseline.

Statistical analysis. Baseline patient characteristics are presented as the mean ± SD for continuous variables and the number (percent) of patients for categorical variables. Characteristics were compared across Mediterranean diet score categories using the chi-square test for categorical variables and analysis of variance for continuous variables. Missing variables were imputed to the mode and the median, for categorical and continuous variables, respectively, if they occurred in <5% of subjects; otherwise, a "missing" category was created. Indeed, previous analyses of this very large cohort have demonstrated identical results with modal/mean compared to multiple linear imputation.

To estimate the risk of RA associated with variables of interest, we used Cox multivariable regression models with age as the time scale to estimate hazard ratios (HRs) and 95% confidence intervals (95% CIs). Thus, women entered the analysis at their baseline age (left truncation) and exited at their event/censoring age (RA diagnosis, last completed questionnaire, death, or loss to follow-up, whichever occurred first).

First, we assessed associations with each food component of the Mediterranean diet score separately, using tertiles of consumption, with the lowest tertiles as the reference category.

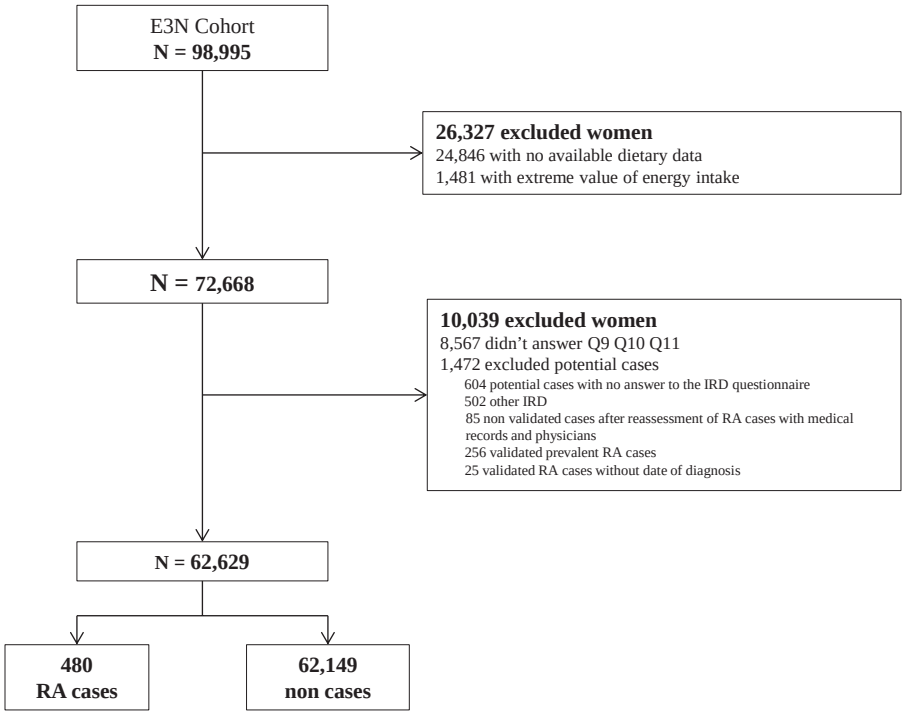


Figure 1. E3N-EPIC (Etude Epidémiologique Auprès des Femmes de la Mutuelle Générale de l'Education Nationale) cohort enrollment. Q9 = 9th questionnaire (2007); IRD = inflammatory rheumatic disease; RA = rheumatoid arthritis.

Tests for linear trend were performed on a semiquantitative variable based on the medians of the tertiles for each food group. For alcohol, we categorized alcohol consumption as low (<5 gm/day), moderate (5–25 gm/day), and high (>25 gm/day) intake.

Next, we investigated the association between the Mediterranean diet score and RA risk using diet adherence score categories (i.e., low [score 0–3], medium [score 4–5], and high [score 6–9]), with the lowest as the reference category. Tests for linear trend were performed using an ordinal variable across the 3 categories. Then, we considered a continuous Mediterranean diet score (1-point increments in score). Model 1 used age as the time scale and was adjusted for energy intake. Model 2 was further adjusted for other potential cofounders (BMI, education level, and physical activity) and known or suspected risk factors for RA (smoking status, passive smoking in childhood, and gastrointestinal transit time).

In order to investigate potential interactions between smoking status, a major risk factor for RA, and the Mediterranean diet score, we first tested an interaction term between the diet score and smoking. We then stratified the analyses according to smoking status (ever-smokers and never-smokers). Finally, we built a model that considered all combinations of the Mediterranean diet adherence score and smoking status. In that model, exposure was categorized as follows: never-smoker and high adherence score, never-smoker and medium adherence score, never-smoker and low adherence score, ever-smoker and high adherence score, ever-smoker and medium adherence score, and ever-smoker and low adherence score. Model 2 was used to calculate the absolute risks of RA associated with combinations of smoking status and adherence score.

All statistical analyses were carried out using SAS software, version 9.3. *P* values less than 0.05 (2-tailed) were considered significant.

RESULTS

Characteristics of the study population. Among the 98,995 women in the cohort, 72,668 women filled in the dietary questionnaire, and 62,629 met the inclusion criteria, including 480 patients with incident RA (Figure 1). The mean ± SD age at the time of Q3 (baseline) was 52.5 ± 6.5 years (Table 1). Patients with incident RA were diagnosed at a mean ± SD of 11.7 ± 5.8 years after baseline. The mean ± SD age at the time of RA diagnosis was 65.2 ± 8.3 years. Antibody status was known for 165 patients (34.4%), including 153 (31.9%) seropositive RA cases.

Characteristics of the overall study population according to RA status are presented in Table 1. Among the entire study population, 18,308 women (29.2%) had a low Mediterranean

Table 1. Baseline characteristics of the study population*

	All (n = 62,629)	Subjects without RA (n = 62,149)	RA cases (n = 480)	*P*
Age at Q3, mean ± SD years	52.5 ± 6.5	52.5 ± 6.5	53.5 ± 6.4	0.0009
BMI at Q3, mean ± SD kg/m^2	22.9 ± 3.2	22.9 ± 3.2	23.3 ± 3.4	0.008
Occupation category				0.015
Teacher	44,885 (71.7)	44,562 (71.7)	323 (67.3)	
Higher-professional occupations	1,737 (2.8)	1,715 (2.8)	22 (4.6)	
Intermediate occupation	9,940 (15.9)	9,852 (15.9)	88 (18.3)	
Unemployed	1,602 (2.6)	1,589 (2.6)	13 (2.7)	
Other	399 (0.5)	392 (0.5)	7 (1.5)	
Not available	4,066 (6.5)	4,039 (6.5)	27 (5.6)	
Education level				0.047
Less than high school	8,322 (13.3)	8,240 (13.3)	82 (17.1)	
Up to 2-level university	32,032 (51.1)	31,794 (51.2)	238 (49.6)	
3–4-level university	22,275 (35.6)	22,115 (35.5)	160 (33.3)	
Smoking status				0.057
Current smoker	8,269 (13.2)	8,188 (13.2)	81 (16.9)	
Nonsmoker	33,558 (53.6)	33,314 (53.6)	244 (50.8)	
Former smoker	20,802 (33.2)	20,647 (33.2)	155 (32.3)	
Passive smoking in childhood				0.063
No	53,657 (85.7)	53,260 (85.7)	397 (82.7)	
Yes	8,972 (14.3)	8,889 (14.3)	83 (17.3)	
Gastrointestinal transit				0.064
Normal	45,104 (72.0)	44,775 (72.1)	329 (68.5)	
Diarrhea	1,720 (2.8)	1,698 (2.7)	22 (4.6)	
Constipation	8,579 (13.7)	8,507 (13.7)	72 (15.0)	
Alternating diarrhea/constipation	7,226 (11.5)	7,169 (11.5)	57 (11.9)	
Physical activity, mean ± SD MET hours/week	44.8 ± 28.7	44.8 ± 28.7	46.0 ± 30.1	0.38
Total daily intake, mean ± SD kcal	2,136.3 ± 542.4	2,136.3 ± 542.3	2,132.3 ± 555.9	0.87

* *P* values were obtained using logistic regression for categorical variables and using Student's *t*-test for continuous variables. Except where indicated otherwise, values are the number (%) of subjects. RA = rheumatoid arthritis; Q3 = third questionnaire (baseline); BMI = body mass index; MET = metabolic equivalent.

diet adherence score (between 0 and 3), 28,324 (45.2%) had a medium score (between 4 and 5), and 15,997 (25.5%) had a high score (between 6 and 9).

Food groups and risk of RA. Associations between the tertiles of consumption of each food group included in the Mediterranean diet score are reported in Table 2. Considered individually, no food group was singularly associated with RA risk. The only exception was for fish consumption, where a medium consumption of fish (9–25 gm/day on average) was associated with a decreased risk of RA, compared to low consumption (<9 gm/day) (HR 0.74 [95% CI 0.59–0.94]). However, no association was found between a high consumption of fish (>25 gm/day) and risk of RA (HR 0.99 [95% CI 0.80–1.22]), thus leading to a nonsignificant linear trend.

Mediterranean diet score and risk of RA. Associations between adherence to the Mediterranean diet and the risk of RA are presented in Table 3. Among the whole study population, adherence to the diet was not associated with a decreased risk of RA in models 1 or 2 (HR 0.86 [95% CI 0.67–1.10] for high adherence versus low adherence, using model 2).

However, we found an interaction between the diet score and smoking status (*P* for interaction = 0.009). Therefore, we stratified our analyses according to smoking status (ever-smoker versus never-smoker). Among ever-smokers, we found an inverse association between diet adherence score and the risk of RA, with a higher diet adherence score being associated with a decreased risk of RA (HR 0.91 [95% CI 0.84–0.99] for 1-point increase in Mediterranean diet score, using model 1; *P* = 0.03).

Table 2. RA risk according to tertiles of food group consumption in the study population*

	Subjects without RA (n = 62,149)	RA cases (n = 480)	Model 1 HR (95% CI)	Model 2 HR (95% CI)
Raw vegetables				
<146 gm/day	20,701 (33.31)	154 (32.08)	Referent	Referent
146–278 gm/day	20,744 (33.38)	175 (36.46)	1.12 (0.90–1.39)	1.13 (0.91–1.41)
>278 gm/day	20,704 (33.31)	151 (31.46)	0.95 (0.76–1.20)	0.95 (0.76–1.20)
Legumes				
<7 gm/day	21,300 (34.27)	178 (37.08)	Referent	Referent
7–20 gm/day	20,142 (32.41)	140 (29.17)	0.84 (0.67–1.05)	0.85 (0.68–1.06)
>20 gm/day	20,707 (33.32)	162 (33.75)	0.98 (0.78–1.22)	0.98 (0.79–1.23)
Fruits				
<157 gm/day	20,694 (33.30)	161 (33.54)	Referent	Referent
157–278 gm/day	21,087 (33.93)	160 (33.33)	0.94 (0.75–1.17)	0.97 (0.78–1.20)
>278 gm/day	20,368 (32.77)	159 (33.13)	0.92 (0.74–1.15)	0.95 (0.75–1.18)
Cereal products				
<120 gm/day	20,692 (33.29)	159 (33.13)	Referent	Referent
120–196 gm/day	20,827 (33.51)	174 (36.25)	1.09 (0.87–1.36)	1.10 (0.88–1.38)
>196 gm/day	20,630 (33.19)	147 (30.63)	0.92 (0.71–1.20)	0.93 (0.72–1.22)
Fish				
<9 gm/day	20,509 (33.00)	172 (35.83)	Referent	Referent
9–25 gm/day	19,628 (31.58)	121 (25.21)	0.74 (0.58–0.93)	0.74 (0.59–0.94)
>25 gm/day	22,012 (35.42)	187 (38.96)	0.99 (0.80–1.22)	0.99 (0.80–1.22)
Meat				
<51 gm/day	20,694 (33.30)	162 (33.75)	Referent	Referent
51–104 gm/day	20,762 (33.41)	155 (32.29)	0.97 (0.78–1.21)	0.97 (0.77–1.21)
>104 gm/day	20,693 (33.30)	163 (33.96)	1.06 (0.84–1.33)	1.03 (0.82–1.30)
Dairy products				
<120 gm/day	20,830 (33.52)	148 (30.83)	Referent	Referent
120–253 gm/day	20,628 (33.19)	167 (34.79)	1.13 (0.91–1.42)	1.14 (0.91–1.43)
>253 gm/day	20,691 (33.29)	165 (34.38)	1.11 (0.89–1.39)	1.12 (0.90–1.41)
Unsaturated fat: saturated fat ratio				
<1.15	20,689 (33.20)	166 (34.58)	Referent	Referent
1.15–1.38	20,764 (33.41)	154 (32.08)	0.93 (0.74–1.15)	0.92 (0.74–1.14)
>1.38	20,695 (33.30)	160 (33.33)	0.96 (0.77–1.20)	0.94 (0.75–1.17)
Alcohol				
<5 gm/day	26,365 (42.42)	209 (43.54)	Referent	Referent
5–25 gm/day	27,439 (44.15)	207 (43.13)	0.95 (0.78–1.15)	0.93 (0.77–1.13)
>25 gm/day	8,345 (13.43)	64 (13.33)	0.97 (0.74–1.29)	0.90 (0.68–1.20)

* Model 1 adjusted for total daily food intake (except for alcohol) and age. Model 2 adjusted for total daily food intake (except for alcohol), age, body mass index (<18.5 kg/m^2, 18.5–<25 kg/m^2, 25–30 kg/m^2, or ≥30 kg/m^2), smoking status (current smoker, nonsmoker, or former smoker), passive smoking in childhood (no or yes), gastrointestinal transit (normal, diarrhea, constipation, or alternating diarrhea/constipation), education level (less than high school, up to 2-level university, or 3–4-level university), and physical activity (quartiles). *P* for trend was not significant for any of the food groups assessed. Values are the number (%) of subjects. RA = rheumatoid arthritis; HR = hazard ratio; 95% CI = 95% confidence interval.

Table 3. RA risk according to smoking status and tertile of Mediterranean diet adherence score in the study population*

Mediterranean diet score	Subjects without RA	RA cases	Model 1 HR (95% CI)	Model 2 HR (95% CI)
Total population, no.	62,149	480	–	–
Low (0–3)	18,156 (29.21)	152 (31.67)	Referent	Referent
Medium (4–5)	28,113 (45.23)	211 (43.96)	0.88 (0.72–1.09)	0.89 (0.72–1.10)
High (6–9)	15,880 (25.55)	117 (24.38)	0.85 (0.67–1.09)	0.86 (0.67–1.10)
Per 1-point increase in score	–	–	0.95 (0.90–1.01)	0.96 (0.90–1.01)
Never-smokers, no.	33,314	244	–	–
Low (0–3)	10,078 (30.25)	73 (29.92)	Referent	Referent
Medium (4–5)	14,906 (44.74)	110 (45.08)	0.99 (0.74–1.33)	1.00 (0.74–1.34)
High (6–9)	8,330 (25.00)	61 (25.00)	0.95 (0.67–1.34)	0.96 (0.68–1.36)
Per 1-point increase in score	–	–	0.99 (0.91–1.07)	1.00 (0.92–1.08)
Ever-smokers, no.	28,835	236	–	–
Low (0–3)	8,078 (28.01)	79 (33.47)	Referent	Referent
Medium (4–5)	13,207 (45.80)	101 (42.80)	0.78 (0.58–1.05)	0.78 (0.58–1.05)
High (6–9)	7,550 (26.18)	56 (23.73)	0.75 (0.53–1.06)	0.76 (0.54–1.08)
Per 1-point increase in score	–	–	0.91 (0.84–0.99)†	0.92 (0.85–0.99)‡

* Model 1 adjusted for total daily food intake (except for alcohol) and age. Model 2 adjusted for total daily food intake (except for alcohol), age, body mass index (<18.5 kg/m^2, 18.5–<25 kg/m^2, 25–30 kg/m^2, or ≥30 kg/m^2), smoking status (current smoker, nonsmoker, or former smoker), passive smoking in childhood (no or yes), gastrointestinal transit (normal, diarrhea, constipation, or alternating diarrhea/constipation), education level (less than high school, up to 2-level university, or 3–4-level university), and physical activity (quartiles). *P* for trend was not significant in the total population or in the never-smoker or ever-smoker group. Except where indicated otherwise, values are the number (%) of subjects. See Table 2 for definitions.
† $P = 0.03$.
‡ $P = 0.04$.

There was no association among never-smokers (HR 0.99 [95% CI 0.91–1.07] for 1-point increase in score, using model 1; $P = 0.74$).

In addition, when restricting the analyses to the 153 confirmed seropositive subjects, the association between Mediterranean diet score and RA risk among ever-smokers, although no longer statistically significant due to reduced power, remained of the same magnitude (HR 0.76 [95% CI 0.90–1.08] for high adherence versus low adherence) (see Supplementary Table 1, on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41487/abstract).

Finally, by combining Mediterranean diet adherence score categories (low, medium, or high) with smoking status (ever-smoker or never-smoker), we observed that ever-smokers with a low diet adherence score were at the highest risk for RA. The absolute risk of RA was lowest in never-smokers with a high score (35.8 per

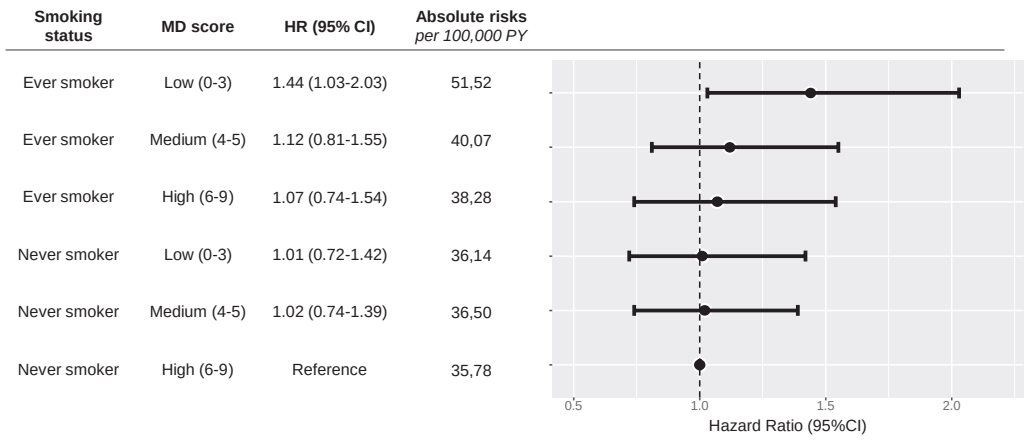


Figure 2. Risk of incident rheumatoid arthritis (RA) according to smoking status and Mediterranean diet (MD) adherence score. Results are expressed as hazard ratios (HRs) and 95% confidence intervals (95% CIs). Models were adjusted for total daily food intake (kcal/day; except for alcohol), age (as the time scale), body mass index (<18.5 kg/m^2, 18.5–<25 kg/m^2, 25–30 kg/m^2, or ≥30 kg/m^2), gastrointestinal transit (normal, diarrhea, constipation, alternating diarrhea/constipation), education level (less than high school, up to 2-level university, or 3–4-level university), and physical activity (in quartiles). PY = person-years.

100,000 person-years), while it was highest in ever-smokers (51.5 per 100,000 person-years) (Figure 2). In ever-smokers, having a high diet adherence score strongly reduced the risk associated with smoking, with an absolute risk of 38.3 per 100,000 person-years, similar to the risk observed in never-smokers with a similar diet.

DISCUSSION

In this large, population-based prospective cohort study of French women, we observed an inverse association between adherence to the Mediterranean diet and RA risk among ever-smokers but not among never-smokers. The association between diet and risk of RA has been previously studied, but most studies focused on a single food component, such as olive oil, fish consumption, or ω-3 fatty acids (11–13,26). However, interactions between food components and smoking status have already been reported, such as in the Nurses' Health Study (NHS and NHSII), in which ever-smokers with infrequent fish intake had a highly elevated risk of RA (HR 2.59) versus never-smokers with frequent fish intake (*P* for interaction = 0.039) (26).

To our knowledge, only 3 studies have investigated the association between the Mediterranean diet and RA risk (27–30). The only prospective study of RA, involving 174,638 female nurses from the NHS and NHSII and 913 incident cases, did not demonstrate any overall association between the Mediterranean diet score and the risk of RA (27). However, these researchers used an alternate Mediterranean diet score, which did not include dairy products (31), and their results may only apply to American women whose dietary habits might differ from their European counterparts. Indeed, since the Mediterranean diet score is based on the median consumption within a study population, it highly depends on the location of the assessed population, and results might differ between cohorts with different dietary habits. In a Swedish nested case–control study in the Västerbotten Intervention Program that compared 396 RA cases to 1,886 controls, there was no association between the Mediterranean diet score and RA risk, although the score was associated with a nonsignificant risk reduction among smokers (28).

Finally, a recent case–control study from the Swedish Epidemiological Investigation of RA that included 1,721 incident RA cases and 3,667 controls demonstrated an inverse association between the Mediterranean diet score and RA risk (odds ratio [OR] 0.79 [95% CI 0.65–0.96]) (29). Interestingly, this inverse association was only observed in men and in RF-positive RA patients, but not among women or RF-negative RA patients. The association was also observed in smokers (OR 0.62 [95% CI 0.40–0.95]), which is consistent with the known association between smoking and the risk of RF-positive RA. However, case–control studies are prone to recall and reverse causation bias, as patients with early RA might have changed their dietary habits. Thus, although reported associations were consistent with our findings, there is a need for further prospective studies.

Although the benefits of the Mediterranean diet have been shown to reduce overall mortality, cardiovascular diseases, or cancers (10,23,31,32), its mechanism is not fully understood and might include decreasing inflammation or increasing antioxidant levels (30). In the present study, we found an inverse association between a high adherence to the Mediterranean diet and RA risk only among ever-smoking women but not among nonsmoking women. This could be explained by the differences in RA pathophysiologic mechanisms between smokers and nonsmokers (6,33). Increased oxidant effect of smoking might be counterbalanced by the antioxidant effect of the Mediterranean diet. Thus, a strong adherence to this diet could reduce the increased risk of RA associated with smoking.

We acknowledge some limitations to our study. First, our population included only French women. However, as the incidence of RA is higher in women than in men, this was the appropriate population to test our hypothesis. Also, dietary habits were assessed only a single time. Nevertheless, the dietary questionnaire has been shown to have high validity and to be reproducible among a subset of participants (21). In addition, the onset of autoimmunity is a long process, and once triggered may be little modified by small changes in the diet. Here, the mean follow-up duration between administering the questionnaire and RA diagnosis was 11.7 years, which is consistent with the suggested delay between the triggering of autoimmunity and the onset of RA symptoms (34). Modifications in dietary habits may also induce reverse causality bias, because subjects with symptoms may try modifying their diets. We were not able to account for the intensity and duration of smoking in our models. Only 153 subjects (32%) were confirmed to be seropositive for RA. This is due to an important lack of knowledge about the serologic status of the patients (available for only 165 RA subjects), despite our attempts to retrieve antibody status by contacting rheumatologists and general practitioners. Thus, the autoantibody status of the 315 other subjects is unknown. When restraining our analysis to the 153 seropositive subjects, the association between the Mediterranean diet score and RA risk was the same magnitude but was no longer statistically significant because of reduced power. Finally, identification of RA cases relied on self-reported data. However, as previously discussed, we highly improved the accuracy of our case identification by using a specific questionnaire and a medication database, with a positive predictive value of 72% and 90.1%, respectively (17).

In conclusion, the Mediterranean diet could reduce the excess risk of RA in ever-smoking women. Our findings must be confirmed in other prospective cohorts.

ACKNOWLEDGMENTS

The authors are indebted to all participants for their continued participation. We would like to thank Pascale Gerbouin-Rerolle, Maxime Valdenaire, and Roselyn Rima Gomes for their help with data management. The present work was performed using data from the INSERM E3N cohort and

support from the MGEN, Gustave Roussy, and the Ligue Contre le Cancer for setting up and maintaining the cohort.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Nguyen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Nguyen, Salliot, Mariette, Boutron-Ruault, Seror.

Acquisition of data. Nguyen, Gelot, Gambaretti.

Analysis and interpretation of data. Salliot, Gelot, Gambaretti, Mariette, Boutron-Ruault, Seror.

REFERENCES

1. Klareskog L, Padyukov L, Rönnelid J, Alfredsson L. Genes, environment and immunity in the development of rheumatoid arthritis. Curr Opin Immunol 2006;18:650–5.
2. Karlson EW, Chang SC, Cui J, Chibnik LB, Fraser PA, de Vivo I, et al. Gene–environment interaction between HLA-DRB1 shared epitope and heavy cigarette smoking in predicting incident rheumatoid arthritis. Ann Rheum Dis 2010;69:54–60.
3. Linn-Rasker SP, van der Helm-van Mil AH, van Gaalen FA, Kloppenburg M, de Vries RR, le Cessie S, et al. Smoking is a risk factor for anti-CCP antibodies only in rheumatoid arthritis patients who carry HLA-DRB1 shared epitope alleles. Ann Rheum Dis 2006;65:366–71.
4. Lee HS, Irigoyen P, Kern M, Lee A, Batliwalla F, Khalili H, et al. Interaction between smoking, the shared epitope, and anti–cyclic citrullinated peptide: a mixed picture in three large North American rheumatoid arthritis cohorts. Arthritis Rheum 2007;56:1745–53.
5. Bang SY, Lee KH, Cho SK, Lee HS, Lee KW, Bae SC. Smoking increases rheumatoid arthritis susceptibility in individuals carrying the HLA–DRB1 shared epitope, regardless of rheumatoid factor or anti–cyclic citrullinated peptide antibody status. Arthritis Rheum 2010;62:369–77.
6. Willemze A, van der Woude D, Ghidey W, Levarht EW, Stoeken-Rijsbergen G, Verduyn 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: 1823–32.
7. Too CL, Yahya A, Murad S, Dhaliwal JS, Larsson PT, Muhamad NA, et al. Smoking interacts with HLA-DRB1 shared epitope in the development of anti-citrullinated protein antibody-positive rheumatoid arthritis: results from the Malaysian Epidemiological Investigation of Rheumatoid Arthritis (MyEIRA). Arthritis Res Ther 2012;14:R89.
8. Guillemin F, Saraux A, Guggenbuhl P, Roux CH, Fardellone P, Bihan EL, et al. Prevalence of rheumatoid arthritis in France: 2001. Ann Rheum Dis 2005;64:1427–30.
9. UNESCO. Multimedia video and sound collections: the Mediterranean diet. URL: http://www.unesco.org/archives/multimedia/?pg=33&s=films_details&id=1680&vl=Eng&vo=2.
10. Sofi F, Macchi C, Abbate R, Gensini GF, Casini A. Mediterranean diet and health status: an updated meta-analysis and a proposal for a literature-based adherence score. Public Health Nutr 2014;17: 2769–82.
11. Linos A, Kaklamanis E, Kontomerkos A, Koumantaki Y, Gazi S, Vaiopoulos G, et al. The effect of olive oil and fish consumption on rheumatoid arthritis: a case control study. Scand J Rheumatol 1991;20:419–26.
12. Linos A, Kaklamani VG, Kaklamani E, Koumantaki Y, Giziaki E, Papazoglou S, et al. Dietary factors in relation to rheumatoid arthritis: a role for olive oil and cooked vegetables? Am J Clin Nutr 1999;70:1077–82.
13. Shapiro JA, Koepsell TD, Voigt LF, Dugowson CE, Kestin M, Nelson JL. Diet and rheumatoid arthritis in women: a possible protective effect of fish consumption. Epidemiol Camb Mass 1996;7:256–63.
14. Hu FB. Dietary pattern analysis: a new direction in nutritional epidemiology. Curr Opin Lipidol 2002;13:3–9.
15. Sparks JA, Costenbader KH. Rheumatoid arthritis in 2017: protective dietary and hormonal factors brought to light [review]. Nat Rev Rheumatol 2018;14:71–2.
16. Clavel-Chapelon F, van Liere MJ, Giubout C, Niravong MY, Goulard H, Corre CL, et al, on behalf of the E3N Group. E3N, a French cohort study on cancer risk factors: epidemiological study among women from National Education. Eur J Cancer Prev 1997;6:473–8.
17. Nguyen Y, Salliot C, Gusto G, Descamps E, Mariette X, Boutron-Ruault MC, et al. Improving accuracy of self-reported diagnoses of rheumatoid arthritis in the French prospective E3N-EPIC cohort: a validation study. BMJ Open 2019;9:e033536.
18. Guillemin F, Saraux A, Fardellone P, Guggenbuhl P, Behier JM, Coste J, et al. Detection of cases of inflammatory rheumatic disorders: performance of a telephone questionnaire designed for use by patient interviewers. Ann Rheum Dis 2003;62:957–63.
19. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
20. Seror R, Henry J, Gusto G, Aubin HJ, Boutron-Ruault MC, Mariette X. Passive smoking in childhood increases the risk of developing rheumatoid arthritis. Rheumatology (Oxford) 2019;58:1154–62.
21. Van Liere MJ, Lucas F, Clavel F, Slimani N, Villeminot S. Relative validity and reproducibility of a French dietary history questionnaire. Int J Epidemiol 1997;26 Suppl 1:S128–36.
22. Trichopoulou A, Kouris-Blazos A, Wahlqvist ML, Gnardellis C, Lagiou P, Polychronopoulos E, et al. Diet and overall survival in elderly people. BMJ 1995;311:1457–60.
23. Trichopoulou A, Costacou T, Bamia C, Trichopoulos D. Adherence to a Mediterranean diet and survival in a Greek population. N Engl J Med 2003;348:2599–608.
24. Couto E, Boffetta P, Lagiou P, Ferrari P, Buckland G, Overvad K, et al. Mediterranean dietary pattern and cancer risk in the EPIC cohort. Br J Cancer 2011;104:1493–9.
25. Nguyen Y, Mariette X, Salliot C, Gusto G, Boutron-Ruault MC, Seror R. Chronic diarrhoea and risk of rheumatoid arthritis: findings from the French E3N-EPIC Cohort Study. Rheumatology (Oxford) 2020. E-pub ahead of print.
26. Sparks JA, O'Reilly ÉJ, Barbhaiya M, Tedeschi SK, Malspeis S, Lu B, et al. Association of fish intake and smoking with risk of rheumatoid arthritis and age of onset: a prospective cohort study. BMC Musculoskelet Disord 2019;20:2.
27. Hu Y, Costenbader KH, Gao X, Hu FB, Karlson EW, Lu B. Mediterranean diet and incidence of rheumatoid arthritis in women. Arthritis Care Res 2015;67:597–606.
28. Sundström B, Johansson I, Rantapää-Dahlqvist S. Diet and alcohol as risk factors for rheumatoid arthritis: a nested case–control study. Rheumatol Int 2015;35:533–9.
29. Johansson K, Askling J, Alfredsson L, Giuseppe DD, for the EIRA Study Group. Mediterranean diet and risk of rheumatoid arthritis: a population-based case-control study. Arthritis Res Ther 2018;20:175.
30. Forsyth C, Kouvari M, D'Cunha NM, Georgousopoulou EN, Panagiotakos DB, Mellor DD, et al. The effects of the Mediterranean diet on

rheumatoid arthritis prevention and treatment: a systematic review of human prospective studies. Rheumatol Int 2018;38:737–47.

31. Fung TT, Rexrode KM, Mantzoros CS, Manson JE, Willett WC, Hu FB. Mediterranean diet and incidence of and mortality from coronary heart disease and stroke in women. Circulation 2009;119:1093–100.
32. Mahamat-Saleh Y, Cervenka I, al Rahmoun M, Savoye I, Mancini FR, Trichopoulou A, et al. Mediterranean dietary pattern and skin cancer risk: a prospective cohort study in French women. Am J Clin Nutr 2019;110:993–1002.
33. Sundstrom B, Johansson I, Rantapaa-Dahlqvist S. Interaction between dietary sodium and smoking increases the risk for rheumatoid arthritis: results from a nested case-control study. Rheumatology (Oxford) 2015;54:487–93.
34. Nielen MM, van Schaardenburg D, Reesink HW, van de Stadt RJ, van der Horst-Bruinsma IE, de Koning MH, et al. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. Arthritis Rheum 2004;50:380–6.

American College of Rheumatology
Empowering Rheumatology Professionals

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 78–88
DOI 10.1002/art.41483

Association of a Serum Protein Signature With Rheumatoid Arthritis Development

Liam J. O'Neil,[1] Victor Spicer,[1] Irene Smolik,[1] Xiaobo Meng,[1] Rishi R. Goel,[2] Vidyanand Anaparti,[1] John Wilkins,[1] and Hani S. El-Gabalawy[1]

Objective. The pathophysiologic events that precede the onset of rheumatoid arthritis (RA) remain incompletely understood. This study was undertaken to identify changes in the serum proteome that precede the onset of RA, with the aim of providing new insights into the pathogenic mechanisms that lead to its development.

Methods. In a cohort of first-degree relatives of Indigenous North American RA patients, the SomaScan proteomics platform was used to determine the levels of 1,307 proteins in multiple longitudinal serum samples from 17 individuals who were followed up prospectively to the time of disease onset. Proteomic signatures from this group of individuals (designated the progressor group) were compared to those in a group of individuals who were considered at risk of developing RA, stratified as either positive (n = 63) or negative (n = 47) for anti–citrullinated protein antibodies (ACPAs) (designated the at-risk group). Machine learning was used to identify a protein signature that could accurately classify those individuals at highest risk of future RA development.

Results. A preclinical proteomic signature that differentiated RA progressors from at-risk individuals, irrespective of ACPA status, was identified (area under the curve 0.913, accuracy 91.2%). Importantly, the predictive preclinical proteomic signature was present not only in serum samples obtained close to the onset of RA, but also in serum samples obtained a median of 30.9 months prior to onset. Network analysis implicated the activation of Toll-like receptor 2 and production of tumor necrosis factor and interleukin-1 as key events that precede RA progression.

Conclusion. Alterations in the serum proteome in the preclinical phase of RA can emerge years prior to the onset of disease. Our findings suggest that the serum proteome provides a rich source of proteins serving both to classify at-risk individuals and to identify molecular pathways involved in the development of clinically detectable RA.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease that affects ~1% of the population worldwide; it leads to systemic and synovial joint inflammation, physical disability, and increased mortality (1). In the majority of patients with established RA, a spectrum of autoantibodies is detectable, particularly antibodies targeting endogenous proteins that have been posttranslationally modified by processes such as citrullination and carbamylation (2). Serologic studies of archival samples obtained from individuals who ultimately developed RA have shown that these autoantibodies, specifically anti–citrullinated protein antibodies (ACPAs), are detectable months to years prior to the onset of clinically identifiable disease (3–5). In turn, this has served as an impetus to better characterize the preclinical stages of RA, with the hope of developing effective prevention strategies for this lifelong autoimmune disease (6).

To address this challenge, and considering the limitations of retrospective studies, we undertook a prospective longitudinal study of the unaffected first-degree relatives (FDRs) of Indigenous North Americans, a population known to have a high prevalence and familial clustering of RA (7,8). Based on this prospective study design spanning almost 15 years, we recently showed that despite an ACPA seroprevalence of almost 10% in the unaffected FDR, most ACPA-positive individuals do not develop RA, and indeed in a substantial proportion of these individuals, reversion to a seronegative state occurs after a prolonged observation period (3). Furthermore, as demonstrated in other studies, we showed

Supported by the Canadian Institutes of Health Research (grant MOP 77700 to Dr. El-Gabalawy).

[1]Liam J. O'Neil, MD, Victor Spicer, BSc, Irene Smolik, PhD, Xiaobo Meng, PhD, Vidyanand Anaparti, PhD, John Wilkins, PhD, Hani S. El-Gabalawy, MD: University of Manitoba, Winnipeg, Manitoba, Canada; [2]Rishi R. Goel, MSc: University of Pennsylvania Perelman School of Medicine, Philadelphia.

No potential conflicts of interest relevant to this article were reported.

Address correspondence to Liam J. O'Neil, MD, University of Manitoba, Department of Internal Medicine and Immunology, Division of Rheumatology, Room RR149E, Health Sciences Centre, 820 Sherbrook Street, Winnipeg MB R3A 1R9, Canada. Email: liam.oneil@umanitoba.ca.

Submitted for publication December 27, 2019; accepted in revised form August 4, 2020.

that individuals who ultimately developed RA exhibited progressive maturation of the ACPA response and accrual of multiple autoreactivities (9). These observations have emphasized the need for additional biomarker approaches with which to identify individuals at highest risk for future RA development, with the aim of undertaking potential prevention strategies in an ethical and cost-effective manner.

SomaScan is an aptamer-based proteomics platform that allows the detection of hundreds of proteins simultaneously (10–12). It has distinct advantages over antibody-based assays, and has previously been used to identify biomarkers in patients with established RA (13). Using the SomaScan platform, we aimed to identify changes in the serum proteome that precede the onset of RA, in order to provide new insights into the pathogenic mechanisms that lead to its development. In the current study, we were able to identify a rich data set of proteins based on the SomaScan platform in serum samples from FDRs of Indigenous North American RA patients. We applied machine-learning algorithms to analyze preclinical serum samples from individuals who ultimately developed RA, designated the progressor group, and compared the findings to those in serum samples obtained from a group of individuals considered at risk of developing RA, designated the at-risk group. Our results indicate that a small proteomic signature could be used to predict future RA onset with a high degree of accuracy, and that the differences in the proteomic signature between the progressor group and the at-risk group were demonstrable years prior to disease onset, irrespective of baseline ACPA status.

SUBJECTS AND METHODS

Cohort overview and sample selection. The methods and protocols used for patient recruitment for this study were described in a previous report (14). In brief, Indigenous North American RA probands who met the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for RA (15) were approached to help recruit their eligible FDR for longitudinal follow-up. We expected that a proportion of these patients would go on to develop inflammatory arthritis. Both RA probands and FDRs were required to have at least 3 grandparents with Indigenous North American ethnicity, ascertained by self-report. All participants had to be age 18 years or older.

All study participants provided informed consent, in accordance with the Declaration of Helsinki. The Biomedical Research Ethics Board of the University of Manitoba approved all aspects of the study (approval no. HS14453). Specific community research agreements were put in place with the study communities. Consistent with the guidelines from the Canadian Institutes of Health Research for conducting research involving indigenous people in Canada, we established an arthritis advisory board, to provide oversight with regard to the indigenous cohort used in this study.

At baseline, all FDRs were examined by a rheumatologist (HSE) to confirm the absence of clinical synovitis. Participants were then entered into the study and underwent annual examinations for the presence of clinical synovitis. Between the annual evaluations, FDRs were instructed to report any new symptoms suggestive of arthritis, and clinical assessment by a member of the research team (HSE) took place as soon as possible, to assess the reported symptoms. If synovitis was unequivocally detected in one or more joints by a rheumatologist, the individual was deemed as having "progressed" to having inflammatory arthritis.

Serum samples were collected at all study visits and stored at –20°C for future studies. Serum rheumatoid factor (RF) was measured by nephelometry. Anti–cyclic citrullinated peptide (anti-CCP) antibodies were detected using CCP2 and/or CCP3 enzyme-linked immunosorbent assays (Inova), with the manufacturer's cutoff value used to determine antibody positivity. At the time of the current SomaScan study, progression to inflammatory arthritis was unequivocal in 17 individuals.

Longitudinal serum samples were selected for serum proteomics analysis, with the aim of enriching for multiple preclinical samples as well as samples obtained after the onset of arthritis. For the purposes of the present study, the at-risk group included both ACPA-positive and ACPA-negative individuals (FDRs of RA probands) who did not develop inflammatory arthritis. The progressor group of FDRs were distinguished from the at-risk group as being individuals who ultimately developed inflammatory arthritis. At baseline, progressors and at-risk individuals were clinically indistinguishable.

SomaScan proteomics assay. SomaScan, a proteomics assay that measures >1,300 proteins using an aptamer library, was performed to assess a total of 127 serum samples available from our FDR cohort (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract). Briefly, for this assay, a library of aptamers is incubated with serum, and proteins that bind are isolated and hybridized to a DNA microarray for detection. The identity and relative concentration of the detected proteins are revealed by localization and measurement of fluorescence intensity. Results of protein quantification are reported in relative fluorescence units (RFU), an arbitrary value.

The RFU values for SOMAmer protein expression observed in the serum from the study patients were transformed into a $\log_2$ scale for differential analysis. A previous study indicated that agreement between aptamer and antibody-based assay results is high (11). When performing the SomaScan assay with our SOMAmer data, batch effects were removed with the use of internal controls. The overall data set was run in 3 plates spanning 2 batches of the same version of reagents (SOMAmer kit version 1.3). We did not observe any batch-level effects manifesting on the principle components analysis (PCA) level with the data from

the $\log_2$ expression matrix. On the advice of the SomaScan manufacturer (SomaLogic), which reviewed the data set, we proceeded with the analysis without attempting to apply computational batch correction. Further details regarding the SomaScan assay are reported elsewhere (16).

Study population, sample selection, and experimental design. We focused our longitudinal proteomics analysis on serum samples from 17 individuals characterized as progressors (see Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract) who ultimately developed inflammatory arthritis after a median duration of preclinical follow-up of 30.8 months. Although samples were not available over the entire disease spectrum for all progressors, we reasoned that using all available samples for our analyses would increase the power of our analyses and would reduce possible bias. Of the 15 individuals with data available from the pre-progression period, 11 (73.3%) were already ACPA positive. At the time of disease onset, nearly all patients were ACPA positive (93.3%), and 81.3% were RF positive. Symptoms of arthritis in the joints, as ascertained using a self-report questionnaire tool, were highly prevalent in all groups at baseline and were not enriched in the progressor group. In fact, the frequency of patient-reported joint symptoms was universally lower in patients who progressed compared to their at-risk counterparts (Table 1).

To characterize changes in 1,307 serum proteins using the SomaScan platform, we interrogated 2 preclinical serum samples, with 1 of the samples being relatively close to RA onset (designated ON –1; median duration prior to onset 23.4 months) and the other sample being more remote from onset (designated ON –2; median duration prior to onset 30.8 months). We classified the ON –1 serum samples as imminent, as they represented the sample obtained closest to the onset of inflammatory arthritis. In parallel, we analyzed serum samples at the time of onset of clinically identifiable inflammatory arthritis (designated ON), and samples obtained after inflammatory arthritis onset (designated ON +1). For longitudinal data trends, this nomenclature (relative to onset) is used to bin samples and identify broad trends over time. Thus, for each progressor, we were able to characterize the evolution of the serum proteome over an extended timeframe, spanning the period prior to RA onset through to clinical onset and finally to the time after RA had become established. In order to bring an appropriate context to this longitudinal proteomics analysis of the progressors, we compared their respective SomaScan protein profiles to those in ACPA-negative (n = 63) and ACPA-positive (n = 47) at-risk individuals. Details on the experimental design are outlined in Supplementary Figure 2 (available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract).

Table 1. Baseline characteristics of the study subjects with serum samples available for aptamer-based proteomics assay*

	At-risk		
	ACPA negative (n = 47)	ACPA positive (n = 63)	Progressor (n = 17)
Age, mean ± SD years	41.3 ± 14.1	43.3 ± 12.9	31.4 ± 11.8
Age range, years	18.7–66.2	20.6–63.5	20.1–65.4
Female, %	58.7	76.2	73.3
BMI, mean ± SD kg/m^2	29.6 ± 7.4	30.9 ± 7.3	26.5 ± 7.2
CRP, mean ± SD mg/dl	4.2 ± 5.0	6.7 ± 8.6	4.1 ± 3.8
ACPA positive, %	0	100	73.3
RF positive, %	14.9	27.0	53.3
Median time to arthritis onset, months	–	–	30.8
Hand joint pain, %	35.7	30.0	20.0
Joint pain excluding hands, %	71.4	69.5	60.0
Hand joint swelling, %	54.8	43.3	33.3
Joint swelling excluding hands, %	38.1	45.0	40.0
Morning hand stiffness, %	58.1	63.3	40.0
Morning stiffness excluding hands, %	72.1	65.0	60.0

* ACPA = anti–citrullinated protein antibody; BMI = body mass index; CRP = C-reactive protein; RF = rheumatoid factor.

Statistical analysis. Descriptive results (as reported in Table 1) are expressed as the mean ± SD, unless specified otherwise. We aimed to identify group differences between pre- and post-progression, as well as differences between pre-progression and at-risk individuals. Group comparisons of the proteomics data were performed using a Welch's *t*-test. Lists of differentially expressed proteins were generated based on identification of significant differences in gene expression between groups, according to *P* values less than 0.05. The differentially expressed protein lists were visualized using ggplot2 (version 3.1.1; R program).

To get an overview of samples in 2-dimensional space, we used multidimensional scaling (MDS), a method of PCA. Using the Pythagorean theorem, the linear distance was calculated based on an arbitrary point in the MDS plot, as a method of quantifying distance by defined groups of samples. Differences in specific proteins were visualized as box plots, and these were generated using R statistical software (version 3.5.3) and the ggplot2 package. Heatmaps were generated using scaled expression data (scaled per gene) and plotted using the R program pheatmap (version 1.0.12). Venn diagrams were generated using the web tool Venny (17). We also produced trajectory maps of specific proteins over the course of the pre-progression period up to the onset of inflammatory arthritis. The geom_smooth ggplot function was used to draw smoothed trend lines for protein expression for select analytes. Correlation analyses were performed for select proteins using Pearson's correlation tests. To analyze proteins associated with progression to RA onset, linear regression was performed in base R.

To better understand the biologic role of these proteins, select sets of proteins were further analyzed using clusterProfiler (version 3.10.1) to enrich for Gene Ontology (GO) terms. Select protein sets were also uploaded to Ingenuity Pathway Analysis (IPA) software (Qiagen), primarily for analysis of upstream targets that were exported based on relevance. Upstream regulators were filtered based on detectable expression in our protein sets, in an attempt to reduce the false discovery rate.

Three LASSO (least absolute shrinkage and selection operator) regression models were designed to explore the minimum set of proteins that would allow classification of pre-progression samples and at-risk individuals, both those without ACPAs (model 1) and those with ACPAs (model 2). Given the exploratory nature of these models, they were trained on 100% of the data, and a lambda 1 standard error was chosen, such that the error is within 1 standard error above the minimum. This method was used for practical purposes, as it tends to avoid overfitting to produce the most regularized model with the minimum set of variables.

We then built a LASSO regression model with the aim of finding a minimum set of proteins that would successfully discriminate pre-progression samples from at-risk samples, regardless of ACPA status. For this model (model 3), we split the entire data set into a training cohort (75%) and a validation cohort (25%). The models were run in R using the package Glmnet (version 2.0). Cross-validation was performed to determine the tuning parameter (lambda), which identified a minimum and 1 standard error of the minimum variables for the model. LASSO regression allows for both shrinkage and selection of variables. Furthermore, it provides a quantifiable output (i.e., a progression score, in arbitrary units) for each sample included in its synthesis, calculated using weighted coefficients of the proteins included in the trained model. Proteins from the LASSO regression model were analyzed for stability based on the resources provided by Candia et al (18).

RESULTS

Association of pre–clinical progression to RA onset with distinct changes in the serum proteome. For each of our 17 individuals classified as progressors, we compared the proteomic profile delineated in the 2 preclinical serum samples (ON −1 and ON −2) to that in the sample obtained at the time of clinically detectable synovitis in one or more joints (ON or ON +1). Importantly, the primary clinical difference between these 2 selected time points is the development of arthritis and systemic inflammation. Concurrent with this, following progression to inflammatory arthritis (ON), the overall mean C-reactive protein level increased from 4.0 to 9.34 mg/dl (P = 0.02), reflecting the onset of general systemic inflammation. Furthermore, the median Disease Activity Score in 28 joints, a measure of arthritis disease activity (19), was found to be 3.8 in the progressors at the time of their onset visit (ON), reflecting clear clinical evidence of active synovial inflammation. Indeed, the majority of progressors (64.3%) had a diagnosis of RA meeting the 2010 ACR/EULAR classification criteria (15,20). Thus, the differences detected in the serum proteome between these 2 time periods are reflective of the uncontrolled inflammatory response that is observed in the very early stages of RA.

Based on this analysis, we observed up-regulation of 149 proteins and down-regulation of 96 proteins in serum samples from the 17 individuals in the progressor group. A listing of these proteins can be found in Supplementary Table 2 (available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract). Proteins with the highest up-regulation reflect common pathways associated with a proinflammatory state (Figure 1A). Our analysis indicated that, among the proteins with the highest up-regulation, JAK2, interleukin-36A (IL-36A), and inter-alpha-trypsin inhibitor heavy chain 4 were identified (Figure 1B). These findings highlight the known up-regulation of proinflammatory proteins associated with inflammatory arthritis. Of note, the down-regulated proteins included protein tyrosine phosphatase non-receptor type 1 and complement component 1r.

Our longitudinal data set allowed us to map the longitudinal trajectory of the proteins as a function of time. Figure 1C displays examples of how the levels of individual proteins changed over the course of disease progression in the progressors as a whole, relative to the onset of arthritis. Interestingly, these proteomic changes paralleled an increase in the ACPA titers, a hallmark of RA development (21). The observed up-regulated proteins after progression to inflammatory arthritis also overlapped to some extent with biomarkers, discovered using the same technology, in Japanese patients with early RA, in particular inflammatory proteins such as serum amyloid A1 (13). These data provide an overview of the proinflammatory serum proteome profile across several years as the condition in individuals progresses from a state of well-being to inflammatory arthritis.

Evidence of broad proteomic changes in progressors years before the onset of RA. Preclinical RA has been characterized by the detection of autoantibodies such as ACPAs and RF. We aimed to explore the proteomic differences that potentially parallel the pathologic immune mechanisms. Although we studied at-risk FDRs of Indigenous North American RA patients, who are known to have an inherently increased risk of RA development based on epidemiologic studies (8), we reasoned that serum samples obtained from at-risk FDRs who did not develop RA, irrespective of ACPA status, could serve as an appropriate comparator for the pre–RA onset serum samples from the progressors.

We compared the preclinical serum proteome signature in the progressors to that in the control group, aiming to identify patterns that could differentiate unaffected at-risk individuals who are likely to develop RA from those who are not. We identified 669 proteins that were differentially expressed between these

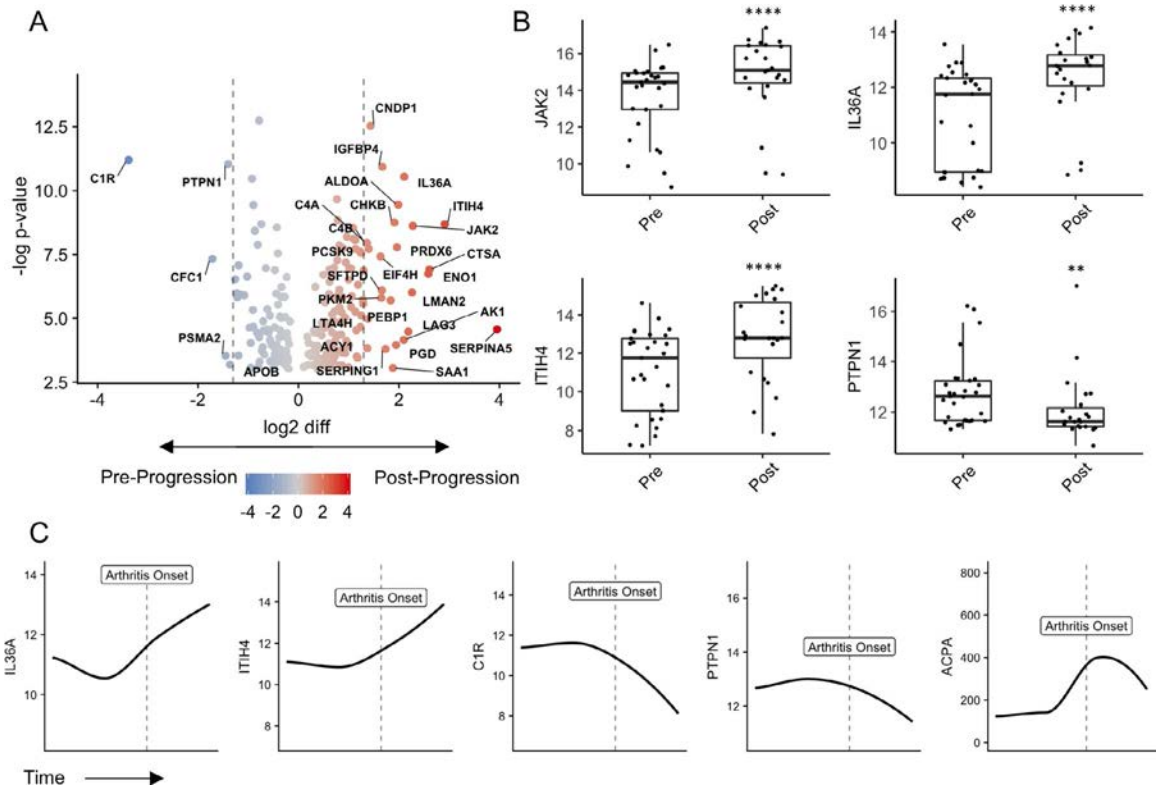


Figure 1. Serum proteins implicated in the pathogenesis of inflammatory arthritis in longitudinal progressor samples. **A**, Volcano plot of differentially expressed proteins between pre-progression (n = 15 subjects, 29 samples) and onset of inflammatory arthritis (post-progression) (n = 14 subjects, 23 samples). Annotated analytes have the highest $\log_2$ differential (diff) expression, and the color scale shows grading based on expression differences. Vertical broken lines indicate the cutoffs for defining the differentially expressed proteins. **B**, Box plots of $\log_2$ expression of JAK2, interleukin-36A (IL-36A), inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4), and protein tyrosine phosphatase non-receptor type 1 (PTPN1) in serum samples pre- and post-progression. Symbols represent individual samples. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. **C**, Smoothed trend lines of serum protein kinetic expression in progressors, mapped over time relative to the onset of clinical arthritis, for expression of IL-36A, ITIH4, complement C1r (C1R), PTPN1, and anti–citrullinated protein antibodies (ACPAs) (n = 17 subjects, 52 samples). ** = $P < 0.01$; **** = $P < 0.001$. CNDP1 = carnosine dipeptidase 1; IGFBP4 = insulin-like growth factor binding protein 4; ALDOA = aldolase, fructose-bisphosphate A; CHKB = choline kinase beta; PCSK9 = proprotein convertase subtilisin/kexin type 9; PRDX6 = peroxiredoxin 6; CTSA = cathepsin A; CFC1 = crypto, FRL-1, cryptic family 1; SFTPD = surfactant protein D; EIF4H = eukaryotic translation initiation factor 4H; ENO1 = enolase 1; PKM2 = pyruvate kinase M2; LMAN2 = lectin, mannose binding 2; PEBP1 = phosphatidylethanolamine binding protein 1; AK1 = adenylate kinase 1; LTA4H = leukotriene A_4 hydrolase; LAG3 = lymphocyte activating gene 3 protein; PSMA2 = proteasome 20S subunit alpha 2; ACY1 = aminoacylase 1; PGD = prostaglandin D; SERPINA5 = serpin family A member 5; APOB = apolipoprotein B; SAA1 = serum amyloid A1. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract.

2 groups of samples, with 260 proteins being up-regulated and 409 being down-regulated (Figure 2A). Among the proteins showing the highest up-regulation, integrins (in this case, α2 integrin 2B [ITGA2B]) potentially play a role in mediating the cell–cell relationships that are required for immune maturation (22). Histone H3.1 was also highly expressed in the progressor samples prior to onset (Figure 2B), suggesting the possibility that uncontrolled neutrophil extracellular trap formation, a common source of histones, may mediate pathogenic immunologic responses that increase the risk for disease development (23).

Samples plotted by MDS and with the linear distance calculated in the MDS plot (see Subjects and Methods) revealed differences between pre-progression samples and at-risk samples (Figures 2C and D), without any indication that these differences were driven by the proximity of the sample acquisition time to arthritis onset (imminent or remote). Indeed, k-means clustering was enriched for both pre-progression (cluster 1, 80.8%) and at-risk samples (cluster 2, 83.7%) (see Supplementary Figure 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract). Furthermore, we found 60% overlap of the differentially expressed proteins between progressors and at-risk individuals based on time relative to arthritis onset (Figure 2E). These findings suggest that the differences between at-risk and progressor individuals were not merely driven by their temporal relationship to arthritis onset.

We then developed 3 models using shrinkage regression to classify pre-progression samples from at-risk samples.

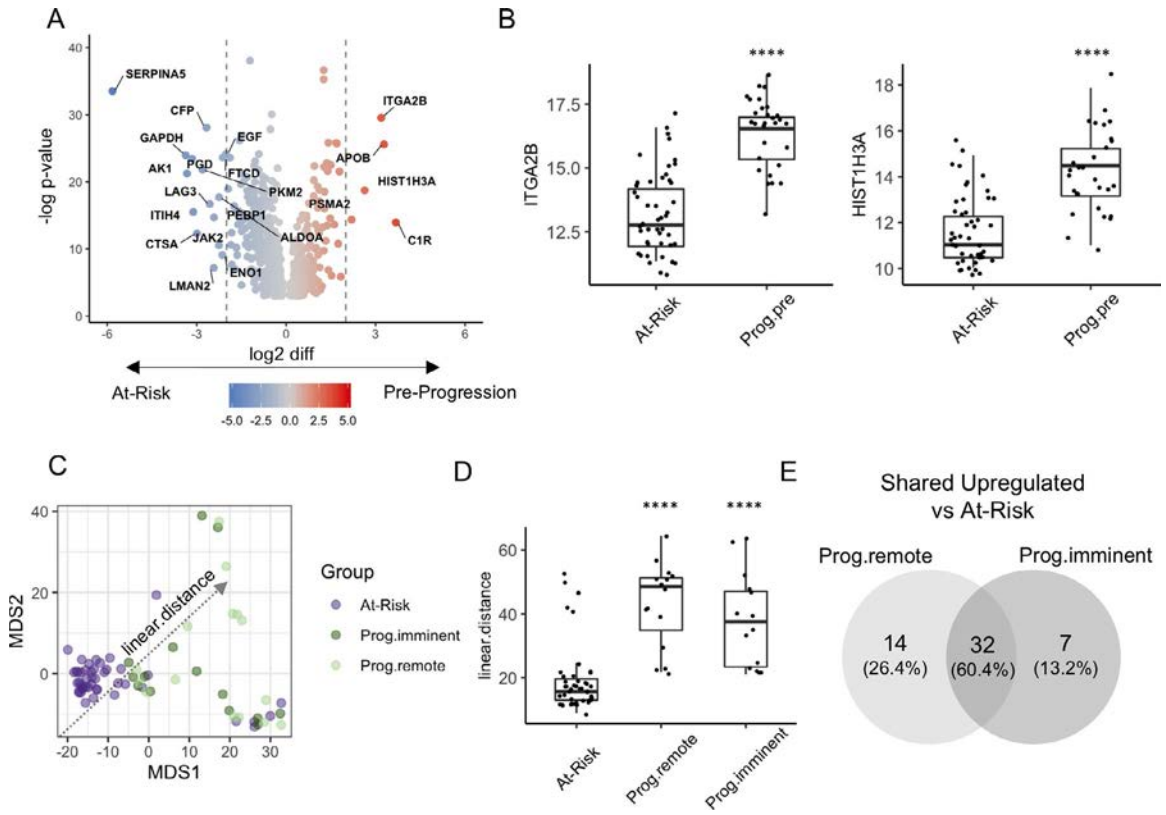


Figure 2. Differences in serum proteomic signatures in pre-progression serum samples compared to samples from at-risk individuals obtained either remote or imminent from the time of clinical arthritis onset. **A**, Volcano plot of differentially expressed proteins between pre-progression (Prog.pre) (n = 15 subjects, 29 samples) and at-risk samples (n = 110 subjects). Annotated analytes have the highest $\log_2$ differential expression, and the color scale shows grading based on expression differences. Vertical broken lines indicate the cutoffs for defining the differentially expressed proteins. **B**, Box plots of $\log_2$ expression of α2 integrin 2B (ITGA2B) and histone H3.1 (HIST1H3A) in at-risk and pre-progression serum samples. **C**, Two-dimensional representation of serum proteome expression using multidimensional scaling (MDS) of proteomics data according to linear distance from time to rheumatoid arthritis (RA) onset. Serum samples were obtained from at-risk individuals (healthy first-degree relatives [FDRs] of RA patients; n = 110), FDRs relatively close to the time of RA onset (median 23.4 months) (Prog.imminent) (n = 13), and FDRs more remote from the time of RA onset (median 30.8 months) (Prog.remote) (n = 14). Linear distance is the distance calculated from the coordinates x = –20, y = –15. **D**, Box plots of linear distance as determined using MDS, indicating differences in serum proteome expression. In **B** and **D**, symbols represent individual samples. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. **E**, Venn diagram of numbers of up-regulated proteins in serum samples from at-risk individuals compared to samples obtained either remote or imminent from the time of RA onset. **** = $P < 0.001$ versus at-risk. CFP = complement factor properdin; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; EGF = epidermal growth factor; FTCD = formimidoyltransferase cyclodeaminase; PEBP1 = phosphatidylethanolamine binding protein 1 (see Figure 1 for other definitions). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract.

Progressors were distinguished from ACPA-negative at-risk individuals using a set of 17 proteins (model 1, accuracy 100%) (Supplementary Tables 3 and 4, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract), while 8 proteins distinguished progressors from ACPA-positive at-risk individuals (model 2, accuracy 86.9%) (Supplementary Tables 4 and 5, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract).

We then developed a 23-protein panel that classified, with 100% accuracy, the progressor samples from all at-risk individuals in a training cohort (n = 105) (Supplementary Figure 4 and Supplementary Tables 4 and 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract). We tested a validation cohort using this model (n = 34), which classified pre-progression samples with 91.2% accuracy, with an area under the curve (AUC) of 0.931 (Figures 3A and B).

Two of the proteins overlapped between all 3 models, ficolin 2 and calreticulin, the levels of which were lower in progressors compared to at-risk individuals (Supplementary Figure 5, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract). Previous work on the SomaScan platform indicated that there was

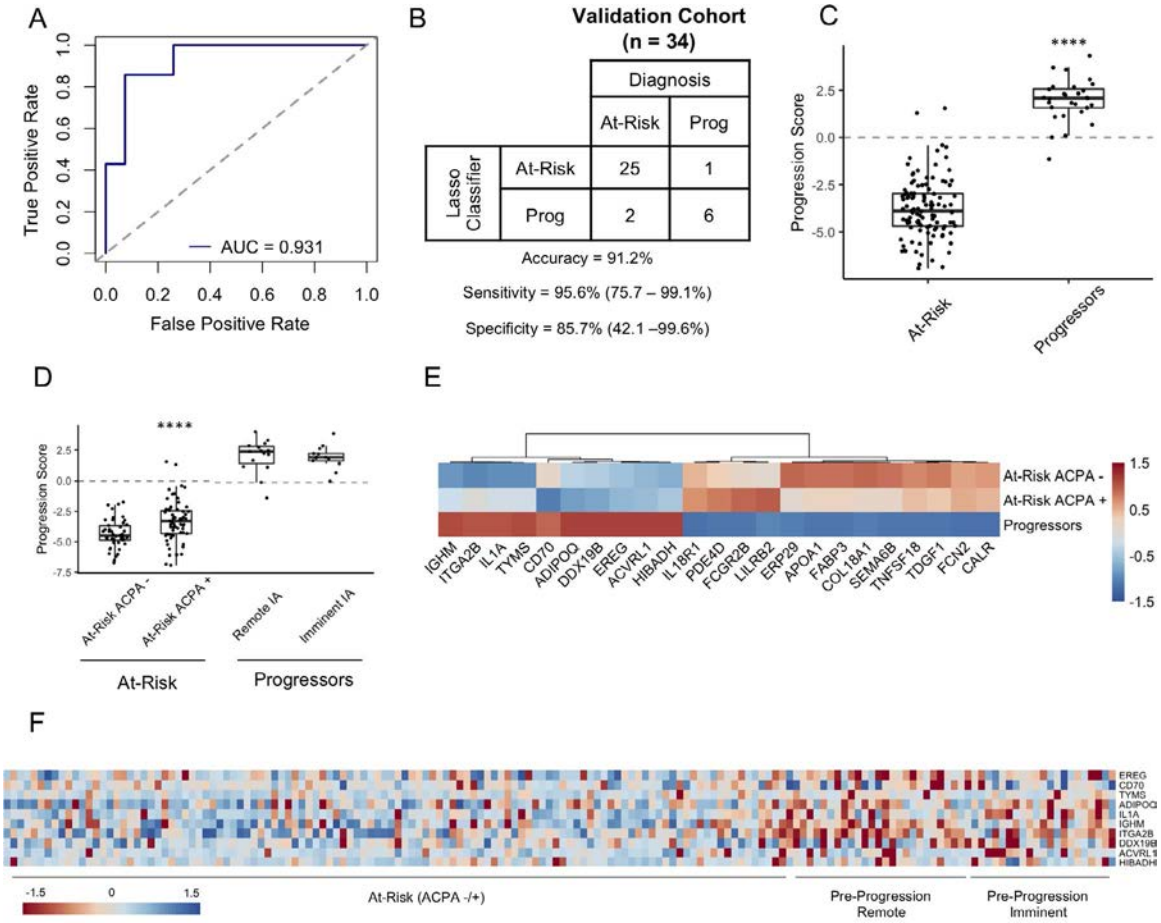


Figure 3. Machine-learning LASSO model identifying a 23-biomarker serum protein signature that accurately classifies pre-progression individuals from either anti–citrullinated antibody (ACPA)–positive or ACPA-negative at-risk individuals. **A**, Area under the curve (AUC) from the LASSO linear model to identify pre-progression samples distinguishable from samples obtained from at-risk individuals with or without ACPA positivity (AUC 0.931) in a validation cohort. **B**, Sensitivity and specificity of the linear model in the validation cohort of at-risk individuals and progressors (Prog). Values in parentheses are the 95% confidence intervals. **C**, Progression scores from the model classifying all individuals included in the development and validation of the LASSO model. The horizontal broken line represents the cutoff classifier score (y = 0) to identify subjects who experienced progression to inflammatory arthritis (IA). **D**, Progression scores for at-risk individuals stratified as ACPA positive or ACPA negative and progressors stratified according to distance from inflammatory arthritis onset (remote [n = 13] versus imminent [n = 14]; P = 0.897). In **C** and **D**, symbols represent individual samples. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. **E**, Representative heatmap of the average protein expression by group, normalized for each protein included in the progression score. **F**, Representative heatmap of individualized protein expression for the progression signature of up-regulated analytes. **** = $P < 0.001$ versus all at-risk or ACPA-negative at-risk individuals. IGHM = immunoglobulin heavy chain constant mu; ITGA2B = α2 integrin 2B; TYMS = thymidylate synthetase; ADIPOQ = adiponectin, C1Q and collagen domain containing; DDX19B = DEAD-box helicase 19B; EREG = epiregulin; ACVRL1 = activin A receptor like type 1; HIBADH = 3-hydroxyisobutyrate dehydrogenase; PDE4D = phosphodiesterase 4D; FCGR2B = Fc fragment of IgG receptor IIb; LILRB2 = leukocyte immunoglobulin like receptor B2; ERP29 = endoplasmic reticulum protein 29; FABP3 = fatty acid binding protein 3; COL18A1 = collagen type XVIII alpha 1 chain; SEMA6B = semaphoring 6B; TNFSF18 = tumor necrosis factor superfamily member 18; TDGF1 = teratocarcinoma-derived growth factor 1; FCN2 = ficolin 2; CALR = calreticulin (see Figure 1 for other definitions).

some variability in analytes across multiple measurements in a limited number of samples (18). Reassuringly, we found that within the top 10 protein contributors to our serum proteomic signature, the majority of them were determined to be reproducible and stable (Supplementary Table 7, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract), as determined using the resource provided by Candia et al (18).

Mean differences in the progression score were higher in ACPA-positive at-risk individuals compared to those who were ACPA negative ($P < 0.001$) (Figure 3D). Consistent with the findings from our initial analysis, the serum progression score was high in both remote and imminent samples in relation to progression to clinical arthritis (Figure 3D).

We then used the mean expression score to depict the expression of each protein within the signature by group

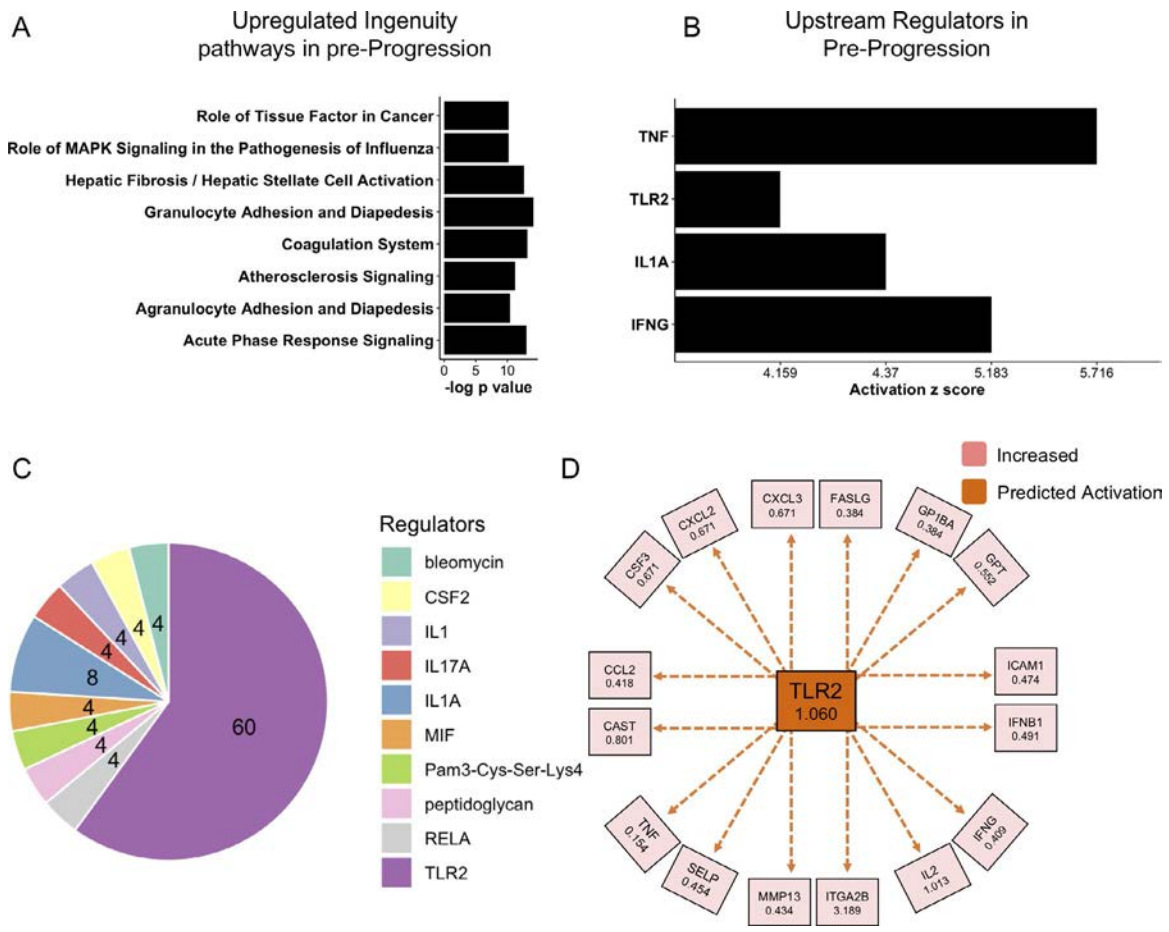


Figure 4. Network analysis of up-regulated proteins in pre-progression samples compared with at-risk individuals. **A**, Top Ingenuity Pathway Analysis (IPA) canonical pathways in the up-regulated set of proteins, with quantified –log *P* values. **B**, Predicted upstream regulators of up-regulated proteins, including tumor necrosis factor (TNF), Toll-like receptor 2 (TLR-2), interleukin-1A (IL-1A), and interferon-γ (IFNG). **C**, A representative pie chart of regulator effects that were enhanced in the pre-progressor samples. Regulators are color coded, and values indicate their percentage representation in the top 25, sorted by consistency score. **D**, The TLR-2 IPA pathway, and downstream proteins included in the data set. Values under each protein are the log difference. CSF2 = colony-stimulating factor 2; MIF = macrophage migration inhibitory factor; RELA = RELA proto-oncogene, NF-κB subunit; FASLG = Fas ligand; GP1BA = glycoprotein Ib platelet subunit α; GPT = glutamic–pyruvic transaminase; ICAM1 = intercellular adhesion molecule 1; IFNB1 = interferon-β1; ITGA2B = α2 integrin 2B; MMP13 = matrix metalloproteinase 13; SELP = selectin P; CAST = calpastatin. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract.

(Figure 3E), and the expression of up-regulated proteins in individual subjects (Figure 3F). Correlation for each of these markers was tested against time to arthritis onset for the samples available, and we found a significant correlation between several protein members of the signature (Supplementary Figure 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract). Using linear regression, we found that, after controlling for individual signature members, apolipoprotein A1 was found to be significantly associated with time to arthritis onset (Supplementary Table 8, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract). Overall, these data suggest that at-risk individuals who experience progression to inflammatory arthritis can be identified using a serum proteomic signature, even if samples are obtained several years prior to the onset of clinical disease.

Role of Toll-like receptor 2 (TLR-2) activation and production of tumor necrosis factor (TNF) and IL-1 in the pre-RA state. We next aimed to better define the underlying pathways that are activated in the preclinical state. We extracted the set of 260 up-regulated proteins and performed a network analysis using IPA and clusterProfiler. IPA canonical pathways that were activated in the pre-progression state included MAPK signaling, granulocyte adhesion, atherosclerosis signaling, and acute-phase response signaling (Figure 4A). Up-regulated GO pathways included response to lipopolysaccharide, humoral immune response, blood coagulation, and leukocyte migration, among others (Supplementary Figure 7, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract).

IPA canonical pathways that were down-regulated are listed in Supplementary Figure 8 (available on the *Arthritis & Rheumatology*

website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract), and include cardiac hypertrophy signaling and high mobility group box chromosomal protein 1 signaling. Upstream regulators were filtered based on inclusion within our set of up-regulated proteins (see Subjects and Methods), but included a host of proteins that suggest an early, subclinical inflammatory response highlighted by up-regulation of TNF, IL-1A, and interferon-γ (IFNγ). TLR-2 was also an upstream regulator identified by our protein set (Figure 4B).

We then used the regulator effects function, which integrates both up-regulators and downstream effects based on relevant biologic pathways. After filtering the top 25 downstream pathways, we observed that the majority of the pathways (60%) were derived from TLR-2 activation (Figure 4C, and Supplementary Table 9 available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41483/abstract), suggesting that this pathway is biologically activated.

Finally, we analyzed the upstream regulator pathway to identify which of the molecules in our data set were closely connected with TLR-2 activation. Our results indicated that all of the top upstream regulators (IFNγ, TNF, and IL-1A) along with 1 of our highest up-regulated proteins, ITGA2B, were located downstream of TLR-2 activation (Figure 4D). These findings suggest that innate immune responses are highly active in the preclinical state.

DISCUSSION

The underlying pathophysiologic events that precede the onset of RA continue to be explored, but remain incompletely understood. We sought to identify serum proteins that are differentially expressed in individuals who ultimately develop inflammatory arthritis compared to individuals who do not develop inflammatory arthritis. Importantly, at the time of sample collection, all of these individuals were clinically indistinguishable from one another. We mapped changes in the expression of several proteins over time relative to the onset of arthritis in our progressor group of FDRs of Indigenous North American RA patients. We used machine learning to define a serum proteomic signature that could distinguish pre–RA progression samples from their at-risk counterparts, regardless of ACPA status. Network analysis identified biologic pathways that were activated in the pre-progression state, suggesting that innate immune responses are triggered well before disease onset. Thus, the analysis of our rich proteomic data set, derived from both longitudinal and cross-sectional serum samples from at-risk individuals, provides new information that enhances the understanding of the preclinical events that lead to the development of clinically defined RA.

It has been shown that preclinical RA encompasses a lengthy time period (24). Despite the daunting logistic challenges inherent in the study of preclinical RA, they remain important for understanding its pathogenesis, for classifying individuals at high risk for future disease, and for defining potential preventative interventions. ACPAs have served as a key biomarker for identifying individuals who are at risk of RA development. In support of this, we and others have shown that ACPA seropositivity clearly increases the risk of future RA. Epitope spreading (21) as well as increased avidity (9) and glycosylation changes are all key events that occur prior to disease onset (25–27). Serum cytokines have also been shown to serve as potential preclinical biomarkers; however, the specificity of individual cytokines with regard to the risk of RA relative to healthy controls is low (28–30). Thus, important gaps remain in our capacity to identify individuals who are at highest risk of future RA development and in whom prevention studies can be ethically undertaken.

Our study design has several important strengths. First, we prospectively recruited at-risk study participants, which allowed us to follow up individuals to the time of disease onset. This reduces several unmeasured biases that tend to burden retrospective sample collection.

Second, our selection process for study participants was driven solely by identifying individuals who were relatives of RA patients, and not by the presence of arthritis symptoms such as arthralgia. It is well appreciated by clinicians and investigators alike that defining a single time point as the "onset" is difficult, with many individuals having a stuttering onset to RA. Recently, we have shown that ACPA seropositivity, particularly at modest titers, reverts, not uncommonly, to a seronegative state after prolonged observation (3). Furthermore, we have shown that glycosylation of ACPAs in RA patients is a key event that occurs prior to disease onset in at-risk individuals. Our proteomic data provide a new layer of discovery to these observations, highlighting the possibility of understanding relevant mechanisms that distinguish individuals who ultimately go on to develop disease.

Several important limitations regarding this study should be noted. The number of progressors who were included in our analysis is relatively low, as there are inherent difficulties in recruiting study subjects to be followed up for the development of a rare clinical event. Our study focused exclusively on FDRs of Indigenous North American RA patients in order to enrich our cohort with individuals who are considered to have the highest risk of future RA development, based on previous work from our group and from others. As such, this may limit the generalizability of the findings to individuals who are neither an FDR of an Indigenous North American RA patient nor an Indigenous North American. Having said this, it is now well established that there are extensive commonalities in seropositive RA worldwide.

Machine learning is an emerging tool for clinical decision-making in high-dimensional data analysis, but there are limitations to models that are generated using this technique—and our study is no exception. In particular, overfitting of the data is a potential impediment to machine learning, and results may not be generalizable. LASSO is a shrinkage technique that tends to reduce overfitting, and we used modest methods to attempt to sacrifice lower accuracy for a more modest output (higher error with lower

number of analytes). SomaScan has its own inherent limitations, including that it covers only a small portion of the entire proteome. Although our classifying biomarker panel is modest in size, these results should be replicated in an unrelated cohort to investigate its generalizability. In order to use the serum proteome as a clinical tool, an alternative method for measuring analytes will be needed to ensure high throughput and low cost.

Aptamer-based proteomics analysis represents an advanced tool for analyzing proteins from biologic samples (31). This technology uses single-stranded oligonucleotides that bind to targets with high affinity, to generate quantitative levels of hundreds of proteins simultaneously (32). Furthermore, it has high specificity and reproducibility, providing several distinguished advantages over antibody-based assays (11). Our data set represents the first high-dimensional, semi-supervised analysis of the preclinical RA proteome. By using shrinkage-based machine learning (33), we were able to define a minimum set of proteins that could distinguish preclinical RA from healthy at-risk individuals who did not develop RA, regardless of ACPA status. These differences are apparent several years prior to the onset of disease, suggesting that they may provide important new tools for the detection of at-risk individuals.

Fewer total proteins were included in our model comparing progressors to APCA-positive at-risk individuals (model 2), relative to ACPA-negative at-risk individuals (model 1). This suggests that the ACPA-positive at-risk proteome profile (lower number of distinguishing proteins, lower accuracy) is more difficult to classify than the ACPA-negative at-risk proteome profile (higher number of distinguishing proteins, higher accuracy) when compared to progressors. As a proof of concept, our study results hold promise that serum proteomics analysis is a valuable tool for distinguishing at-risk individuals in whom RA may develop in the future.

It is well understood that the loss of immune tolerance, epitope spreading through T and B cell interactions, and modifications to pathogenic immunoglobulin (4,25,26,28) are interrelated but distinct pathophysiologic features that occur prior to the onset of disease. Much less is understood about the fundamental systemic perturbations that drive these immunologic responses. Our network analyses suggested that canonical inflammatory pathways are activated in the preclinical state (28), and may involve a key innate immune trigger that signals through TLRs, leading to production of proinflammatory cytokines such as TNF and IL-1. TLRs are innate pattern-recognition receptors that are activated by a wide array of molecules that are generally derived from human pathogens (34). TLR activation and TNF/IL-1 production are all implicated in the systemic and articular inflammatory response in RA (35,36), although their role in the preclinical response is much less well defined.

Our data do not rule out the possibility that other TLRs or innate receptors may be activated in the preclinical state. Pathway analysis of our data suggests that up-regulation of integrin (ITGA2B) may occur downstream of TLR-2 signaling. This protein is associated with platelet activation and acts as a receptor for coagulation products (37), reflecting a prothrombotic and proinflammatory state. Our data suggest that understanding the role of innate immunity in the development of RA remains an important area of research.

Overall, we describe a serum proteomic signature that potentially identifies pre-RA patients years before the onset of clinically detectable disease. Considering what is known about the serologic changes predating RA onset, it may be possible to develop highly specific combinations with other biomarkers that would increase our precision in defining preclinical RA. Our results also provide novel insights into some of the underlying pathophysiologic pathways leading to the development of RA, and perhaps point to interventions that could have a direct impact on these pathways and, in turn, reduce the risk of developing future disease.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. O'Neil had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. O'Neil, Spicer, Meng, Goel, Anaparti, Wilkins, El-Gabalawy.

Acquisition of data. O'Neil, Smolik, Meng, El-Gabalawy.

Analysis and interpretation of data. O'Neil, Goel, Wilkins, El-Gabalawy.

REFERENCES

1. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. Lancet 2016;388:2023–38.
2. Verheul MK, Böhringer S, van Delft MA, Jones JD, Rigby WF, Gan RW, et al. Triple positivity for anti–citrullinated protein autoantibodies, rheumatoid factor, and anti–carbamylated protein antibodies conferring high specificity for rheumatoid arthritis: implications for very early identification of at-risk individuals. Arthritis Rheumatol 2018;70:1721–31.
3. Tanner S, Dufault B, Smolik I, Meng X, Anaparti V, Hitchon C, et al. A prospective study of the development of inflammatory arthritis in the family members of Indigenous North American people with rheumatoid arthritis. Arthritis Rheumatol 2019;71:1494–503.
4. Deane KD, El-Gabalawy H. Pathogenesis and prevention of rheumatic disease: focus on preclinical RA and SLE [review]. Nat Rev Rheumatol 2014;10:212–28.
5. Deane KD. Preclinical rheumatoid arthritis and rheumatoid arthritis prevention [review]. Curr Rheumatol Rep 2018;20:50.
6. Holers VM, Demoruelle MK, Kuhn KA, Buckner JH, Robinson WH, Okamoto Y, et al. Rheumatoid arthritis and the mucosal origins hypothesis: protection turns to destruction [review]. Nat Rev Rheumatol 2018;14:542–57.
7. Scally SW, Law SC, Ting YT, Heemst JV, Sokolove J, Deutsch AJ, et al. Molecular basis for increased susceptibility of Indigenous North Americans to seropositive rheumatoid arthritis. Ann Rheum Dis 2017;76:1915–23.
8. Peschken CA, Hitchon CA, Robinson DB, Smolik I, Barnabe CR, Prematilake S, et al. Rheumatoid arthritis in a North American Native population: longitudinal followup and comparison with a white population. J Rheumatol 2010;37:1589–95.
9. Suwannalai P, van de Stadt LA, Radner H, Steiner G, El-Gabalawy HS, Jol-van der Zijde CM, et al. Avidity maturation of anti–citrullinated protein antibodies in rheumatoid arthritis. Arthritis Rheum 2012;64:1323–8.

10. Altieri A, Piyadasa H, Recksiedler B, Spicer V, Mookherjee N. Cytokines IL-17, TNF and IFNγ alter the expression of antimicrobial peptides and proteins disparately: a targeted proteomics analysis using SOMAscan technology. Vaccines (Basel) 2018;6:51.
11. Lollo B, Steele F, Gold L. Beyond antibodies: new affinity reagents to unlock the proteome. Proteomics 2014;14:638–44.
12. Gold L, Walker JJ, Wilcox SK, Williams S. Advances in human proteomics at high scale with the SOMAscan proteomics platform. N Biotechnol 2012;29:543–9.
13. Murota A, Suzuki K, Kassai Y, Miyazaki T, Morita R, Kondo Y, et al. Serum proteomic analysis identifies interleukin 16 as a biomarker for clinical response during early treatment of rheumatoid arthritis. Cytokine 2016;78:87–93.
14. Smolik I, Robinson DB, Bernstein CN, El-Gabalawy HS. First-degree relatives of patients with rheumatoid arthritis exhibit high prevalence of joint symptoms. J Rheumatol 2013;40:818–24.
15. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO III, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum 2010;62:2569–81.
16. Gold L, Ayers D, Bertino J, Bock C, Bock A, Brody EN, et al. Aptamer-based multiplexed proteomic technology for biomarker discovery. PLoS One 2010;5:e15004.
17. Oliveros JC. Venny: an interactive tool for comparing lists with Venn diagrams. URL: https://bioinfogp.cnb.csic.es/tools/venny/index.html.
18. Candia J, Cheung F, Kotliarov Y, Fantoni G, Sellers B, Griesman T, et al. Assessment of variability in the SOMAscan assay. Sci Rep 2017;7:14248.
19. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight–joint counts: development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44–8.
20. Villeneuve E, Nam J, Emery P. 2010 ACR-EULAR classification criteria for rheumatoid arthritis [editorial]. Rev Bras Reumatol 2010;50:481–3.
21. Sokolove J, Bromberg R, Deane KD, Lahey LJ, Derber LA, Chandra PE, et al. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. PLoS One 2012;7:e35296.
22. Cordeiro OG, Chypre M, Brouard N, Rauber S, Alloush F, Romera-Hernandez M, et al. Integrin-α IIb identifies murine lymph node lymphatic endothelial cells responsive to RANKL. PLoS One 2016;11:e0151848.
23. Carmona-Rivera C, Carlucci PM, Moore E, Lingampalli N, Uchtenhagen H, James E, et al. Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis. Sci Immunol 2017;2:eaag3358.
24. El-Gabalawy H. The preclinical stages of RA: lessons from human studies and animal models. Best Pract Res Clin Rheumatol 2009;23:49–58.
25. Ercan A, Cui J, Chatterton DE, Deane KD, Hazen MM, Brintnell W, et al. Aberrant IgG galactosylation precedes disease onset, correlates with disease activity, and is prevalent in autoantibodies in rheumatoid arthritis. Arthritis Rheum 2010;62:2239–48.
26. Hafkenscheid L, de Moel E, Smolik I, Tanner S, Meng X, Jansen BC, et al. N-linked glycans in the variable domain of IgG anti–citrullinated protein antibodies predict the development of rheumatoid arthritis. Arthritis Rheumatol 2019;71:1626–33.
27. Kissel T, van Schie KA, Hafkenscheid L, Lundquist A, Kokkonen H, Wuhrer M, et al. On the presence of HLA-SE alleles and ACPA-IgG variable domain glycosylation in the phase preceding the development of rheumatoid arthritis. Ann Rheum Dis 2019;78:1616–20.
28. Deane KD, O'Donnell CI, Hueber W, Majka DS, Lazar AA, Derber LA, 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;62:3161–72.
29. Nielen MM, van Schaardenburg D, Reesink HW, Twisk JW, van de Stadt RJ, van der Horst-Bruinsma IE, et al. Increased levels of C-reactive protein in serum from blood donors before the onset of rheumatoid arthritis. Arthritis Rheum 2004;50:2423–7.
30. El-Gabalawy HS, Robinson DB, Smolik I, Hart D, Elias B, Wong K, et al. Familial clustering of the serum cytokine profile in the relatives of rheumatoid arthritis patients. Arthritis Rheum 2012;64:1720–9.
31. Lehallier B, Gate D, Schaum N, Nanasi T, Lee SE, Yousef H, et al. Undulating changes in human plasma proteome profiles across the lifespan. Nat Med 2019;25:1843–50.
32. Zhou J, Rossi J. Aptamers as targeted therapeutics: current potential and challenges. Nat Rev Drug Discov 2017;16:440.
33. Li Z, Sillanpaa MJ. Overview of LASSO-related penalized regression methods for quantitative trait mapping and genomic selection. Theor Appl Genet 2012;125:419–35.
34. Elshabrawy HA, Essani AE, Szekanecz Z, Fox DA, Shahrara S. TLRs, future potential therapeutic targets for RA [review]. Autoimmun Rev 2017;16:103–13.
35. Connell L, McInnes IB. New cytokine targets in inflammatory rheumatic diseases. Best Pract Res Clin Rheumatol 2006;20:865–78.
36. Asquith DL, McInnes IB. Emerging cytokine targets in rheumatoid arthritis. Curr Opin Rheumatol 2007;19:246–51.
37. Yan SL, Russell J, Harris NR, Senchenkova EY, Yildirim A, Granger DN. Platelet abnormalities during colonic inflammation. Inflamm Bowel Dis 2013;19:1245–53.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 89–99
DOI 10.1002/art.41486

American College of Rheumatology
Empowering Rheumatology Professionals

Synergistic Roles of Macrophages and Neutrophils in Osteoarthritis Progression

Ming-Feng Hsueh, Xin Zhang, Samuel S. Wellman, Michael P. Bolognesi, and Virginia B. Kraus

Objective. To evaluate the role of immune cells and their effector cytokines in the pathogenesis and progression of knee osteoarthritis (OA) in matched OA synovial fluid (SF) and synovial tissue samples.

Methods. Cells from matched samples of synovial tissue and SF acquired from individuals undergoing total knee replacement for OA (n = 39) were characterized for immune cell–associated surface markers and intracellular cytokine expression using polychromatic flow cytometry. Additional individuals with radiographic knee OA (Kellgren/Lawrence severity grades ≥1) who had available etarfolatide (inflammatory cell) imaging (n = 26) or baseline and 3-year data on progression of radiographic knee OA (n = 85) were also assessed. SF cytokine concentrations in all cohorts were evaluated for associations with synovial tissue and SF cell phenotypes and severity of radiographic knee OA.

Results. Macrophages (predominant in the synovial tissue, 53% of total cells) and neutrophils (predominant in the SF, 26% of total cells) were the major immune cell populations identified in the OA knee joints, exhibiting expression of or association with transforming growth factor β1 (TGFβ1) and elastase, respectively, in the SF. Expression levels of TGFβ1 and elastase were significantly associated with severity of radiographic knee OA. Baseline SF concentrations of TGFβ1 and elastase along with radiographic knee OA severity scores were predictive of knee OA progression, with areas under the receiver operating characteristic curves of 0.806 (for TGFβ1), 0.810 (for elastase), and 0.846 (for both TGFβ1 and elastase combined), with greater stability of prediction when both markers were utilized.

Conclusion. Our findings demonstrate the hitherto underappreciated role of neutrophils in the sterile inflammatory process and progression of OA. Two soluble mediators, SF elastase and TGFβ1, are strong predictors of knee OA progression, reflecting a synergistic role of neutrophil and macrophage populations in the pathogenesis and worsening of OA that could potentially be utilized to identify patients who may have a greater risk of more rapid disease progression.

INTRODUCTION

Osteoarthritis (OA) is considered an organ disease of the whole joint with an important inflammatory component, involving immune cells, such as macrophages, and their effector cytokines (1–3). Magnetic resonance imaging (MRI) and ultrasonography have been used to confirm a high prevalence of joint inflammation in OA (4,5). Moreover, the presence of MRI-detected inflammation has recently been shown to be predictive of incident radiographic OA within 1 year thereafter (6). Low-grade inflammation induced by metabolic syndromes, innate immunity, and manifestations of systemic inflammaging have all been suggested to play a role in the initiation and perpetuation of the OA process (1). Taken together, the findings from these studies highlight the critical role of inflammation in the pathogenesis of OA.

Our pilot study using etarfolatide imaging of the knee joints of OA patients to visualize activated, but not resting, cells demonstrated the presence of immune cells with functional folate receptor (FR), traditionally considered an indicator of activated macrophages, in the majority (76%) of the OA knees studied (7). The presence and number (based on the intensity of etarfolatide uptake) of FR-positive immune cells was strongly correlated with knee joint symptoms (ascertained as the severity of knee pain, aching, and stiffness). Strikingly, other sites commonly affected by OA (shoulders, hands, and

Supported by NIH grants R01-AR-48769 and P30-AG-028716 awarded to Dr. Kraus. The primary etarfolatide imaging study was supported by a grant from Eli Lilly, Inc.

Ming-Feng Hsueh, PhD, Xin Zhang, MD, Samuel S. Wellman, MD, Michael P. Bolognesi, MD, Virginia B. Kraus, MD, PhD: Duke University, Durham, North Carolina.

No potential conflicts of interest relevant to this article were reported.

Address correspondence to Virginia B. Kraus, MD, PhD, Duke Molecular Physiology Institute, Box 104775, Durham, NC 27701. Email: vbk@duke.edu.

Submitted for publication November 1, 2019; accepted in revised form August 6, 2020.

ankles) also showed a high frequency of FR-positive immune cells whose abundance was positively associated with patient-reported joint symptoms (7). We subsequently determined that FR-bearing cells included not only activated macrophages, as previously described in the literature (8), but also a subset of neutrophils (9).

Furthermore, we found that the synovial fluid (SF) concentrations of 2 macrophage-generated soluble proteins, CD14 and CD163, were associated with radiographic knee OA progression (10). CD14 can be found on various cell types, including monocytes and macrophages (11). Cell surface CD14, used as part of the basis for macrophage identification in this study, is clearly linked to activation of innate immune responses, including production of the inflammatory mediators tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, IL-8, IL-10, and IL-12 (12–15).

Previous studies have also suggested that macrophages and macrophage-produced mediators help drive the inflammatory and destructive responses in the OA synovium (16,17). These results suggest that macrophages are involved in the pathogenesis of OA. However, the role of other major immune cells in the pathogenesis of OA has not been fully evaluated. Although prior investigation of the role of neutrophils has generally been confined to rheumatoid arthritis (18), a few studies have provided some indications of their involvement in OA. For example, matrix metalloproteinase 9 and neutrophil gelatinase-associated lipocalin form a complex in OA SF that is relevant to cartilage degradation (19). In experimental arthritis in mice, neutrophils and natural killer cells interact to promote arthritis following intraarticular collagenase injection (20). Colchicine, which inhibits neutrophil production of superoxide and neutrophil adhesion, mobilization, recruitment, and chemotaxis (21), has to date yielded both positive results (22–25) and negative results (26) in clinical trials evaluating the treatment of OA symptoms or progression.

To advance the understanding of the role of immune cells in the pathogenesis of OA, we characterized the major immune cells in synovial tissue and SF and their effector cytokines in a total of 150 individuals with knee OA. Available etarfolatide and radiographic imaging data were used to assess for associations of joint inflammation and progression with immune cell populations in OA. Our findings in this study provide justification to utilize the immune cell effectors transforming growth factor β1 (TGFβ1) and elastase as potential tools for identifying patients with a higher risk of OA progression.

PATIENTS AND METHODS

Study cohorts. Details on the characteristics of the patients with knee OA are summarized in Supplementary Table 1 (available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41486/abstract).

Total knee replacement (TKR) cohort. With Institutional Review Board (IRB) approval, biospecimens were collected from patients with knee OA undergoing TKR at Duke University Hospital. Samples of synovial tissue and matched SF samples were collected as anonymized waste surgical specimens from 39 patients. Cells were isolated from the synovial tissue and SF for polychromatic flow cytometry analysis. Samples from 17 patients were used for immune cell profiling and quantification of soluble cytokines. Samples from 8 patients were used for intracellular cytokine profiling, and additional samples from 14 patients were used for FR specificity testing.

Etarfolatide scan cohort. In our pilot study using etarfolatide imaging of the knee joints of OA patients (ClinicalTrials.gov identifier: NCT01237405), patients with unilateral or bilateral radiographic knee OA, with Kellgren/Lawrence (K/L) radiographic OA severity grades (27) of 1–4, underwent aspiration of knee SF. Directly aspirated SF was available from 26 knees of 18 patients for utilization in the present analyses.

Prediction of OA progression (POP) cohort. A previously described cohort of patients with radiographic knee OA in at least 1 knee (28), with K/L severity grades of 1–3, underwent aspiration of knee SF at baseline. Directly aspirated SF from 85 knees of 60 patients with radiographic knee OA and 3-year follow-up clinical data were available from the POP cohort for utilization in the present analyses.

Radiographic scoring and definition of OA progression. Participants in the POP and etarfolatide scan cohorts underwent knee radiography. Each knee radiograph was scored for K/L severity grade, and radiographic features of joint space narrowing (JSN) and osteophyte severity were assessed using the Osteoarthritis Research Society International (OARSI) standardized atlas (29). Based on the change over 3 years in radiographic features of knee OA (JSN and osteophyte severity scores) and occurrence of TKR after the baseline evaluation, participants from the POP cohort were categorized into 1 of 4 mutually exclusive, successively more severe OA progression outcome categories, as follows: non-progression, osteophyte progression (increased osteophyte severity scores/no change in JSN scores), both osteophyte and JSN progression (increased osteophyte and JSN severity scores, or progression to TKR).

Cell isolation from synovial tissue. All biospecimens were collected from patients after Duke IRB approval. Synovial tissue and SF samples, obtained at the time of TKR surgery for knee OA, were processed within 2 hours of acquisition. After removing the adjoining tissue, synovial tissue cells were isolated using an enzyme-free method, with high cell viability (~95%) and fewer than 5% of the cells being fibroblasts (see Supplementary Methods and Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://online

library.wiley.com/doi/10.1002/art.41486/abstract); we have previously shown that this method preserves FR presence and function on macrophages (9). Cells from matched SF samples were isolated by centrifugation. The cell-free SF supernatants were stored at −80°C until analyzed.

Polychromatic flow cytometry analysis. Synovial tissue and SF cells were stained for the following surface markers: HLA–DR, CD14, CD19, CD16, CD3, CD11c, and CD11b (see Supplementary Methods [http://onlinelibrary.wiley.com/doi/10.1002/art.41486/abstract]). To quantify cell type–specific production of cytokines, cells isolated from the OA synovial tissue and SF were cultured with brefeldin A for 2 hours, and then phenotyped with the following human surface marker antibodies: CD11b, CD11c, CD14, CD16, and CD3. Thereafter, the cells were stained with antibodies against human TGFβ1 (eBiocience) and elastase (Novus) according to the manufacturers' protocols.

SF cytokine measurements. Cytokines in the OA SF, including interferon-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF (comprising human proinflammatory panel 1) as well as IL-17A, IL-21, IL-22, IL-23, IL-27, IL-31, and macrophage inflammatory protein 3α (MIP-3α) (comprising human Th17 panel 1) were measured by immunoassay (MSD) in accordance with the manufacturer's instructions (30). Neutrophil elastase, TGFβ1, and monocyte chemoattractant protein 1 (MCP-1) were measured by Platinum enzyme-linked immunosorbent assay (ThermoFisher). All of these effectors in the OA SF were measured in samples obtained from the TKR cohort; only TGFβ1 and elastase were measured in the etarfolatide scan and POP cohorts. In addition, archival data on the SF concentrations of IL-6 and IL-8, measured using a Bioplex Human Cytokine 17-plex assay (Bio-Rad), were available from the POP cohort.

Statistical analysis. Pearson's correlation tests were used to assess associations of immune cell types and cytokines with progression of radiographic knee OA. Ordinal logistic regression was used to estimate the association between SF cytokines and OA progression based on the above-defined outcome groups. Receiver operating characteristic (ROC) curve analysis was employed to evaluate the performance of SF TGFβ1 and elastase levels as well as archival data on SF IL-6 and IL-8 levels alone or in combination with demographic covariates (age, sex, and body mass index [BMI]) or radiographic covariates (total JSN and osteophyte severity scores) to discriminate between patients at high risk and those at low risk of any knee OA progression (31,32). Areas under the ROC curves (AUCs) were determined with bootstrap validations using 95% bias-corrected and accelerated bootstrap intervals (95% BCa). The analyses were performed using JMP Pro software version 13 (SAS).

RESULTS

Patient cohorts. A total of 3 knee OA cohorts were used for this research: a TKR cohort (n = 39), a radiographic knee OA cohort (K/L grades ≥1) with available etarfolatide (inflammatory cell) imaging (n = 26), and a natural longitudinal radiographic knee OA progression cohort (n = 85) with baseline and 3-year knee radiographic OA progression data (see Supplementary Table 1 [http://onlinelibrary.wiley.com/doi/10.1002/art.41486/abstract]). The majority of patients in these cohorts were female, and most of the patients were older (mean age >60 years) and obese (mean BMI >30 kg/m^2). Of note, in the natural longitudinal radiographic knee OA progression cohort, a total of 36 individuals met the criteria for any progression (>1-unit increase in JSN or osteophyte severity scores or progression to TKR), and 49 individuals were classified with an outcome of nonprogression.

Three major immune cell populations in OA joints. Using flow cytometry analyses, we determined that macrophages (CD14+CD11c+HLA–DR+CD11b+CD16^{low}), neutrophils (CD14–CD11c–HLA–DR–CD11b+CD16^{high}), and T cells (CD45+CD3+) were the 3 major immune cell populations in OA synovial tissue and SF (n = 17 patients) (Figure 1A; see also Supplementary Figures 1 and 2 [http://onlinelibrary.wiley.com/doi/10.1002/art.41486/abstract]). Macrophages were the most abundant population in both the SF and synovial tissue. Overall, neutrophils were less abundant than macrophages and T cells.

Although all 3 cell types were present in all OA knee joints, only 35% of the joints had proportions of neutrophils representing more than 5% of the total cell population. Neutrophils were more abundant in the SF than in the synovial tissue (mean 26% versus 8% of total cells). In contrast, macrophages were more abundant in the synovial tissue than in the SF (mean 53% versus 38% of total cells). The number of T cells was not significantly different between the SF and synovial tissue (mean 36% versus 35% of total cells) (Figures 1A and B).

A subset of immune cells (macrophages, neutrophils, and T cells) was identified as being FR positive in both the SF and synovial tissue (9% and 8% of the total live cells, respectively), based on their high uptake of folic acid (Figures 1A and B). We confirmed the functional specificity of the FR on macrophages and neutrophils by observing a significant reduction in the amount of fluorescent folic acid uptake by the cells with the addition of nonlabeled folic acid (see results in Supplementary Figure 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41486/abstract). In contrast, the small amount of folic acid uptake by the small number of FR-positive T cells was not specific.

Linkage of representative effectors in the SF to macrophages and neutrophils in patients with knee OA. In the TKR cohort (n = 17), we identified profiles of soluble SF cytokines that were associated with neutrophil, macrophage, and

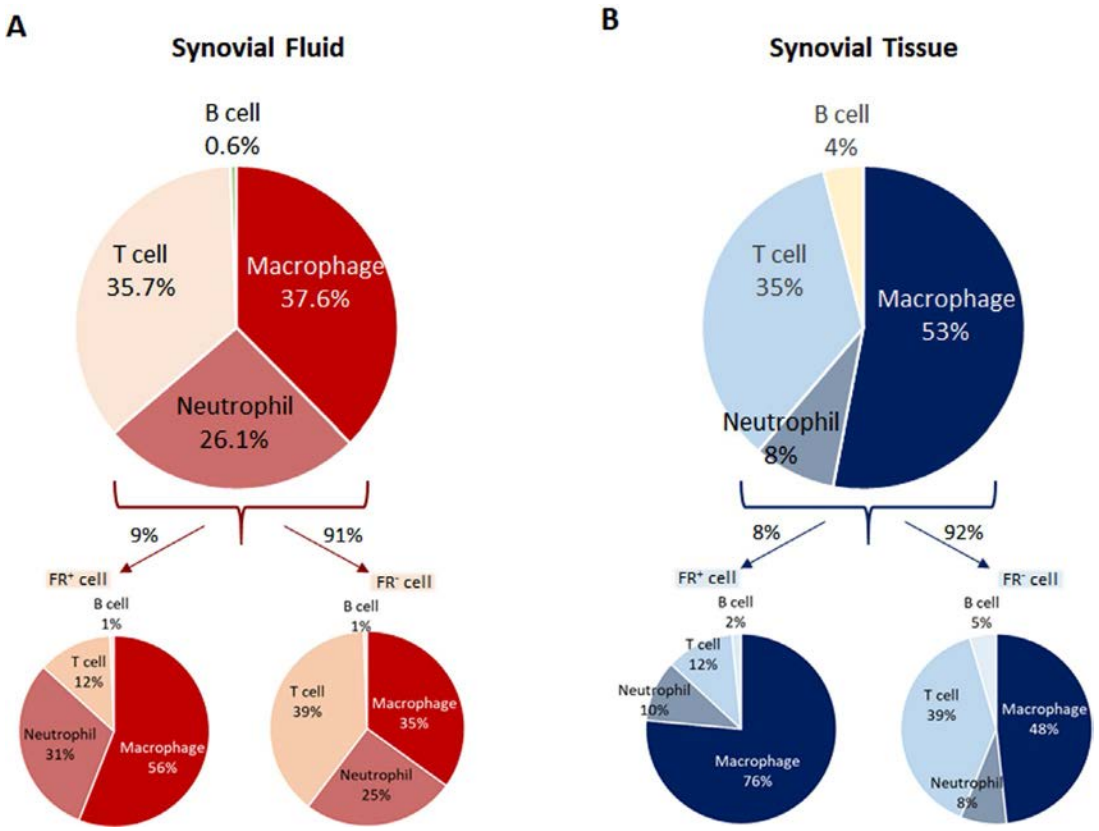


Figure 1. Phenotypic characterization of immune cells by flow cytometry in the synovial fluid (SF) (**A**) and synovial tissue (**B**) of patients with knee osteoarthritis. Macrophages (CD14+CD11c+HLA–DR+CD11b+CD16^{low}), neutrophils (CD14–CD11c–HLA–DR–CD11b+CD16^{high}), and T cells (CD45+CD3+) were the major immune cell populations detected in the SF and synovial tissue (n = 17 patients per group). A higher mean percentage of neutrophils was present in the SF compared to the synovial tissue, whereas a higher mean percentage of macrophages was present in the synovial tissue compared to the SF. Overall, a mean 8–9% of synovial tissue and SF cells were positive for functional folate receptor (FR+), with the FR-positive cell subtype primarily enriched for macrophages and neutrophils but with fewer T cells as compared to the FR-negative majority cell populations.

T cell populations in knee OA joints (Figure 2). The levels of soluble neutrophil-secreted elastase (r = 0.728, P = 0.001) and neutrophil-attracting MIP-3α (r = 0.235, P = 0.364) were selectively positively associated with the numbers of SF neutrophils. SF elastase levels were inversely associated with the numbers of T cells in the OA SF (r = –0.397, P = 0.115) and OA synovial tissue (r = –0.241, P = 0.369). SF neutrophil numbers also showed a positive, but not significant, association with SF IL-6 levels (r = 0.451, P = 0.069). In flow cytometry analyses, we confirmed the expression of elastase in both SF and synovial tissue neutrophils (results in Supplementary Figure 4, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41486/abstract).

In the TKR cohort, the total number of SF macrophages was positively associated with SF concentrations of IL-6 (r = 0.503, P = 0.04). The total number of synovial tissue macrophages was positively, but not significantly, associated with SF concentrations of any single soluble cytokine: IL-6 (r = 0.127, P = 0.639), TGFβ1 (r = 0.253, P = 0.344), TNF (r = 0.304, P = 0.291), and IL-27 (r = 0.288, P = 0.279) (Figure 2). In contrast to the positive but weak associations between the total number of macrophages in the synovial tissue and the levels of TGFβ1, IL-27, and TNF in the SF, the associations between the total number of macrophages in the SF and the SF levels of each of these cytokines were negative (for TGFβ1, r = –0.320, P = 0.21; for TNF, r = –0.399, P = 0.216);

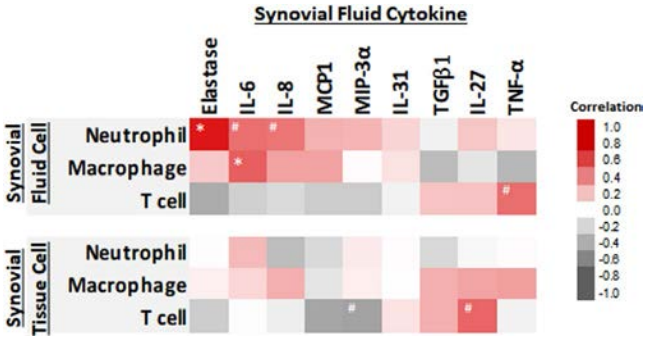


Figure 2. Heatmaps representing the associations between total numbers of synovial fluid (SF) and synovial tissue immune cell types and SF levels of cytokines, including elastase, interleukin-6 (IL-6), IL-8, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 3α (MIP-3α), IL-31, transforming growth factor β1 (TGFβ1), IL-27, and tumor necrosis factor (TNF), in samples from 17 patients with knee osteoarthritis. Correlations were assessed by Pearson's correlation analysis. * = P < 0.05; # = P < 0.1 versus the other cell types.

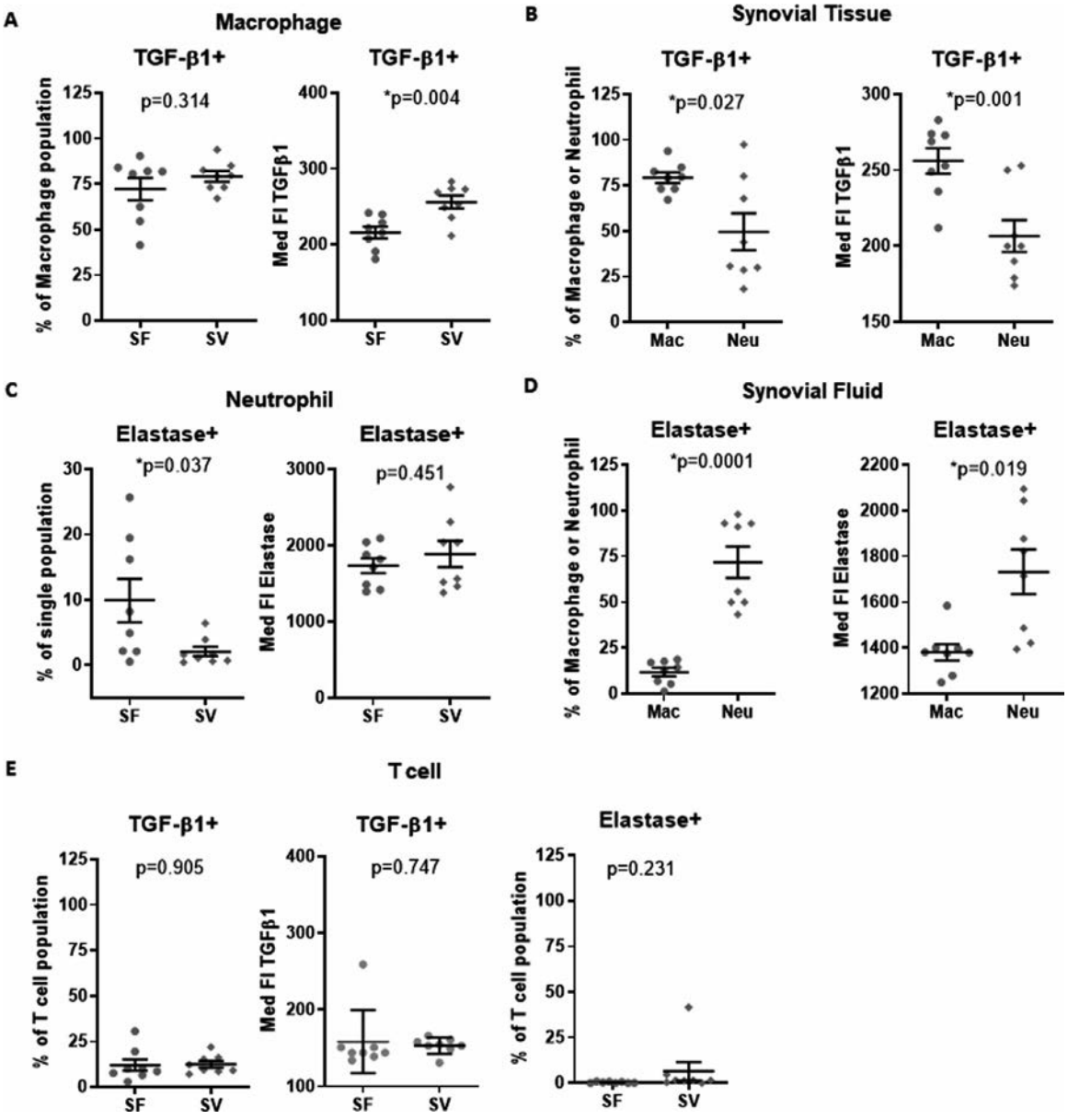


Figure 3. Intracellular cytokine production by macrophages (Mac) and neutrophils (Neu) in synovial fluid (SF) and synovial tissue (SV) from patients with knee osteoarthritis. **A**, **B**, and **E**, Production of transforming growth factor β1 (TGFβ1) was evaluated by flow cytometry. Percentages of TGFβ1+ macrophages among total macrophages (CD3–CD14+CD16^{low}) in the SF and synovial tissue (**A**), TGFβ1+ macrophages or neutrophils among total macrophages or total neutrophils (CD3–CD14–CD16^{high}) in the synovial tissue (**B**), and TGFβ1+ T cells among total T cells in the SF and synovial tissue (**E**) were determined. **C–E**, Production of elastase was evaluated by flow cytometry. Percentages of elastase-positive neutrophils among total neutrophils in the SF and synovial tissue (**C**), elastase-positive macrophages or neutrophils among total macrophages or total neutrophils in the SF (**D**), and elastase-positive T cells among total T cells in the SF and synovial tissue (**E**) were determined. Results are also expressed as the median fluorescence intensity (Med FI) of cytokine expression. Symbols represent individual samples; horizontal lines with bars show the mean ± SEM (n = 8 samples per group). * Significant *P* value.

for IL-27, r = –0.124, *P* = 0.637). Results of flow cytometry showed that macrophages expressed, but were not a sole source of, TGFβ1 (Supplementary Figure 4 [http://onlinelibrary.wiley.com/doi/10.1002/art.41486/abstract]). Although macrophages were also a source of IL-6, only a minority of SF and synovial tissue macrophages expressed this cytokine (Supplementary Figure 4 [http://onlinelibrary.wiley.com/doi/10.1002/art.41486/abstract]).

Identification of TGFβ1 and elastase as immune cell phenotypic markers for macrophages and neutrophils. To assess the role of various immune cell types in the pathogenesis and progression of OA, we aimed to identify biomarkers characteristic of each cell type. Based on the results of flow cytometry analyses (n = 8 patients), elastase and TGFβ1 were found to be representative of the neutrophil and macrophage

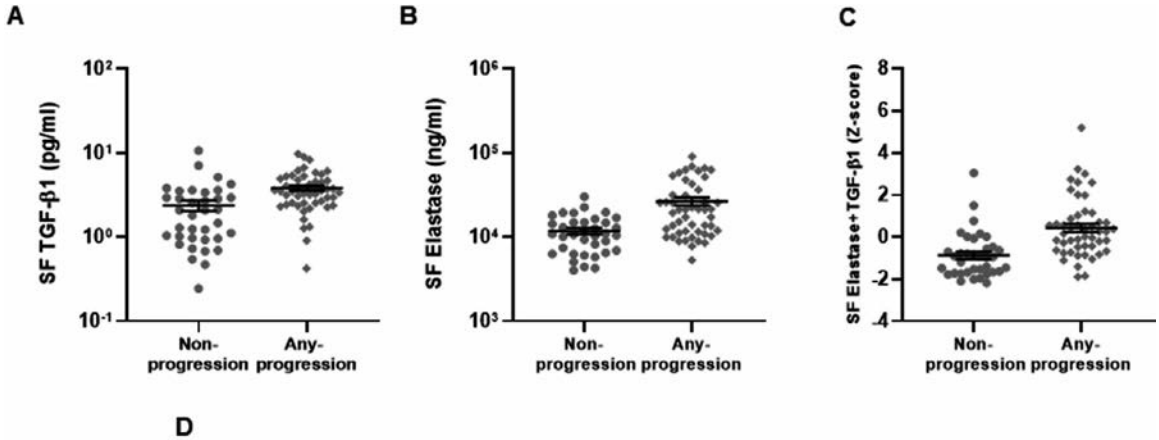
populations, respectively, in the OA knee joints. Macrophages were the predominant source of TGFβ1 in the SF (72%) and synovial tissue (79%); the amount of TGFβ1 produced per cell (based on the median fluorescence intensity [MFI]) was significantly higher in macrophages from the synovial tissue compared to macrophages from the SF (Figure 3A).

In the synovial tissue, the proportion of TGFβ1+ macrophages was significantly higher than the proportion of TGFβ1+ neutrophils; the amount of TGFβ1 per cell was also significantly higher in macrophages compared to neutrophils (Figure 3B).

In flow cytometry analyses, neutrophils, particularly SF neutrophils, appeared to be the predominant source of elastase (mean 72% of SF neutrophils expressing elastase). Elastase-positive neutrophils were more abundant in the SF than in the synovial tissue, but the amount per cell was similar in both microenvironments (Figure 3C). Although a mean 12% of macrophages expressed elastase in the SF and a mean 23% of macrophages expressed elastase in the synovial tissue (Supplementary Figure 4 [http://onlinelibrary.wiley.com/doi/10.1002/art.41486/abstract]), the MFI of elastase-positive SF macrophages was significantly lower than the MFI of elastase-positive SF neutrophils (Figure 3D).

The number of TGFβ1+ T cells was much lower than the number of TGFβ1+ macrophages, and no or very few elastase-expressing T cells were detected (Figure 3E). The amount of TGFβ1 produced by T cells (based on the MFI) was also lower than that produced by macrophages (Figure 3E).

Positive association of SF TGFβ1 and elastase levels with radiographic knee OA severity. To determine the role of these 2 immune cell types in the pathogenesis and progression of OA, we measured TGFβ1 and elastase in SF biospecimens from highly phenotyped cohorts of patients who had a wide range of knee OA severity (the etarfolatide scan and POP cohorts). After adjustment for age, sex, and BMI, the SF levels of TGFβ1 and elastase in the SF were both significantly associated with baseline knee radiographic OA osteophyte severity in both the etarfolatide scan cohort (for TGFβ1, β = 10.31, $P = 0.006$; for elastase, β = 2.54, $P = 0.041$) and the POP cohort (for TGFβ1,



D

	Non-progression (n = 36)		Progression (n = 49)		p value
Age	60.3	(56.5–64.2)	62.9	(59.7–66.0)	0.189
Sex	12M 24F	66.7% F	16M 33F	67.3% F	0.959
BMI	32.5	(30.4–34.5)	33.9	(31.6–36.2)	0.392
TOT JSN	1.6	(1.3–2.0)	2.3	(1.9–2.7)	*0.009
TOT OST	3.3	(2.2–4.5)	4.3	(3.6–5.2)	0.141

Figure 4. Prediction of radiographic knee osteoarthritis (OA) progression based on baseline synovial fluid (SF) levels of cytokines. Knee OA progression was defined as either nonprogression (n = 36) or any progression (n = 49) based on the absence versus presence of either a 1-unit increase in the total (TOT) osteophyte (OST) severity score, 1-unit increase in the total joint space narrowing (JSN) severity score, or progression to total knee replacement (TKR) during a 3-year follow-up interval among patients in the prediction of OA progression cohort. **A–C**, Scatter plots by knee OA progression status demonstrate that samples from the any progression group as compared to the nonprogression group had significantly higher mean SF levels of transforming growth factor β1 (TGFβ1) (mean 3.8 pg/ml, 95% confidence interval [95% CI] 3.3–4.4 versus mean 2.4 pg/ml, 95% CI 1.7–3.1) (**A**), significantly higher mean SF levels of elastase (mean 26,770 ng/ml, 95% CI 20,845–32,695 versus mean 11,850 ng/ml, 95% CI 9,852–13,847) (**B**), and significantly higher mean SF levels of the 2 cytokines combined (Z score 0.4, 95% CI 0.03–0.84 versus Z score −0.88, 95% CI −1.24 to −0.51) (**C**). Symbols represent individual samples; horizontal lines with bars show the mean ± SEM. Significant differences between the groups were as follows: $P = 7 \times 10^{-5}$ in **A**, $P = 2.9 \times 10^{-5}$ in **B**, and $P = 1.0 \times 10^{-6}$ in **C**. **D**, Demographic variables and radiographic knee OA features were compared between the nonprogression and progression groups. Mean values with 95% CIs are shown for age (in years), body mass index (BMI) (in kg/m^2), total JSN severity score, and total osteophyte severity score. Values for sex are the number of males (M), number of females (F), and percentage of females in each group. * Significant P value.

β = 2.29, $P = 0.012$; for elastase, β = 2.74, $P = 0.001$). In addition, the SF levels of TGFβ1 and elastase were both significantly associated with baseline knee radiographic OA JSN severity in the POP cohort (for TGFβ1, β = 0.66, $P = 0.068$; for elastase, β = 0.95, $P = 0.005$).

Evidence of SF TGFβ1 and elastase levels, singly and in combination, as strong predictors of knee OA progression. The baseline SF elastase level also showed a significant positive association with osteophyte progression (β = 1.04, $P = 0.048$) among patients in the POP cohort, for whom 3-year longitudinal follow-up data were available. The baseline levels of TGFβ1 and elastase in the SF were associated with the severity of OA progression according to the 4 mutually exclusive, successively more severe OA outcome progression categories. Successively worse outcomes were associated with a higher mean baseline biomarker concentration of SF TGFβ1 (Z score β = 0.90, 95% confidence interval [95% CI] 0.45–1.39, $P < 0.0001$; n = 85), SF elastase (Z score β = 1.10, 95% CI 0.51–1.70, $P = 0.0005$; n = 85), and their combination (Z score β = 1.54, 95% CI 0.89–2.27, $P < 0.0001$; n = 85). Since the beta values were standardized, higher beta values yielded by the combination of SF TGFβ1 levels with SF elastase levels suggested that patients with high concentrations of TGFβ1 and elastase in the SF have the highest likelihood of more severe disease progression.

Mean baseline concentrations of SF TGFβ1 (mean 3.8 pg/ml, 95% CI 3.3–4.4 pg/ml; $P < 0.0001$), SF elastase (mean 26,770 ng/ml, 95% CI 20,845–32,695 ng/ml; $P < 0.01$), and both SF TGFβ1 and SF elastase combined (Z score 0.4, 95% CI 0.03–0.84; $P < 0.0001$) were significantly higher in the groups with any progression (either osteophyte progression, JSN progression, or progression to TKR during a 3-year follow-up interval) than those in the SF samples from the nonprogression group (for TGFβ1, mean 2.4 pg/ml, 95% CI 1.7–3.1; for elastase, mean 11,850 ng/ml, 95% CI 9,852–13,847; for both combined, Z score –0.88, 95% CI –1.24 to 0.51) (Figures 4A–C).

We used ROC curves to further investigate the prognostic capability of these biomarkers to predict any OA progression as compared to an outcome of nonprogression. Both SF TGFβ1 levels and SF elastase levels were predictive of any OA progression in the knee joints (Table 1 and Supplementary Table 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41486/abstract), yielding high AUC values with bootstrap validations for SF elastase levels (AUC 0.776, 95% BCa 0.662–0.863) and for SF TGFβ1 levels (AUC 0.768, 95% BCa 0.639–0.858). The combination (Z score) of the 2 cytokines yielded a higher AUC (AUC 0.827, 95% BCa 0.729–0.903) than that with either cytokine alone for the prediction of radiographic knee OA progression.

Demographic covariates (age, sex, and BMI) assessed as predictors yielded an AUC of 0.596 (95% BCa 0.512–0.633). The addition of demographic covariates to the model with SF TGFβ1 levels and SF elastase levels slightly improved the predictive capability of the model (increased AUC values), but did not improve the model stability (i.e., decreased Akaike's information criterion [AIC] values) (Table 1). Baseline radiographic features (JSN and osteophyte severity scores) yielded an AUC of 0.704 (95% BCa 0.631–0.769). The combination of radiographic features and SF cytokine levels improved the model performance for predicting knee OA progression (AUC 0.806, 95% BCa 0.675–0.874).

The model combining the 2 cytokines (SF TGFβ1 levels and SF elastase levels), demographic covariates, and radiographic

Table 1. Comparison of baseline demographics, SF cytokine levels, and radiographic progression variables for the ability to predict progression of radiographic knee osteoarthritis*

Model	AUCs with bootstrap validations†			AIC	Lower order of AUC‡	Upper order of AUC‡	Specificity§
	AUC	Lower BCa	Upper BCa				
Age + sex + BMI	0.596	0.512	0.633	179.56	0.49	1,642.58	34.5
JSN + OST	0.704	0.631	0.769	167.70	56.70	2,432.45	50.4
SF elastase levels	0.776	0.662	0.863	67.34	48.14	2,421.53	55.4
SF elastase levels + JSN + OST	0.810	0.670	0.874	68.32	3.84	2,132.20	62.2
SF TGFβ1 levels	0.768	0.639	0.858	70.37	36.48	2,402.03	61.1
SF TGFβ1 levels + JSN + OST	0.806	0.675	0.874	71.06	6.97	2,230.58	74.1
SF elastase + SF TGFβ1 levels	0.827	0.729	0.903	64.88	43.72	2,415.91	70.4
JSN + OST + SF elastase levels + SF TGFβ1 levels	0.846	0.740	0.907	66.03	11.25	2,293.65	75.9
All combined	0.854	0.715	0.904	70.74	0.64	1,852.23	77.8

* Models include age (in years), sex, and body mass index (BMI) (in kg/m^2), joint space narrowing (JSN) and osteophyte (OST) severity scores, synovial fluid (SF) elastase levels alone or with JSN and osteophyte severity scores, SF transforming growth factor β1 (TGFβ1) levels alone or with JSN and osteophyte severity scores, SF elastase and SF TGFβ1 levels, JSN and osteophyte severity scores with SF elastase and SF TGFβ1 levels, or all variables combined. AIC = Akaike's information criterion (based on the likelihood ratio test).
† Areas under the receiver operating characteristic curves (AUCs) were expressed with 95% bias-corrected and accelerated confidence intervals (BCa) for the 95% confidence in the bootstrap (2,500 repetitions) confidence limit.
‡ The lower and upper orders of the AUCs refer to the order statistic used to obtain the lower and upper 95% confidence intervals for the AUCs. Order statistics of <1 (lower) or >2,500 (upper) indicate that the model was unstable.
§ Specificity was based on the cutoff value when sensitivity of the model was 80%.

severity variables performed very well as a predictor of any radiographic knee OA progression, yielding an AUC of 0.854 (95% BCa 0.715–0.904). However, a value of <1 for the lower order of the AUC in this model indicated that this model was unstable. The best predictive model (high AUC, low AICs, and adequate lower order/upper order values) was provided by the combination of baseline SF TGFβ1 levels, baseline SF elastase levels, and baseline radiographic features (JSN and osteophyte severity scores), yielding an AUC of 0.846 (95% BCa 0.740–0.907) (Table 1). The mean specificity of this model was 75.9% and the sensitivity was 80% (Table 1). Each of these markers yielded higher AUCs for knee OA progression when compared to models with SF levels of IL-6 and IL-8 (see Supplementary Table 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41486/abstract).

DISCUSSION

The results of the present study demonstrate that the inflammatory microenvironment of the OA joint is orchestrated by macrophages, neutrophils, and multiple inflammatory cytokines. Previous studies have shown the importance of macrophages and macrophage-related mediators in driving inflammatory and destructive responses in OA (16,17). However, the evaluation of the role of neutrophils in chronic arthritis inflammation has generally been confined to rheumatoid arthritis (18) or indirectly related to OA (19,26). Our findings of the presence of neutrophils in OA SF and OA synovial tissue, their production of SF elastase, and the association of SF elastase with radiographic knee OA progression all clearly demonstrate the involvement of neutrophils in the sterile inflammatory process and progression of OA. Whereas macrophages were localized to both the synovial tissue and the SF, we found that neutrophils were predominantly localized to the SF. The levels of elastase and TGFβ1, singly or combined, in the SF performed very well in discriminating between knee OA patients at high risk and those at low risk of any knee OA progression.

TGFβ1 was predominantly produced by synovial tissue macrophages. This result is consistent with previous findings demonstrating the production of TGFβ1 predominantly within the synovial lining layer (3,8), and is also consistent with an animal model study in which depletion of synovial macrophages resulted in a significant reduction in osteophyte formation (33), shown to be a TGFβ1-driven process (34). Our findings are congruent with a prior study demonstrating a predominance of macrophage proliferation and the absence of neutrophils in OA synovial tissue (3). Macrophages express the α4β7 integrin (35), enabling their tethering in OA synovium via vascular cell adhesion molecule 1 (VCAM-1), expressed by synovial tissue fibroblasts. These α4 integrins are constitutively expressed on all human leukocyte subtypes except neutrophils (36). The lack of α4 integrins on human neutrophils may, at least in part, account for the general lack of neutrophils in OA synovium. Additionally, transmigrating neutrophils secrete localized elastase, a serine protease with broad specificity that is protected from plasma inhibitors (37). The expression of elastase enables neutrophils to transmigrate extracellular matrices and even modulate trafficking of other leukocyte subsets, such as T cells, by altering their endothelial-associated chemotactic activities (37). In our study, we observed that the SF concentration of elastase was inversely correlated with SF and synovial tissue T cell numbers; elastase-modulated leukocyte trafficking may contribute to these inverse associations (37).

In this study, the number of SF macrophages was positively associated with SF IL-6 levels. Although macrophages were a source of IL-6, only a minority of macrophages expressed this cytokine, according to the results of flow cytometry analysis. The SF IL-6 level was also positively associated with the number of neutrophils. A recent study demonstrated that IL-6 was produced by a variety of types of OA synovial tissue cells, including fibroblasts, macrophages, neutrophils, T cells, and B cells (38). Taken together, these findings show that IL-6 is generally involved in OA inflammatory responses but may not be representative of a specific cell type.

We also observed positive associations of SF TGFβ1, IL-27, and TNF levels with the number of macrophages in the synovial tissue, but negative associations of these cytokine levels with the number of macrophages in the SF. Several prior studies provided potential explanations for this finding. TGFβ induces synovial lining cells to produce inflammatory factors, including TNF (39). An in vitro study also found that IL-27 induces higher fibroblast-like synoviocyte surface expression of VCAM-1, which is known to tether migratory macrophages to the synovial tissue (35,40). In a mouse model of arthritis, TNF was found to be significantly reduced in IL-27$^{-/-}$ mice compared to IL-27$^{+/+}$ mice (41). These studies provide evidence that both TGFβ and IL-27 are linked to TNF and interact with macrophages in the synovial tissue, thereby providing evidence to explain the observed differences in their association, in this study, between the macrophages in the synovial tissue and the macrophages in the SF.

Neutrophils contribute to the cytokine and chemokine cascades that accompany inflammation and regulate immune responses via cell–cell interactions (18). Based on analogous findings in studies of rheumatoid arthritis (18), and owing to their ability to release degradative enzymes and reactive oxygen species, neutrophils possess the most significant cytotoxic potential of the cells implicated in the pathologic development of OA. The strong association of SF neutrophil numbers with SF elastase concentrations in our study supports the notion of an SF origin, as opposed to a synovial tissue origin, of activated neutrophils and demonstrates the destructive potential of this enzyme in OA joints (18,42).

Neutrophil elastase has been implicated in both joint inflammation and pain in mouse models, through its ability to activate

proteinase-activated receptor 2 (PAR-2), resulting in the activation of transient receptor potential ion channels (2). Inhibition of neutrophil elastase, PAR-2, or p44/42 MAPK activity all reduced inflammation and pain in a mouse model of arthritis (2). In the intraarticular monoiodoacetate (MIA) murine model system, in which neutrophil elastase proteolytic activity and transient inflammation were induced, early treatment with a PAR-2 antagonist, GB83, reversed the inflammation (43). MIA-induced synovitis and joint pain were both attenuated in PAR-2–knockout mice (43). Neutrophil elastase has also been implicated in osteophyte formation through PAR-2 (44), with a significant reduction of osteophyte formation in PAR-2–knockout mice (44). The re-expression of PAR-2 by adenovirus in PAR-$2^{-/-}$ mice recapitulated osteophyte formation (44). Taken together, these data suggest that elastase plays a critical role in the PAR-2 pathway related to arthritis pain and inflammation (2), which is consistent with our findings of an association of neutrophils with osteophyte severity and OA progression. Given the role of neutrophils and elastase in human OA, elastase inhibitors may be a potential therapeutic strategy for slowing OA progression and reducing symptoms.

We favored evaluation of radiographic knee OA progression in the POP cohort by assessing JSN and osteophyte severity scores separately, as they represent catabolic and anabolic phenomena, respectively. As previously discussed by Ratzlaff et al (45), separate evaluation of these radiographic manifestations of OA may better distinguish pathologic processes that could potentially be critical to understanding etiologic pathways of risk factors and interventions in knee OA. As demonstrated by Ratzlaff et al, a 1-grade increase in the OARSI JSN score (used in defining OA progression in this study) was associated with a mean decrease in the minimum joint space width (JSW) of 0.89 mm to 1.13 mm or a decrease in the fixed JSW of 0.75 mm to 0.97 mm (the amount being dependent on the baseline level of JSN) (45). Importantly, based on a study by Bruyere et al (46), a loss of 0.7 mm in the minimum JSW after 3 years provided the best numeric overall efficiency for predicting the incidence of future knee surgery; notably, a loss in the minimum JSW of between 0.5 mm and 0.8 mm after 3 years was linked to a 4–5-fold increase in the risk of future knee surgery (P = 0.003–0.004) over the subsequent 8 years. Thus, these studies confirmed the clinical relevance of a 1-unit change in categorical JSN, underscoring the potential clinical relevance of using this biomarker for the prediction of radiographic knee OA progression in our study.

It is possible that cell types in addition to macrophages may produce TGFβ1, which would thereby lead to the lower association between SF TGFβ1 concentrations and the number of synovial tissue macrophages. For example, in the peripheral blood, previous studies have shown that TGFβ1 was detected both in CD4+ T cells (47) and in neutrophils (48). In the joint, TGFβ1 has been detected in the synovial tissue (8) and chondrocytes (49). However, the SF TGFβ1 level was very low or absent in normal joints, and elevated during development of joint disease, such as in knee OA (50). Although cell types other than macrophages in the peripheral blood could also be contributing to the increased concentrations of TGFβ1 in the SF, our results suggest that synovial tissue macrophages were primarily responsible, based on the stronger associations of SF TGFβ1 with macrophage cell number and with the MFI as compared to associations with other cell types evaluated (T cells and neutrophils).

The synovial tissue biospecimens provided for this study were available at the discretion of the collaborating surgeons. Although not designated a priori from a specific location, the tissue biospecimens we obtained represented synovial tissue still attached to the tibial plateau. However, the anatomic location of the synovial tissue was not standardized across patients. Synovial tissue cell numbers and SF biomarkers might have correlated even more strongly had we been able to more precisely control the synovial tissue harvest. Nevertheless, given the results showing that effector cytokines associated with the synovial tissue and SF cell types were predictive of OA severity and progression in samples from cohorts of patients with longitudinal follow-up data and displaying the full spectrum of radiographic knee OA severity, we do not believe that the location of the synovial tissue sample was a major confounder, although it may have accounted for a certain degree of variance in the inflammatory status between patient samples. Because the synovial tissue was collected at the time of surgery, fresh blood sometimes covered the tissue surface. The synovial tissue was rinsed with 70% ethanol and then washed with phosphate buffered saline to remove potential peripheral blood contaminants. With these wash steps, we believe that the cells isolated were predominantly resident within the synovial tissue, instead of being a remnant from peripheral blood contamination. We therefore expect that the cells that were analyzed would be minimally contaminated by peripheral blood leukocytes.

In summary, we have demonstrated that the baseline SF levels of elastase and TGFβ1, singly or combined, are strongly predictive of the risk of knee OA progression, reflecting the hitherto underappreciated role of neutrophils in the sterile inflammatory process and progression of OA, and the synergism of neutrophils with macrophage populations in the pathogenesis and worsening of OA. Both SF TGFβ1 and SF elastase might be utilized to identify patients at greater risk of more rapid and severe disease progression. A separate sample cohort with longitudinal follow-up data will be required to validate these findings. These results provide evidence that therapies targeting pathogenic immune cell populations might be used to attenuate the severe joint inflammation that occurs in OA.

ACKNOWLEDGMENTS

We thank Dr. Y. Li and staff members at Endocyte for providing the fluorescently labeled folic acid for the study. We also thank Alexander Reed for conducting the confirmatory bootstrap analysis.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kraus had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Hsueh, Zhang, Kraus.
Acquisition of data. Hsueh, Zhang, Wellman, Bolognesi.
Analysis and interpretation of data. Hsueh, Zhang, Kraus.

ROLE OF THE STUDY SPONSOR

Eli Lilly, Inc. provided funding for the primary etarfolatide imaging study from which biospecimens were obtained for one of the cohorts in the present study, but Eli Lilly, Inc. 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 Eli Lilly, Inc.

REFERENCES

1. Berenbaum F. Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). Osteoarthritis Cartilage 2013;21:16–21.
2. Muley MM, Reid AR, Botz B, Bolcskei K, Helyes Z, McDougall JJ. Neutrophil elastase induces inflammation and pain in mouse knee joints via activation of proteinase-activated receptor-2. Br J Pharmacol 2016;173:766–77.
3. De Lange-Brokaar BJ, Ioan-Facsinay A, van Osch GJ, Zuurmond AM, Schoones J, Toes RE, et al. Synovial inflammation, immune cells and their cytokines in osteoarthritis: a review. Osteoarthritis Cartilage 2012;20:1484–99.
4. Tarhan S, Unlu Z. Magnetic resonance imaging and ultrasonographic evaluation of the patients with knee osteoarthritis: a comparative study. Clin Rheumatol 2003;22:181–8.
5. Song IH, Althoff CE, Hermann KG, Scheel AK, Knetsch T, Schoenharting M, et al. Knee osteoarthritis: efficacy of a new method of contrast-enhanced musculoskeletal ultrasonography in detection of synovitis in patients with knee osteoarthritis in comparison with magnetic resonance imaging. Ann Rheum Dis 2008;67:19–25.
6. Atukorala I, Kwoh CK, Guermazi A, Roemer FW, Boudreau RM, Hannon MJ, et al. Synovitis in knee osteoarthritis: a precursor of disease? Ann Rheum Dis 2016;75:390–5.
7. Kraus VB, McDaniel G, Huebner JL, Stabler TV, Pieper CF, Shipes SW, et al. Direct in vivo evidence of activated macrophages in human osteoarthritis. Osteoarthritis Cartilage 2016;24:1613–21.
8. Tsuneyoshi Y, Tanaka M, Nagai T, Sunahara N, Matsuda T, Sonoda T, et al. Functional folate receptor β-expressing macrophages in osteoarthritis synovium and their M1/M2 expression profiles. Scand J Rheumatol 2012;41:132–40.
9. Hsueh MF, Lu Y, Wheeler L, Wellman SS, Bolognesi MP, Kraus VB. Functional folate receptor cells within synovium and fluid as therapeutic targets for osteoarthritis. Osteoarthritis Cartilage 2017;25:S42–3.
10. Daghestani HN, Pieper CF, Kraus VB. Soluble macrophage biomarkers indicate inflammatory phenotypes in patients with knee osteoarthritis. Arthritis Rheumatol 2015;67:956–65.
11. Landmann R, Müller B, Zimmerli W. CD14, new aspects of ligand and signal diversity. Microbes Infect 2000;2:295–304.
12. Bazil V, Strominger JL. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. J Immunol 1991;147:1567–74.
13. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990;249:1431–3.
14. Akashi S, Ogata H, Kirikae F, Kirikae T, Kawasaki K, Nishijima M, et al. Regulatory roles for CD14 and phosphatidylinositol in the signaling via Toll-like receptor 4-MD-2. Biochem Biophys Res Commun 2000;268:172–7.
15. Krutzik SR, Sieling PA, Modlin RL. The role of Toll-like receptors in host defense against microbial infection. Curr Opin Immunol 2001;13:104–8.
16. Bondeson J, Blom AB, Wainwright S, Hughes C, Caterson B, van den Berg WB. The role of synovial macrophages and macrophage-produced mediators in driving inflammatory and destructive responses in osteoarthritis [review]. Arthritis Rheum 2010;62:647–57.
17. Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. Arthritis Res Ther 2006;8:R187.
18. Wright HL, Moots RJ, Edwards SW. The multifactorial role of neutrophils in rheumatoid arthritis [review]. Nat Rev Rheumatol 2014;10:593–601.
19. Gupta K, Shukla M, Cowland JB, Malemud CJ, Haqqi TM. Neutrophil gelatinase–associated lipocalin is expressed in osteoarthritis and forms a complex with matrix metalloproteinase 9. Arthritis Rheum 2007;56:3326–35.
20. Benigni G, Dimitrova P, Antonangeli F, Sanseviero E, Milanova V, Blom A, et al. CXCR3/CXCL10 axis regulates neutrophil-NK cell cross-talk determining the severity of experimental osteoarthritis. J Immunol 2017;198:2115–24.
21. Leung YY, Hui LL, Kraus VB. Colchicine: update on mechanisms of action and therapeutic uses. Semin Arthritis Rheum 2015;45:341–50.
22. Das SK, Mishra K, Ramakrishnan S, Srivastava R, Agarwal GG, Singh R, et al. A randomized controlled trial to evaluate the slow-acting symptom modifying effects of a regimen containing colchicine in a subset of patients with osteoarthritis of the knee. Osteoarthritis Cartilage 2002;10:247–52.
23. Das SK, Ramakrishnan S, Mishra K, Srivastava R, Agarwal GG, Singh R, et al. A randomized controlled trial to evaluate the slow-acting symptom-modifying effects of colchicine in osteoarthritis of the knee: a preliminary report. Arthritis Rheum 2002;47:280–4.
24. Aran S, Malekzadeh S, Seifirad S. A double-blind randomized controlled trial appraising the symptom-modifying effects of colchicine on osteoarthritis of the knee. Clin Exp Rheumatol 2011;29:513–8.
25. Srivastava R, Das SK, Goel G, Asthana A, Agarwal GG. Does long term colchicine prevent degradation of collagen fiber network in osteoarthritis? Int J Rheum Dis 2018;21:114–7.
26. Leung YY, Haaland B, Huebner JL, Wong SB, Tjai M, Wang C, et al. Colchicine lack of effectiveness in symptom and inflammation modification in knee osteoarthritis (COLKOA): a randomized controlled trial. Osteoarthritis Cartilage 2018;26:631–40.
27. Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. Ann Rheum Dis 1957;16:494–502.
28. Addison S, Coleman RE, Feng S, McDaniel G, Kraus VB. Whole-body bone scintigraphy provides a measure of the total-body burden of osteoarthritis for the purpose of systemic biomarker validation. Arthritis Rheum 2009;60:3366–73.
29. Altman RD, Gold GE. Atlas of individual radiographic features in osteoarthritis, revised. Osteoarthritis Cartilage 2007;15 Suppl A:A1–56.
30. Leung YY, Huebner JL, Haaland B, Wong SB, Kraus VB. Synovial fluid proinflammatory profile differs according to the characteristics of knee pain. Osteoarthritis Cartilage 2017;25:1420–7.
31. Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 1993;39:561–77.

32. Kraus VB, Feng S, Wang S, White S, Ainslie M, Brett A, et al. Trabecular morphometry by fractal signature analysis is a novel marker of osteoarthritis progression. Arthritis Rheum 2009;60:3711–22.
33. Blom AB, van Lent PL, Holthuysen AE, van der Kraan PM, Roth J, van Rooijen N, et al. Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis. Osteoarthritis Cartilage 2004;12:627–35.
34. Van Beuningen HM, van der Kraan PM, Arntz OJ, van den Berg WB. Transforming growth factor-β 1 stimulates articular chondrocyte proteoglycan synthesis and induces osteophyte formation in the murine knee joint. Lab Invest 1994;71:279–90.
35. Prieto-Potin I, Largo R, Roman-Blas JA, Herrero-Beaumont G, Walsh DA. Characterization of multinucleated giant cells in synovium and subchondral bone in knee osteoarthritis and rheumatoid arthritis. BMC Musculoskelet Disord 2015;16:226.
36. Davenpeck KL, Sterbinsky SA, Bochner BS. Rat neutrophils express α4 and β1 integrins and bind to vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). Blood 1998;91:2341–6.
37. Rao RM, Betz TV, Lamont DJ, Kim MB, Shaw SK, Froio RM, et al. Elastase release by transmigrating neutrophils deactivates endothelial-bound SDF-1α and attenuates subsequent T lymphocyte transendothelial migration. J Exp Med 2004;200:713–24.
38. Labinsky H, Panipinto PM, Ly KA, Khuat DK, Madarampalli B, Mahajan V, et al. Multiparameter analysis identifies heterogeneity in knee osteoarthritis synovial responses. Arthritis Rheumatol 2020;72:598–608.
39. Cheon H, Yu SJ, Yoo DH, Chae IJ, Song GG, Sohn J. Increased expression of pro-inflammatory cytokines and metalloproteinase-1 by TGF-β1 in synovial fibroblasts from rheumatoid arthritis and normal individuals. Clin Exp Immunol 2002;127:547–52.
40. Wong CK, Chen DP, Tam LS, Li EK, Yin YB, Lam CW. Effects of inflammatory cytokine IL-27 on the activation of fibroblast-like synoviocytes in rheumatoid arthritis. Arthritis Res Ther 2010;12:R129.
41. Cao Y, Doodes PD, Glant TT, Finnegan A. IL-27 induces a Th1 immune response and susceptibility to experimental arthritis. J Immunol 2008;180:922–30.
42. Barrett AJ. The possible role of neutrophil proteinases in damage to articular cartilage. Agents Actions 1978;8:11–8.
43. Muley MM, Krustev E, Reid AR, McDougall JJ. Prophylactic inhibition of neutrophil elastase prevents the development of chronic neuropathic pain in osteoarthritic mice. J Neuroinflammation 2017;14:168.
44. Huesa C, Ortiz AC, Dunning L, McGavin L, Bennett L, McIntosh K, et al. Proteinase-activated receptor 2 modulates OA-related pain, cartilage and bone pathology. Ann Rheum Dis 2016;75:1989–97.
45. Ratzlaff C, Ashbeck EL, Guermazi A, Roemer FW, Duryea J, Kwoh CK. A quantitative metric for knee osteoarthritis: reference values of joint space loss. Osteoarthritis Cartilage 2018;26:1215–24.
46. Bruyere O, Richy F, Reginster JY. Three year joint space narrowing predicts long term incidence of knee surgery in patients with osteoarthritis: an eight year prospective follow up study. Ann Rheum Dis 2005;64:1727–30.
47. Zhang X, Tao Y, Chopra M, Ahn M, Marcus KL, Choudhary N, et al. Differential reconstitution of T cell subsets following immunodepleting treatment with alemtuzumab (anti-CD52 monoclonal antibody) in patients with relapsing-remitting multiple sclerosis. J Immunol 2013;191:5867–74.
48. Grotendorst GR, Smale G, Pencev D. Production of transforming growth factor beta by human peripheral blood monocytes and neutrophils. J Cell Physiol 1989;140:396–402.
49. Villiger PM, Lotz M. Differential expression of TGF beta isoforms by human articular chondrocytes in response to growth factors. J Cell Physiol 1992;151:318–25.
50. Van der Kraan PM. Differential role of transforming growth factor-β in an osteoarthritic or a healthy joint [review]. J Bone Metab 2018;25:65–72.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 100–109
DOI 10.1002/art.41473

American College of Rheumatology
Empowering Rheumatology Professionals

Multi-Tissue Epigenetic and Gene Expression Analysis Combined With Epigenome Modulation Identifies *RWDD2B* as a Target of Osteoarthritis Susceptibility

Eleanor Parker,[1] Ines M. J. Hofer,[1] Sarah J. Rice,[1] Lucy Earl,[1] Sami A. Anjum,[1] David J. Deehan,[2] and John Loughlin[1]

Objective. Osteoarthritis (OA) is polygenic, with more than 90 risk loci currently mapped, including at the single-nucleotide polymorphism rs6516886. Previous analysis of OA cartilage DNA identified 6 CpG dinucleotides with methylation levels that correlated with the rs6516886 genotype, forming methylation quantitative trait loci (mQTLs). We undertook this study to investigate these mQTLs and to map expression quantitative trait loci (eQTLs) across joint tissues in order to identify a particular gene as a target of the rs6516886 association effect.

Methods. Nucleic acids were extracted from the cartilage, fat pad, synovium, and peripheral blood from OA patients. Methylation of CpGs and allelic expression imbalance of potential target genes were assessed by pyrosequencing. A chondrocyte cell line expressing deactivated Cas9 (dCas9)–TET1 was used to directly alter CpG methylation levels, with effects on gene expression quantified by polymerase chain reaction.

Results. Multiple mQTLs were detected, with effects strongest in joint tissues and methylation at CpG cg20220242 correlating most significantly with the rs6516886 genotype. CpG cg20220242 is located upstream of *RWDD2B*. Significant rs6516886 eQTLs were observed for this gene, with the OA risk–conferring allele of rs6516886 correlating with reduced expression. CpG methylation also correlated with allelic expression of *RWDD2B*, forming methylation–expression QTLs (meQTLs). Deactivated Cas9–TET1 reduction in the methylation of cg20220242 increased expression of *RWDD2B*.

Conclusion. The rs6516886 association signal is a multi-tissue meQTL involving cg20220242 and acting on *RWDD2B*. Modulating CpG methylation reverses the impact of the risk allele. *RWDD2B* codes for a protein about which little is currently known. Further analysis of *RWDD2B* as a target of OA genetic risk will provide novel insight into this complex disease.

INTRODUCTION

Osteoarthritis (OA) is the most common musculoskeletal disorder, affecting 250 million people worldwide (1). OA is characterized by loss of articular cartilage in addition to other joint disruptions, including osteophyte formation, inflammation of the synovium, meniscal damage, and bone remodeling. These structural changes lead to chronic pain, decreased quality of life, and comorbidities, including type 2 diabetes mellitus, cardiovascular disease, and premature death (2–4). OA is polygenic, and multiple genome-wide association studies (GWAS) have been conducted to identify osteoarthritis risk loci, with >90 independent signals identified so far in Europeans (5–11). The overwhelming majority of these loci are intergenic or intronic and are known or predicted to mediate their effect via altered target gene expression, acting as expression quantitative trait loci (eQTLs) (12). Additionally, OA-associated polymorphisms have been shown to correlate with methylation levels at CG dinucleotides (CpGs) in *cis*,

Supported by the MRC and Versus Arthritis as part of the Centre for Integrated Research into Musculoskeletal Ageing (grants JXR 10641, MR/P020941/1, and MR/R502182/1, and Versus Arthritis grants 20679 and 20771). The Genotype-Tissue Expression Project was supported by the Common Fund of the Office of the Director of NIH and by the National Cancer Institute, National Human Genome Research Institute, National Heart, Lung, and Blood Institute, National Institute on Drug Abuse, National Institute of Mental Health, and National Institute of Neurological Disorders and Stroke. Funds to purchase the pyrosequencer were provided by The Ruth and Lionel Jacobson Charitable Trust. Dr. Parker's work was supported by The Ruth and Lionel Jacobson Charitable Trust.

[1]Eleanor Parker, BSc, MSc, PhD, Ines M. J. Hofer, BSc, MRes, Sarah J. Rice, MRPharmS, MRes, PhD, Lucy Earl, BSc, MRes, Sami A. Anjum, MBBS, MRes, MRCS, John Loughlin, BSc, PhD: Newcastle University, Newcastle-upon-Tyne, UK; [2]David J. Deehan, MBChB, MSc, MD, FRCS (Eng), DSc, FRCS (Tr&Orth): Newcastle University Teaching Hospitals NHS Trust, Freeman Hospital, Newcastle-upon-Tyne, UK.

No potential conflicts of interest relevant to this article were reported.

Address correspondence to Eleanor Parker, BSc, MSc, PhD, or John Loughlin, BSc, PhD, Newcastle University, Biosciences Institute, Newcastle-upon-Tyne NE1 3BZ, UK. Email: eleanorlouiseparker@gmail.com or john.loughlin@ncl.ac.uk.

Submitted for publication April 27, 2020; accepted in revised form July 28, 2020.

a mechanism known to regulate gene expression, thereby acting as methylation QTLs (mQTLs) (13). Indeed, several of these OA loci have been shown to correlate with expression in addition to methylation, known as methylation–expression QTLs (meQTLs), where methylation regulates expression (14). It is therefore clear that DNA methylation drives a significant share of the functional consequences of OA genetic risk loci, and identification and investigation of eQTLs, mQTLs, and meQTLs at disease loci can prioritize genes and gene-regulatory elements, for subsequent study, as targets of the association effect.

One such identified OA risk locus is marked by single-nucleotide polymorphism (SNP) rs6516886 (T>A; minor allele frequency [MAF] of 0.29), which is located on chromosome 21q21.2 and where the major allele (T) was found to be associated with both knee and hip OA in Europeans at genome-wide significance (8). Previously, we have reported that the genotype at rs6516886 correlated with methylation levels at 6 CpG sites, using an Illumina Infinium HumanMethylation450 genome-wide CpG array and cartilage DNA samples from 87 patients (15). The CpGs were located at an interval of <90 kb and mapped upstream and downstream of rs65168886, with the T risk allele associated with increased methylation at 5 CpGs, (cg00065302, cg05468028, cg18001427, cg20220242, and cg24751378) and with decreased methylation at the most distal CpG, cg16140273 (15). The associated region encompasses several genes, including the following: *LTN1*, which codes for E3 ubiquitin-protein ligase (listerin); *RWDD2B*, which codes for RWD domain–containing protein 2B; *USP16*, which codes for ubiquitin carboxyl-terminal hydrolase 16; *CCT8*, which codes for chaperonin containing TCP1 subunit 8; and *MAP3K7CL*, which codes for MAP3K7 C-terminal–like protein.

Although cartilage is the central tissue involved in OA, there is joint-wide involvement of other tissues in the disease etiology (16). Therefore, to determine the potential functional role of rs6516886 in OA, we aimed to investigate whether the identified mQTLs are active across different joint tissues and, further, to identify potential gene targets of the association signal to understand how this locus increases OA risk.

MATERIALS AND METHODS

Patient samples. Joint tissue samples, including cartilage, synovium, and infrapatellar fat pad, were obtained from 348 patients who had primary hip or knee OA and who had undergone joint replacement surgery at the Newcastle-upon-Tyne NHS Foundation Trust hospitals. The Newcastle and North Tyneside Research Ethics Committee granted ethical approval for the collection, with each donor providing verbal and written informed consent (REC reference no. 14/NE/1212). For 55 patients, peripheral blood samples were also collected prior to surgery, using EDTA vacutainers for DNA extraction and Tempus tubes for RNA extraction (ThermoFisher Scientific). Patient details are available in Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract.

Nucleic acid extraction from tissue samples. Joint tissue samples were frozen and ground to a powder using a mixer mill (Retsch Limited) under liquid nitrogen, prior to nucleic acid extraction. Cartilage DNA and RNA were extracted using an EZNA Tissue DNA isolation kit (VWR; Omega Biotek) and TRIzol (Life Technologies), respectively. Nucleic acids from synovium and fat pad were extracted using an EZNA DNA/RNA isolation kit and from blood using a QIAamp DNA blood mini kit (Qiagen) and a Tempus Spin RNA isolation kit (ThermoFisher Scientific).

Genotyping. SNPs were genotyped using pyrosequencing, as previously described (12,15). Briefly, pyrosequencing assays were designed using PyroMark assay design SW 2.0 (Qiagen), polymerase chain reactions (PCRs) were performed using a G-Storm GS4 Q4 Quad Block Thermal Cycler (Somerton Biotechnology Centre), and sequencing was performed using the PyroMark Q24 Advanced platform and reagents kit, according to the instructions of the manufacturer (Qiagen). Primer sequences are listed in Supplementary Table 2 (http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract).

Targeted CpG methylation analysis. DNA samples were bisulfite-converted using an EZ DNA methylation kit according to the instructions of the manufacturer (Zymo Research). CpG site methylation analysis was then performed by pyrosequencing as described above (primer sequences are listed in Supplementary Table 2, http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract). Methylation analysis was performed in duplicate for each sample, with a 5% variance between replicates used as a quality control threshold; samples exceeding this threshold were excluded from analysis. For CpG sites with a global average methylation level of ≤15%, a quality control threshold of 1% was used.

Chromatin interactions. The UCSC Genome Browser (http://genome.ucsc.edu/) (17) was mined to identify long-range chromatin interactions stemming from the CpG sites within the locus and the association SNP. All publicly available Hi-C and long-range chromatin interaction data sets were utilized.

Quantitative gene expression. Complementary DNA (cDNA) was synthesized with a SuperScript First-Strand synthesis kit and random hexamers, according to the manufacturer's instructions (Invitrogen), and quantitative PCR (qPCR) was subsequently performed using predesigned TaqMan assays (Integrated DNA Technologies), as previously described (12). Investigated genes were normalized to the housekeeping genes *HPRT1*, *18S*, and *GAPDH*, and the relative expression of each gene was calculated using the $2^{-\Delta C_t}$ method (12).

Allelic expression imbalance (AEI). AEI at transcript SNPs located in the exons of investigated genes was quantified by pyrosequencing (primer sequences are listed in Supplementary Table 2, http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract), as previously described (18). Analysis was performed in triplicate on DNA and cDNA for each sample, and a quality control threshold of 5% variance across replicates was applied. Allelic expression for each sample was produced using PyroMark Advanced software, and the output for cDNA was normalized to that of its respective DNA.

Generation of a stable chondrocyte cell line with inducible deactivated Cas9 (dCas9)–TET1 expression. The PiggyBac 138-dCas9-TET1 vector (19) and modified 137-transposase "helper" vector (20) were kind gifts from the laboratory of R. Jaenisch, MIT, Boston, MA. The PiggyBac vector was digested with NsiI-HF and NdeI enzymes (New England Biolabs) to remove the 2-kb sequence encoding the Neo^r/kanamycin resistance gene. Following this, a 1.7-kb sequence, including the puromycin resistance gene and complementary overhangs to the vector backbone, was ligated into the plasmid. The sequence of the resulting construct was confirmed by Sanger sequencing (Source BioScience). The modified dCas9-TET1 and the transposase constructs were transfected into Tc28a2 immortalized chondrocytes by nucleofection (Amaxa 4D; Lonza) at a PiggyBac:helper ratio of 3:1. Post-nucleofection cells were cultured with 1 μg/ml puromycin for 2 weeks. Doxycycline-inducible expression of dCas9-TET1 protein was confirmed and optimized by immunoblotting using a Cas9 antibody (7A9-3A3; Cell Signaling Technology).

Clustered regularly interspaced short palindromic repeat (CRISPR)/dCas9. Guide RNA (gRNA) sequences were designed using the Integrated DNA Technologies design tool (https://eu.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM) to target the region encompassing and flanking cg20220242 (Supplementary Figure 1, http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract). Identified gRNA sequences were assessed for off-target risk and on-target potential using the design tool. Low scoring gRNAs were rejected, resulting in 2 gRNAs being selected for testing. The gRNAs were then purchased as synthetic Alt-R CRISPR-Cas9 CRISPR RNAs (crRNAs) alongside Alt-R CRISPR-Cas9 *trans*-activating CRISPR RNA (tracrRNA) (Integrated DNA Technologies). A nontargeting Alt-R CRISPR-Cas9 negative control crRNA (Integrated DNA Technologies) was used to exclude TET1 off-target effects.

TC28a2-dCas9-TET1 cells were plated at subconfluence (100,000 cells/well) in 6-well plates (VWR) and allowed to adhere for 24 hours. Deactivated Cas9–TET1 expression was induced by incubation with 2 μ*M* doxycycline hyclate (Sigma-Aldrich) in 2 ml complete medium for a further 24 hours. CRISPR RNAs were annealed to tracrRNA in a 1:1:2 ratio for paired gRNA and at a 1:1 ratio for single guides at 95°C for 5 minutes, while the nontargeting negative control crRNA was annealed to tracrRNA. Complexes of crRNA/tracrRNA were separately combined with Dharmafect1 (Dharmacon) in serum-free medium (PCS-500-030; ATCC). Next, 1.2 ml of the doxycycline-containing medium was aspirated from each well and replaced with 200 μl transfection mix for 24 hours, after which the medium was changed to complete growth medium, and the cells expanded until confluence in T25 flasks (VWR) was reached (between 2 and 5 days). Guide RNA transfection efficiency was previously confirmed using green fluorescent protein–labeled gRNAs (Parker E et al: unpublished observations). Cells were collected and pelleted by trypsinization, and the cell pellets were frozen. RNA was extracted from pellets using a NucleoSpin TriPrep Kit (Macherey-Nagel, supplied by Thermo Fisher Scientific), and qPCR was undertaken as described above. DNA was extracted using a PureLink Genomic DNA Purification Kit (Invitrogen, supplied by ThermoFisher Scientific), and methylation analysis of cg20220242 (and of upstream and downstream flanking CpGs) was performed as described above and using the

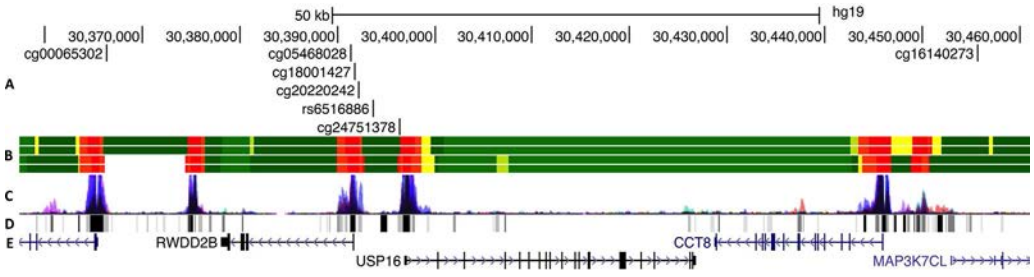


Figure 1. In silico investigation of the rs6516886 locus visualized using the UCSC Genome Browser (http://genome.ucsc.edu/). **A** and **B**, The positions of the 6 CpGs and rs6516886 (**A**) and Roadmap ChIP-seq data in adipose-derived mesenchymal stem cell–cultured cells and mesenchymal stem cell–derived chondrocyte–cultured cells, displaying peaks in this region (**B**). Yellow = enhancer; green = strong transcription; dark green = weak transcription; red = active transcription start site (TSS); orange-red = flanking active TSS; yellow-green = genic enhancers. Data were interpreted using the 15-state model. **C**, H3K27Ac marks, an indicator of active regulatory elements. **D**, DNase I hypersensitivity clusters, a mark of open chromatin. Gray boxes indicate the area of the hypersensitive region. The darkness of the box is proportional to the maximum signal strength observed. In **C** and **D**, data were obtained from the ENCODE project. **E**, UCSC reference genes. Genomic location and scale are denoted at the top.

primers listed in Supplementary Table 2 (http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract).

Statistical analysis. Kruskal-Wallis tests were used to assess the significance of the association between genotype and methylation of CpGs. For AEI, *P* values were calculated using Wilcoxon's matched pairs signed rank test for *LTN1*, *RWDD2B*, *USP16,* and *CCT8* and an unpaired *t*-test for *MAP3K7CL* and *BACH1*. For two-way analyses, *P* values were calculated using Mann-Whitney 2-tailed exact tests. Correlations between genotype, age, and methylation, and the significance of the correlations, were determined using a standard least squares linear regression model.

RESULTS

SNP rs6516886 acting as an mQTL across multiple tissues. Previous work (15) has identified 6 CpG sites that correlate with the genotype at the OA risk–conferring SNP rs6516886 (Supplementary Table 3, http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract). In silico analysis of this region revealed that cg00065302, cg05468028, cg18001427, cg20220242, and cg24751378 all reside in areas marked as promoters in relevant cell types (Figure 1). Additionally, these sites are enriched with H3K27ac, an indicator of active regulatory elements, and DNase I hypersensitivity regions, which indicate accessible chromatin regions, a mark of a transcribed region. In contrast, the most distal of the CpGs, cg16140273, resides in a region with no notable epigenetic marks of a transcriptionally active or regulatory region.

Methylation analysis of cartilage, fat pad, synovium, and blood DNA samples from OA patients showed a correlation between methylation and the rs6516886 genotype, generating significant rs6516886 mQTLs at cg00065302, cg05468028, cg18001427, cg20220242, and cg16140273 across the tissues (Figure 2). We were unable to develop a pyrosequencing assay for cg24751378, which was therefore excluded from further investigation, whereas the cg05468028 assay captured an additional CpG site 2 bp upstream of cg05468028, which we termed cg05468028_2. This CpG was also an mQTL (Figure 2). The OA risk–conferring T allele of rs6516886 correlated with increased methylation across all tissues at cg00065302, cg05468028, cg05468028_2, cg18001427, and cg20220242,

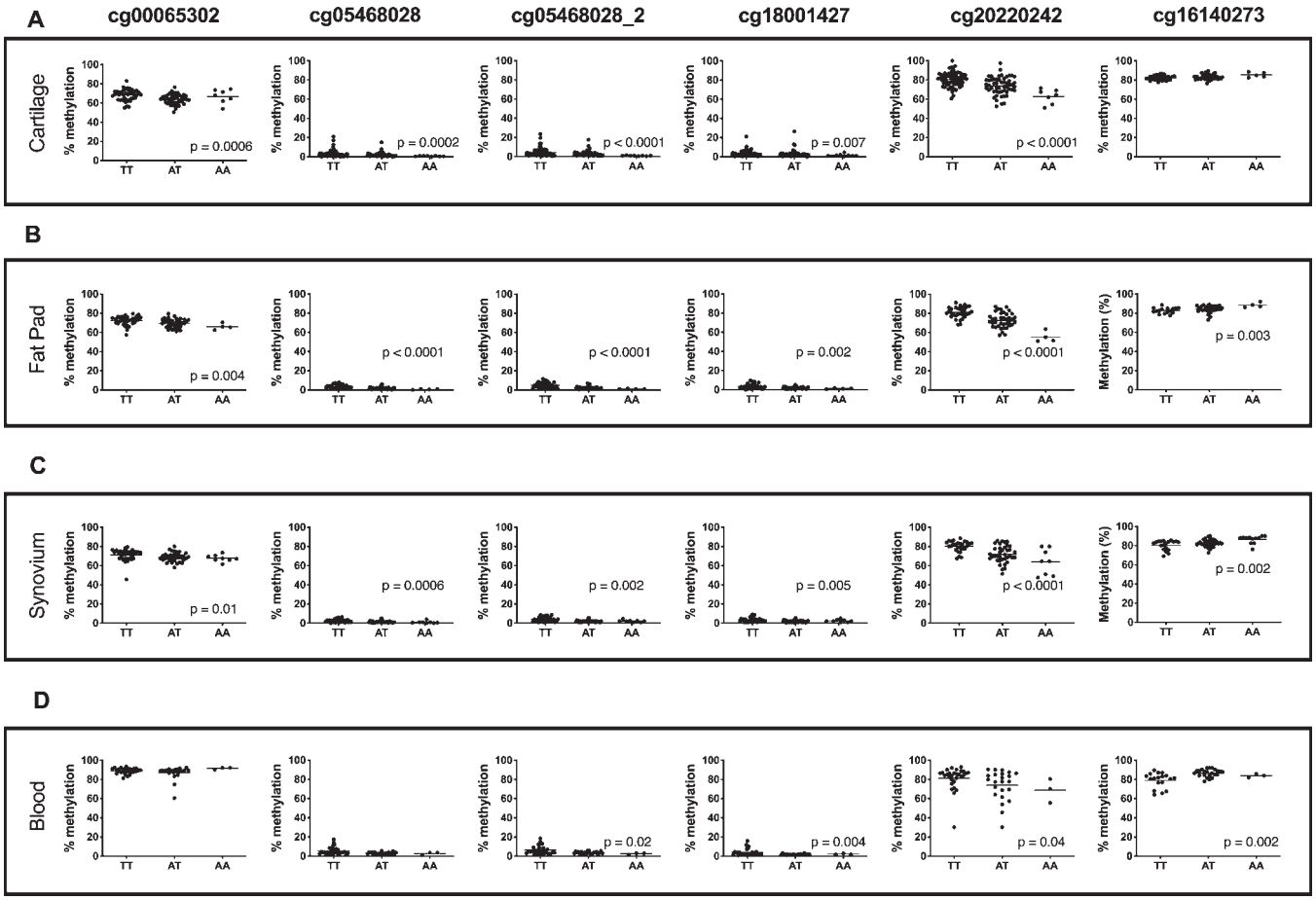


Figure 2. Relationship between rs6516886 genotype and methylation at CpGs cg00065302, cg05468028, cg05468028_2, cg18001427, cg20220242, and cg16140273 in DNA from cartilage (**A**), fat pad (**B**), synovium (**C**), and blood (**D**). Each symbol represents an individual patient. *P* values were calculated using the Kruskal-Wallis test. Bars show the mean.

and with decreased methylation across all tissues at cg16140273. These are the same directions of effect that we had previously observed in our array analysis of cartilage DNA (15). The most significant results obtained in the mQTL analysis were for cg20220242, with *P* values of <0.0001 in cartilage, fat pad, and synovium.

In summary, we replicated the mQTLs that we had previously reported in cartilage and demonstrated that they were also active across other joint tissues and blood. The most striking effect was for cg20220242.

Methylation data independent of genotype. When methylation data was plotted independent of genotype, we observed that there was a significantly higher methylation in blood for cg00065302, cg05468028, cg05468028_2, and cg00065302, compared to the other tissues (Supplementary Figure 2, http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract). Additionally, cg00065302 showed a clear decrease in methylation in cartilage samples. This effect can be seen in Supplementary Figure 3 (http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract), where data on patients for whom ≥2 tissue samples were available are plotted together. For the majority of patients, it can be clearly observed that at cg00065302, methylation values were the highest in blood and the lowest in cartilage. A striking example was in patient 333, who displayed a 24% difference between methylation in cartilage and blood. Analysis of methylation data stratified by joint revealed no significant differences between hip and knee cartilage (Supplementary Figure 4, http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract).

Quantifying the effect of rs6516886 genotype on methylation. We next determined how much of the observed interpatient variability in methylation was determined by genotype alone, with the magnitude of the genotypic effect calculated by linear regression analysis and represented in a heatmap (Supplementary Figure 5A, http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract). As with the mQTL analysis, cg20220242 displayed the greatest genotypic effect size across the joint tissues, with a 23.1% contribution in cartilage, 41.9% in fat pad, and 28.5% in synovium. Conversely, genotype had a relatively small effect on cg20220242 methylation variability in blood, with only a 6.5% contribution. Across the CpG sites, the greatest genotypic contribution to methylation was observed in the fat pad, except for cg16140273, in which the greatest contribution was observed in blood (20.2%). A linear regression analysis was also carried out to determine whether age contributed to the interpatient methylation variation, which confirmed that genotype had a much stronger role, and age had at most a minimal effect on methylation (0–3.8%) (Supplementary Figure 5B, http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract).

Candidate genes at the association locus. In silico analysis of chromatin interactions identified several examples of chromatin looping between areas containing the CpGs and genes at the locus (Figure 3). The cluster of cg05468028, cg05468028_2, cg18001427, and cg20220242, which are <1 kb apart, is located within the promoter/upstream region of *RWDD2B* but also shows interactions with *LTN1*, *USP16*, and *CCT8*. Both cg00065302 and cg16140273 show interactions with promoter regions of *BACH1*, with cg16140273 also interacting with *CCT8* and *MAP3K7CL*. Similarly, cg00065302, which resides on the edge of the *LTN1* promoter region, also shows interactions with *RWDD2B*, *USP16*, *CCT8*, and *MAP3K7CL*.

Quantification of the expression of these genes revealed that all 6 were expressed in the joint tissues and in blood (Supplementary Figure 6, http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract). Blood showed the highest level of expression

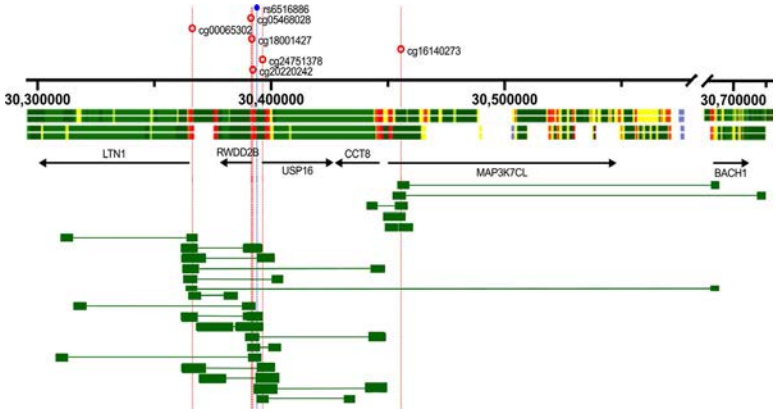


Figure 3. In silico investigation of chromatin interactions at the rs6516886 locus. The positions of CpGs and rs6516886 are shown by red and blue lines, respectively. Roadmap ChIP-seq data in adipose-derived mesenchymal stem cell–cultured cells and mesenchymal stem cell–derived chondrocyte–cultured cells, displaying chromatin state data, are shown. Yellow = enhancer; green = strong transcription; dark green = weak transcription; red = active transcription start site (TSS); orange-red = flanking active TSS; yellow-green = genic enhancers; white = quiescent/low. Data were interpreted using the 15-state model. Gene positions are indicated by **arrows**. Chromatin interactions mined from all publicly available Hi-C and long-range chromatin interaction data sets are indicated by green boxes and lines linking the interacting regions.

for all investigated genes, except for *RWDD2B*, which was most highly expressed in fat pad ($P = 0.0004$).

An rs6516886 eQTL at *RWDD2B*. To test for differential allelic expression, transcript SNPs were identified for the 6 genes (Supplementary Table 4, http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract), and AEI analysis was conducted. For *RWDD2B*, the transcript SNP was in perfect linkage disequilibrium (LD; $r^2 = 1$) with rs6516886, while for *LTN1*, *USP16*, and *CCT8*, the transcript SNPs were in complete or near complete LD ($D' = 1$). As such, the phase between the transcript SNP alleles and the rs6516886 alleles could be determined unambiguously for these 4 genes. Patients' compound heterozygotes for the respective transcript SNP and rs6516886 were therefore used in analyses of each of these genes. For *MAP3K7CL* and *BACH1*, the transcript SNPs were not in LD with rs6516886 (Supplementary Table 4). AEI at these 2 genes was therefore assessed by comparing the variance in compound heterozygotes to that measured in patients who were homozygotic at rs6516886 (21,22). In this instance, AEI driven by genotype at rs6516886 would be evidenced by compound heterozygotes forming bidirectional clusters at ratios higher and lower than 1.

Significant AEI in *RWDD2B* was detected in all 3 joint tissues, with the OA risk–conferring T allele of rs6516886 correlating with a decrease in expression in the combined analysis of all patients ($P < 0.0001$; Figure 4). In blood, for which the number of patients available for analysis was much smaller, AEI approached significance ($P = 0.062$) in the same direction (Figure 4D). Pairwise analysis of the magnitude of *RWDD2B* AEI between the tissues revealed that cartilage showed the least AEI and blood showed the most, with risk allele expression reduced on average by 15% in cartilage and 25% in blood (Figure 4E). Fat pad and synovium also had a greater average AEI compared to cartilage, but these were not significantly different compared to blood.

For the other 5 genes (Supplementary Figures 7–11, http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract), AEI was detected in combined analyses of all patients for *LTN1* in cartilage ($P = 0.01$; Supplementary Figure 7A), *CCT8* in synovium ($P = 0.021$; Supplementary Figure 9C), and *BACH1* in synovium ($P = 0.04$; Supplementary Figure 11C).

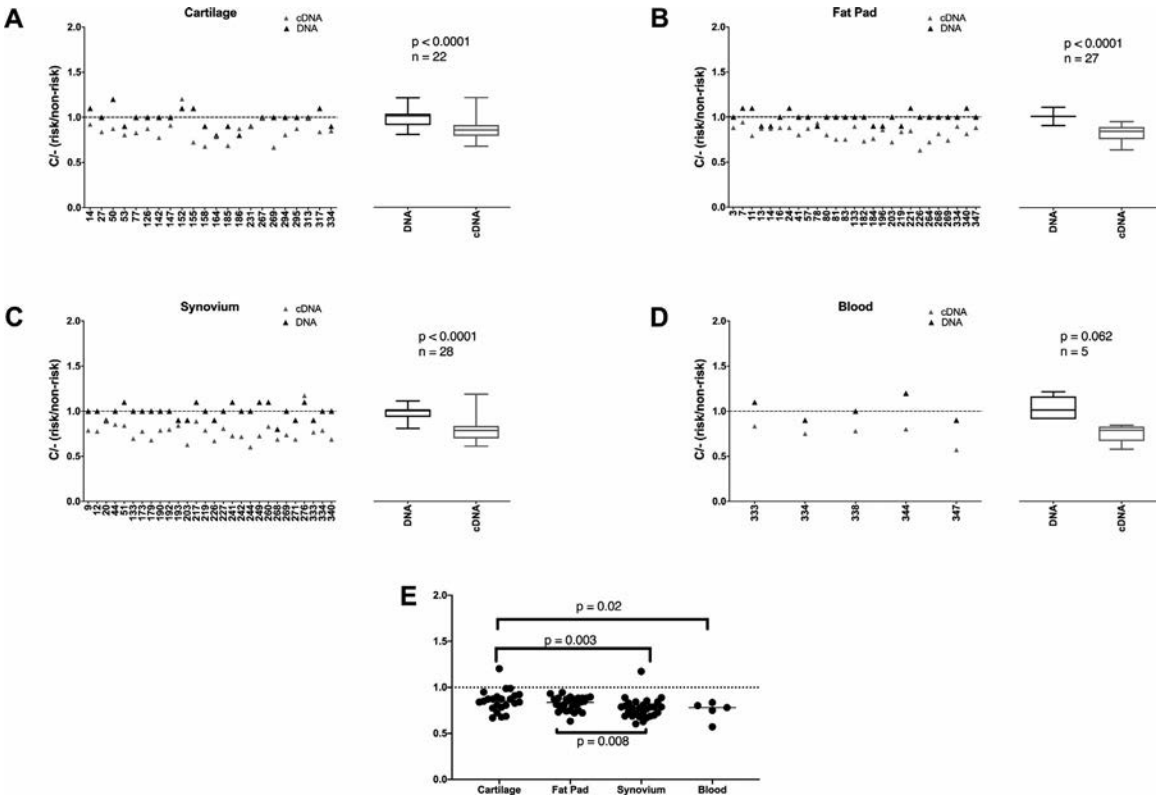


Figure 4. *RWDD2B* allelic expression imbalance (AEI) in cartilage (**A**), fat pad (**B**), synovium (**C**), and blood (**D**), and the comparison of the scale of AEI between tissues (**E**). AEI analysis was conducted using transcript single-nucleotide polymorphism rs112411829. In **A**–**D**, the risk: nonrisk allelic ratio is plotted, with a ratio <1 indicating decreased *RWDD2B* expression from the risk allele. For each patient, the mean of the DNA ratio (black = 3 technical repeats) and the mean of the cDNA ratio (gray = 3 technical repeats) are plotted. Numbers on the x-axis refer to the anonymized patient identification number. Box plots show the values of DNA and cDNA for all patients combined. Lines within the box represent the median, the box represents the 25th to 75th percentiles, and the whiskers represent the maximum and minimum values. *P* values were calculated using Wilcoxon's matched pairs signed rank test in **A–D** and using the Mann-Whitney 2-tailed exact test in **E**.

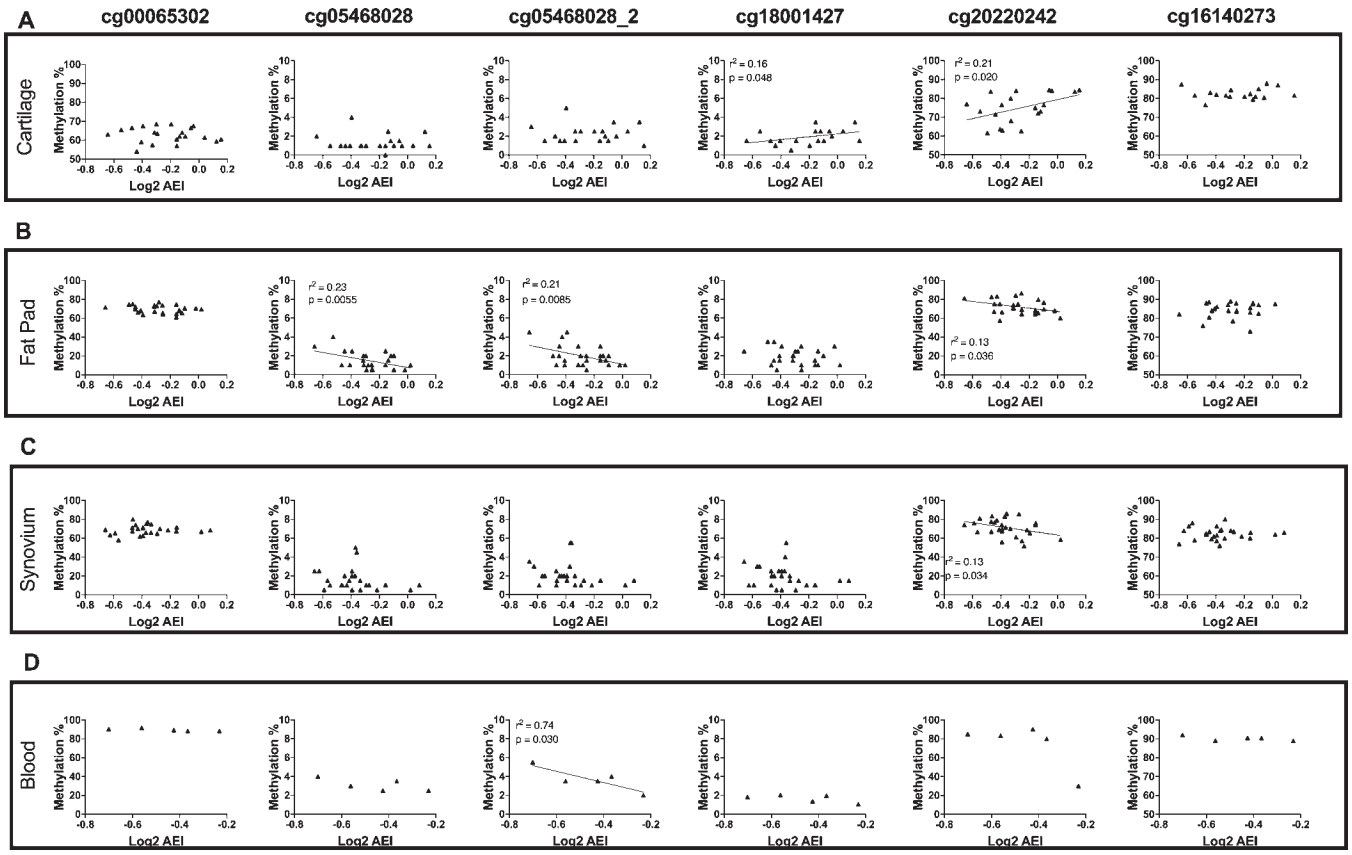


Figure 5. Methylation–expression quantitative trait locus analyses for *RWDD2B* in cartilage (**A**), fat pad (**B**), synovium (**C**), and blood (**D**). *RWDD2B* $\log_2$ allelic expression imbalance (AEI) ratios were plotted against methylation at cg00065302, cg05468028, cg05468028_2, cg18001427, cg20220242, and cg16140273. Each symbol represents an individual patient. The square of the correlation coefficient (r^2) and *P* values were calculated by linear regression analysis using a standard least squares model.

To summarize, there is compelling evidence of AEI across all joint tissues and therefore of an rs6516886 eQTL acting on *RWDD2B*. For other genes, any positive evidence was much less significant and was not detected in more than 1 tissue.

Observation of rs6516886 meQTLs at *RWDD2B*. We next plotted the *RWDD2B* AEI data for the heterozygotes against their individual methylation data. The most striking effects were seen at cg20220242, with significant correlations seen across all 3 joint tissues (Figure 5). Interestingly, the effects were not all in the same direction, with lower AEI levels observed with increasing methylation in cartilage ($P = 0.02$) and higher levels in fat pad ($P = 0.036$) and synovium ($P = 0.034$). Methylation at other CpGs also correlated with *RWDD2B* AEI. These included cg18001427 in cartilage ($P = 0.048$) and cg05468028 and cg05468028_2 in fat pad ($P = 0.0055$ and $P = 0.0085$, respectively), all with the same tissue-specific directionality as seen with cg20220242 (Figure 5).

***RWDD2B* expression regulated by cg20220242 methylation.** CpG cg20220242 was hypermethylated in chondrocytes, with median methylation levels >80% (Figure 6A). To determine whether methylation of cg20220242 is a direct regulator of *RWDD2B* expression, we reduced the methylation of the CpG, and of flanking CpGs, in the immortalized chondrocyte cell line Tc28a2, using CRISPR gRNAs and dCas9 fused to the DNA demethylating enzyme TET1. Using 2 different gRNAs, individually and in combination, we observed that guide 2, which localizes −15 to +5 bp across cg20220242 (Supplementary Figure 1, http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract), induced a significant decrease in methylation at cg20220242 (Figure 6A), with a reduction of 21.5% compared to control ($P = 0.0022$). Guide 1 had no significant effect ($P > 0.05$), and using both guides together did not reduce methylation greater than guide 2 alone (Figure 6A). We therefore focused on guide 2.

To determine the limits of the methylation modulation, we analyzed methylation at all CpGs 100 bp upstream and downstream of cg20220242 (Supplementary Figure 1, http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract). Methylation was significantly decreased ($P < 0.05$) at all 5 CpGs upstream of cg20220242 and at 3 of the 8 CpGs downstream of cg20220242 (Figure 6B). The largest decrease in methylation was 36.6% at the CpG positioned −41 bp from cg20220242. There was a 3.8-fold increase in expression of *RWDD2B* in the demethylated cells compared to control ($P = 0.0004$; Figure 6C). No significant

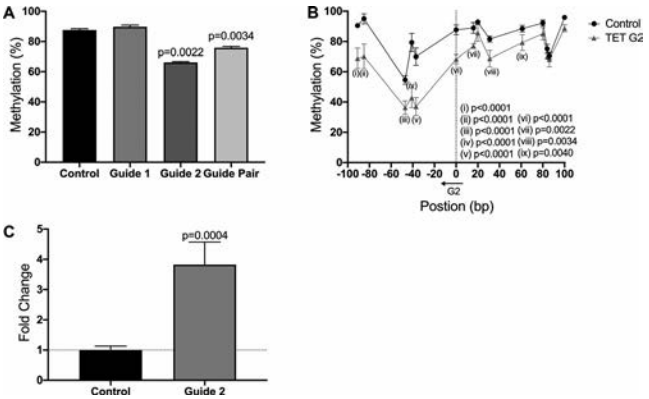


Figure 6. Methylation modulation of cg20220242 in Tc28a2–deactivated Cas9 (dCas9)–TET1 cells. **A**, Percentage methylation at cg20220242. Data were obtained from 3 biologic replicates. **B**, Percentage methylation at CpG sites 100 bp upstream and downstream of cg20220242 in control cells and cells transfected with guide 2 (G2). The position of CpGs was plotted relative to cg20220242 (dotted line) on the x-axis. Negative values are upstream, and positive values are downstream. Data were obtained from 6 biologic replicates. **C**, Gene expression of *RWDD2B* in control cells and cells transfected with guide 2. *RWDD2B* mRNA levels were measured by quantitative polymerase chain reaction and plotted as fold change in expression relative to control (red dotted line). Data were obtained from 6 biologic replicates. In all panels, values represent the mean ± SEM. *P* values were calculated using the Mann-Whitney 2-tailed exact test.

changes in expression were observed in the flanking genes ($P > 0.05$ for all; Supplementary Figure 12, http://onlinelibrary.wiley.com/doi/10.1002/art.41473/abstract), indicating a specific effect on *RWDD2B*.

DISCUSSION

The number of GWAS conducted on human diseases has increased exponentially over the past 15 years. However, there is currently a huge disparity between the number of GWAS reports in the literature each year and the number of published functional follow-up studies investigating likely mechanisms of action of causal variants (23). One approach to functional analysis of signals obtained by GWAS is to study the epigenetics of the associated regions. DNA methylation is the best described and most studied epigenetic modification, and can regulate gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factor(s) to DNA (24). As OA presents with pathologic changes in all of the joint tissues, it should be considered a disease of the whole joint (16), and investigation of genetic risk signals should therefore not be limited to cartilage alone. As such, when investigating the functional mechanisms of OA signals described in the GWAS literature, it is important to consider that the potential mechanisms may function differentially between tissues. The present study therefore aimed to increase understanding of the functional role of the OA risk locus marked by rs6516886, focusing on the CpGs previously identified as correlating with the genotype at this SNP in cartilage array data (15) and expanding the analysis to include multiple joint tissues and an analysis of gene expression.

Multi-tissue mQTLs operating at rs6516886 were initially discovered. The majority of these had consistently larger rs6516886 genotypic effects in the examined joint tissues compared to blood. The largest effects were seen at CpG cg20220242. However, none of the calculated genotypic effects were at or even approaching 100%, implying that rs6516886 genotype alone does not account for all of the variation in methylation that was observed. Other DNA polymorphisms or nongenetic factors presumably also influence the levels of methylation of the tested CpGs.

An rs6516886 *RWDD2B* eQTL was subsequently observed across the joint tissues using an AEI approach, with the OA risk–conferring allele of the SNP correlating with reduced expression of the gene. Unlike for the mQTL data, this eQTL effect was as strong, if not stronger, in blood than in the joint tissues. A search of the Genotype-Tissue Expression portal (25; https://www.gtexportal.org/) revealed that there are ≥40 tissues in which *RWDD2B* eQTLs have been reported to correlate with rs6516886 genotype. These tissues include blood, but joint tissues were not examined. However, in each case, the OA risk–conferring T allele of rs6516886 correlated with decreased expression of *RWDD2B*, a pattern we observed in joint tissues. While no other significant eQTL effects were observed, effects at the other genes cannot be ruled out, as the number of heterozygotes available for analysis varied between genes.

We next identified meQTLs between CpGs and *RWDD2B,* with cg20220242 methylation correlating significantly with *RWDD2B* expression across all 3 joint tissues. The direction of the meQTL effects varied between cartilage (lower AEI levels correlated with increasing methylation) and fat pad and synovium (higher AEI levels correlated with increased methylation), implying that although the meQTLs are active across the tissues, they may not necessarily operate in the same manner.

Interestingly, the *RWDD2B* eQTL and meQTL effects observed in our study were not restricted to cartilage but were also active in the other joint tissues. The strongest genotypic effect on methylation in fat pad was observed with rs6516886, which displayed significantly increased expression of *RWDD2B* compared to the other tissues. Once again, this highlights the importance of considering OA as a whole-joint disease (16) and indicates a role for fat pad in OA pathology (26,27).

All of the data generated previously have been correlative, with the OA risk–conferring T allele of rs6516886 corresponding with increased methylation of cg20220242 and decreased expression of *RWDD2B*. To directly determine whether methylation levels of cg20220242 alter *RWDD2B* expression, we modulated the epigenome at and flanking the CpG using TET1-mediated

demethylation in a chondrocyte cell line. This resulted in altered expression of the gene and, consistent with what we would have predicted from our correlative data, the decrease in methylation caused an increase in expression. We can therefore conclude that there is a direct link between a change in methylation and a subsequent change in *RWDD2B* expression. As far as we are aware, this is the first time that such a link has been experimentally demonstrated at an OA susceptibility locus using targeted methylome editing tools. It is also noteworthy that we have modulated the epigenome to counteract the effect of an OA risk allele by stimulating increased expression of a gene whose expression is typically reduced in carriers of the risk allele.

In conclusion, we have highlighted the expression of *RWDD2B* as a target of the rs6516886 OA association signal with differential methylation of cg20220242 and other CpGs in its vicinity as intermediaries in the regulation of the gene. The OA risk–conferring allele at the locus correlates with reduced expression of the gene, implying that a reduction in the level of its encoded protein is detrimental to joint health. *RWDD2B* codes for the RWD domain–containing protein 2B, about which very little is currently known. Proteins containing RWD domains have the capacity to bind to other cellular proteins, including ubiquitin ligases (28). The current dearth of knowledge regarding the biologic function of RWD domain–containing protein 2B means that further analysis of it and of its gene as a target of OA genetic risk will provide novel insight into this complex disease.

ACKNOWLEDGMENTS

The authors would like to acknowledge Dr Louise Reynard, Ms Melissa Kaye, and Ms Maria Tselepi for their technical advice and support.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Parker had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Parker, Loughlin.
Acquisition of data. Parker, Hofer, Rice, Earl, Anjum, Deehan.
Analysis and interpretation of data. Parker, Loughlin.

REFERENCES

1. Hunter DJ, Bierma-Zeinstra S. Osteoarthritis. Lancet 2019;393: 1745–59.
2. Glyn-Jones S, Palmer AJ, Agricola R, Price AJ, Vincent TL, Weinans H, et al. Osteoarthritis. Lancet 2015;386:376–87.
3. Kendzerska T, Jüni P, King LK, Croxford R, Stanaitis I, Hawker GA. The longitudinal relationship between hand, hip and knee osteoarthritis and cardiovascular events: a population-based cohort study. Osteoarthritis Cartilage 2017;25:1771–80.
4. Hawker GA, Croxford R, Bierman AS, Harvey PJ, Ravi B, Stanaitis I, et al. All-cause mortality and serious cardiovascular events in people with hip and knee osteoarthritis: a population-based cohort study. PLoS One 2014;9:e91286.
5. Zeggini E, Panoutsopoulou K, Southam L, Rayner NW, Day-Williams AG, Lopes MC, et al, for the arcOGEN Consortium and arcOGEN Collaborators. Identification of new susceptibility loci for osteoarthritis (arcOGEN); a genome-wide association study. Lancet 2012; 380:815–23.
6. Reynard LN. Analysis of genetics and DNA methylation in osteoarthritis: What have we learnt about the disease? Semin Cell Dev Biol 2017;62:57–66.
7. Styrkarsdottir U, Helgason H, Sigurdsson A, Norddahl GL, Agustsdottir AB, Reynard LN, et al. Whole-genome sequencing identifies rare genotypes in COMP and CHADL associated with high risk of hip osteoarthritis. Nat Genet 2017;49:801–5.
8. Zengini E, Hatzikotoulas K, Tachmazidou I, Steinberg J, Hartwig FP, Southam L, et al. Genome-wide analyses using UK Biobank data provide insights into the genetic architecture of osteoarthritis. Nat Genet 2018;50:549–58.
9. Styrkarsdottir U, Lund SH, Thorleifsson G, Zink F, Stefansson OA, Sigurdsson JK, et al. Meta-analysis of Icelandic and UK data sets identifies missense variants in SMO, IL11, COL11A1 and 13 more new loci associated with osteoarthritis. Nat Genet 2018;50: 1681–7.
10. Tachmazidou I, Hatzikotoulas K, Southam L, Esparza-Gordillo J, Haberland V, Zheng J, et al. Identification of new therapeutic targets for osteoarthritis through genome-wide analyses of UK Biobank data. Nat Genet 2019;51:230–6.
11. Styrkarsdottir U, Stefansson OA, Gunnarsdottir K, Thorleifsson G, Lund SH, Stefansdottir L, et al. GWAS of bone size yields twelve loci that also affect height, BMD, osteoarthritis or fractures. Nat Commun 2019;10:2054.
12. Shepherd C, Reese AE, Reynard LN, Loughlin J. Expression analysis of the osteoarthritis genetic susceptibility mapping to the matrix Gla protein gene MGP. Arthritis Res Ther 2019;21:149.
13. Rice SJ, Cheung K, Reynard LN, Loughlin J. Discovery and analysis of methylation quantitative trait loci (mQTLs) mapping to novel osteoarthritis genetic risk signals. Osteoarthritis Cartilage 2019;27:1545–56.
14. Reynard LN, Bui C, Syddall CM, Loughlin J. CpG methylation regulates allelic expression of GDF5 by modulating binding of SP1 and SP3 repressor proteins to the osteoarthritis SNP rs143383. Hum Genet 2014;133:1059–73.
15. Rice SJ, Tselepi M, Sorial AK, Aubourg G, Shepherd C, Almarza D, et al. Prioritization of PLEC and GRINA as osteoarthritis risk genes through the identification and characterization of novel methylation quantitative trait loci. Arthritis Rheumatol 2019;71:1285–96.
16. Loeser RF, Goldring SR, Scanzello CR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. Arthritis Rheum 2012;64:1697–707.
17. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, et al. The human genome browser at UCSC. Genome Res 2002;12:996–1006.
18. Gee F, Clubbs CF, Raine EV, Reynard LN, Loughlin J. Allelic expression analysis of the osteoarthritis susceptibility locus that maps to chromosome 3p21 reveals cis-acting eQTLs at GNL3 and SPCS1. BMC Med Genet 2014;15:53–9.
19. Liu XS, Wu H, Ji X, Steizer Y, Wu X, Czauderna S, et al. Editing DNA methylation in the mammalian genome. Cell 2016;167:233–47.
20. Wilson MH, Coates CJ, George AL Jr. PiggyBac transposon-mediated gene transfer in human cells. Mol Ther 2007;15: 139–45.
21. Teare MD, Pinyakorn S, Heighway J, Koref MF. Comparing methods for mapping cis acting polymorphisms using allelic expression ratios. PLoS One 2011;6:e28636.

22. Rice SJ, Aubourg G, Sorial AK, Almarza D, Tselepi M, Deehan DJ, et al. Identification of a novel, methylation-dependent, RUNX2 regulatory region associated with osteoarthritis risk. Hum Mol Genet 2018;27:3464–74.
23. Gallagher MD, Chen-Plotkin AS. The post-GWAS era: from association to function. Am J Hum Genet 2018;102:717–30.
24. Zhu H, Wang G, Qian J. Transcription factors as readers and effectors of DNA methylation [review]. Nat Rev Genet 2016;17:551–65.
25. Lonsdale J, Thomas J, Salvatore M, Phillips R, Lo E, Shad S, et al, for the GTex Consortium. The genotype-tissue expression (GTex) project. Nat Genet 2013;45:580–5.
26. Clockaerts S, Bastiaansen-Jenniskens YM, Runhaar J, van Osch GJ, van Offel JF, Verhaar JA, et al. The infrapatellar fat pad should be considered as an active osteoarthritic joint tissue: a narrative review. Osteoarthritis Cartilage 2010;15:876–82.
27. Ioan-Facsinay A, Kloppenburg M. An emerging player in knee osteoarthritis: the infrapatellar fat pad [review]. Arthritis Res Ther 2013;15:225.
28. Alontaga AY, Ambaye ND, Li YJ, Vega R, Chen CH, Bzymek KP, et al. RWD domain as an E2 (Ubc9)-interaction module. J Biol Chem 2015;290:16550–9.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 110–120
DOI 10.1002/art.41477

American College of Rheumatology
Empowering Rheumatology Professionals

Improvement of Signs and Symptoms of Nonradiographic Axial Spondyloarthritis in Patients Treated With Secukinumab: Primary Results of a Randomized, Placebo-Controlled Phase III Study

Atul Deodhar,[1] Ricardo Blanco,[2] Eva Dokoupilová,[3] Stephen Hall,[4] Hideto Kameda,[5] Alan J. Kivitz,[6] Denis Poddubnyy,[7] Marleen van de Sande,[8] Anna S. Wiksten,[9] Brian O. Porter,[10] Hanno B. Richards,[9] Sibylle Haemmerle,[9] and Jürgen Braun[11]

Objective. To report the primary (1-year) results from PREVENT, the first phase III study evaluating secukinumab in patients with active nonradiographic axial spondyloarthritis (SpA).

Methods. A total of 555 patients were randomized (1:1:1) to receive subcutaneous secukinumab 150 mg with a loading dose (loading dose [LD] group), secukinumab 150 mg without a loading dose (non–loading dose [NL] group), or placebo weekly and then every 4 weeks starting at week 4. The NL group received placebo at weeks 1, 2, and 3 to maintain blinding. Switch to open-label secukinumab or standard of care was permitted after week 20. The study had 2 independent analysis plans, per European Union and non-US (plan A; week 16) and US (plan B; week 52) regulatory requirements. The primary end point was 40% improvement in disease activity according to the Assessment of SpondyloArthritis international Society (ASAS40) criteria at week 16 (in the LD group) and at week 52 (in the NL group) in tumor necrosis factor inhibitor (TNFi)–naive patients. Safety analyses included all patients who received ≥1 dose of study treatment.

Results. Overall, 481 patients completed 52 weeks of treatment, including 84.3% (156 of 185) in the LD group, 89.7% (165 of 184) in the NL group, and 86.0% (160 of 186) in the placebo group. The proportion of patients who switched to open-label or standard of care between weeks 20 and 48 was 50.8% in the LD group, 47.3% in the NL group, and 64.0% in the placebo group. Both primary and all secondary end points were met at week 16. The proportion of TNFi-naive patients who met ASAS40 was significantly higher for LD at week 16 (41.5%) and NL at week 52 (39.8%) versus placebo (29.2% at week 16 and 19.9% at week 52; both $P < 0.05$). No new safety findings were reported.

Conclusion. Our findings indicate that secukinumab 150 mg provides significant and sustained improvement in signs and symptoms of nonradiographic axial SpA through 52 weeks. Safety was consistent with previous reports.

INTRODUCTION

Axial spondyloarthritis (SpA) is a chronic inflammatory disease of the spine, which includes nonradiographic axial SpA and ankylosing spondylitis (AS) (1–6). The prevalence of axial SpA is reported to be 0.32–1.4% (1,2,4,5,7). Patients with AS have structural damage in the sacroiliac (SI) joints and/or the spine that is visible on radiographs (1,3,4,8). Patients with nonradiographic axial SpA do not exhibit definitive radiographic sacroiliitis but have a disease burden comparable to that of patients with AS, including inflammatory back pain (IBP; predominantly in the pelvis and lower back), morning stiffness, nocturnal awakening, fatigue, and

A video abstract of this article can be found at https://players.brightcove.net/656326989001/default_default/index.html?videoId=6178634034001".

ClinicalTrials.gov identifier: NCT02696031.
Supported by Novartis Pharma, Basel, Switzerland.

[1]Atul Deodhar, MD: Oregon Health & Science University, Portland; [2]Ricardo Blanco, MD, PhD: Hospital University Marqués de Valdecilla, IDIVAL, Santander, Spain; [3]Eva Dokoupilová, MD: Medical Plus, s.r.o., Uherské Hradiště, and University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic; [4]Stephen Hall, MD: Monash University, Melbourne, Victoria, Australia; [5]Hideto Kameda, MD, PhD: Toho University, Tokyo, Japan; [6]Alan J. Kivitz, MD, CPI: Altoona Center for Clinical Research, Duncansville, Pennsylvania; [7]Denis Poddubnyy, MD, PhD: Charité Universitätsmedizin, Berlin, Germany; [8]Marleen van de Sande, MD, PhD: Amsterdam UMC, University of Amsterdam, Amsterdam Rheumatology and Immunology Center, Amsterdam, The Netherlands; [9]Anna S. Wiksten, MSc, Hanno B. Richards, MD, PhD, Sibylle Haemmerle, PhD: Novartis Pharma, Basel, Switzerland; [10]Brian O. Porter, MPH, MBA, MD, PhD: Novartis Pharmaceuticals, East Hanover, New Jersey; [11]Jürgen Braun, MD, PhD: Rheumazentrum Ruhrgebiet, Herne, and Ruhr-University Bochum, Bochum, Germany.

Dr. Deodhar has received consulting fees, speaking fees, and/or honoraria from AbbVie, Boehringer Ingelheim, GlaxoSmithKline, and Janssen (less than $10,000 each), and from Eli Lilly, Novartis, Pfizer, and UCB (more than $10,000 each) and research grants from those companies. Dr. Blanco has received consulting fees, speaking fees, and/or honoraria

reduced spinal mobility (1,3,4,6,8). The prevalence of nonradiographic axial SpA is reported to be ~0.1–0.4% in the general population, more prevalent in women, and ~16–37% in patients with IBP (1,4,7,9,10). The epidemiology of nonradiographic axial SpA is evolving due to heterogeneity in definition, slow progression, and diagnostic delays (1,6,7,9,10).

The average delay in diagnosis of nonradiographic axial SpA is estimated to be 6–8 years (4,6,7,11,12). The reported rate of progression from nonradiographic axial SpA to AS varies from ~10% to 40% of patients over 2–10 years, with a lifetime risk of progression of ~50% (6,8–10,13). Early diagnosis of nonradiographic axial SpA is important for the management of disease symptoms and to potentially limit spinal damage. The Assessment of SpondyloArthritis international Society (ASAS) criteria have been developed for the classification of axial SpA and include patients with early disease, with or without radiographic evidence of sacroiliitis (1–4).

According to the ASAS/European League Against Rheumatism (EULAR) (14) and the American College of Rheumatology (ACR)/Spondylitis Association of America (SAA)/Spondyloarthritis Research and Treatment Network (SPARTAN) (15) treatment guidelines, nonsteroidal antiinflammatory drugs (NSAIDs) are recommended as first-line pharmacologic therapy in patients with nonradiographic axial SpA. Biologic disease-modifying antirheumatic drugs are recommended in patients with active disease and objective signs of inflammation (elevated C-reactive protein [CRP] level and/or evidence of sacroiliitis on magnetic resonance imaging [MRI]) despite treatment with NSAIDs. Interleukin-17 (IL-17) is expressed by multiple cells in both the innate and adaptive immune systems and plays a crucial role in the pathogenesis of axial SpA, driving inflammation, enthesitis, and structural damage (16,17). According to the 2019 update of the ACR/SAA/SPARTAN treatment guidelines, IL-17 inhibitors are recommended over the use of a second tumor necrosis factor inhibitor (TNFi) agent in patients with AS with primary nonresponse to the first TNFi agent (15). Secukinumab, a human monoclonal antibody that directly inhibits IL-17A, has provided significant and sustained improvement in the signs and symptoms of AS, as evidenced in the phase III MEASURE studies (18–20).

PREVENT is the first phase III study evaluating the efficacy, safety, and tolerability of secukinumab 150 mg, with or without loading doses, in patients with active nonradiographic axial SpA. Here, we report the efficacy up to week 52 and the safety results for the entire treatment period (including at least 52 weeks of exposure for all patients and up to 104 weeks of exposure for some patients) from the PREVENT study.

PATIENTS AND METHODS

Patients. Patients with a clinical diagnosis of nonradiographic axial SpA who were age ≥18 years were included if they met the ASAS classification criteria for axial SpA (IBP ≥6 months, disease onset at <45 years of age, and sacroiliitis on MRI with ≥1 SpA feature or HLA–B27 positive with ≥2 SpA features) plus objective signs of inflammation (MRI with SI joint inflammation [by central reading] and/or high-sensitivity CRP [hsCRP] greater than the upper limit of normal [ULN; as defined by the central laboratory]). Patients previously treated with a TNFi (no more than 1) could participate if they had an inadequate response or were intolerant. Patients could continue to receive the following medications at a stable dose: sulfasalazine (≤3 gm/day), methotrexate (≤25 mg/week), corticosteroids (≤10 mg/day prednisone or equivalent), and NSAIDs. At randomization, patients were stratified according to objective signs of inflammation based on their CRP and MRI status (positive or negative) at screening. A positive CRP was defined as a value greater than the ULN (hsCRP >5 mg/liter) by the central laboratory. MRI positivity was defined as the presence of inflammatory lesions in the SI joints on MRI according to the ASAS/Outcome Measures in Rheumatology definition (21) as assessed by a central reader.

from AbbVie, Pfizer, Roche, Bristol Myers Squibb, Janssen, Sanofi, Eli Lilly, and MSD (less than $10,000 each) and research support from AbbVie, MSD, and Roche. Dr. Dokoupilová has received research support from AbbVie, Eli Lilly, GlaxoSmithKline, Novartis, Pfizer, UCB, Sanofi-Aventis, and Hexal. Dr. Hall has received consulting fees, speaking fees, and/or honoraria from Novartis, Merck, Janssen, Pfizer, Eli Lilly, and UCB (less than $10,000 each) and research support from AbbVie, UCB, Janssen, and Merck. Dr. Kameda has received consulting fees, speaking fees, and/or honoraria from AbbVie, Astellas, Bristol Myers Squibb, Chuagi, Eisai, Gilead, Janssen, Novartis, Pfizer, and UCB (less than $10,000 each) and from Asahi-Kasei, Eli Lilly, and Mitsubishi-Tanabe (more than $10,000 each), and research support from AbbVie, Asahi-Kasei, Astellas, Chugai, Eisai, Mitsubishi-Tanabe, and Novartis. Dr. Kivitz has received consulting fees and/or honoraria from AbbVie, Janssen, Boehringer Ingeleheim, Pfizer, Sanofi, Genzyme, Flexion, and Regeneron (less than $10,000 each) and owns stock or stock options in Pfizer, Sanofi, Regeneron, Amgen, GlaxoSmithKline, and Gilead. Dr. Poddubnyy has received consulting fees, speaking fees, and/or honoraria from AbbVie, Bristol Myers Squibb, Celgene, Janssen, Eli Lilly, MSD, Novartis, Pfizer, Roche, and UCB (less than $10,000 each) and research support from AbbVie, MSD, Novartis, and Pfizer. Dr. van de Sande has received consulting fees, speaking fees, and/or honoraria from AbbVie, Novartis, Eli Lily, and MSD (less than $10,000 each) and research support from Novartis, Eli Lilly, Boehringer Ingelheim, Janssen, and UCB. Ms Wiksten and Drs. Porter, Richards, and Haemmerle own stock or stock options in Novartis. Dr. Braun has received consulting fees, speaking fees, and/or honoraria from AbbVie, Amgen, Celgene, Chugai, MSD, Novartis, Eli Lilly, and UCB (less than $10,000 each) and research support from AbbVie, Amgen, Bristol Myers Squibb, Boehringer, Celgene, Celltrion, Centocor, Chugai, Medac, MSD, Mundipharma, Novartis, Pfizer, Roche, Sanofi-Aventis, and UCB.

The data sets generated during and/or analyzed at the end of the present study are not publicly available. Novartis is committed to sharing with qualified external researchers' access to patient-level data and supporting clinical documents from eligible studies. These requests are reviewed and approved on the basis of scientific merit. All data provided is anonymized to respect the privacy of patients who have participated in the trial in line with applicable laws and regulations. The data may be requested from the corresponding author of the manuscript.

Address correspondence to Atul Deodhar, MD, Oregon Health & Science University, Division of Arthritis & Rheumatic Diseases (OP-09), 3181 SW Sam Jackson Park Road, Portland, OR 97239-3098. Email: deodhara@ohsu.edu.

Submitted for publication March 28, 2020; accepted in revised form July 31, 2020.

Key exclusion criteria included evidence of sacroiliitis meeting the modified New York criteria for AS (22) (assessed centrally), active ongoing inflammatory conditions other than axial SpA, including active inflammatory bowel disease (IBD) or uveitis, evidence of ongoing infection or malignant process on chest radiograph, active systemic infection within 2 weeks before randomization, history of ongoing, chronic, or recurrent infectious disease or evidence of tuberculosis infection, known infection with HIV, hepatitis B, or hepatitis C at screening or randomization, history of lymphoproliferative disease or any known malignancy or malignancy of any organ system within the past 5 years, and previous treatment with biologic agents other than TNFi. Detailed eligibility criteria are listed in Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41477/abstract.

The study protocol was reviewed and approved by the Independent Ethics Committee or Institutional Review Board for each center. The study was conducted according to ICH E6 Guideline for Good Clinical Practice that has its origin in the Declaration of Helsinki (23). Written informed consent was obtained from all enrolled patients.

Study design. PREVENT (ClinicalTrials.gov identifier: NCT02696031) is an ongoing randomized, double-blind, placebo-controlled 2-year phase III study with an extension of up to 2 years in patients with nonradiographic axial SpA. The study had 2 independent analysis plans per European Union and non-US regulatory requirements (plan A [week 16]) and US regulatory requirements (plan B [week 52]). The study was initiated on April 29, 2016 (first patient's first visit) and is being conducted across 130 sites in 24 countries.

Randomization and blinding. Eligible patients were randomized (1:1:1) via Interactive Response Technology to receive subcutaneous secukinumab 150 mg with a loading dose (150 mg loading dose [LD] group), 150 mg without a loading dose (150 mg non–loading dose [NL] group), or placebo at baseline and weeks 1, 2, and 3, followed by every 4 weeks starting at week 4 (Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41477/abstract). The 150 mg NL group received placebo at weeks 1, 2, and 3 to maintain blinding. Study treatment was self-administered throughout the study using syringes prefilled with 150 mg/1 ml secukinumab or 1 ml placebo.

Switch to open-label subcutaneous secukinumab 150 mg or standard of care was permitted after week 20 for inadequate responders based on clinical judgment of disease activity by the investigator and the patient. No specific efficacy parameter for inadequate response was mandated. In cases in which the chosen standard of care was a TNFi, a 12-week washout period was required. All investigators, site personnel, and patients remained blinded with regard to the originally randomized treatment assignment until the week 52 database lock. Starting at week 52, all patients (except those who switched to standard of care) received open-label secukinumab 150 mg up to week 100, unless they had discontinued study treatment. Starting at week 104, all patients who complete the core phase of the trial can continue in an additional 2-year extension phase. A follow-up visit is conducted 12 weeks after the last administration of study treatment for all patients.

Data were collected in accordance with Good Clinical Practice guidelines by the study investigators and analyzed by the sponsor. Efficacy data up to week 52 and safety data for the entire treatment period up to the data cutoff date of July 1, 2019 are presented here.

Outcome measures. Based on differences in regional regulatory requirements, there were 2 predefined hierarchical analysis plans for the primary and secondary objectives. An interim analysis was conducted for the week 16 end points when all patients had completed week 24 (analysis plan A). A separate firewalled team (to maintain blinding) conducted the study up to the second interim analysis, which was conducted when all patients had completed week 52 (analysis plan B). Full details on the outcome measures are provided in Supplementary Table 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41477/abstract.

Primary objective. The primary objective was to demonstrate that secukinumab 150 mg LD at week 16 (analysis plan A) and 150 mg NL at week 52 (analysis plan B) were superior to placebo in TNFi-naive patients with active nonradiographic axial SpA, based on the proportion of patients achieving an ASAS40 response (24).

Secondary objectives. Secondary objectives comprised week 16 end points (analysis plan A) and a combination of week 16 and week 52 end points (analysis plan B) (Supplementary Table 2). These were assessed in the overall population and included ASAS40 response (40% improvement in disease activity according to the ASAS criteria), ASAS5/6 response (20% improvement in 5 of 6 domains) (24), change from baseline in total Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (25), BASDAI50 response (50% decrease in BASDAI score from baseline), change from baseline in hsCRP level, change from baseline in Bath Ankylosing Spondylitis Functional Index (BASFI), SI joint edema score (Berlin Active Inflammatory Lesions Scoring, range 0–24) on MRI (oblique coronal views of the pelvis including both SI joints were obtained for each patient; scores of 2 central readers were averaged), ASAS20 response, change from baseline in Short Form 36 (SF-36) physical component summary (PCS) (26), change from baseline in Ankylosing Spondylitis Quality of Life (ASQoL) (27), ASAS partial remission response (24), and inactive disease according to the Ankylosing Spondylitis Disease Activity Score using the CRP level (ASDAS-CRP) (28).

The overall safety and tolerability of secukinumab versus placebo for the entire treatment period was assessed by adverse events (AEs), serious AEs (SAEs), adjudicated major adverse cardiovascular events (MACE), laboratory assessments, and vital signs. Safety data are presented separately for individual treatment groups (secukinumab 150 mg LD or NL and placebo) and for the "any secukinumab" group, which included all patients originally randomized to receive secukinumab and all placebo patients who had started open-label secukinumab treatment.

Statistical analysis. The sample sizes for analysis plans A and B were calculated so as to have 91% and 97% power, respectively, for the primary end point, with a 5% Type I error rate for comparison between secukinumab 150 mg and placebo. The assumed ASAS40 response rates (primary end point) for the corresponding plans were 47.1% and 43.0%, respectively, for secukinumab 150 mg compared with 27.9% and 21.7%, respectively, for placebo. Based on this estimation, at least 185 patients were needed to have 90% power to show superiority versus placebo. Efficacy analyses were performed on the full analysis set, which comprised all patients who were randomized and had study treatment assigned.

Primary and secondary end points were analyzed according to a predefined statistical hierarchy (Supplementary Figures 2 and 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41477/abstract). End points are shown in the order of the testing strategy. The family-wise Type I error rate was set to 5% and was controlled with the applied sequential testing strategy. All end points are shown with unadjusted *P* values with statistical significance only claimed for end points within the predefined hierarchy which met significance based on adjusted *P* values corrected for multiplicity of testing. For all exploratory end points unadjusted *P* values are shown. The primary analysis in the TNFi-naive population was conducted via logistic regression with treatment group and stratification (CRP level or MRI) as factors and weight as a covariate.

Missing values were imputed as nonresponders (by nonresponder imputation [NRI]) for binary variables and via a mixed-effects model repeated measures (MMRM; valid under the missing at random assumption) for continuous variables up to week 20. MMRM analysis included treatment group, CRP level or MRI stratification group, TNFi therapy status, and analysis visit as factors and baseline score of the respective end point and weight as continuous covariates. Treatment-by–analysis visit and baseline score–by–analysis visit were included as interaction terms in the model. An unstructured covariance structure was assumed for the model. The significance of treatment effect for the secukinumab regimens was determined from the pairwise comparisons

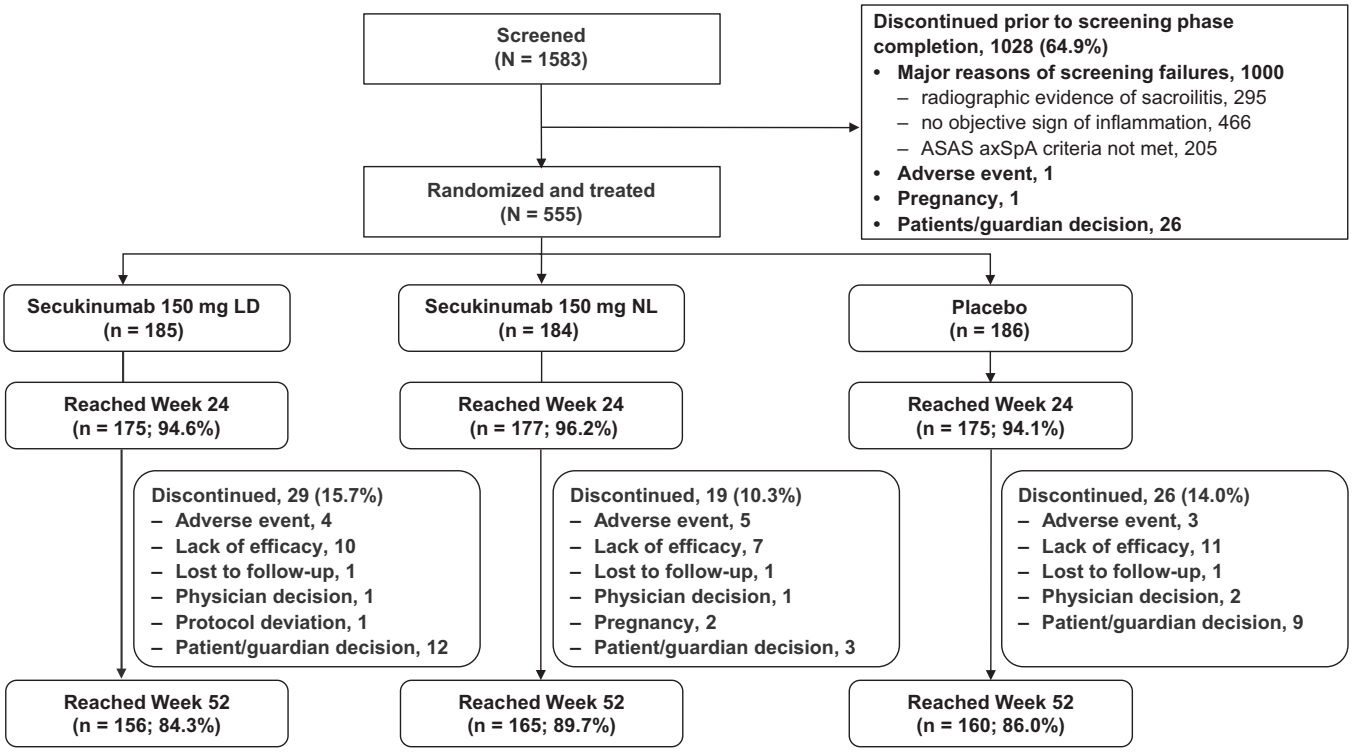


Figure 1. Patient disposition through week 52. Of 1,583 patients screened, 555 (35.1%) were randomized. A patient can have more than 1 reason for screening failure. The main reasons for screening failure based on inclusion and exclusion criteria are presented in Supplementary Table 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41477/abstract. The majority (84–89%) of patients completed week 52. Discontinuations are presented for the whole treatment period from baseline to week 52. ASAS = Assessment of SpondyloArthritis international Society; axSpA = axial spondyloarthritis; LD = with loading; NL = without loading.

performed between secukinumab regimens and placebo at week 16. For the change in hsCRP level, the log(e) ratio of the post-baseline value to the baseline value was used to normalize the distribution of the hsCRP level at each assessment time point.

Safety analyses included all patients who received ≥1 dose of study medication. AEs are reported as exposure-adjusted incidence rates (EAIR) per 100 patient-years over the entire treatment period, which refers to the cumulative treatment period (i.e., events started after the first dose of study treatment or events present prior to the first dose of study treatment but increased in severity based on preferred term and on or before last dose plus 84 days). Patients switching to standard of care were counted in their previous treatment until the end of the washout phase.

RESULTS

A total of 1,583 patients were screened for eligibility, and 1,028 patients (64.9%) discontinued prior to the completion of the screening phase, either due to not meeting the eligibility criteria or for other reasons such as patient decision. The main reasons for screen failures based on inclusion and exclusion criteria are presented in Supplementary Table 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41477/abstract. A total of 555 patients were randomized, of which 94.6% of the patients in the secukinumab 150 mg LD group (175 of 185), 96.2% of the patients in the secukinumab 150 mg NL group (177 of 184), and 94.1% of the patients in the placebo group (175 of 186) completed 24 weeks of treatment. Completion rates at week 52 in the corresponding groups were 84.3% (156 of 185), 89.7% (165 of 184), and 86.0% (160 of 186). The detailed patient disposition through week 52 is presented in Figure 1. Demographic and baseline disease characteristics were comparable across treatment groups (Table 1). The majority of randomized patients (90.3%) were TNFi-naive. The proportion of patients who switched to either open-label secukinumab or standard of care between weeks 20 and 52, based on clinical judgment of disease activity by the investigator and the patient, was 50.8% in the 150 mg LD group (94 of 185, with 94 switching to open-label secukinumab and 2 subsequently switched to

Table 1. Demographic and baseline disease characteristics of the patients with nonradiographic axial SpA*

Variable	Secukinumab 150 mg with loading (n = 185)	Secukinumab 150 mg without loading (n = 184)	Placebo (n = 186)
Age, mean ± SD years	39.10 ± 11.45	39.80 ± 11.68	39.30 ± 11.47
Sex, no. (%) men	80 (43.2)	84 (45.7)	91 (48.9)
Race, no. (%) white	176 (95.1)	165 (89.7)	167 (89.8)
BMI, mean ± SD kg/m^2	27.13 ± 5.50	27.17 ± 5.75	26.87 ± 5.61
Time since diagnosis, mean ± SD years	2.75 ± 4.63	2.12 ± 3.05	2.96 ± 5.01
Symptom duration, mean ± SD years	8.72 ± 9.27	8.57 ± 8.64	8.39 ± 8.34
HLA–B27 positive, no. (%)	136 (73.5)	117 (63.6)	129 (69.4)
Elevated hsCRP (>5 mg/liter), no. (%)	104 (56.2)	107 (58.2)	105 (56.5)
hsCRP, mean ± SD mg/liter	13.17 ± 27.21	9.67 ± 15.82	10.76 ± 21.34
Historic or current SI joint inflammation on MRI, no. (%)	132 (71.4)	134 (72.8)	139 (74.7)
SI joint inflammation on MRI score, mean ± SD	2.80 ± 3.83	2.24 ± 3.29	2.70 ± 3.96
TNFi-naive, no. (%)	164 (88.6)	166 (90.2)	171 (91.9)
Smoker at baseline, no. (%)	45 (24.3)	40 (21.7)	47 (25.3)
History of uveitis, no. (%)	21 (11.4)	26 (14.1)	18 (9.7)
History of IBD, no. (%)	2 (1.1)	3 (1.6)	5 (2.7)
Total back pain (0–100-mm VAS), mean ± SD	73.30 ± 13.02	72.0 ± 14.48	70.90 ± 12.52
Nocturnal back pain (0–100-mm VAS), mean ± SD	70.90 ± 17.42	70.80 ± 16.43	70.10 ± 14.72
BASDAI score, mean ± SD	7.08 ± 1.33	6.93 ± 1.45	6.76 ± 1.24
BASFI score, mean ± SD	6.24 ± 2.04	5.92 ± 2.04	5.89 ± 1.90
ASDAS-CRP score, mean ± SD	3.70 ± 0.87	3.59 ± 0.78	3.49 ± 0.81
Concomitant NSAIDs, no. (%)	154 (83.2)	153 (83.2)	156 (83.9)
Concomitant MTX			
No. (%)	17 (9.2)	15 (8.2)	23 (12.4)
Median mg/week	15	15	20
Concomitant sulfasalazine			
No. (%)	29 (15.7)	24 (13.0)	29 (15.6)
Median gm/day	2	2	2
Concomitant steroids			
No. (%)	14 (7.6)	17 (9.2)	17 (9.1)
Median mg/day	5	10	6.7

* SpA = spondyloarthritis; BMI = body mass index; hsCRP = high-sensitivity C-reactive protein; SI = sacroiliac; MRI = magnetic resonance imaging; TNFi = tumor necrosis factor inhibitor; IBD = inflammatory bowel disease; VAS = visual analog scale; BASDAI = Bath Ankylosing Spondylitis Disease Activity Index; BASFI = Bath Ankylosing Spondylitis Functional Index; ASDAS-CRP = Ankylosing Spondylitis Disease Activity Score using the CRP level; NSAIDs = nonsteroidal antiinflammatory drugs; MTX = methotrexate.

standard of care treatment with TNFi), 47.3% in the 150 mg NL group (87 of 184; 86 switched to open-label secukinumab and 1 to standard of care), and 64.0% (119 of 186 to open-label secukinumab) in the placebo group.

Efficacy. Results of hypothesis tests according to the predefined testing strategy in analysis plans A and B are presented in Supplementary Tables 4 and 5, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41477/abstract.

Primary objectives. The primary end points as per analysis plan A and analysis plan B were met (Figure 2A); ASAS40 response in TNFi-naive patients was significantly higher in the secukinumab 150 mg LD group (41.5%) compared with the placebo group (29.2%) at week 16 ($P = 0.0197$) and significantly higher in the secukinumab 150 mg NL group (39.8%) compared with the placebo group (19.9%) at week 52 ($P = 0.0021$).

Secondary objectives. The secukinumab 150 mg LD and NL regimens showed significant improvement versus placebo across all predefined secondary end points for analysis plan A at week 16 (Table 2). The total BASDAI score (Figure 2B) was significantly improved from baseline in patients treated with 150 mg LD (–2.35) or NL (–2.43) versus placebo (–1.46; $P = 0.0006$ and $P = 0.0002$, respectively), with improvement versus placebo seen as early as week 1 (–0.87 in the LD group and –0.82 in the NL group versus –0.48 in the placebo group). The proportion of BASDAI50 responders (Figure 2C) was significantly higher in patients treated with 150 mg LD (37.3%) or 150 mg NL (37.5%) versus placebo

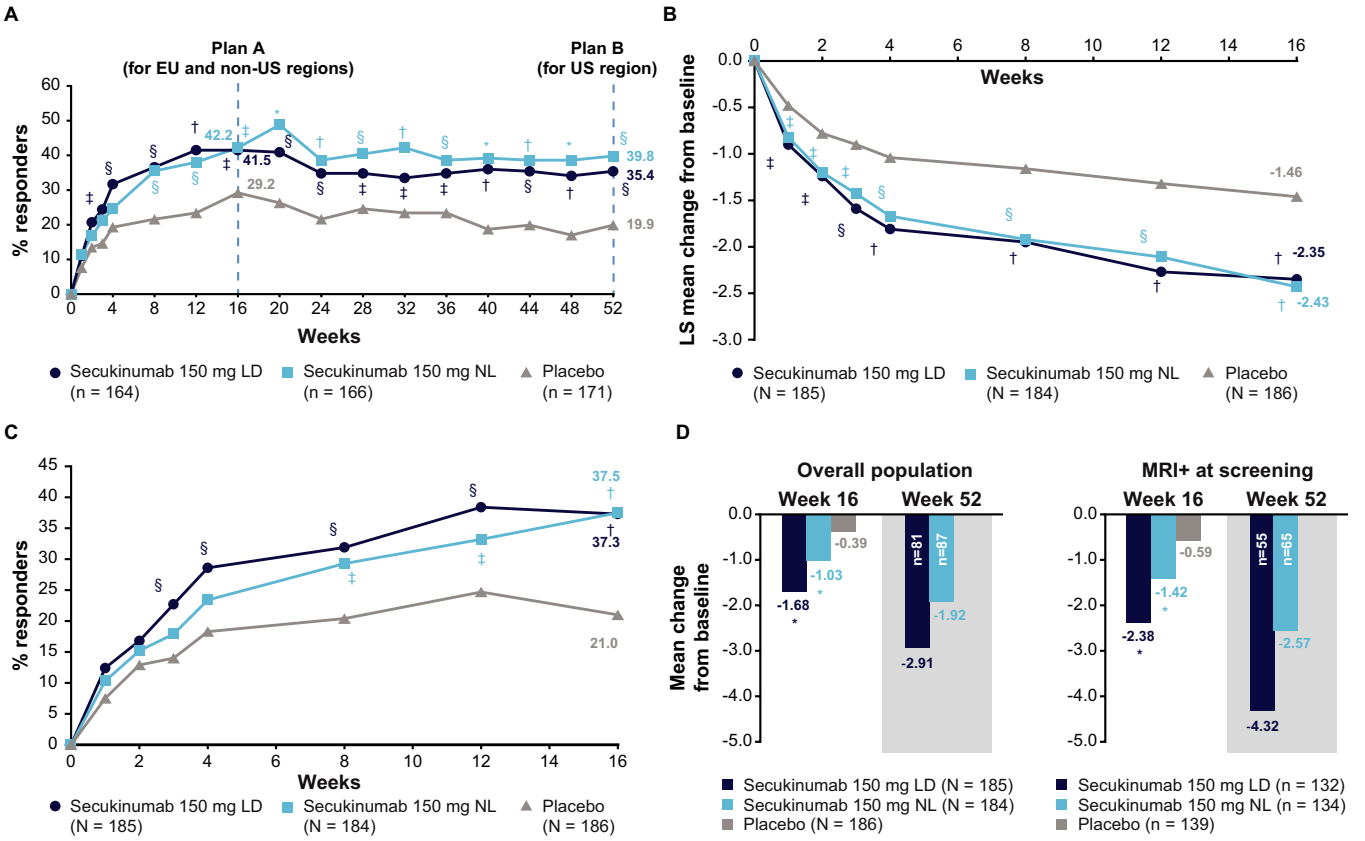


Figure 2. Primary and key secondary outcomes through week 52 based on statistical hierarchy. **A**, Assessment of SpondyloArthritis international Society criteria for 40% improvement (ASAS40) response at week 16 (analysis plan A for European Union [EU] and non-US region regulatory requirements) and week 52 (analysis plan B for US regulatory requirements) in tumor necrosis factor inhibitor–naive patients randomized to receive secukinumab 150 mg with loading (LD), secukinumab 150 mg without loading (NL), or placebo (primary objective). **B**, Total Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score in each treatment group through week 16. **C**, BASDAI criteria for 50% improvement response in each treatment group through week 16. **D**, Sacroiliac (SI) joint edema score on magnetic resonance imaging (MRI) in the overall population and in the subgroup of patients who were MRI-positive at screening (defined as the presence of inflammatory lesions in the SI joints on MRI according to the ASAS/Outcome Measures in Rheumatology definition). The mean baseline SI joint edema score was 3.56 in the group with loading and 2.64 in the group without loading in the overall population and 5.23 in the group with loading and 3.48 in the group without loading in the subgroup of patients who were MRI-positive at screening. For SI joint edema score at week 16, *P* values were determined by an analysis of covariance model based on multiple imputation (missing at random assumption), and data are presented as the least squares (LS) mean change. Observed data (shaded) for SI joint edema score at week 52 are shown for secukinumab-treated patients who did not switch treatment. * = $P < 0.0001$; † = $P < 0.001$; § = $P < 0.01$; ‡ = $P < 0.05$, versus placebo.

Table 2. Secondary end points according to the statistical hierarchy of analysis plans A and B*

Variable	Secukinumab 150 mg with loading (n = 185)	Secukinumab 150 mg without loading (n = 184)	Placebo (n = 186)	*P*, with loading versus placebo	*P*, without loading versus placebo
ASAS40 (overall population), % responders					
Week 16	40.0	40.8	28.0	0.0108	0.0087
Week 52	33.5	38.0	19.4	0.0015	0.0016
ASAS5/6 at week 16, % responders	40.0	35.9	23.7	0.0005	0.0094
BASDAI at week 16, LSM ± SEM change from baseline	−2.35 ± 0.20	−2.43 ± 0.20	−1.46 ± 0.21	0.0006	0.0002
BASDAI50, % responders					
Week 16	37.3	37.5	21.0	0.0001	0.0002
Week 52	30.8	35.3	19.9	0.0056	0.0061
High-sensitivity CRP at week 16, LSM ± SEM change from baseline†	0.64 ± 1.08	0.64 ± 1.08	0.91 ± 1.08	0.0002	0.0002
BASFI at week 16, LSM ± SEM change from baseline	−1.75 ± 0.20	−1.64 ± 0.20	−1.01 ± 0.21	0.0041	0.0143
SI joint edema score on MRI at week 16, LSM ± SEM change from baseline‡§	−1.68 ± 0.24	−1.03 ± 0.18	−0.39 ± 0.15	<0.0001¶	<0.0001¶
ASAS20 at week 16, % responders	56.8	58.2	45.7	0.0260	0.0149
SF-36 PCS at week 16, LSM ± SEM change from baseline	5.71 ± 0.68	5.57 ± 0.69	2.93 ± 0.71	0.0006	0.0011
ASQoL at week 16, LSM ± SEM change from baseline‡§	−3.45 ± 0.41	−3.62 ± 0.41	−1.84 ± 0.42	0.0008	0.0002
ASAS partial remission at week 16, % responders	21.6	21.2	7.0	<0.0001	0.0001
ASDAS-CRP inactive disease (<1.3) at week 52, % responders§	15.7	23.9	10.2	0.0577	0.0003

* The study included 2 independent analysis plans: plan A (week 16) per European Union and non-US regulatory requirements, and plan B (week 52) per US regulatory requirements. Nonresponder imputation analysis was used for binary variables and a mixed-effects model repeated measures was used for continuous variables. *P* values are unadjusted. Data are presented only for the secondary end points assessed according to the statistical hierarchy as shown in Supplementary Figures 2 and 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41477/abstract. ASAS40 = Assessment of SpondyloArthritis international Society criteria for 40% improvement; BASDAI = Bath Ankylosing Spondylitis Disease Activity Index; ASAS5/6 = 20% improvement in 5 of 6 domains of the ASAS criteria; BASDAI 50 = 50% decrease in BASDAI score from baseline; BASFI = Bath Ankylosing Spondylitis Functional Index; SF-36 = Short Form 36; PCS = physical component summary.

† Exponentially transformed least squares mean (LSM) for the geometric mean ratio of postbaseline value to baseline value. A value of <1 indicates a reduced C-reactive protein (CRP) value postbaseline.

‡ Continuous end points at week 52 were analyzed using nonparametric methods; detailed results are presented in Supplementary Table 9, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41477/abstract.

§ For the secukinumab 150 mg with loading dose group at week 52, sacroiliac (SI) joint edema score on magnetic resonance imaging (MRI), Ankylosing Spondylitis Quality of Life (ASQoL) score, and inactive disease according to the Ankylosing Spondylitis Disease Activity Score using the CRP level (ASDAS-CRP) were not significant according to the testing hierarchy.

¶ *P* values were determined by an analysis of covariance model based on multiple imputation (missing at random assumption).

(21.0%; $P = 0.0001$ and $P = 0.0002$, respectively). Secukinumab 150 mg LD and NL regimens significantly reduced the SI joint edema score on MRI (Figure 2D) in the overall study population versus placebo (−1.68 and −1.03, respectively, versus −0.39; both $P < 0.0001$).

For analysis plan B, significance versus placebo was achieved for the majority of secondary end points for both the LD and NL regimens at weeks 16 and 52 (Table 2). Significance versus placebo was not achieved for the 150 mg LD group at week 52 for inactive disease according to ASDAS-CRP (Table 2). Therefore, the subsequent end points in the hierarchical testing sequence, SI joint edema score on MRI and ASQoL for the 150 mg LD group, were not tested. Of the patients who switched to open-label secukinumab between weeks 20 and 52, 16.0% in the 150 mg LD group (15 of 94), 24.4% in the 150 mg NL group (21 of 86), and 10.9% in the placebo group (13 of 119) had actually achieved ASAS40 at the time of treatment switch. Notably, these patients were imputed as nonresponders for the week 52 analyses of binary end points.

Exploratory outcomes. In the overall population, the mean change from baseline in ASDAS-CRP score (by MMRM) was −1.07 for the 150 mg LD group and −1.12 for the 150 mg NL group versus −0.60 for the placebo group at week 16 (both $P < 0.0001$). An ASDAS-CRP major improvement response (by NRI) at week 16 was achieved in 24.9% of the patients in the 150 mg LD group and 25.5% of the patients in the 150 mg NL group versus 9.7% of the patients in the placebo group ($P = 0.0008$ and $P = 0.0001$, respectively). ASDAS-CRP clinically important improvement (by NRI) at week 16 was achieved in 49.7% of the patients in the 150 mg LD group and 53.3%

Table 3. Safety profile up to week 20 and over the entire treatment period*

	Secukinumab 150 mg with loading (n = 185)	Secukinumab 150 mg without loading (n = 184)	Any secukinumab (n = 369)†	Placebo (n = 186)
Up to week 20 (safety set)				
Any AE, no. (%)	119 (64.3)	107 (58.2)	226 (61.2)	101 (54.3)
Any serious AE, no. (%)	2 (1.1)	4 (2.2)	6 (1.6)	5 (2.7)
Discontinuation due to any AE, no. (%)	0 (0)	3 (1.6)	3 (0.8)	3 (1.6)
Death	0 (0)	0 (0)	0 (0)	0 (0)
Most common AEs, no. (%)‡				
Nasopharyngitis	27 (14.6)	19 (10.3)	46 (12.5)	23 (12.4)
Diarrhea	14 (7.6)	9 (4.9)	23 (6.2)	7 (3.8)
Headache	17 (9.2)	5 (2.7)	22 (6.0)	7 (3.8)
Upper respiratory tract infection	11 (5.9)	11 (6.0)	22 (6.0)	7 (3.8)
Selected AEs, no. (%)				
Serious infections	1 (0.5)	1 (0.5)	2 (0.5)	0 (0)
IBD (preferred term)	0 (0)	1 (0.5)	1 (0.3)	0 (0)
MACE	0 (0)	0 (0)	0 (0)	1 (0.5)
Uveitis	2 (1.1)	0 (0)	2 (0.5)	1 (0.5)
Entire treatment period (safety set)§				
Any AE, no. (%)	162 (87.6)	156 (84.8)	431 (79.4)	121 (65.1)
Any serious AE, no. (%)	20 (10.8)	12 (6.5)	39 (7.2)	8 (4.3)
Discontinuation due to any AE, no. (%)	7 (3.8)	13 (7.1)	24 (4.4)	3 (1.6)
Death	0 (0)	0 (0)	0 (0)	0 (0)
Most common AEs, no. (EAIR/100 patient-years)¶				
Nasopharyngitis	56 (25.4)	43 (17.6)	122 (19.4)	32 (32.5)
Upper respiratory tract infection	25 (9.6)	24 (9.0)	59 (8.4)	13 (12.4)
Diarrhea	23 (8.8)	20 (7.4)	50 (7.1)	10 (9.5)
Headache	26 (10.1)	12 (4.3)	46 (6.5)	9 (8.6)
Selected AEs, no. (EAIR/100 patient-years)				
Serious infections	5 (1.8)	5 (1.7)	12 (1.6)	1 (0.9)
IBD	3 (1.1)	1 (0.3)	7 (0.9)	0 (0)
MACE	0 (0)	0 (0)	0 (0)	1 (0.9)
Uveitis	5 (1.8)	2 (0.7)	9 (1.2)	2 (1.8)
Malignancies	0 (0)	0 (0)	3 (0.4)	0 (0)
Suicide attempt	0 (0)	1 (0.3)	1 (0.1)	0 (0)

* IBD = inflammatory bowel disease; MACE = major adverse cardiovascular event.
† The "any secukinumab" group (n = 369 for up to week 20 and n = 543 for the entire treatment period) included patients originally randomized to receive secukinumab and patients originally randomized to receive placebo who switched to open-label secukinumab 150 mg.
‡ Adverse events (AEs) with a frequency of >5% up to week 20, presented in descending order in the "any secukinumab" group. Events are listed according to preferred term in the Medical Dictionary for Regulatory Activities (MedDRA), version 21.1.
§ The entire treatment period includes safety data up to the cutoff date July 1, 2019 and includes at least 52 weeks of exposure for all patients and up to 104 weeks of exposure for some patients. The cumulative exposure was 286.1 patient-years for the secukinumab 150 mg with loading group, 291.3 patient-years for the secukinumab 150 mg without loading group, 757.9 patient-years for the "any secukinumab" group, and 109.3 patient-years for the placebo group.
¶ AEs that occurred with an exposure-adjusted incidence rate (EAIR) of >5.0 cases per 100 patient-years in the "any secukinumab" group over the entire treatment period. Events are listed according to preferred term in the MedDRA, version 21.1.

of the patients in the 150 mg NL group versus 30.6% of the patients in the placebo group (P = 0.0009 and P < 0.0001, respectively). In patients with a positive MRI at screening, the SI joint edema score on MRI using multiple imputation was –2.38 for the 150 mg LD group and –1.42 for the 150 mg NL group versus –0.59 for the placebo group (both P < 0.0001) at week 16 (Figure 2D). The corresponding score (observed data) at week 52 in patients originally randomized to receive secukinumab who did not switch treatment was –2.91 for the 150 mg LD group (n = 81) and –1.92 for the 150 mg NL group (n = 87) in the overall population and –4.32 for the 150 mg LD group (n = 55) and –2.57 for the 150 mg NL group (n = 65) with MRI positivity at screening (Figure 2D).

Observed data across all prespecified efficacy end points for the overall population at week 16 and at week 52 for patients who did not switch treatment are presented in Supplementary Table 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41477/abstract. Additional efficacy data (observed) at week 52 for all secukinumab-treated patients (including patients who did not switch treatment and those who switched to open-label secukinumab or standard of care) and for placebo patients who switched to open-label secukinumab are presented in Supplementary Tables 7 and 8, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41477/abstract. The change from baseline to week 52 in SI joint total edema score on MRI

and ASQoL scores are presented in Supplementary Table 9, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41477/abstract.

Safety. Table 3 shows safety results for this study up to week 20, when all patients were still receiving the treatment to which they were originally randomized, and for the entire treatment period (up to the data cutoff date of July 1, 2019). All patients remaining in the study had completed the week 52 visit by the data cutoff date, with many having completed up to 2 years of treatment. The mean duration of exposure was 564.8 days (286.1 patient-years in total), 578.3 days (291.3 patient-years in total), and 214.6 days (109.3 patient-years in total) for the 150 mg LD, 150 mg NL, and placebo groups, respectively. The mean exposure in the "any secukinumab" group (all patients randomized to receive secukinumab and patients who switched to open-label secukinumab after originally being randomized to receive placebo) was 509.8 days, with a cumulative exposure of 757.9 patient-years over the entire treatment period.

The overall incidence of treatment-emergent AEs up to week 20 was 61.2% for the "any secukinumab" group and 54.3% for placebo. Most AEs reported up to week 20 were mild or moderate in severity for all treatment groups. The most frequent treatment-emergent AEs in terms of crude incidence rates up to week 20 were nasopharyngitis, diarrhea, headache, and upper respiratory tract infection in both the secukinumab and placebo groups (Table 3). Most AEs reported during the entire treatment period were mild or moderate in severity across all treatment groups.

The most frequent treatment-emergent AEs and selected AEs of interest are shown in Table 3. A total of 14 cases of uveitis in 11 patients were reported; 9 in the secukinumab groups (4 de novo cases) and 2 in the placebo group. All uveitis cases were mild to moderate in severity, none of them were reported as SAEs, and none led to treatment interruption or discontinuation. A total of 7 patients receiving secukinumab reported IBD (5 Crohn's disease and 2 ulcerative colitis). Two patients had a history of IBD. Three of the IBD cases led to treatment interruption or discontinuation. No cases of IBD were reported in the placebo group. Suicide attempts were reported in 2 patients with a history of depression; 1 in a patient who had switched to a TNFi as standard of care ~10 months before the event and 1 in a patient in the secukinumab 150 mg NL group. Three malignancy cases were reported in patients in the placebo group who switched to open-label secukinumab: a malignant melanoma (reported as an SAE), a squamous cell carcinoma of the tongue, and a basal cell carcinoma. All malignancy events led to discontinuation of study medication as required by the protocol, although none of these cases were considered by the investigator to be related to study medication. Grade 3 neutropenia was reported in 3 patients: 1 patient in the secukinumab 150 mg LD group and 2 patients in the placebo group who switched to open-label secukinumab. Grade 4 neutropenia was reported in 1 patient in the placebo group. There were no MACE events reported in the secukinumab groups, with 1 case of myocardial infarction in the placebo group. No deaths, tuberculosis reactivation, esophageal candidiasis, or hepatitis B reactivation were reported.

DISCUSSION

PREVENT is the first randomized placebo-controlled phase III study evaluating the efficacy and safety of secukinumab treatment in patients with nonradiographic axial SpA and the largest randomized controlled trial of a biologic therapy in nonradiographic axial SpA to date. The retention rate was high, with 95.0% of randomized patients completing week 24 and 86.7% completing week 52. Secukinumab 150 mg met both primary end points (ASAS40 response) at weeks 16 and 52 in TNFi-naive patients with nonradiographic axial SpA. ASAS40 and all predefined secondary end points in the overall study population were met at week 16 and the majority were met at week 52, demonstrating that secukinumab provided significant improvement in disease activity, physical function, quality of life, and objective signs of inflammation in nonradiographic axial SpA patients who were either naive to prior biologic therapy or had demonstrated an inadequate response to TNF inhibition. The treatment effect of both secukinumab regimens (LD and NL) was observed early and was sustained through week 52. While the study was not powered to compare differences between dose regimens, the LD regimen was associated with a more rapid onset of action compared with the NL regimen for most efficacy end points up to week 16.

The efficacy outcomes of this study are consistent with previous phase III studies, which evaluated the efficacy of TNF or IL-17 inhibitors in patients with nonradiographic axial SpA over a shorter duration, ranging from 12 to 16 weeks (29–33). The ASAS40 response of 29.2% for placebo in the present study is higher than that observed in trials with other biologics. In the ABILITY-1 study, 36.0% of adalimumab-treated patients with nonradiographic axial SpA achieved an ASAS40 response at week 12 compared with 15.0% of placebo-treated patients (29). The ASAS40 responses at week 16 were 56.7% (golimumab) versus 23.0% (placebo) in the GO-AHEAD study (30). In the EMBARK study, the ASAS40 response rate was 32.0% in the etanercept group versus 16.0% in the placebo group at week 12 (31). In the C-axSpAnd study, the ASAS40 response rate was 47.8% in the certolizumab pegol group versus 11.4% in the placebo group at week 12 (32). In a recently published study, the ASAS40 response rates were 35.0% with ixekizumab versus 19.0% with placebo at week 16 in patients with nonradiographic axial SpA (33).

High response rates to placebo in clinical studies is the subject of ongoing debate and research. The expectation for the efficacy of newer biologics, particularly in biologic-naive patients, and the subjective nature of the majority of the outcome measures used in axial SpA studies may be potential reasons for the high response to

placebo observed in the present study. This would be expected to be reflected particularly in end points such as ASAS20 and ASAS40 responses, with high hurdle efficacy end points having a lower placebo response. This is indeed reflected in the present study, with lower placebo response rates and greater differentiation observed for partial remission according to ASAS and inactive disease according to the ASDAS-CRP. Moreover, low responses to placebo were also observed for end points using objective measures, in particular hsCRP levels and SI joint edema reduction on MRI.

Overall, treatment with secukinumab 150 mg (LD or NL) was well tolerated in patients with nonradiographic axial SpA. No new or unexpected safety signals were identified during the entire treatment period. The safety profile was consistent with the established safety profile across approved indications (34), with rates of IBD and uveitis being consistent with previously reported data with secukinumab in patients with AS (18,20).

The strengths of this study include the fact that it is the largest interventional phase III study to date in patients with nonradiographic axial SpA and allowed for the inclusion of patients with previous exposure to TNFi. The study is also notable for its 52-week placebo-controlled treatment period. However, the ability of patients to switch to open-label secukinumab or standard of care (TNFi) treatment based on the judgment of the physician and the patient after week 20 (as requested by regulatory authorities) led to the limitation that by week 52 many patients were no longer receiving the treatment that they were originally randomized to receive. In turn, while the efficacy analysis took the most conservative approach for the primary and all binary secondary end point analyses by defining these patients as nonresponders, a proportion of these patients across all treatment groups had achieved ASAS40 at the time of switch to open-label secukinumab or standard of care treatment.

In conclusion, secukinumab 150 mg demonstrated rapid and significant improvement in the signs and symptoms of nonradiographic axial SpA in both TNFi-naive patients and the overall study population by week 16, which was sustained through week 52. Secukinumab was well tolerated, with no new or unexpected safety signals identified. The PREVENT study results, combined with the results from the MEASURE program (18,20) in patients with radiographic axial SpA, demonstrate that secukinumab can be a viable option to treat the entire spectrum of axial SpA, i.e., from early to late stage or from nonradiographic axial SpA to radiographic axial SpA.

ACKNOWLEDGMENTS

The authors thank the patients and the investigators who participated in the study. The authors also thank John Gallagher (Novartis Pharmaceuticals UK Ltd., UK) and Abhijit Shete (Novartis Pharma AG, Basel, Switzerland) for medical guidance and editorial support.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Deodhar had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Deodhar, Wiksten, Porter, Richards, Haemmerle, Braun.

Acquisition of data. Deodhar, Blanco, Dokoupilová, Hall, Kameda, Kivitz, van de Sande, Braun.

Analysis and interpretation of data. Deodhar, Blanco, Dokoupilová, Hall, Kameda, Kivitz, Poddubnyy, van de Sande, Wiksten, Porter, Richards, Haemmerle, Braun.

ROLE OF THE STUDY SPONSOR

The study was designed by the scientific steering committee and Novartis personnel. All authors had access to the data, contributed to the interpretation, and collaborated in the development of the manuscript. The first draft of the manuscript was written by a medical writer, employed by the study sponsor (Niladri Maity, Novartis Healthcare Pvt., Ltd., Hyderabad, India), under the guidance of the authors. All authors critically reviewed and provided feedback on subsequent versions for important intellectual content. All authors approved the final version of the manuscript to be submitted for publication and vouch for the accuracy and completeness of the data and fidelity of this report to the study protocol. Statistical analyses were performed by statisticians employed by the study sponsor (Novartis Pharma AG, Basel, Switzerland). Publication of this article was not contingent upon approval by Novartis.

REFERENCES

1. Sieper J, Poddubnyy D. Axial spondyloarthritis. Lancet 2017;390: 73–84.
2. Sieper J, Braun J, Dougados M, Baeten D. Axial spondyloarthritis [review]. Nat Rev Dis Primers 2015;1:15013.
3. Deodhar A, Strand V, Kay J, Braun J. The term 'non-radiographic axial spondyloarthritis' is much more important to classify than to diagnose patients with axial spondyloarthritis. Ann Rheum Dis 2016; 75:791–4.
4. Lockwood MM, Gensler LS. Nonradiographic axial spondyloarthritis. Best Pract Res Clin Rheumatol 2017;31:816–29.
5. Wang R, Ward MM. Epidemiology of axial spondyloarthritis: an update. Curr Opin Rheumatol 2018;30:137–43.
6. Malaviya AN, Rawat R, Agrawal N, Patil NS. The nonradiographic axial spondyloarthritis, the radiographic axial spondyloarthritis, and ankylosing spondylitis: the tangled skein of rheumatology [review]. Int J Rheumatol 2017;2017:1824794.
7. Burgos-Varga R, Wei JC, Rahman MU, Akkoc N, Haq SA, Hammoudeh M, et al. The prevalence and clinical characteristics of nonradiographic axial spondyloarthritis among patients with inflammatory back pain in rheumatology practices: a multinational, multicenter study. Arthritis Res Ther 2016;18:132.
8. Poddubnyy D, Rudwaleit M. Early spondyloarthritis. Rheum Dis Clin North Am 2012;38:387–403.
9. Reveille JD, Weisman MH. The epidemiology of back pain, axial spondyloarthritis and HLA-B27 in the United States. Am J Med Sci 2013;345:431–6.
10. Protopopov M, Poddubnyy D. Radiographic progression in nonradiographic axial spondyloarthritis. Expert Rev Clin Immunol 2018;14:525–33.
11. Redeker I, Callhoff J, Hoffmann F, Haibel H, Sieper J, Zink A, et al. Determinants of diagnostic delay in axial spondyloarthritis: an analysis based on linked claims and patient-reported survey data. Rheumatology (Oxford) 2019;58:1634–8.
12. Sykes MP, Doll H, Sengupta R, Gaffney K. Delay to diagnosis in axial spondyloarthritis: are we improving in the UK? Rheumatology (Oxford) 2015;54:2283–4.

13. Poddubnyy D, Rudwaleit M, Haibel H, Listing J, Marker-Hermann E, Zeidler H, et al. Rates and predictors of radiographic sacroiliitis progression over 2 years in patients with axial spondyloarthritis. Ann Rheum Dis 2011;70:1369–74.
14. Van der Heijde D, Ramiro S, Landewe R, Baraliakos X, van den Bosch F, Sepriano A, et al. 2016 update of the ASAS-EULAR management recommendations for axial spondyloarthritis. Ann Rheum Dis 2017;76:978–91.
15. Ward MM, Deodhar A, Gensler LS, Dubreuil M, Yu D, Khan MA, et al. 2019 update of the American College of Rheumatology/Spondylitis Association of America/Spondyloarthritis Research and Treatment Network recommendations for the treatment of ankylosing spondylitis and nonradiographic axial spondyloarthritis. Arthritis Rheumatol 2019;71:1599–613.
16. Robinson PC, Sengupta R, Siebert S. Nonradiographic axial spondyloarthritis (nr-axSpA): advances in classification, imaging and therapy. Rheumatol Ther 2019;6:165–77.
17. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. N Engl J Med 2009;361:888–98.
18. Baeten D, Sieper J, Braun J, Baraliakos X, Dougados M, Emery P, et al. Secukinumab, an interleukin-17A inhibitor, in ankylosing spondylitis. N Engl J Med 2015;373:2534–48.
19. Baraliakos X, Deodhar A, Poddubnyy D, Kivitz A, Tahir H, van den Bosch F, et al. Long-term efficacy and safety of secukinumab 150 mg in ankylosing spondylitis: 5-year results from the phase III MEASURE 1 extension study. RMD Open 2019;5:e001005.
20. Pavelka K, Kivitz A, Dokoupilova E, Blanco R, Maradiaga M, Tahir H, et al. Efficacy, safety, and tolerability of secukinumab in patients with active ankylosing spondylitis: a randomized, double-blind phase 3 study, MEASURE 3. Arthritis Res Ther 2017;19:285.
21. Rudwaleit M, Jurik AG, Hermann KG, Landewé R, van der Heijde D, Baraliakos X, et al. Defining active sacroiliitis on magnetic resonance imaging (MRI) for classification of axial spondyloarthritis: a consensual approach by the ASAS/OMERACT MRI group. Ann Rheum Dis 2009;68:1520–7.
22. Van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis: a proposal for modification of the New York criteria. Arthritis Rheum 1984;27:361–8.
23. World Medical Association. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA 2013;310:2191–4.
24. Sieper J, Rudwaleit M, Baraliakos X, Brandt J, Braun J, Burgos-Vargas R, et al. The Assessment of SpondyloArthritis international Society (ASAS) handbook: a guide to assess spondyloarthritis. Ann Rheum Dis 2009;68:ii1–44.
25. Garrett S, Jenkinson T, Kennedy LG, Whitelock H, Gaisford P, Calin A. A new approach to defining disease status in ankylosing spondylitis: the Bath Ankylosing Spondylitis Disease Activity Index. J Rheumatol 1994;21:2286–91.
26. Ware JE Jr. SF-36 health survey update. Spine (Phila Pa 1976) 2000;25:3130–9.
27. Doward LC, Spoorenberg A, Cook SA, Whalley D, Helliwell PS, Kay LJ, et al. Development of the ASQoL: a quality of life instrument specific to ankylosing spondylitis. Ann Rheum Dis 2003;62:20–6.
28. Lukas C, Landewe R, Sieper J, Dougados M, Davis J, Braun J, et al. Development of an ASAS-endorsed disease activity score (ASDAS) in patients with ankylosing spondylitis. Ann Rheum Dis 2009;68:18–24.
29. Sieper J, van der Heijde D, Dougados M, Mease PJ, Maksymowych WP, Brown MA, et al. Efficacy and safety of adalimumab in patients with nonradiographic axial spondyloarthritis: results of a randomised placebo-controlled trial (ABILITY-1). Ann Rheum Dis 2013;72:815–22.
30. Sieper J, van der Heijde D, Dougados M, Maksymowych WP, Scott BB, Boice JA, et al. A randomized, double-blind, placebo-controlled, sixteen-week study of subcutaneous golimumab in patients with active nonradiographic axial spondyloarthritis. Arthritis Rheumatol 2015;67:2702–12.
31. Dougados M, van der Heijde D, Sieper J, Braun J, Maksymowych WP, Citera G, et al. Symptomatic efficacy of etanercept and its effects on objective signs of inflammation in early nonradiographic axial spondyloarthritis: a multicenter, randomized, double-blind, placebo-controlled trial. Arthritis Rheumatol 2014;66:2091–102.
32. Deodhar A, Gensler LS, Kay J, Maksymowych WP, Haroon N, Landewé R, et al. A fifty-two–week, randomized, placebo-controlled trial of certolizumab pegol in nonradiographic axial spondyloarthritis. Arthritis Rheumatol 2019;71:1101–11.
33. Deodhar A, van der Heijde D, Gensler LS, Kim TH, Maksymowych WP, Ostergaard M, et al. Ixekizumab for patients with nonradiographic axial spondyloarthritis (COAST-X): a randomised, placebo-controlled trial. Lancet 2020;395:53–64.
34. Deodhar A, Mease PJ, McInnes IB, Baraliakos X, Reich K, Blauvelt A, et al. Long-term safety of secukinumab in patients with moderate-to-severe plaque psoriasis, psoriatic arthritis, and ankylosing spondylitis: integrated pooled clinical trial and post-marketing surveillance data. Arthritis Res Ther 2019;21:111.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 121–131
DOI 10.1002/art.41466

American College of Rheumatology
Empowering Rheumatology Professionals

Phase II Randomized Trial of Rituximab Plus Cyclophosphamide Followed by Belimumab for the Treatment of Lupus Nephritis

Yemil Atisha-Fregoso,[1] Susan Malkiel,[2] Kristina M. Harris,[3] Margie Byron,[4] Linna Ding,[5] Sai Kanaparthi,[3] William T. Barry,[4] Wendy Gao,[5] Kristin Ryker,[3] Patti Tosta,[3] Anca D. Askanase,[6] Susan A. Boackle,[7] W. Winn Chatham,[8] Diane L. Kamen,[9] David R. Karp,[10] Kyriakos A. Kirou,[11] S. Sam Lim,[12] Bradley Marder,[13] Maureen McMahon,[14] Samir V. Parikh,[15] William F. Pendergraft III,[16] Amber S. Podoll,[7] Amit Saxena,[17] David Wofsy,[18] Betty Diamond,[2] Dawn E. Smilek,[3] Cynthia Aranow,[2] and Maria Dall'Era[18]

Objective. To assess the safety, mechanism of action, and preliminary efficacy of rituximab followed by belimumab in the treatment of refractory lupus nephritis (LN).

Methods. In a multicenter, randomized, open-label clinical trial, 43 patients with recurrent or refractory LN were treated with rituximab, cyclophosphamide (CYC), and glucocorticoids followed by weekly belimumab infusions until week 48 (RCB group) or with rituximab and CYC but no belimumab infusions (RC group). Patients were followed up until week 96. Percentages of total and autoreactive B cell subsets in the patients' peripheral blood were analyzed by flow cytometry.

Results. Treatment with belimumab did not increase the incidence of adverse events in patients with refractory LN. At week 48, a complete or partial renal response occurred in 11 (52%) of 21 patients receiving belimumab, compared to 9 (41%) of 22 patients in the RC group who did not receive belimumab ($P = 0.452$). Lack of improvement in or worsening of LN was the major reason for treatment failure. B cell depletion occurred in both groups, but the percentage of B cells remained lower in those receiving belimumab (geometric mean number of B cells at week 60, 53 cells/μl in the RCB group versus 11 cells/μl in the RC group; $P = 0.0012$). Percentages of total and autoreactive transitional B cells increased from baseline to week 48 in both groups. However, percentages of total and autoreactive naive B cells decreased from baseline to week 48 in the belimumab group compared to the no belimumab RC group ($P = 0.0349$), a finding that is consistent with impaired maturation of naive B cells and enhanced censoring of autoreactive B cells.

Conclusion. The addition of belimumab to a treatment regimen with rituximab and CYC was safe in patients with refractory LN. This regimen diminished maturation of transitional to naive B cells during B cell reconstitution, and enhanced the negative selection of autoreactive B cells. Clinical efficacy was not improved with rituximab and CYC in combination with belimumab when compared to a therapeutic strategy of B cell depletion alone in patients with LN.

ClinicalTrials.gov identifier: NCT02260934.

Research reported in this publication was conducted by the Immune Tolerance Network and supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (award no. UM1-AI-109565). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Study medication and financial support were also provided by Genentech, Inc.

[1]Yemil Atisha-Fregoso, MD: Feinstein Institutes for Medical Research, and Elmezzi Graduate School of Molecular Medicine at Northwell Health, Manhasset, New York; [2]Susan Malkiel, PhD, Betty Diamond, MD, Cynthia Aranow, MD: Feinstein Institutes for Medical Research, Manhasset, New York; [3]Kristina M. Harris, PhD, Sai Kanaparthi, MS, Kristin Ryker, MPH, Patti Tosta, RN, MS, Dawn E. Smilek, MD, PhD: Immune Tolerance Network, San Francisco, California; [4]Margie Byron, MStat, William T. Barry, PhD: Rho, Durham, North Carolina; [5]Linna Ding, MD, PhD, Wendy Gao, BSN, MS: National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland; [6]Anca D. Askanase, MD, MPH: Columbia University Medical Center, New York, New York; [7]Susan A. Boackle, MD, Amber S. Podoll, MD, FACP, FASN: University of Colorado, Denver; [8]W. Winn Chatham, MD: University of Alabama at Birmingham; [9]Diane L. Kamen, MD, MSCR: Medical University of South Carolina, Charleston; [10]David R. Karp, MD, PhD: UT Southwestern Medical Center, Dallas, Texas; [11]Kyriakos A. Kirou, MD: Hospital for Special Surgery, New York, New York; [12]S. Sam Lim, MD, MPH: Emory University, Atlanta, Georgia; [13]Bradley Marder, MD: Medical Center of Aurora, Aurora, Colorado; [14]Maureen McMahon, MD, MS: University of California, Los Angeles; [15]Samir V. Parikh, MD: Ohio State University Wexner Medical Center, Columbus; [16]William F. Pendergraft III, MD, PhD: University of North Carolina Kidney Center, Chapel Hill; [17]Amit Saxena, MD: New York University School of Medicine, New York, New York; [18]David Wofsy, MD, Maria Dall'Era, MD: University of California, San Francisco.

Drs. Atisha-Fregoso, Malkiel, and Harris contributed equally to this work. Drs. Smilek, Aranow, and Dall'Era contributed equally to this work.

Dr. Askanase has received consulting fees, speaking fees, and/or honoraria from GlaxoSmithKline (less than $10,000). Dr. McMahon has received consulting fees, speaking fees, and/or honoraria from GlaxoSmithKline (less than $10,000). Dr. Parikh has received consulting fees from Aurinia, Bristol Myers Squibb, and GlaxoSmithKline (less than $10,000 each). Dr. Pendergraft has received consulting fees and/or honoraria from GlaxoSmithKline (less than $10,000). Dr. Saxena has received consulting fees and/or honoraria from GlaxoSmithKline (less than $10,000). Dr. Wofsy has received consulting fees from GlaxoSmithKline

INTRODUCTION

Lupus nephritis (LN) is the most common organ-threatening manifestation of systemic lupus erythematosus (SLE), resulting in significant morbidity and mortality (1,2). Despite the generation of data from multiple clinical trials, there are currently no US Food and Drug Administration (FDA)–approved therapies for LN. Current treatment for LN consists of an induction phase followed by a maintenance phase. During induction, intensive treatment with glucocorticoids in combination with an immunosuppressive agent, such as cyclophosphamide (CYC) or mycophenolate mofetil (MMF), is used to suppress renal inflammation and induce immune quiescence (3). The aim of induction is to achieve a complete renal response and minimize early damage, thereby preserving long-term kidney health. The goals of the maintenance phase are to prevent renal flares while minimizing exposure to glucocorticoids and toxicity from immunosuppressive agents. Current treatment regimens have demonstrated incomplete efficacy and have been associated with substantial toxicity and low levels of adherence (4). Results of a recent study suggested that the risk of end-stage kidney disease in patients with class IV LN is ~30% (5).

Because of the evidence supporting a critical role of B cells in the pathogenesis of SLE, some therapeutic strategies have focused on targeting the B cell compartment. Rituximab (anti-CD20) was the first biologic B cell–targeted therapy to be studied in SLE and LN. Although the potential efficacy of B cell depletion has been demonstrated in several observational open-label studies, 2 randomized, placebo-controlled trials of rituximab in SLE, one of which was conducted in patients with LN, did not meet their primary end points (6,7). One possible explanation for this is that levels of BAFF rise following B cell depletion (8). In murine studies, an elevated BAFF level promotes maturation of autoreactive B cells, thereby allowing them to enter a reconstituted B cell repertoire. B cell reconstitution in the absence of elevated BAFF levels results in fewer autoreactive cells in the reconstituted B cell repertoire (9). The monoclonal antibody belimumab targets soluble BAFF and might help prevent reemergence of autoreactive B cells following B cell depletion. Belimumab is approved by the FDA for the treatment of nonrenal manifestations of SLE.

We initiated a randomized trial of a B cell–targeted sequential combination regimen of rituximab and belimumab for refractory LN. The goals of this preliminary investigation were to assess the safety of this regimen, examine its mechanism of action, and generate preliminary efficacy data.

PATIENTS AND METHODS

Study design and treatment protocol. The Combination of Antibodies in Lupus Nephritis: Belimumab and Rituximab Assessment of Tolerance and Efficacy (CALIBRATE) trial was a phase II multicenter, randomized, controlled, open-label trial of CYC plus rituximab followed by belimumab in patients with active LN who had previously been treated with CYC or MMF. Randomization, initiated at week 4, was distributed 1:1 using a permuted block design, and due to the small planned sample size, no stratification factors were incorporated. For randomization, sites used a secure interactive web response system developed and maintained at the Statistical and Clinical Coordinating Center (Rho, Durham, NC). The trial was conducted at 14 clinical sites in the United States. Enrollment opened in November 2014 and concluded in April 2017 and was conducted in compliance with the Declaration of Helsinki. Institutional review boards at all sites approved the study design; all participants provided written informed consent.

In the treatment phase, all participants received methylprednisolone at a dose of 100 mg, rituximab at a dose of 1,000 mg, and CYC at a dose of 750 mg intravenously (IV) at weeks 0 and 2, based on the regimen described by Ng and colleagues (10). Prednisone at a dosage of 40 mg/day was initiated, with a prescribed taper to 10 mg/day by week 12, followed by ≤10 mg/day through week 96.

At week 4, trial participants were randomized to receive rituximab and CYC followed by weekly belimumab infusions (RCB group), or to receive rituximab and CYC but no belimumab infusions (RC group). Patients in the RCB group received belimumab IV at a dose of 10 mg/kg at weeks 4, 6, and 8 and every 4 weeks thereafter through week 48, whereas patients in the RC group received no additional treatment and also did not receive a placebo infusion. Treatment with hydroxychloroquine was allowed throughout the study.

Immunosuppressive medications, including additional doses of rituximab, were not permitted unless the participant met a criterion for study regimen discontinuation, which included the following: <25% improvement in the urine protein-to-creatinine ratio (UPCR) on a 24-hour urine sample collection at week 24, occurrence of a renal flare, emergence of selected adverse events, or an investigator's decision to discontinue treatment. Participants who were discontinued from the study regimen received standard of care therapy, as determined by their physician, and were followed up for treatment safety through week 96.

Study participants. Eligible participants were age ≥18 years, had a diagnosis that fulfilled the American College of Rheumatology or Systemic Lupus International Collaborating Clinics criteria for SLE (11,12), and were required to have serum positivity

and Genentech (less than $10,000 each). Dr. Diamond has received consulting fees from NextCure, Janssen, and GlaxoSmithKline (less than $10,000 each). Dr. Dall'Era has received consulting fees from AstraZeneca, GlaxoSmithKline, Eli Lilly, and Janssen (less than $10,000 each). No other disclosures relevant to this article were reported.

Address correspondence to Betty Diamond, MD, Feinstein Institute, North Shore Hospital, 350 Community Drive, Manhasset, NY 11030. Email: bdiamond@northwell.edu.

Submitted for publication March 2, 2020; accepted in revised form July 28, 2020.

for antinuclear antibodies (ANAs) and/or anti–double-stranded DNA (anti-dsDNA) antibodies at the time of screening. All participants had recurrent or refractory LN and had been treated previously with either CYC or MMF. Key exclusion criteria included prior treatment with rituximab at any time or treatment with another B cell biologic therapy within the prior 12 months. All participants had a UPCR of >1 based on a 24-hour urine sample collection and had undergone a kidney biopsy within the 18 months prior to documentation of International Society of Nephrology/Renal Pathology Society class III or class IV LN or class III/IV in combination with class V LN. If the kidney biopsy was conducted >3 months prior to screening, a laboratory finding of active urinary sediment, a UPCR of >3, or an increasing UPCR over the 3 months prior to screening was required.

Study end points and assessments. The primary end point of the study was safety of the study treatment, reported as the proportion of participants who had at least 1 infectious adverse event of grade 3 or higher at or prior to week 48. Grading of the severity of adverse events was carried out using the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE), version 4.03 (grade scale 0–5). Secondary end points were 1) the proportion of participants with evidence of B cell reconstitution, defined according to the baseline B cell count or a B cell count in the lower limit of normal, whichever value was lower; and 2) the proportion of participants with grade 4 hypogammaglobulinemia, defined as an IgG level of <300 mg/dl associated with an infectious adverse event of CTCAE grade 3 or higher.

Efficacy end points, which were prospectively defined, included the proportion of participants who achieved a complete response or overall (complete plus partial) response at weeks 24, 48, and 96. Complete response was defined as the presence of all of the following criteria: 1) a UPCR of <0.5 based on a 24-hour urine sample collection; 2) an estimated glomerular filtration rate (eGFR) of ≥120 ml/minute/1.73 m^2, or if the value was <120 ml/minute/1.73 m^2, then >80% of the eGFR recorded at the time of study entry; and 3) adherence to the prednisone dosing provisions. Partial response was defined as the presence of the same criteria as used for the complete response, except that the UPCR component of the partial response definition required only >50% improvement from baseline. Nonresponders were those who did not meet the renal response criteria.

In addition, other measures of disease activity were assessed, including anti-dsDNA antibody levels, presence of hypocomplementemia, and frequency of nonrenal flares. For identification of nonrenal flares, the British Isles Lupus Assessment Group criteria (13) were used.

Mechanistic assessments. In evaluating the mechanisms of action of the treatment regimen, mechanistic outcomes were assessed as the percentages of ANA+ B cells and B cell subsets, as determined by flow cytometry in the patients' peripheral blood. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation 1 day following collection of heparinized blood samples. The cells were cryopreserved by controlled-rate freezing, and stored in liquid nitrogen until used. Vials of 5–10 × 10^6 frozen PBMCs were thawed in warmed RPMI 1640 medium (Gibco) with 15% fetal bovine serum (FBS), and then washed and resuspended in cold Hanks' balanced salt solution (HBSS) with 5% FBS. Cells were incubated on ice for 30 minutes in HBSS with 1.5% nonfat dry milk (LabScientific) with biotinylated nuclear extract, as described previously (14).

After washing, cells were incubated with a cocktail of BV421-conjugated streptavidin (BioLegend), eFluor 506–labeled fixable viability dye (ThermoFisher), and the following anti-human antibodies in HBSS with 2% FBS: BV785-conjugated IgD (IA6-2), allophycocyanin (APC)–Fire 750–conjugated CD3 (UCHT1), APC–Fire 750–conjugated CD14 (M5E2), APC–Fire 750–conjugated CD16 (3G8), PerCP–Cy5.5–conjugated IgM (MHM-88), phycoerythrin (PE)–Cy7–conjugated CD10 (HI10a), and APC-conjugated CD19 (HIB19) (all from BioLegend); PE-conjugated CD27 (CLB-27/1) and PE–eFluor 610–conjugated CD38 (HIT2) (both from ThermoFisher); and fluorescein isothiocyanate–conjugated IgG (G18-145) (from BD Biosciences). Events were acquired using a Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star) (for the gating strategies, see Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41466/abstract). Transitional (CD19+CD27–IgD+CD38^{high}CD10^{high}), naive (CD19+CD27–IgD+CD38^{intermediate}CD10$^{intermediate/low}$IgM$^{intermediate/high}$), anergic (CD19+CD27–IgD+CD38^{intermediate}CD10$^{intermediate/low}$IgM^{low}), switched memory (CD19+CD27+IgD–), IgD+ memory (CD19+CD27+IgD+), and double-negative (CD19+CD27–IgD–) B cells were assessed.

Mechanistic studies were restricted to blood samples from participants in the week 24 and/or week 48 per-protocol (PP) populations, as defined below. Global B cell subpopulations and ANA+ B cells with <50 events at all of the time points evaluated were excluded from the analyses. Consequently, the distribution of data from each analysis varied. Numbers of samples assessed in the between–treatment group comparisons are specified in Supplementary Tables 1–6 (available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41466/abstract).

Statistical analysis. Statistical analyses were performed in the modified intent-to-treat (MITT) population, defined as all randomized participants who received 1 dose each of methylprednisolone, rituximab, and CYC, and 1 dose of belimumab if in the RCB group. Analyses were also performed in the PP population, defined as participants from the MITT population who received the study regimen through week 24, week 48, or week 96. Treatment group comparisons for the proportion of MITT participants who experienced at least 1 infectious adverse event

of CTCAE grade 3 or higher by week 48 were performed using Clopper-Pearson 95% confidence intervals (95% CIs) and a logistic regression model with an indicator of whether the participant experienced at least 1 infectious adverse event of CTCAE grade 3 or higher as the dependent variable and treatment group as the independent variable.

The sample size was selected by evaluating the width of a CI surrounding the point estimate of the safety primary end point (proportion of participants meeting the primary end point), and was not powered for between-group comparisons. Data from the Immune Tolerance Network ACCESS study (Abatacept and Cyclophosphamide Combination Efficacy and Safety Study) and a review of the literature (7,15–19) suggested that this proportion could range from 0.05 to 0.35. With 20 participants per group and an observed proportion of patients meeting the safety primary end point of 0.15, the Clopper-Pearson 95% CI would range from 0.032 to 0.379.

Treatment group comparisons for the proportion of participants meeting secondary end points were performed using Clopper-Pearson 95% CIs and a logistic regression model, similar to the methods for the primary end point analysis. Fisher's exact test was used when 1 treatment group had 0 events. Treatment group comparisons for the level of B cells, B cell subpopulations, or ANA+ B cell subpopulations at a given visit were performed using repeated-measures analysis of variance on log values with baseline adjustment. Tukey-Kramer post hoc tests were done to adjust for multiple comparisons. Treatment group comparisons for directional change from week 0 at week 48 in the percentage of ANA+ transitional and ANA+ naive B cell subpopulations were performed using Fisher's exact tests. *P* values less than 0.05 were considered significant. All analyses were performed using SAS version 9.4.

Data availability statement. Data sets for these analyses are accessible through TrialShare, a public website managed by the Immune Tolerance Network (https://www.itntrialshare.org/CALIBRATE.url). This website allows the user to filter the underlying data and generate figures and results from the analysis, in addition to those submitted as part of the published reports.

RESULTS

Study population. Forty-three participants were enrolled in the trial, and these patients comprised the MITT population used for the safety and efficacy analyses. Twenty-one participants were randomized to the RCB group, and 22 were randomized to the RC group.

Table 1 shows the demographic and clinical characteristics of the study population. The median baseline UPCR in a 24-hour urine sample collection was 3.1 (minimum 1.08, maximum 10.76). A greater number of participants in the RC group compared to those in the RCB group entered the study with a UPCR of >3; however, the mean UPCR, eGFR, and serum albumin levels were similar between the groups. Eighty-four percent of the participants had LN for more than 1 year. At study entry, 72% of participants were taking hydroxychloroquine and 72% of participants were taking either an angiotensin-converting enzyme or an angiotensin receptor blocker; 54% were taking both. The distribution of the study subjects from initial assessment through week 96 is shown in Figure 1.

Table 1. Baseline characteristics of the patients in each treatment group*

	RC group (n = 22)	RCB group (n = 21)
Demographic		
Age, mean ± SD years	32.3 ± 11.43	34.5 ± 9.14
Female sex	18 (81.8)	19 (90.5)
Race/ethnicity		
White	7 (31.8)	9 (42.9)
Black	9 (40.9)	9 (42.9)
Asian	3 (13.6)	2 (9.5)
Other/unknown	3 (13.6)	1 (4.8)
Hispanic or Latino	10 (45.5)	5 (23.8)
Clinical		
Time from renal biopsy to week 0, mean ± SD months	3.6 ± 4.57	2.9 ± 3.30
ISN/RPS lupus nephritis classification		
Class III	1 (4.5)	1 (4.8)
Class IV	8 (36.4)	7 (33.3)
Class III with class V	3 (13.6)	5 (23.8)
Class IV with class V	10 (45.5)	8 (38.1)
UPCR†		
Mean ± SD	3.4 ± 1.5	3.3 ± 2.5
Ratio >3	14 (63.6)	8 (38.1)
SCr, mean ± SD mg/dl	1.02 ± 0.41	1.04 ± 0.47
eGFR, mean ± SD, ml/minute/1.73 m^2	92.7 ± 36.0	89.1 ± 33.9
Serum albumin, mean ± SD mg/dl	2.96 ± 0.50	2.89 ± 0.61
B cell count, median no. cells/μl	105.5	143.0
Hypogammaglobulinemia‡	2 (9.1)	4 (19.0)
Anti-dsDNA positive	20 (90.9)	19 (90.5)
Hypocomplementemia		
C3	18 (81.8)	16 (76.2)
C4	10 (45.5)	8 (38.1)

* Demographic and clinical characteristics were assessed among participants treated with rituximab and cyclophosphamide but no belimumab infusions (RC group) or with rituximab, cyclophosphamide, and glucocorticoids followed by weekly belimumab infusions until week 48 (RCB group) in the modified intent-to-treat population. Except where indicated otherwise, values are the number (%) of subjects. ISN/RPS = International Society of Nephrology/Renal Pathology Society; SCr = serum creatinine; eGFR = estimated glomerular filtration rate; anti-dsDNA = anti–double-stranded DNA.
† Urinary protein-to-creatinine ratio (UPCR) from 24-hour urine sample collection.
‡ Defined as an IgG level of <450 mg/dl.

Safety and adverse events. The primary end point was treatment safety, defined as the proportion of participants with at least 1 infectious adverse event of CTCAE grade 3 or higher at or prior to week 48. In the MITT population, the proportion of participants with at least 1 infectious adverse event of CTCAE grade 3 or higher at or prior to week 48 was 5 (23%) of 22 patients in

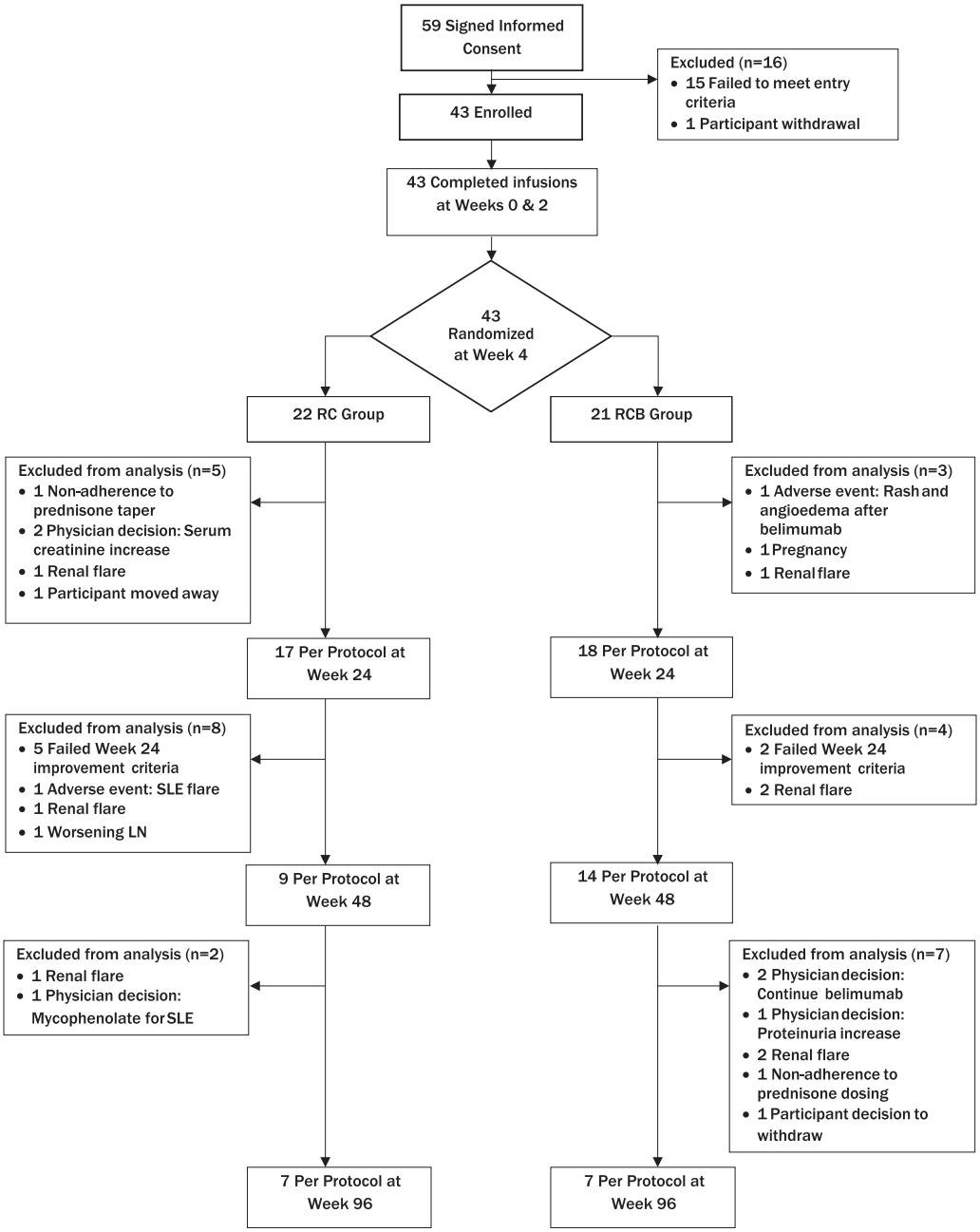


Figure 1. Flow diagram according to the Consolidated Standards of Reporting Trials (CONSORT) statement, showing the distribution of patients with recurrent or refractory lupus nephritis (LN) at each stage of the study from the time of informed consent to week 96. Reasons for exclusion of patients at each stage are provided. Samples from the per-protocol population were evaluated at weeks 24, 48, and 96. RC = treatment with rituximab and cyclophosphamide but no belimumab infusions; RCB = treatment with rituximab, cyclophosphamide, and glucocorticoids followed by weekly belimumab infusions until week 48; SLE = systemic lupus erythematosus.

the RC group and 2 (9.5%) of 21 patients in the RCB group. The difference was not statistically significant (Table 2).

The infectious adverse events in the RC group included pneumonia (n = 3, of whom 1 had respiratory syncytial virus [RSV] pneumonia), urinary tract infection (n = 1), cystitis (n = 1), cellulitis (n = 1), and sepsis (n = 1). The cellulitis, RSV pneumonia, and sepsis occurred in the same participant. The infectious adverse events in the RCB group included soft tissue abscess (n = 1), cellulitis (n = 1), and urinary tract infection (n = 1). The soft tissue abscess and cellulitis occurred in the same participant. All infectious adverse events resolved. Table 2 summarizes the infectious adverse events of grade 3 or higher, adverse events of grade 2 or higher, and serious adverse events that occurred in the MITT population of participants while they were receiving study treatment and during the full study follow-up. All participants experienced at least

Table 2. Summary of TEAEs*

	RC group (n = 22)		RCB group (n = 21)	
	Participants	Events	Participants	Events
Primary safety end point, infectious TEAEs grade 3 or higher	5 (23) (7.82–45.37)	7	2 (10) (1.17–30.38)	3
Secondary safety end points				
Infectious TEAEs				
Grade 3 or higher	6 (27) (10.73–50.22)	10	2 (10) (1.17–30.38)	5
Grade 3 or higher on protocol therapy†	4 (18) (5.19–40.28)	5	2 (10) (1.17–30.38)	2
TEAEs				
Grade 2 or higher	22 (100) (0.00–15.44)	287	21 (100) (0.00–16.11)	202
Grade 2 or higher on protocol therapy†	22 (100) (0.00–15.44)	218	21 (100) (0.00–16.11)	172
Serious TEAEs	11 (50) (28.22–71.78)	40	4 (19) (5.45–41.91)	7
Serious TEAEs on protocol therapy†	6 (27) (10.73–50.22)	10	4 (19) (5.45–41.91)	4

* Values are the number (%) of participants (95% confidence interval) with the specified treatment-emergent adverse event (TEAE) and the number of TEAEs occurring among participants in the modified intent-to-treat population. RC = treatment with rituximab and cyclophosphamide but no belimumab infusions; RCB = treatment with rituximab, cyclophosphamide, and glucocorticoids followed by weekly belimumab infusions until week 48.
† On protocol therapy includes all TEAEs reported through 30 days after the participants had discontinued protocol-specified treatment. The confidence interval bounds were calculated using the Clopper-Pearson (exact) method for binomial proportions.

1 adverse event. There were no deaths and no opportunistic infections.

Efficacy results. Fourteen participants in the PP analysis population completed the study through week 96, while 29 participants were excluded due to having left the study or having met a criterion for study regimen discontinuation. Table 3 shows the number of participants in the PP population who had a renal response to treatment that was designated as either a complete response, partial response, or nonresponse at weeks 24, 48, and 96. Table 3 also shows the number of participants who did not meet the requirements for inclusion in the PP population due to LN treatment failures and other reasons.

The numbers of participants in the PP population exhibiting an overall renal response (defined as a complete response plus partial response) were similar between the RC group and the RCB group at all time points. The highest frequency of PP participants with an overall renal response occurred at week 48, in which 9 (41%) of 22 patients in the RC group and 11 (52%) of 21 patients in the RCB group had an overall renal response ($P = 0.452$).

Treatment failure in most participants was attributable to lack of improvement in or worsening of LN (Figure 1). By week 48, 10 of 22 subjects in the RC group and 5 of 21 in the RCB group had been removed from the PP analysis due to a renal flare, worsening nephritis, or failure to show improvement in LN (Figure 1). Fewer participants in the RCB group than in the RC group exhibited C3 hypocomplementemia at week 96 in the MITT analysis population (61% versus 28%; $P = 0.049$). There were no differences in other prespecified clinical efficacy end points in the MITT analysis population (see Supplementary Table 1 [http://onlinelibrary.wiley.com/doi/10.1002/art.41466/abstract]), and there were no differences between the groups in the PP analysis population (data

Table 3. Renal response among participants at major study time points*

	Complete response	Partial response	Nonresponse	Withdrawal
Week 24				
RC group (n = 22)	5 (23)	4 (18)	8 (36)	5 (23)
RCB group (n = 21)	5 (24)	5 (24)	8 (38)	3 (14)
Week 48				
RC group (n = 22)	7 (32)	2 (9)	0	13 (59)
RCB group (n = 21)	8 (38)	3 (14)	3 (14)	7 (33)
Week 96				
RC group (n = 21)†	4 (19)	2 (10)	0	15 (71)
RCB group (n = 21)	5 (24)	1 (5)	1 (5)	14 (67)

* Participants in the complete response, partial response, and nonresponse categories were included in the per-protocol (PP) population for the time point. Participants in the withdrawal category did not meet the requirements for inclusion in the PP population, but are included in the modified intent-to-treat (MITT) population. Values are the number (%) of participants according to each renal response category analyzed in the MITT population. RC = treatment with rituximab and cyclophosphamide but no belimumab infusions; RCB = treatment with rituximab, cyclophosphamide, and glucocorticoids followed by weekly belimumab infusions until week 48.
† One participant in the RC treatment group completed the study treatment regimen per protocol but did not complete the renal response assessments at week 96, and therefore was unevaluable.

not shown). Nonrenal flares were infrequent, and there were no between-group differences in the frequency of nonrenal flares (Supplementary Table 1).

Although the parameters of renal disease (mean eGFR and UPCR) were comparable between the treatment groups at baseline (Table 1), there were some notable differences. Fourteen participants in the RC group entered the study with nephrotic levels of proteinuria (UPCR >3), compared to 8 participants in the RCB group. Among this subset, the response rate (complete response plus partial response) at week 48 was 43% (6 of 14) in the RC group and 88% (7 of 8) in the RCB group, suggesting that belimumab may be exerting a beneficial effect among participants with more severe LN. Furthermore, 3 participants in the RC group (14%) subsequently required dialysis and progressed to end-stage renal disease (ESRD) within 2 years of study entry, as compared to 1 (5%) in the RCB group. This single participant in the RCB group who progressed to ESRD had a rapidly deteriorating condition at study entry, and was withdrawn at week 8 due to rising serum creatinine levels and proteinuria. The 3 participants in the RC group who progressed to ESRD were removed from the PP analysis at week 27 (2 participants) or week 44 (1 participant). They progressed to ESRD by week 64.

Peripheral B cell reconstitution and B cell subset redistribution. B cell depletion was achieved in the PP analysis population of participants in both treatment groups by week 12 (geometric mean number of B cells, 3 cells/μl [95% CI 1–10] in the RC group versus 2 cells/μl [95% CI 1–3] in the RCB group) (Figure 2A, and Supplementary Table 2 [http://onlinelibrary.wiley.com/doi/10.1002/art.41466/abstract]). At later time points, B cells counts were consistently lower in the RCB group. This difference remained significant at week 60, 12 weeks after belimumab treatment was discontinued (geometric mean number of B cells, 53 cells/μl [95% CI 26–109] in the RC group versus 11 cells/μl [95% CI 6–20] in the RCB group; $P = 0.0012$) (Figure 2A, and Supplementary Table 2).

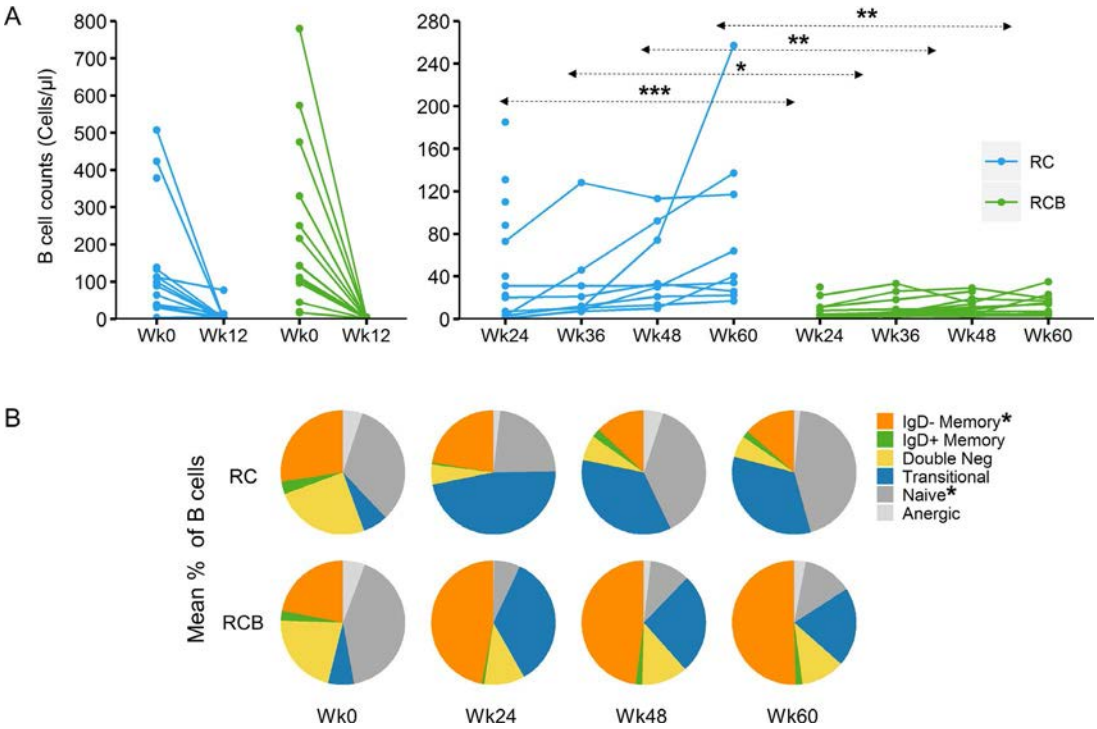


Figure 2. Total numbers of B cells within peripheral blood mononuclear cells (PBMCs) and relative frequencies of B cell subpopulations following treatment with RC versus RCB in samples from the per-protocol population of patients with lupus nephritis. **A**, B cell counts before treatment and at week 12 (left) and during reconstitution in the peripheral blood at weeks 24–60 (right) following RC or RCB treatment. Each data point represents CD19+ B cell counts as determined by clinical laboratory testing in the peripheral blood from individual patients. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ by analysis of variance (ANOVA) on log values for comparisons at week 12, and by repeated-measures ANOVA on log values (with baseline adjustment) for comparisons at weeks 24 through 60. Tukey-Kramer post hoc adjustment was applied for multiple comparisons. **B**, Mean frequencies of each B cell subpopulation within total B cells from individual patients, including a per-protocol sample analyzed at weeks 0 and 24 and per-protocol sample analyzed at weeks 48 and 60, in each treatment group at each time point, as determined by flow cytometric analysis of cryopreserved PBMCs. B cell subpopulation data were analyzed for subpopulations with >50 cells at each of the time points evaluated. * = $P < 0.01$ between treatment groups. For more details, see Supplementary Tables 2 and 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41466/abstract. Double neg = CD27–IgD– double-negative (see Figure 1 for other definitions).

Consistent with this observation, the proportion of participants in the PP analysis who met the prespecified criteria for B cell reconstitution at week 24 was 5 of 14 in the RC group and 0 of 14 in the RCB group ($P = 0.041$). By week 48, 2 of 8 participants in the RC group and 0 of 12 in the RCB group met the prespecified criteria for B cell reconstitution. In the week 24 PP sample of participants in the RC group, the mean number of B cells was higher in nonresponders compared to those who exhibited either a complete response or partial response at week 24 (geometric mean 74.5 cells/μl versus 17.3 cells/μl).

The median IgG level was lower in the RCB group, but well above the range defining hypogammaglobulinemia, with a median IgG level at week 48 of 1,410.0 mg/dl in the RC group compared to 904.5 mg/dl in the RCB group ($P = 0.022$). No participant in the trial had grade 4 hypogammaglobulinemia, and only 1 participant (in the RC group) had an IgG level of <300 mg/dl, which was not associated with infectious complications.

As BAFF is known to be important for transitional to naive B cell differentiation, we examined the distribution of B cell subsets before and after treatment with rituximab, with or without belimumab. Before treatment, the distribution of B cell subsets was similar between the groups (Figure 2B, and Supplementary Table 3 [http://onlinelibrary.wiley.com/doi/10.1002/art.41466/abstract]). In the RCB group, the percentage of naive B cells was diminished relative to baseline and smaller than that in the RC group, with concomitant increases in the percentage of transitional B cells and class-switched IgD– memory B cells. These differences between the 2 groups were significant at weeks 24, 48, and 60 (each $P < 0.01$) (Figure 2B, and Supplementary Table 3).

Reconstitution and subset redistribution of autoreactive ANA+ B cells. In order to investigate treatment effects on autoreactivity, we examined the percentages of total ANA+ B cells and their subset distributions, using a previously described flow cytometry–based method that identifies B cells bearing a B cell receptor that is capable of binding nuclear antigens (ANA+ B cells) (14). Before treatment, the predominant subpopulation of ANA+ B cells was naive B cells (Figure 3A, and Supplementary Table 4 [http://onlinelibrary.wiley.com/doi/10.1002/art.41466/abstract]). At week 48, the predominant subpopulation of ANA+ B cells in both groups was transitional cells. However, the distribution of other B cell subpopulations within ANA+ B cells differed between the treatment groups, with a diminished percentage of ANA+ naive B cells ($P = 0.0176$) and correspondingly greater percentages of class-switched IgD– memory B cells ($P = 0.0082$) and CD27–IgD– double-negative cells ($P = 0.0026$) in the RCB group compared to the RC group (Figure 3A, and Supplementary Table 4).

At week 48, the percentage of ANA+ naive B cells was increased from baseline in 5 of 7 RC participants and decreased from baseline in 8 of 9 RCB participants ($P = 0.0349$), when assessed in the peripheral blood of patients who could be evaluated at both time points (Figure 3B, and Supplementary Table 5 [http://onlinelibrary.wiley.com/doi/10.1002/art.41466/abstract]). The relative percentages of ANA+ transitional cells increased from baseline to week 48 in all participants evaluated in either group (Figure 3B and Supplementary Table 5). These results support the interpretation that treatment with belimumab delays reconstitution of ANA+ naive B cells by inhibiting maturation of ANA+ transitional B cells.

We also observed a higher percentage of ANA+ anergic cells within peripheral blood B cells at week 48 among patients in the

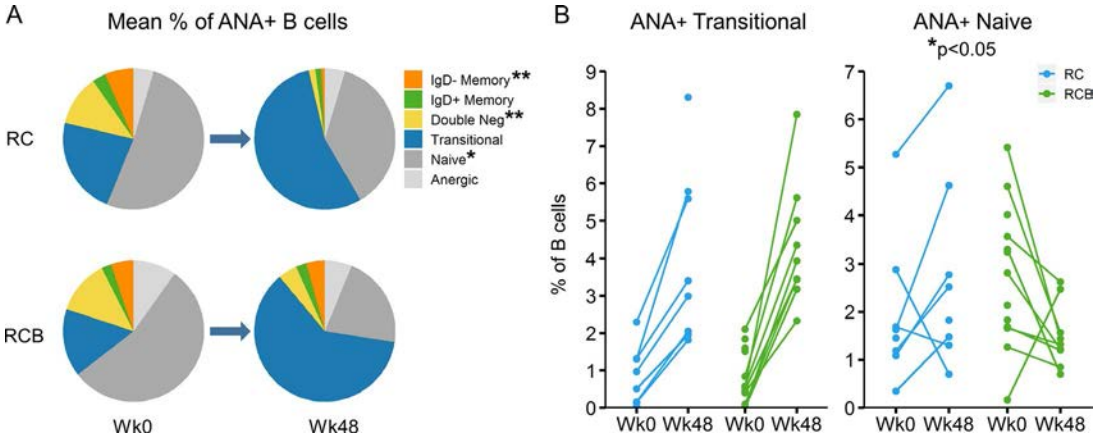


Figure 3. Reconstitution of autoreactive antinuclear antibody–positive (ANA+) B cell subsets following treatment with RC versus RCB in the per-protocol population at week 48. **A**, Mean frequencies of B cell subpopulations within total ANA+ B cells from each group before treatment and at week 48. * = $P < 0.05$; ** = $P < 0.01$ between treatment groups at week 48. **B**, ANA+ transitional and ANA+ naive B cells as a percentage of total B cells in the peripheral blood before treatment and at week 48. Each data point represents the relative frequency of ANA+ transitional B cells (left) or ANA+ naive B cells (right) in an individual patient at each time point *P* value was determined by Fisher's exact test. For more details, see Supplementary Tables 4–6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41466/abstract. Double neg = CD27–IgD– double-negative (see Figure 1 for other definitions).

RCB group, although this was not significantly different from that in patients in the RC group (Supplementary Figure 2 and Supplementary Table 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41466/abstract).

DISCUSSION

This study is the first randomized, controlled trial to examine the safety and efficacy of the combination of rituximab and belimumab in patients with LN. The trial was not powered to fully evaluate efficacy, but rather was designed primarily to evaluate safety. Consistent with published guidelines, the CALIBRATE trial was limited to patients with recurrent or refractory LN who had previously received standard of care treatment with either MMF or CYC (20,21).

Sequential therapy with belimumab was not associated with an increased frequency of adverse events. However, there were no significant differences in efficacy between the 2 treatment groups. Notably, the complete response rates at week 24 and week 48 were comparable to those observed in past trials in patients with LN (22) that included participants with new-onset LN.

Compared to prior treatment trials in LN, we encountered a higher frequency of ESRD (9%). This finding may reflect the fact that enrollment in the CALIBRATE trial was restricted to patients with recurrent or refractory nephritis. All 4 participants who progressed to ESRD entered the trial with nephrotic levels of proteinuria (UPCRs of 3.4, 3.8, 4.2, and 5.3). The fact that 3 of these participants were in the RC group may simply reflect the imbalance between the groups with respect to the UPCR value at trial entry. However, we observed a trend toward 1) better responses among participants with nephrotic levels of proteinuria in the RCB group, 2) an increased frequency of ESRD in the RC group, and 3) an increased number of participants in the RC group who were withdrawn prior to week 48 due to lack of renal response at week 24, or withdrawn for reasons related to LN. All of these findings imply that a maintenance regimen may be important following a single course of rituximab and CYC therapy in patients with recurrent or refractory LN. This is consistent with recent data showing a benefit of adding belimumab to a maintenance regimen for LN (23), and consistent with the practice of administering a second dose of rituximab as reinforcement at 6 months.

For more than a decade, there has been controversy regarding the role of B cell depletion in the treatment of LN. Despite positive anecdotal experiences and case series, controlled trials continue to yield disappointing results. In this respect, the findings from the CALIBRATE trial are consistent with those from past controlled trials. Forty-eight weeks after treatment with rituximab, only one-third of participants in each group achieved a complete response.

Belimumab reduces disease activity in SLE patients without nephritis (24) and produces partial B cell depletion, which is associated with lower circulating levels of BAFF (25). The CALIBRATE trial explored the effects of belimumab after B cell depletion with anti-CD20 therapy. Participants who received belimumab exhibited lower B cell numbers at all time points. Nonetheless, median IgG levels remained within the normal range in both groups, and the addition of belimumab to a regimen of CYC, rituximab, and glucocorticoids did not result in an increase in serious infectious adverse events.

This study employed sequential administration of rituximab and belimumab, with the objective of reducing the emergence of autoreactive B cells during B cell reconstitution, as increased BAFF levels have been associated with the risk of relapse (26). Another potential therapeutic strategy would be to administer belimumab followed by rituximab. Since BAFF enhances the mobilization of B cells into lymphoid follicles (27) and belimumab reduces the number of B cells in lymphoid tissues (28), this sequence might increase systemic depletion of memory B cells by moving them into circulation, where they would be more susceptible to rituximab-mediated cell death. This strategy is being examined in an ongoing clinical trial of nonrenal SLE, the BLISS-BELIEVE study (Study to Evaluate the Efficacy and Safety of Belimumab Administered in Combination With Rituximab to Adult Subjects With Systemic Lupus Erythematosus; ClinicalTrials.gov identifier: NCT03312907). The increased percentage of class-switched IgD– memory B cells that was observed in the RCB group is consistent with previous observations (29), and also with a belimumab-induced release of memory B cells from lymphoid organs, suggesting that a regimen of belimumab prior to rituximab may be of benefit.

We observed a reduced percentage of naive B cells in the RCB group, consistent with the dependence on BAFF for differentiation of transitional to naive B cells. In contrast, the RC group exhibited an increased proportion of naive B cells, presumably due to the unhindered maturation of transitional into naive B cells. This observation is consistent with that in previous studies showing decreased numbers of circulating naive B cells in patients treated with belimumab only (25,30,31). The results of one study demonstrated that belimumab controlled the developmental checkpoint of transitional cells between the T1 and T3 stages, with the conservation of the T1 population and reduction of the late T3 population in SLE patients. Although not studied in this trial, the increased percentages of transitional B cells observed in both treatment groups is likely composed of different subsets, with the T1 phenotype predominating in the RCB group and T2 and T3 subsets in the RC group.

A recent study of lupus patients receiving long-term belimumab therapy showed a reduction in the usage of the V_H4–34 gene associated with anti-dsDNA antibodies in IgM+ B cells (30). We analyzed the percentage of each subpopulation in ANA+ autoreactive B cells, as well as the percentage of each ANA+ B cell subpopulation among B cells, by flow cytometry in the patients' peripheral blood. As expected, we observed a decreased percentage of naive B cells within the autoreactive ANA+ B cell compartment (Figure 3A), and a decreased percentage of ANA+

naive cells among total B cells (Figure 3B) in the RCB treatment group compared to the RC treatment group. We did not, however, observe a decreased percentage of ANA+ B cells in the naive B cell subset in patients receiving belimumab.

This trial adds to the growing body of literature examining B cell combination therapy in SLE. The recently published single-arm, proof-of-concept SynBiOse trial of rituximab and belimumab in 16 patients with active SLE demonstrated clinical efficacy at week 24 (32). Thirteen participants had LN. The median SLE Disease Activity Index score decreased from 18 at baseline to 2 at week 24, and median proteinuria levels decreased from 2.3 gm/24 hours to 0.7 gm/24 hours. These clinical benefits were noted despite the fact that background treatment with MMF and glucocorticoids was tapered to low levels during the study. The rate of complete renal response was slightly higher in the SynBiOse trial participants compared to that in the CALIBRATE trial. The reasons for this difference are not entirely clear, but are likely multifactorial. Notably, the trial designs and study populations were quite different: 1) SynBiOse participants received higher initial doses of steroids and did not receive concomitant CYC; 2) the definition for complete renal response was more stringent in the CALIBRATE trial; and 3) the racial/ethnic composition of the participants differed. In this regard, the CALIBRATE trial included a racially diverse sample, including 40% of patients being African American, a group underrepresented in the first trials of belimumab (33).

The CALIBRATE trial is an important step in understanding the mechanisms of action of combination therapy with rituximab and belimumab for the treatment of LN in SLE. These findings may lay the foundation for larger trials designed to assess efficacy.

ACKNOWLEDGMENTS

The authors thank their colleagues at the Immune Tolerance Network, and their collaborators who contribute in many capacities to Immune Tolerance Network projects and perspectives. The authors are also grateful to the study participants in the ITN055AI CALIBRATE trial.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Diamond had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Ding, Kanaparthi, Tosta, Wofsy, Diamond, Smilek, Aranow, Dall'Era.

Acquisition of data. Atisha-Fregoso, Malkiel, Harris, Byron, Ding, Kanaparthi, Ryker, Tosta, Askanase, Boackle, Chatham, Kamen, Karp, Kirou, Lim, Marder, McMahon, Parikh, Pendergraft, Podoll, Saxena, Wofsy, Smilek, Aranow, Dall'Era.

Analysis and interpretation of data. Atisha-Fregoso, Malkiel, Harris, Byron, Ding, Kanaparthi, Barry, Gao, Wofsy, Diamond, Smilek, Aranow, Dall'Era.

ROLE OF THE STUDY SPONSOR

The National Institute of Allergy and Infectious Diseases of the National Institutes of Health was the study sponsor. Genentech, Inc. provided the study medication and some financial support, but had no role in the study design or in the collection, analysis, or interpretation of the data, or the writing of the manuscript. The manuscript was reviewed by Genentech, Inc. prior to submission, but no revisions were made.

ADDITIONAL DISCLOSURES

Authors Byron and Barry are employees of Rho.

REFERENCES

1. Mok CC, Kwok RC, Yip PS. Effect of renal disease on the standardized mortality ratio and life expectancy of patients with systemic lupus erythematosus. Arthritis Rheum 2013;65:2154–60.
2. Hanly JG, O'Keeffe AG, Su L, Urowitz MB, Romero-Diaz J, Gordon C, et al. The frequency and outcome of lupus nephritis: results from an international inception cohort study. Rheumatology (Oxford) 2016;55:252–62.
3. Dall'Era M. Treatment of lupus nephritis: current paradigms and emerging strategies [review]. Curr Opin Rheumatol 2017;29: 241–7.
4. Feldman CH, Collins J, Zhang Z, Xu C, Subramanian SV, Kawachi I, et al. Azathioprine and mycophenolate mofetil adherence patterns and predictors among Medicaid beneficiaries with systemic lupus erythematosus. Arthritis Care Res (Hoboken) 2019;71: 1419–24.
5. 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: 1432–41.
6. Merrill JT, Neuwelt CM, Wallace DJ, Shanahan JC, Latinis KM, Oates JC, et al. Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. Arthritis Rheum 2010;62:222–33.
7. Rovin BH, Furie R, Latinis K, Looney RJ, Fervenza FC, Sanchez-Guerrero J, et al. Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis Assessment with Rituximab study. Arthritis Rheum 2012;64:1215–26.
8. Ehrenstein MR, Wing C. The BAFFling effects of rituximab in lupus: danger ahead [review]? Nat Rev Rheumatol 2016;12:367–72.
9. Kawabata D, Venkatesh J, Ramanujam M, Davidson A, Grimaldi CM, Diamond B. Enhanced selection of high affinity DNA-reactive B cells following cyclophosphamide treatment in mice. PloS One 2010;5:e8418.
10. Ng KP, Leandro MJ, Edwards JC, Ehrenstein MR, Cambridge G, Isenberg DA. Repeated B cell depletion in treatment of refractory systemic lupus erythematosus. Ann Rheum Dis 2006;65:942–5.
11. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.
12. Petri M, Orbai AM, Alarcon GS, Gordon C, Merrill JT, Fortin PR, et al. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. Arthritis Rheum 2012;64:2677–86.
13. Isenberg DA, Rahman A, Allen E, Farewell V, Akil M, Bruceet IN, et al. BILAG 2004. Development and initial validation of an updated version of the British Isles Lupus Assessment Group's disease activity index for patients with systemic lupus erythematosus. Rheumatology (Oxford) 2005;44:902–6.
14. Malkiel S, Jeganathan V, Wolfson S, Orduño NM, Marasco E, Aranow C, et al. Checkpoints for autoreactive B cells in the peripheral blood of lupus patients assessed by flow cytometry. Arthritis Rheumatol 2016;68:2210–20.

15. Appel GB, Contreras G, Dooley MA, Ginzler EM, Isenberg D, Jayne D, et al. Mycophenolate mofetil versus cyclophosphamide for induction treatment of lupus nephritis. J Am Soc Nephrol 2009;20:1103–12.
16. Diaz-Lagares C, Croca S, Sangle S, Vital EM, Catapano F, Martinez-Berriotxoa A, et al. Efficacy of rituximab in 164 patients with biopsy-proven lupus nephritis: pooled data from European cohorts [review]. Autoimmun Rev 2012;11:357–64.
17. Jonsdottir T, Zickert A, Sundelin B, Henriksson EW, van Vollenhoven RF, Gunnarsson I. Long-term follow-up in lupus nephritis patients treated with rituximab: clinical and histopathological response. Rheumatology (Oxford) 2013;52:847–55.
18. Dooley MA, Jayne D, Ginzler EM, Isenberg D, Olsen NJ, Wofsy D, et al. Mycophenolate versus azathioprine as maintenance therapy for lupus nephritis. N Engl J Med 2011;365:1886–95.
19. The ACCESS Trial Group. Treatment of lupus nephritis with abatacept: the Abatacept and Cyclophosphamide Combination Efficacy and Safety Study. Arthritis Rheumatol 2014;66:3096–104.
20. Hahn BH, McMahon MA, Wilkinson A, Wallace WD, Daikh DI, Fitzgerald JD, et al. American College of Rheumatology guidelines for screening, treatment, and management of lupus nephritis. Arthritis Care Res (Hoboken) 2012;64:797–808.
21. Bertsias GK, Tektonidou M, Amoura Z, Aringer M, Bajema I, Berden JH, et al. Joint European League Against Rheumatism and European Renal Association-European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of adult and paediatric lupus nephritis. Ann Rheum Dis 2012;71:1771–82.
22. Wofsy D, Diamond B, Houssiau FA. Crossing the Atlantic: the Euro-Lupus Nephritis regimen in North America. Arthritis Rheumatol 2015;67:1144–6.
23. GSK. GSK announces positive headline results in phase 3 study of Benlysta in patients with lupus nephritis. December 2019. URL: https://www.gsk.com/en-gb/media/press-releases/gsk-announces-positive-headline-results-in-phase-3-study-of-benlysta-in-patients-with-lupus-nephritis/.
24. Regola F, Piantoni S, Lowin T, Archetti S, Reggia R, Kumar R, et al. Association between changes in BLyS levels and the composition of B and T cell compartments in patients with refractory systemic lupus erythematosus treated with belimumab. Front Pharmacol 2019;10:433.
25. Jacobi AM, Huang W, Wang T, Freimuth W, Sanz I, Furie R, et al. Effect of long-term belimumab treatment on B cells in systemic lupus erythematosus: extension of a phase II, double-blind, placebo-controlled, dose-ranging study. Arthritis Rheum 2010;62:201–10.
26. Carter LM, Isenberg DA, Ehrenstein MR. Elevated serum BAFF levels are associated with rising anti–double-stranded DNA antibody levels and disease flare following B cell depletion therapy in systemic lupus erythematosus. Arthritis Rheum 2013;65:2672–9.
27. Badr G, Borhis G, Lefevre EA, Chaoul N, Deshayes F, Dessirier V, et al. BAFF enhances chemotaxis of primary human B cells: a particular synergy between BAFF and CXCL13 on memory B cells. Blood 2008;111:2744–54.
28. Halpern WG, Lappin P, Zanardi T, Cai W, Corcoran M, Zhong J, et al. Chronic administration of belimumab, a BLyS antagonist, decreases tissue and peripheral blood B-lymphocyte populations in cynomolgus monkeys: pharmacokinetic, pharmacodynamic, and toxicologic effects. Toxicol Sci 2006;91:586–99.
29. Stohl W, Hiepe F, Latinis KM, Thomas M, Scheinberg MA, Clarke A, et al. Belimumab reduces autoantibodies, normalizes low complement levels, and reduces select B cell populations in patients with systemic lupus erythematosus. Arthritis Rheum 2012;64:2328–37.
30. Huang W, Quach TD, Dascalu C, Liu Z, Leung T, Byrne-Steele M, et al. Belimumab promotes negative selection of activated autoreactive B cells in systemic lupus erythematosus patients. JCI Insight 2018;3:e122525.
31. Ramskold D, Parodis I, Lakshmikanth T, Sippl N, Khademi M, Chen Y, et al. B cell alterations during BAFF inhibition with belimumab in SLE. EBioMedicine 2019;40:517–27.
32. Kraaij T, Kamerling SW, de Rooij EN, van Daele PL, Bredewold OW, Bakker JA, et al. The NET-effect of combining rituximab with belimumab in severe systemic lupus erythematosus. J Autoimmun 2018;91:45–54.
33. Anjorin A, Lipsky P. Engaging African ancestry participants in SLE clinical trials. Lupus Sci Med 2018;5:e000297.

American College of Rheumatology
Empowering Rheumatology Professionals

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 132–142
DOI 10.1002/art.41457

Conversion of T Follicular Helper Cells to T Follicular Regulatory Cells by Interleukin-2 Through Transcriptional Regulation in Systemic Lupus Erythematosus

He Hao,[1] Shingo Nakayamada,[2] Kaoru Yamagata,[2] Naoaki Ohkubo,[2] Shigeru Iwata,[2] Yoshino Inoue,[2] Mingzeng Zhang,[1] Tong Zhang,[2] Yurie Kanda Satoh,[2] Yu Shan,[2] Takashi Otsuka,[2] and Yoshiya Tanaka[2]

Objective. This study was undertaken to identify characteristics of follicular regulatory T (Tfr) cells and elucidate the mechanisms by which follicular helper T (Tfh) cells convert to Tfr cells. We probed the phenotype of T helper cells in patients with systemic lupus erythematosus (SLE) and underlying transcriptional regulation using cytokine-induced STAT family factors.

Methods. Peripheral blood mononuclear cells from 41 patients with SLE and 26 healthy donors were used to sort out the memory Tfh cell subset, and Tfh cells were cultured under various conditions. The phenotype of T helper cells and underlying mechanisms of transcriptional regulation were probed using flow cytometry and quantitative polymerase chain reaction analyses. These analyses evaluated the expression of characteristic markers and phosphorylation of STATs. Chromatin immunoprecipitation was used to evaluate histone modifications.

Results. In patients with SLE, the proportion of CD4+CXCR5+FoxP3–PD-1^{high} Tfh cells was increased ($P < 0.01$), whereas the proportion of CD4+CXCR5+CD45RA–FoxP3^{high} activated Tfr cells was decreased ($P < 0.05$). Serum interleukin-2 (IL-2) levels were also reduced in patients with SLE. IL-2 induced conversion of memory Tfh cells to functional Tfr cells, which was characterized by CXCR5+Bcl-6+FoxP3^{high} pSTAT3+pSTAT5+ cells. The loci of *FOXP3* and *BCL6* at STAT binding sites were marked by bivalent histone modifications. Following IL-2 stimulation, STAT3 and STAT5 selectively bound to *FOXP3* and *BCL6* gene loci accompanied by suppression of H3K27me3. Finally, IL-2 stimulation suppressed the generation of CD38+CD27^{high} plasmablasts in Tfh and B cell coculture assays ex vivo.

Conclusion. Impaired function of Tfr cells might be attributed to defective IL-2 production. Exogenous IL-2 restores the function of Tfr cells through the conversion of Tfh cells to Tfr cells in patients with SLE. Thus, restoring balance between Tfh and Tfr cells may provide new therapeutic approaches in SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disorder characterized by dysfunctional innate and adaptive immune responses, leading to a loss of tolerance and autoantibody production. CD4+ T cells play a crucial role in the development and progression of SLE by making major contributions to antibody production and tissue inflammation (1). Follicular helper T (Tfh) cells are a heterogeneous subset of CD4+ T cells that participate in stimulating germinal center (GC) formation and selection of high-affinity B cells in the GC (2). Tfh cells are characterized by expression of the CXCR5, the transcriptional repressor B cell lymphoma 6 (Bcl-6), programmed death 1 (PD-1), and inducible costimulator (ICOS) (3). Besides, Tfh cells produce interleukin-21

Supported in part by the Japan Society for the Promotion of Science Kakenhi (Grants-In-Aid JP17K10010 and JP20K08815) and by the University of Occupational and Environmental Health, Japan (Grants for Advanced Research H29-903 and H30-905).

[1]He Hao, MD, Mingzeng Zhang, MD, PhD: University of Occupational and Environmental Health, Japan, Kitakyushu, Japan, and The Fourth Hospital of Hebei Medical University, Hebei, China; [2]Shingo Nakayamada, MD, PhD, Kaoru Yamagata, PhD, Naoaki Ohkubo, MD, Shigeru Iwata, MD, PhD, Yoshino Inoue, MD, Tong Zhang, MD, Yurie Kanda Satoh, MD, Yu Shan, MD, Takashi Otsuka, MD, Yoshiya Tanaka, MD, PhD: University of Occupational and Environmental Health, Japan, Kitakyushu, Japan.

Dr. Nakayamada has received consulting fees, speaking fees, and/or honoraria from Bristol Myers Squibb, Pfizer, Chugai, Astella, Sanofi, AbbVie, GlaxoSmithKline, and Asahi Kasei (less than $10,000 each) and research support from Mitsubishi Tanabe, Takeda, Novartis, and MSD. Dr. Tanaka has received consulting fees, speaking fees, and/or honoraria from UCB, YL Biologics, Takeda, Eisai, Novartis, Janssen, and Teijin (less than $10,000 each) and from Daiichi-Sankyo, Astellas, Pfizer, Mitsubishi-Tanabe, Bristol Myers Squibb, Chugai, Eli Lilly, and AbbVie (more than $10,000 each) and has received research support from Asahi Kasei, Mitsubishi-Tanabe, Chugai, Takeda, Sanofi, Bristol Myers Squibb, UCB, Daiichi-Sankyo, Eisai, and Ono. No other disclosures relevant to this article were reported.

Address correspondence to Yoshiya Tanaka, MD, PhD, University of Occupational and Environmental Health, Japan, School of Medicine, The First Department of Internal Medicine, 1-1 Iseigaoka, Kitakyushu 807-8555, Japan. Email: tanaka@med.uoeh-u.ac.jp.

Submitted for publication April 3, 2020; accepted in revised form July 21, 2020.

(IL-21), which supports the differentiation and survival of B cells in the GC (2). Moreover, a previous study showed that patients with active SLE can be divided into 3 subgroups based on T cell heterogeneity, with the highest proportion of resistance to treatment observed in the Tfh-dominant group (4).

Follicular regulatory T (Tfr) cells are a newly identified subset of Treg cells that coexpress markers of both Treg cells and Tfh cells. In addition to expressing Tfh-related markers, Tfr cells also express regulatory markers, such as FoxP3, CD25, CTLA-4, IL-10, and transforming growth factor β (TGFβ) (5–7). However, the function of Tfr cells is not well defined. A popular model for the function of Tfr cells is the limitation of Tfh cell activity and GC reaction (8,9), which suppresses the expansion of autoantibodies (10,11). Given that Tfh cells and Tfr cells perform opposing roles in regulating GC responses, the dysregulation of their actions may eventually promote the development of autoimmune diseases. Indeed, recent evidence indicates that patients with autoimmune diseases, such as rheumatoid arthritis (RA), SLE, and systemic sclerosis (SSc), exhibit a disrupted balance of Tfh cells and Tfr cells (12–15).

IL-2 was first identified as a T cell growth factor capable of supporting activated human T cell expansion (16). More recently, IL-2 has also been shown to be crucial for the development and maintenance of Treg cells. IL-2 is primarily produced by activated CD4+ T cells and promotes Treg cell development by activating the transcription factor STAT5, which binds to both promoter and intronic elements of the *FOXP3* gene (17). Although Tfh cells are reportedly repressed by IL-2 via the STAT5-dependent suppression of Bcl-6 (18,19), the role of IL-2 in Tfr cells is inconsistent and requires further exploration (20).

Increasing evidence has revealed that T helper cells possess phenotypic flexibility and transcriptional modification, thereby explaining both the stability and plasticity of these cells (21). Moreover, SLE patients have greater numbers of Tfh cells and potentially altered numbers of Tfr cells, suggesting that the balance between Tfh cells and Tfr cells may be dysregulated. Hence, conversion of Tfh cells to Tfr cells may restore this balance and control the GC reaction, which is a process that can provide important therapeutic approaches for SLE. The present study was designed to assess the characteristics of Tfr cells, the mechanisms of conversion of Tfh cells to Tfr cells, and the regulation of transcription by T helper cells in patients with SLE.

PATIENTS AND METHODS

Study population. The study subjects included 41 patients who were diagnosed as having SLE according to the American College of Rheumatology (ACR) revised criteria (22). The control group included 37 patients who were diagnosed as having RA according to the 2010 ACR/European League Against Rheumatism criteria (23) and 26 healthy donors who did not have an autoimmune or infectious disease. Disease activity was assessed using the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA)–SLE Disease Activity Index (SLEDAI) (24), with a SLEDAI score of <5 indicating low disease activity and a SLEDAI score of ≥5 indicating active disease. Clinical features of patients are listed in Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41457/abstract. The present study was approved by the Institutional Review Board of the University of Occupational and Environmental Health, Japan. Written informed consent was obtained from each subject.

Cell isolation. Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples using a lymphocyte separation medium (Cedarlane Corporation). CD4+ T cells and CD19+ B cells were purified using a CD4+ T Cell Isolation Kit and CD19+ B Cell Isolation Kit (BioLegend), respectively. CD4+CXCR5+CD45RA–CD25–CD127+ Tfh cells, CD4+CXCR5+CD45RA–CD25+CD127– Tfr cells, and CD4+CXCR5–CD45RA–CD25+CD127– Treg cells were sorted from PBMCs obtained from healthy donors, using a FACSAria II (BD Biosciences). Cell purity was always >90%.

Cell stimulation. Cells were cultured in 96-well flat-bottomed plates coated with anti-human CD3, cytokines, and antibodies (Supplementary Table 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41457/abstract) with complete RPMI 1640 medium (Wako) supplemented with 10% fetal calf serum and 5% penicillin/streptomycin. All cells were cultured in a humidified incubator at a temperature of 37ºC in an atmosphere of 5% CO_2. Cytokine production was measured using the Cytokine Bead Array system.

Flow cytometry. Cells were stained for 20–60 minutes with antibodies (Supplementary Table 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41457/abstract). For intracellular staining, cells were fixed and permeabilized for 30 minutes at a temperature of 4°C with a Transcription Factor Buffer Set and then washed with Perm/Wash Buffer (BD Biosciences). For intracytoplasmic staining, cells were stimulated for 5 hours with phorbol 12-myristate 13-acetate (50 ng/ml), ionomycin (1 μg/ml), and brefeldin A (2.5 μg/ml). Isotype-matched control antibodies were used as the background control. Flow cytometric analysis was performed using BD FACSAria and further analyzed with FlowJo software version 10 (TOMY Digital Biology).

Quantitative real-time polymerase chain reaction (PCR). Total RNA was isolated from cells and purified using the RNeasy Mini Kit (Qiagen), and complementary DNA (cDNA) was prepared using the high-capacity RNA-to-cDNA Kit (Applied BioSystems). Quantitative PCR was performed using a sequence detection system with site-specific primers and probes. The

expression level of each messenger RNA (mRNA) was normalized to the level of the endogenous control *GAPDH*. The primers and probes used are shown in Supplementary Table 4, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41457/abstract.

Chromatin immunoprecipitation (ChIP)–PCR. CD4+ T cells from healthy donors were isolated and cultured for 3 days with T cell receptor (TCR). Cells were washed and cultured in fresh cytokine-free medium for 24 hours, then restimulated in the absence or presence of IL-2 for 20 minutes before ChIP was performed. Chromatin was crosslinked with formaldehyde and fragmented to 200–300 bp by sonication after 20 minutes of stimulation in the absence or presence of IL-2. DNA was extracted and purified from cells using an EZ ChIP Kit (Millipore), according to the manufacturer's instructions. DNA was immunoprecipitated with antibodies, and subsequent PCR was performed with specifically designed primers (Supplementary Tables 2 and 4, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41457/abstract).

Cytokine bead array. IL-2, IL-4, IL-6, and IL-21 in serum samples from patients with SLE and healthy donors were analyzed using the respective Human Enhanced Sensitivity Flex Sets (Biosciences) for each cytokine, according to the manufacturer's instructions. By evaluating the standard curve of the assay applied, the theoretical limits of detection in the analyses were 88.9 fg/ml (IL-2), 144.4 fg/ml (IL-4), 68.4 fg/ml (IL-6), and 34.3 pg/ml (IL-21).

Statistical analysis. Differences between the 2 groups were assessed using Student's unpaired *t*-test, and more than 2 groups were evaluated by analysis of variance. Spearman's correlation coefficient analysis was used to examine the relationship between 2 variables of interest. In the in vitro experiments, data

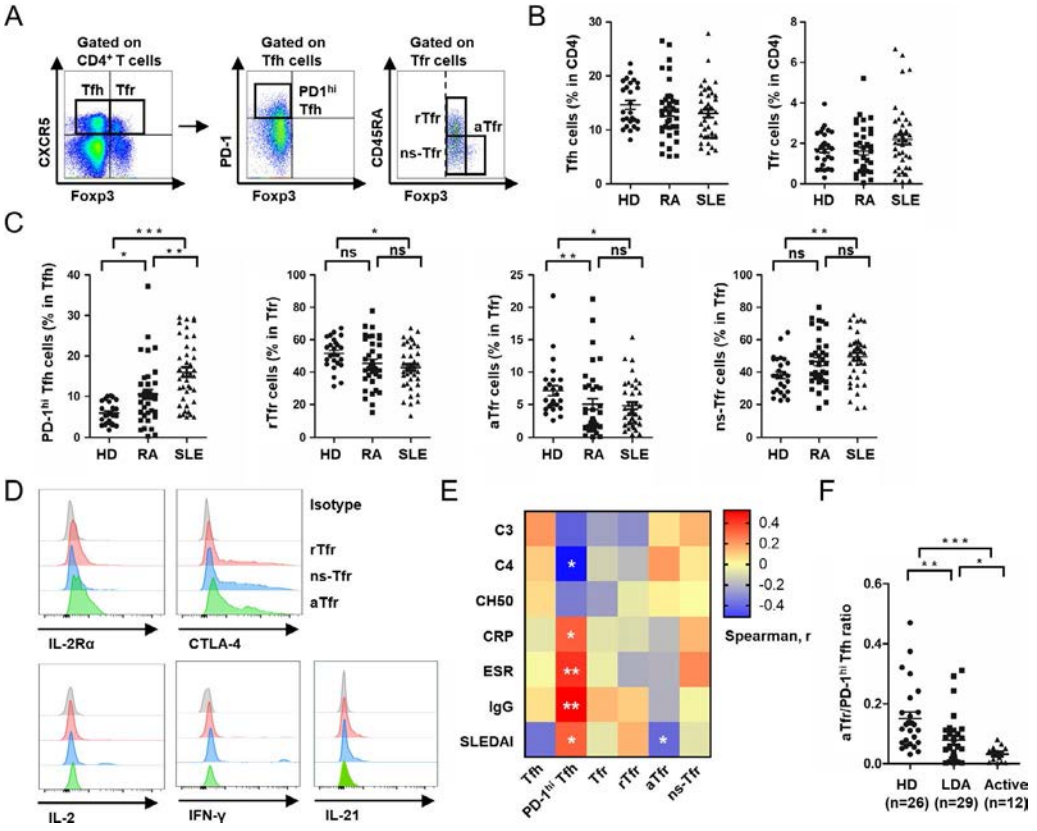


Figure 1. Imbalanced activation of follicular regulatory T (Tfr) cells and follicular helper T (Tfh) cells in systemic lupus erythematosus (SLE) patients with active disease. Peripheral blood mononuclear cells (PBMCs) isolated from the peripheral blood of 41 SLE patients, 37 rheumatoid arthritis (RA) patients, and 26 healthy donors (HD) were analyzed using flow cytometry without incubation. **A**, Gating strategy to identify Tfh cells (CD4+CXCR5+FoxP3–), Tfr cells (CD4+CXCR5+FoxP3+), programmed death 1 (PD-1)^{high} Tfh cells (CD4+CXCR5+FoxP3–PD-1^{high}), resting Tfr (rTfr) cells (CD4+CXCR5+CD45RA+FoxP3^{low}), activated Tfr (aTfr) cells (CD4+CXCR5+CD45RA–FoxP3^{high}), and nonsuppressive Tfr (ns-Tfr) cells (CD4+CXCR5+CD45RA–FoxP3^{low}). **B** and **C**, Percentage of Tfh cells and Tfr cells (**B**) and their subsets (**C**) among healthy donors, RA patients, and SLE patients. **D**, Histograms of interleukin-2 receptor α (IL-2Rα), CTLA-4, IL-2, interferon-γ (IFNγ), and IL-21 expression by Tfr subsets. **E**, Heatmaps showing correlations of C3, C4, CH50, and C-reactive protein (CRP) levels, erythrocyte sedimentation rate (ESR), IgG levels, and SLE Disease Activity Index (SLEDAI) scores with presence of activated Tfh and Tfr cells and their subsets in PBMCs from SLE patients. **F**, The activated Tfr cell:PD-1^{high} Tfh cell ratio among healthy donors, SLE patients with low disease activity (LDA), and SLE patients with active disease. Symbols represent individual subjects; bars show the mean ± SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. NS = not significant.

were expressed as the mean ± SEM of 3 independent experiments using different donors. *P* values less than 0.05 were considered significant. All analyses were performed using GraphPad Prism Software version 8.

RESULTS

Association of imbalanced activation of Tfr cells and Tfh cells with disease activity in patients with SLE. We investigated the frequencies of Tfh cells and Tfr cells in the peripheral blood of patients with SLE, patients with RA, and healthy donors. CD4+CXCR5+FoxP3– Tfh cells and CD4+CXCR5+FoxP3+ Tfr cells were identified using flow cytometry (Figure 1A). A significant difference in the frequency of Tfh cells and Tfr cells from SLE patients, RA patients, and healthy donors was not demonstrated (Figure 1B). Thereafter, we assessed the active phenotypes of Tfh cells and Tfr cells from peripheral blood. The proportion of programmed death 1 (PD-1)^{high} Tfh cells was significantly higher in SLE patients than it was in RA patients and healthy donors (Figures 1A and C).

CD4+FoxP3+ T cells are composed of 3 phenotypically and functionally distinct subpopulations: CD45RA+FoxP3^{low} resting Treg cells and CD45RA–FoxP3^{high} activated Treg cells, both of which are suppressive in vitro, and cytokine-secreting CD45RA–FoxP3^{low} nonsuppressive T cells (25). We considered the change in each fraction of CD4+CXCR5+FoxP3+ Tfr cells in the 3 subpopulations. Representative Tfr cell subsets are shown in Figure 1A. Among Tfr cells, we found that the proportions of CD45RA+FoxP3^{low} resting Tfr cells and CD45RA–FoxP3^{high} activated Tfr cells were significantly decreased in SLE patients compared to healthy donors, whereas CD45RA–FoxP3^{low} nonsuppressive Tfr cells were significantly increased in SLE patients, but not in RA patients, compared to healthy donors (Figure 1C). These differential proportions of Tfr cell subsets were specific to SLE patients.

Next, we analyzed the characteristics of Tfr cell subsets. Activated Tfr cells expressed the highest amount of IL-2 receptor α (IL-2Rα) and CTLA-4, but weakly produced IL-2, interferon-γ (IFNγ), and IL-21. Nonsuppressive Tfr cells expressed the cytokines to the highest degree, but weakly expressed IL-2Rα and CTLA-4 (Figure 1D).

Since PD-1^{high} Tfh cells are known to express high amounts of ICOS, but only small amounts of CCR7, these cells can be regarded as activated Tfh cells (26). Therefore, we examined the correlation of peripheral blood Tfh cells and Tfr cells and their subsets with disease activity, serum autoantibody levels, and inflammation markers. We found that SLEDAI scores negatively correlated with the frequency of activated Tfr cells but positively correlated with PD-1^{high} Tfh cells (Figure 1E and Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41457/abstract). In addition, a negative correlation between C4 levels and the frequency of PD-1^{high} Tfh cells was observed, whereas C-reactive protein (CRP) levels, the erythrocyte sedimentation rate (ESR), and IgG levels were positively correlated with the frequency of PD-1^{high} Tfh cells (Figure 1E). Notably, patients with active disease had a lower ratio of activated Tfr to Tfh cells compared to patients in whom no disease activity was observed and healthy donors (Figure 1F).

We also checked whether treatment status affected the proportions of Tfh cells and Tfr cells. No correlation between

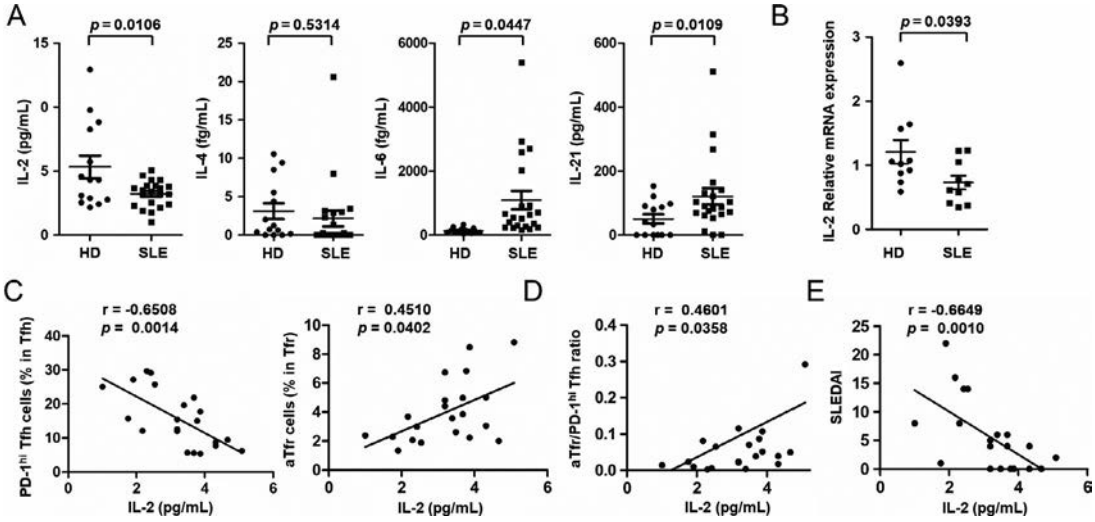


Figure 2. IL-2 defects in SLE patients and their effect on the imbalanced activation of Tfr cells and Tfh cells. **A**, IL-2, IL-4, IL-6, and IL-21 levels in serum from 14 healthy donors and 21 SLE patients assessed using a cytokine bead array. **B**, Relative mRNA expression of *IL2* in isolated CD4+ T cells from 10 healthy donors and 10 SLE patients evaluated using quantitative polymerase chain reaction. **C**, Correlation of serum IL-2 levels with the percentage of PD-1^{high} Tfh cells and activated Tfr cells in 21 SLE patients. **D** and **E**, Correlation of serum IL-2 levels with the ratio of activated Tfr to PD-1^{high} cells (**D**) and with SLEDAI scores (**E**) in 21 SLE patients. Symbols represent individual subjects; bars show the mean ± SEM. See Figure 1 for definitions.

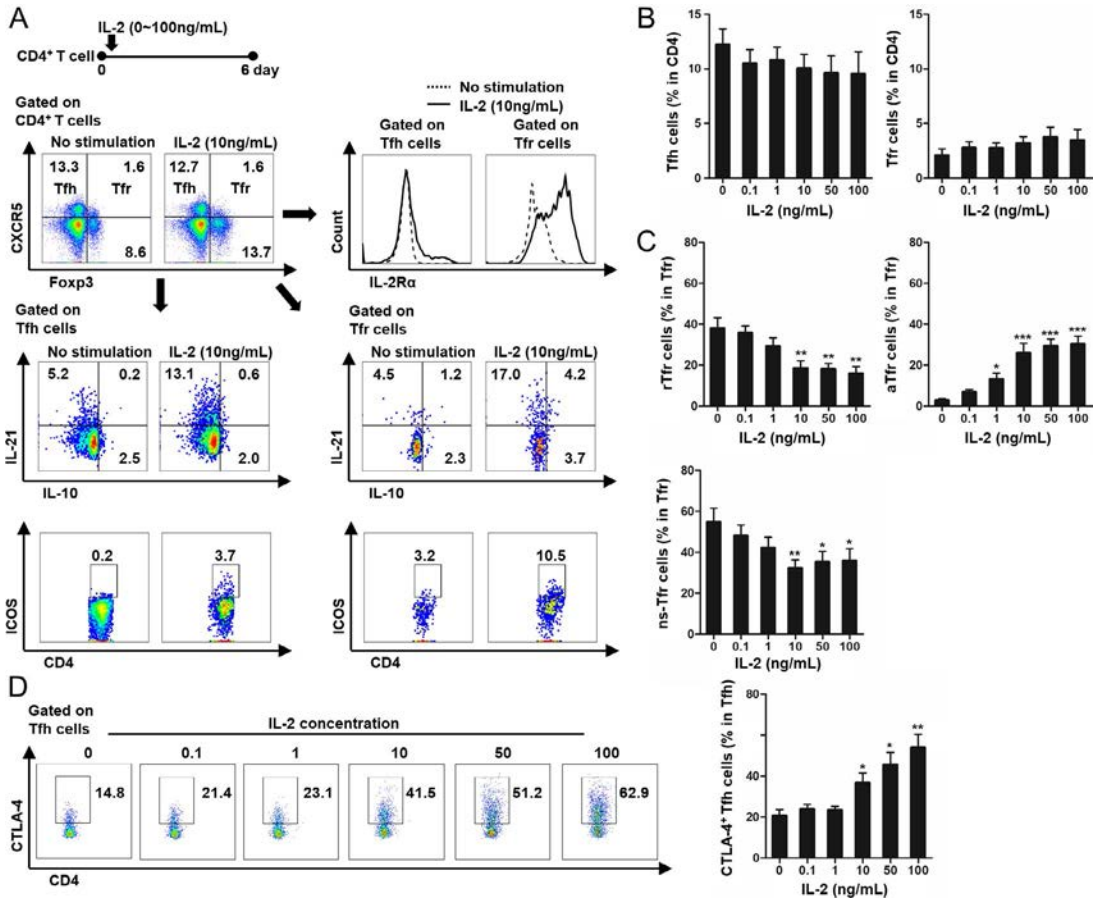


Figure 3. Expansion of activated Tfr cells and CTLA-4+ Tfh cells via IL-2 stimulation in SLE patients. CD4+ T cells from 6 SLE patients were cultured for 6 days without IL-2 or with IL-2 in various concentrations (0.1, 1, 10, 50, or 100 ng/ml) and analyzed using flow cytometry. **A**, Representative flow cytometry plots showing the percentage of Tfh cells and Tfr cells in CD4+ T cells after 6 days of stimulation with 10 ng/ml IL-2 compared to samples that did not receive IL-2 stimulation (top left). Representative histograms showing the expression levels of IL-2Rα in Tfh cells and Tfr cells after 6 days of stimulation with 10 ng/ml IL-2 compared to samples that did not receive IL-2 stimulation (top right). Representative flow cytometry plots show coexpression of IL-21 and IL-10 (middle left and right) and expression of inducible costimulator (ICOS) among CD4+ T cells (bottom left and right) within Tfh and Tfr cell subsets. **B** and **C**, Bar graphs showing the percentage of Tfh cells and Tfr cells among CD4+ T cells (**B**) and the percentage of resting Tfr cells, activated Tfr cells, and nonsuppressive Tfr cells among Tfr cells (**C**) following stimulation with varying IL-2 doses compared to samples that did not receive IL-2 stimulation. **D**, Representative flow cytometry plots showing the expression of CTLA-4 in Tfh cells (left). Results were quantified as the mean ± SEM percentages of CTLA-4+ cells expressed in Tfh cells from different donors (right). * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ versus no stimulation. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41457/abstract.

prednisolone dose and frequency of activated Tfh cells and Tfr cells was observed. However, patients who received immunosuppressive drugs showed a reduced frequency of activated Tfh cells (Supplementary Figures 2A and B, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41457/abstract). Although immunosuppressive drugs decreased the frequency of activated Tfh cells, the levels of these cells were still higher in SLE patients compared to other groups, so therefore the increased frequency of activated Tfh cells was considered to be mainly attributable to the extent of SLE disease activity. Collectively, these findings indicate that the impaired balance of activated Tfr/Tfh cells may lead to a reduced capacity to efficiently counteract autoimmunity.

Association of lack of IL-2 with imbalanced activation of Tfr cells and Tfh cells. IL-2 is essential for the development and maintenance of Treg cells but inhibits Tfh cell development. Therefore, we aimed to investigate whether an IL-2 defect is detectable in patients with SLE under physiologic conditions. We evaluated the expression level of various cytokines, including IL-2, in serum from healthy donors and SLE patients. Serum levels of IL-2 were decreased in SLE patients compared to healthy donors (Figure 2A). As expected, serum levels of IL-6 and IL-21 were increased in SLE patients, while levels of IL-4 were not observed to be significantly different (Figure 2A). The relative expression of *IL2* mRNA in isolated CD4+ T cells was also significantly lower in SLE patients compared to healthy donors (Figure 2B). A negative correlation between serum IL-2 levels

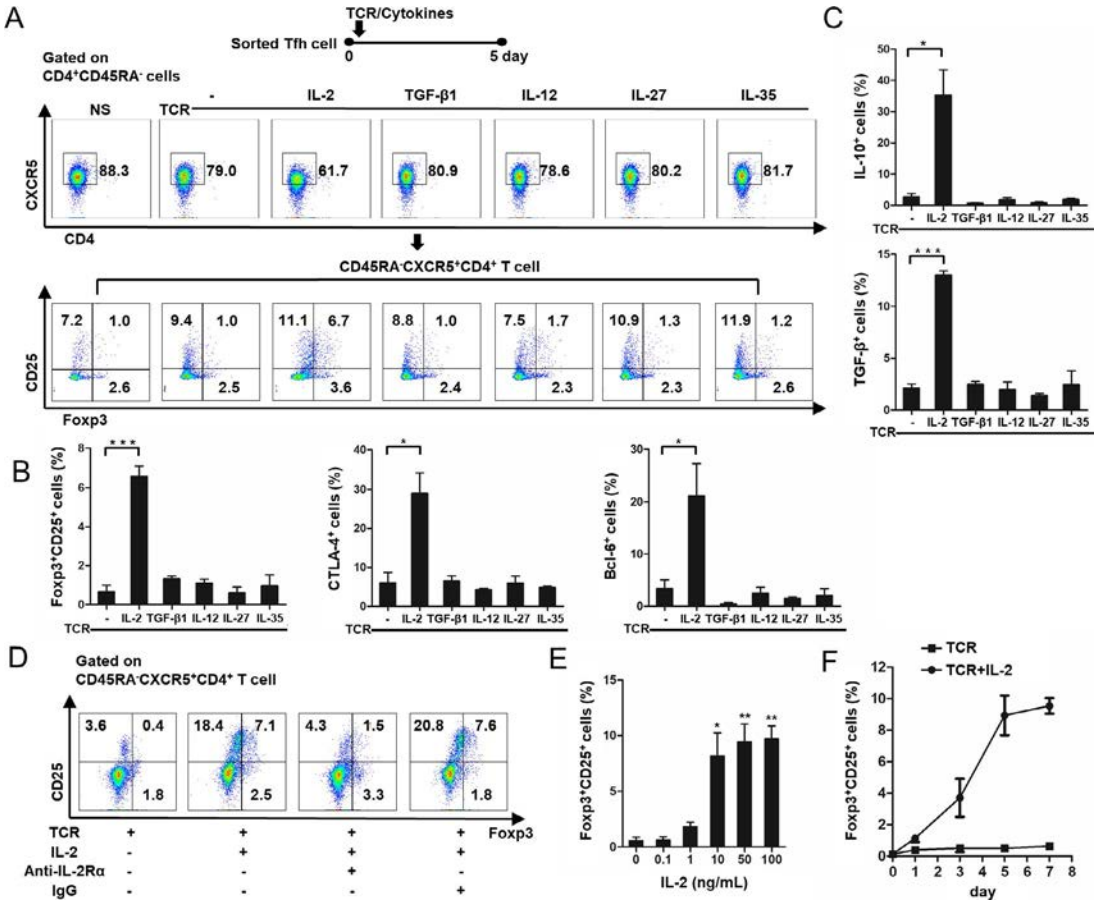


Figure 4. Conversion of Tfh cells to Tfr cells by IL-2 stimulation. Sorted Tfh cells from healthy donors were cultured for 5 days with T cell receptor (TCR) in the presence of the indicated cytokines or antibodies. **A**, Representative flow cytometry plots showing the expression of FoxP3 and CD25 in CD45RA–CXCR5+CD4+ T cells. **B** and **C**, Percentages of FoxP3+CD25+, CTLA-4, and Bcl-6 cells (**B**) as well as cells expressing IL-10 and transforming growth factor β (TGFβ) (**C**) among CD45RA–CXCR5+CD4+ T cells. **D**, Representative flow cytometry plots showing the expression of FoxP3 and CD25 with TCR stimulation in the presence or absence of IL-2 or anti–IL-2Rα antibody. **E**, Percentages of FoxP3+CD25+ cells in CD45RA–CXCR5+CD4+ T cells with TCR stimulation and after incubation with varying doses of IL-2 compared to samples that were stimulated with TCR alone. **F**, Percentages of FoxP3+CD25+ cells at different time points after stimulation with either TCR alone or TCR and IL-2. Data are the mean ± SEM of 3 independent experiments with samples obtained from 3 different donors. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ versus no stimulation. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41457/abstract.

and the percentage of PD-1^{high} Tfh cells was observed, although serum IL-2 levels were shown to be positively correlated with the percentage of activated Tfr cells and the activated Tfr:PD-1^{high} Tfh ratio (Figures 2C and D). Additionally, a negative correlation was shown between serum IL-2 levels and SLEDAI scores (Figure 2E). Taken together, the above results raise the possibility that reduced levels of IL-2 may account for the decreased ratio of activated Tfr cells to activated Tfh cells.

IL-2–induced expansion of activated Tfr cells and CTLA-4+ Tfh cells in SLE patients. We evaluated whether the observed Tfr cell defects in patients with SLE could be reversed in vitro by stimulation with IL-2. CD4+ T cells from SLE patients were cultured for 6 days with various concentrations of IL-2 and analyzed using flow cytometry. Expression levels of IL-2Rα on CD4+CXCR5+FoxP3– Tfh cells and CD4+CXCR5+FoxP3+ Tfr cells were increased following IL-2 stimulation (Figure 3A). We also detected the presence of IL-10, IL-21, and ICOS production on Tfh cells and Tfr cells. We found that levels of IL-10, IL-21, and ICOS were increased among Tfr cells, whereas only IL-21 and ICOS were up-regulated on Tfh cells (Figure 3A). IL-2 stimulation did not elicit a significant effect on the frequencies of Tfh cells and Tfr cells among CD4+ T cells (Figure 3B).

We then analyzed the change in each fraction of Tfr cells. Higher frequencies of activated Tfr cells were observed after IL-2 stimulation. In parallel with this observation, frequencies of both resting and nonsuppressive Tfr cells declined (Figure 3C). Since Tfh cells showed slightly increased IL-2Rα expression after IL-2 stimulation, which may have an impact on Tfh cell phenotype, we assessed the expression of CTLA-4 on Tfh cells. IL-2 significantly

induced the expression of CTLA-4 on Tfh cells in a dose-dependent manner (Figure 3D). Taken together, these findings indicate that IL-2 may restore some Tfr cells and expand the levels of activated Tfr cells in SLE patients. Furthermore, we found that the subset of CTLA-4+ Tfh cells was augmented by IL-2 stimulation.

Conversion of Tfh cells to Tfr cells shaped by IL-2. We evaluated which cytokines are involved in the conversion of circulating Tfh cells to Tfr cells in humans by carrying out a series of cell conversion experiments. IL-2, but not other cytokines such as TGFβ1, IL-12, IL-27, and IL-35, increased the percentage of FoxP3+CD25+, Bcl-6+, CTLA-4+, IL-10+, and TGFβ+ Tfr-like cells among CD45RA–CXCR5+CD4+ T cells (Figures 4A–C). Further, treatment with anti–IL-2Rα antibody inhibited the conversion of Tfh cells to Tfr cells (Figure 4D). Therefore, we sought to assess whether IL-2–mediated conversion of Tfh cells to Tfr cells was dose- and time-dependent. Sorted Tfh cells were cultured for 5 days with TCR in the presence of various IL-2 concentrations. Nearly maximal levels of FoxP3+CD25+ cells were noted after stimulation with 10 ng/ml of IL-2 (Figure 4E).

Next, we checked the 2-day interval change of FoxP3+CD25+ cells. During the 7-day stimulation cycle, the percentage of FoxP3+CD25+ cells among CD4+CXCR5+CD45RA– T cells gradually increased after IL-2 stimulation (Figure 4F). Above all, our findings suggest that IL-2 is the cytokine with the most impact on the conversion of Tfh cells to Tfr cells examined in the present study.

Selective binding of IL-2–activated phosphorylation of STAT3 and STAT5 to *FOXP3* and *BCL6* gene loci, with alteration of histone modification. We investigated the mechanism by which IL-2 promotes the conversion of Tfh cells to Tfr cells. The STAT family mediates cytokine-induced gene expression. Stimulation with TCR and IL-2 induced higher levels of pSTAT3 and pSTAT5 than that of pSTAT1 (Figure 5A). Furthermore, FoxP3 and Bcl-6 were highly expressed in pSTAT3+pSTAT5+ cells

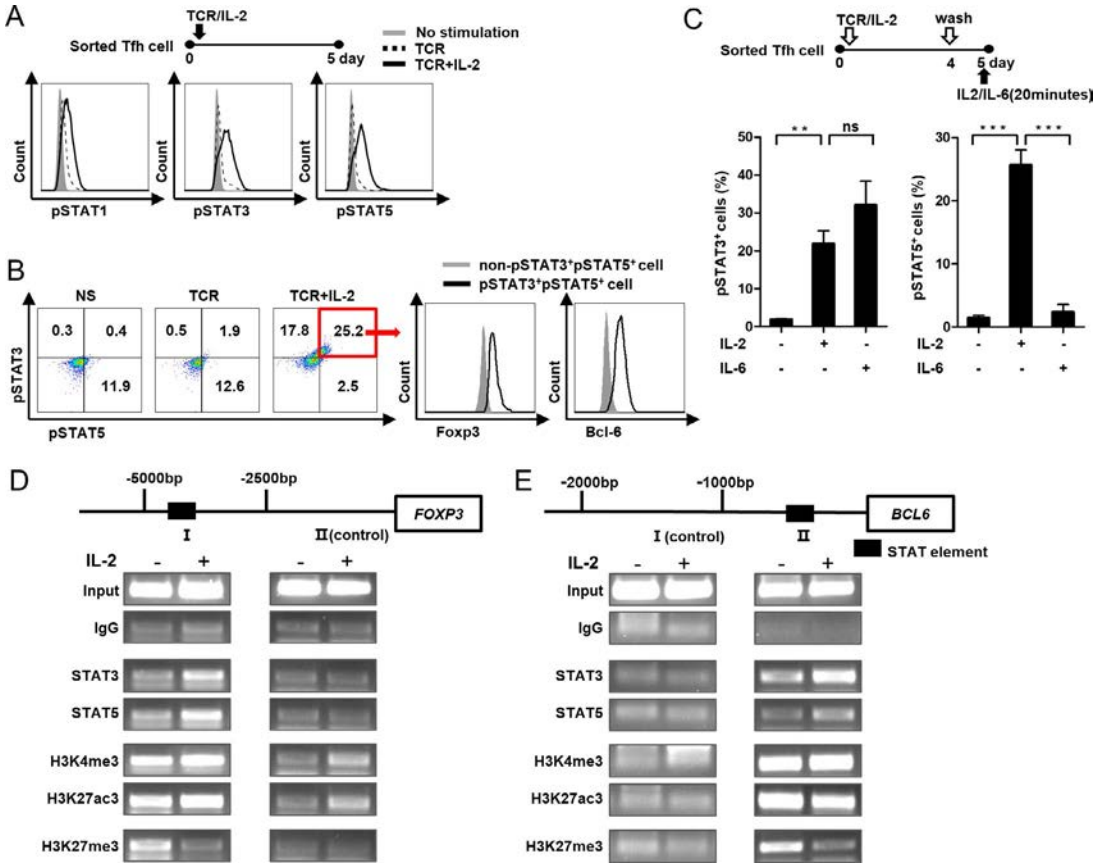


Figure 5. Induction of phosphorylation of STAT3 and STAT5 by IL-2 in Tfh cells. **A** and **B**, Tfh cells were sorted from PBMCs from healthy donors and cultured with T cell receptor (TCR) for 5 days in the presence or absence of IL-2. **A**, Histograms showing pSTAT1, pSTAT3, and pSTAT5 in CD45RA–CXCR5+CD4+ T cells. **B**, Representative flow cytometry plots showing pSTAT3 and pSTAT5 expression in CD45RA–CXCR5+CD4+ T cells (left), and expression of Bcl-6 and FoxP3 in CD45RA–CXCR5+CD4+ T cells with or without phosphorylation of STAT3 and STAT5 (right). **C**, Sorted Tfh cells from healthy donors were cultured with TCR for 4 days. Thereafter, cells were washed 3 times with complete medium, cultured in fresh cytokine-free medium for 24 hours, and then restimulated in the absence or presence of IL-2 or IL-6 for 20 minutes. Expression of pSTAT3 and pSTAT5 was analyzed using flow cytometry. **D** and **E**, Chromatin immunoprecipitation was used to analyze expression of *FOXP3* and *BCL6* at the indicated loci in the presence or absence of IL-2 stimulation. Data are the mean ± SEM of 3 independent experiments with samples obtained from ≥3 different donors. ** = $P < 0.01$; *** = $P < 0.001$. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41457/abstract.

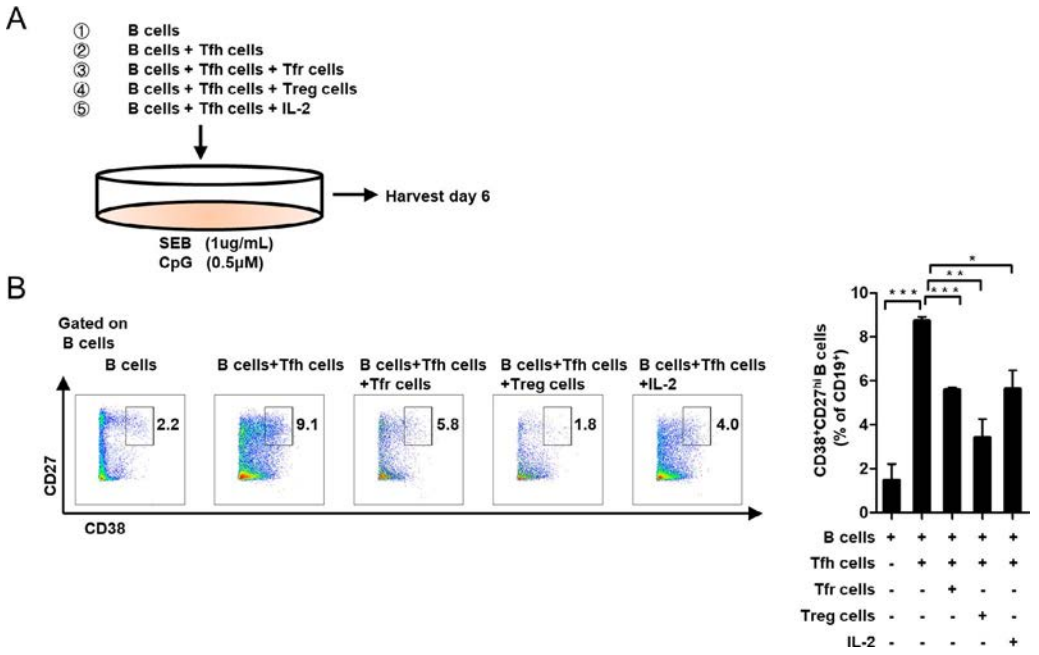


Figure 6. Effects of IL-2 on Tfh-mediated B cell activation. **A**, Sorted Tfh cells (3×10^4) were cocultured with 3×10^4 Tfr cells or Treg cells or stimulated with IL-2 (10 ng/ml) and then cultured with staphylococcal enterotoxin B (SEB) (1 μg/ml) and CpG (0.5 μ*M*) in the presence of 5×10^4 CD19+ B cells for 6 days. **B**, Representative flow cytometry plots showing expression of CD38 and CD27 in CD19+ B cells (left). Bar graphs show the percentages of CD38+CD27^{high} plasmablasts among CD19+ B cells under the various culture conditions (right). Data are the mean ± SEM of 3 independent experiments using samples obtained from 3 different donors. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41457/abstract.

(Figure 5B). To elucidate the direct effect of IL-2 on STAT phosphorylation, cells were washed to remove the effect of endogenous cytokines, and thereafter stimulated with IL-2 for 20 minutes. We included IL-6 as a control, since it generally mediates STAT3 activation. As expected, IL-6 only promoted STAT3 phosphorylation, whereas IL-2 promoted the phosphorylation of both STAT3 and STAT5 (Figure 5C). Collectively, the above results indicate that IL-2 can induce downstream events that lead to STAT3 and STAT5 phosphorylation, resulting in the conversion of Tfh cells to Tfr cells.

ChIP–PCR was performed to investigate whether IL-2–induced pSTAT3 and pSTAT5 directly regulated *FOXP3* and *BCL6* promoter regions. As predicted, STAT5 directly bound to *FOXP3* loci, whereas STAT3 bound to both *BCL6* and *FOXP3* loci around STAT binding sites following stimulation with IL-2 (Figures 5D and E).

Further, we evaluated the effect of IL-2 on transcriptional modification of *FOXP3* and *BCL6* loci. Both *FOXP3* and *BCL6* loci in TCR-stimulated CD4+ T cells exhibited bivalent histone modifications, such as permissive markers (H3K4me3 and H3K27ac3) and repressive markers (H3K27me3). After IL-2 stimulation, no changes in permissive markers H3K4me3 and H3K27ac3 on *FOXP3* and *BCL6* loci were observed. Conversely, the repressive marker H3K27me3 on *FOXP3* and *BCL6* loci was strongly suppressed following IL-2 stimulation (Figures 5D and E). These results suggest that IL-2 promotes the conversion of Tfh cells to Tfr cells by binding STAT3 and STAT5 to *FOXP3* and *BCL6* genes, with simultaneous suppression of H3K27me3.

Suppression of the generation of CD38+CD27^{high} plasmablasts by IL-2 and Tfr cells during coculture of Tfh cells and B cells. Finally, to directly assess the impact of IL-2 on Tfh-mediated B cell activation, CD4+CXCR5+CD45RA–CD25–CD127+ Tfh cells, CD4+CXCR5+CD45RA–CD25+CD127– Tfr cells, and CD4+CXCR5–CD45RA–CD25+CD127– Treg cells were sorted from the peripheral blood of healthy donors, with in vitro T cell–B cell cocultures used in the presence of staphylococcal enterotoxin B (SEB) superantigen and Toll-like receptor 9 agonists (CpG) (Figure 6A). The regulatory capacity of peripheral blood Tfr cells was also assessed. As expected, B cells differentiated into CD38+CD27^{high} plasmablasts in the presence of Tfh cells (Figure 6B). Both Tfr cells and Treg cells impaired the generation of CD38+CD27^{high} plasmablasts (Figure 6B). Moreover, IL-2 suppressed the generation of CD38+CD27^{high} plasmablasts when Tfh cells and B cells were cocultured.

DISCUSSION

In our study, we found that the proportion of CD45RA–FoxP3^{high} activated Tfr cells, which were characterized by high expression of IL-2Rα and CTLA-4, was decreased among CD4+CXCR5+FoxP3+ Tfr cells and negatively correlated with disease activity in SLE patients. We also confirmed that Tfr cells play a suppressive role during the generation of CD38+CD27^{high} plasmablasts. Furthermore, we found that patients with active

disease had a lower ratio of activated Tfr cells to Tfh cells, which suggested that the imbalance of Tfr and Tfh activation eventually led to the promotion of SLE development.

Our results are consistent with findings from a previous study that had shown increased numbers of PD-1^{high} activated Tfh cells in the blood of SLE patients (27). However, there have only been 2 studies concerning Tfr cells and the Tfr:Tfh ratio. A report by Robb et al described a reduction in the numbers of CD4+CXCR5+ CD25+CD127– Tfr cells and decreased ratio of Tfr cells to Tfh cells (16), while a study by Fonseca and colleagues demonstrated an increased level of CD4+CXCR5+FoxP3+ Tfr cells and increased ratio of Tfr cells to Tfh cells (15). In our study, we found that CD4+CXCR5+FoxP3+ Tfr cells would tend to increase in SLE patients, which is similar to the results observed by Fonseca et al (15). Considering that activated Tfr cells highly express CD25, the study by Robb and colleagues demonstrated that CD4+ CXCR5+CD25+CD127– Tfr cells are more likely to be activated Tfr cells, and the results of our study were consistent with these findings. Taken together, a more accurate definition may be more useful to understand the characteristics of Tfr cells.

Defective IL-2 production is commonly observed in SLE patients (28). Our study added to these findings by demonstrating that IL-2 was decreased at the protein and gene level. Proof-of-concept clinical trials have shown that low-dose IL-2 selectively activates and expands Treg cells and has demonstrated clinical efficacy in patients with SLE (29,30). However, the associated mechanisms of action remain uncharacterized. For example, how IL-2 might be involved in the differentiation or maintenance of Tfr cells is unclear. Hence, the role of IL-2 in Tfr cells is complex as it is required for and positively influences the differentiation of Tfr cells in the GC and in vitro cultures (31–33).

Interestingly, Treg cells are precursors of Tfr cells, and high concentrations of IL-2 at the peak of influenza infection precludes Tfr cell development by promoting the B lymphocyte–induced maturation protein 1–mediated repression of Bcl-6. However, as the infection resolves, IL-2 levels decrease, and IL-2Rα^{high} Treg cells lower levels of IL-2Rα and raise levels of Bcl-6 and differentiate into mature Tfr cells in the GC (34). Tfr cells may be partially independent from IL-2, whereas environments with low IL-2 levels may be required to induce the coexpression of FoxP3 and Bcl-6 in tissue Tfr cells. Additionally, distinct from tissue Tfr cells, circulating Tfr cells do not express the transcription factor Bcl-6 (35). We confirmed that the serum levels of IL-2 positively correlated with the levels of activated Tfr cells, and stimulation with IL-2 increased the levels of activated Tfr cells. Thus, IL-2 promotes human blood Tfr cell responses.

Tfh cells can be reprogrammed in vitro to Th1, Th2, and Th17 cells. Conversely, Th1, Th2, and Th17 cells can convert to Tfh cells (36–38). In gut Peyer's patches, Treg cells can convert into Tfh cells (39). Thus, Tfh cells might be treated as a distinct analogous lineage with the potential to convert into other subsets of T helper cells. Our results show that Tfh memory cells are not only compliant to regulation by Tfr cells (40,41), but can indeed become Tfr cells under the influence of the TCR and IL-2. It is worth noting that IL-2 alone did not promote the conversion of Tfh cells to Tfr cells as the sorted Tfh cells slightly express IL-2Rα. Following TCR stimulation, the expression of IL-2Rα on Tfh cells was enhanced (42).

We consider IL-2–activated pSTAT3+pSTAT5+ cells to be critical for the conversion of Tfh cells to Tfr cells. There is common agreement that STAT5 plays an important role in the development of FoxP3-expressing Treg cells. Transduction of FoxP3 can convert Tfh cells to functional Tfr cells without altering the expression of Bcl-6 and CXCR5 (40). In addition, STAT3 promotes Tfr cell differentiation by inducing Bcl-6 expression (43). Although IL-2 has been reported to activate STAT3 (44), the importance of its influence on human T cells remains unclear. Normally, IL-6, IL-10, and IL-23 are involved in STAT3 phosphorylation (45,46), whereas direct action from IL-2 is neglected. However, our results show that IL-2 also activates STAT3 and that expression of pSTAT3 and pSTAT5 is necessary for the conversion of Tfh cells to Tfr cells.

Recent studies have demonstrated that transcriptional regulation, guided by transcription factors, enable T cells to tune the threshold of specific gene expression, thereby helping to determine memory T cell fate and function in response to environmental stimuli (47,48). Our results showed that IL-2–activated STAT3 and STAT5 selectively bind to *FOXP3* and *BCL6* gene loci accompanied by suppression of H3K27me3. Previous studies have demonstrated that STAT5 binds to the *BCL6* promoter and directly represses Bcl-6 expression in response to strong IL-2–STAT5 signaling (49,50), whereas our data showed STAT5 did not bind to the *BCL6* region. Given that the occupation of STAT binding sites is cell-type specific, this could occur because different IL-2–STAT5 signals are received by cells depending on the concentrations of IL-2 in their environment and the duration for which cells are exposed (44). In addition, no changes were observed in H3K4me3 and H3K27ac3 on *FOXP3* and *BCL6* loci. We consider that permissive modifications of the *FOXP3* and *BCL6* loci are initiated by the TCR and maintained after IL-2 stimulation as previous studies have demonstrated that TCR stimulation induces *FOXP3* and *BCL6* gene expression (38,51). Thus, we postulate that IL-2 stimulation removed H3K27me3 modification at the *FOXP3* and *BCL6* regions, leading to enhanced Tfr cell gene expression in CD4+ T cells.

Certain limitations were noted in our research. For instance, peripheral T cell subsets may be affected by treatment with glucocorticoids and/or immunosuppressants (52). We initially tried to collect fresh blood samples from patients who were not receiving treatment, to avoid potential drug effects on Tfh cell and Tfr cell subsets. However, since the number of treatment-naive patients was small, we expanded the scope of patients and assessed the impact on Tfh cells and Tfr cells by treatment. Although our results indicate that immunosuppressive drugs reduced levels of activated Tfh cells, the precise mechanism responsible for

the modulatory effect of immunosuppressive therapy on the Tfh cell and Tfr cell balance requires further investigation. Additionally, characteristics of Tfr cells in the immune organs of SLE patients should be further examined.

In summary, our findings demonstrate that Tfr cells are vitally affected by IL-2 defects and that Tfr cell deficiency is apparent in SLE patients. IL-2 can restore the function of Tfr cells, not only by directly expanding the activated Tfr cell population, but also by indirectly converting Tfh cells to Tfr cells via the regulation of *FOXP3* and *BCL6* genes through histone modification, which could be one of the underlying mechanisms responsible for low-dose IL-2 treatment and could provide potential therapeutic approaches for SLE.

ACKNOWLEDGMENTS

We thank the patients and healthy volunteers for their cooperation and participation in the study.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Hao had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Hao, Nakayamada, Tanaka.

Acquisition of data. Hao, Yamagata, Ohkubo, Inoue, M. Zhang, Kanda Satoh.

Analysis and interpretation of data. Hao, Nakayamada, Iwata, T. Zhang, Shan, Otsuka, Tanaka.

REFERENCES

1. Comte D, Karampetsou MP, Tsokos GC. T cells as a therapeutic target in SLE. Lupus 2015;24:351–63.
2. Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular helper T cells [review]. Annu Rev Immunol 2016;34:335–68.
3. Crotty S. T follicular helper cell differentiation, function, and roles in disease. Immunity 2014;41:529–42.
4. Kubo S, Nakayamada S, Yoshikawa M, Miyazaki Y, Sakata K, Nakano K, et al. Peripheral immunophenotyping identifies three subgroups based on T cell heterogeneity in lupus patients. Arthritis Rheumatol 2017;69:2029–37.
5. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. Nat Med 2011;17:975–82.
6. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. Nat Med 2011;17:983–8.
7. Sage PT, Alvarez D, Godec J, von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. J Clin Invest 2014;124:5191–204.
8. Sage PT, Ron-Harel N, Juneja VR, Sen DR, Maleri S, Sungnak W, et al. Suppression by TFR cells leads to durable and selective inhibition of B cell effector function. Nat Immunol 2016;17:1436–46.
9. Wang L, Shen E, Luo L, Rabe H, Wang Q, Yin J, et al. Control of germinal center localization and lineage stability of follicular regulatory T cells by the Blimp1 transcription factor. Cell Rep 2019;29:1848–61.
10. Clement RL, Daccache J, Mohammed MT, Diallo A, Blazar BR, Kuchroo VK, et al. Follicular regulatory T cells control humoral and allergic immunity by restraining early B cell responses. Nat Immunol 2019;20:1360–71.
11. Yao Y, Wang ZC, Wang N, Zhou PC, Chen CL, Song J, et al. Allergen immunotherapy improves defective follicular regulatory T cells in patients with allergic rhinitis. J Allergy Clin Immunol 2019;144:118–28.
12. Liu C, Wang D, Lu S, Xu Q, Zhao L, Zhao J, et al. Increased circulating follicular Treg cells are associated with lower levels of autoantibodies in patients with rheumatoid arthritis in stable remission. Arthritis Rheumatol 2018;70:711–21.
13. Xu B, Wang S, Zhou M, Huang Y, Fu R, Guo C, et al. The ratio of circulating follicular T helper cell to follicular T regulatory cell is correlated with disease activity in systemic lupus erythematosus. Clin Immunol 2017;183:46–53.
14. Liu C, Wang D, Song Y, Lu S, Zhao J, Wang H. Increased circulating $CD4^+CXCR5^+FoxP3^+$ follicular regulatory T cells correlated with severity of systemic lupus erythematosus patients. Int Immunopharmacol 2018;56:261–8.
15. Fonseca VR, Romão VC, Agua-Doce A, Santos M, López-Presa D, Ferreira AC, et al. The ratio of blood T follicular regulatory cells to T follicular helper cells marks ectopic lymphoid structure formation while activated follicular helper T cells indicate disease activity in primary Sjogren's syndrome. Arthritis Rheumatol 2018;70:774–84.
16. Robb RJ, Munck A, Smith KA. T cell growth factor receptors: quantitation, specificity, and biological relevance. J Exp Med 1981;154:1455–74.
17. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. Immunity 2009;30:616–25.
18. Leon B, Bradley JE, Lund FE, Randall TD, Ballesteros-Tato A. FoxP3+ regulatory T cells promote influenza-specific Tfh responses by controlling IL-2 availability. Nat Commun 2014;5:3495.
19. Papillion A, Powell MD, Chisolm DA, Bachus H, Fuller MJ, Weinmann AS, et al. Inhibition of IL-2 responsiveness by IL-6 is required for the generation of GC-T_{FH} cells. Sci Immunol 2019;4.
20. Spolski R, Li P, Leonard WJ. Biology and regulation of IL-2: from molecular mechanisms to human therapy [review]. Nat Rev Immunol 2018;18:648–59.
21. Nakayamada S, Takahashi H, Kanno Y, O'Shea JJ. Helper T cell diversity and plasticity. Curr Opin Immunol 2012;24:297–302.
22. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.
23. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO III, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum 2010;62:2569–81.
24. Petri M, Kim MY, Kalunian KC, Grossman J, Hahn BH, Sammaritano LR, et al. Combined oral contraceptives in women with systemic lupus erythematosus. N Engl J Med 2005;353:2550–8.
25. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity 2009;30:899–911.
26. Ekman I, Ihantola EL, Viisanen T, Rao DA, Nanto-Salonen K, Knip M, et al. Circulating $CXCR5^-PD\text{-}1^{hi}$ peripheral T helper cells are associated with progression to type 1 diabetes. Diabetologia 2019;62:1681–8.
27. Choi JY, Ho JH, Pasoto SG, Bunin V, Kim ST, Carrasco S, et al. Circulating follicular helper–like T cells in systemic lupus erythematosus: association with disease activity. Arthritis Rheumatol 2015;67:988–99.
28. Von Spee-Mayer C, Siegert E, Abdirama D, Rose A, Klaus A, Alexander T, et al. Low-dose interleukin-2 selectively corrects regulatory T cell defects in patients with systemic lupus erythematosus. Ann Rheum Dis 2016;75:1407–15.

29. He J, Zhang X, Wei Y, Sun X, Chen Y, Deng J, et al. Low-dose interleukin-2 treatment selectively modulates CD4+ T cell subsets in patients with systemic lupus erythematosus. Nat Med 2016;22:991–3.
30. He J, Zhang R, Shao M, Zhao X, Miao M, Chen J, et al. Efficacy and safety of low-dose IL-2 in the treatment of systemic lupus erythematosus: a randomised, double-blind, placebo-controlled trial. Ann Rheum Dis 2020;79:141–9.
31. Li L, Yang SH, Yao Y, Xie YQ, Yang YQ, Wang YH, et al. Block of both TGF-β and IL-2 signaling impedes Neurophilin-1+ regulatory T cell and follicular regulatory T cell development. Cell Death Dis 2016;7:e2439.
32. Xie MM, Liu H, Corn C, Koh BH, Kaplan MH, Turner MJ, et al. Roles of T follicular helper cells and T follicular regulatory cells in autoantibody production in IL-2-deficient mice. Immunohorizons 2019;3:306–16.
33. Schulten V, Tripple V, Seumois G, Qian Y, Scheuermann RH, Fu Z, et al. Allergen-specific immunotherapy modulates the balance of circulating Tfh and Tfr cells [letter]. J Allergy Clin Immunol 2018;141:775–7.
34. Botta D, Fuller MJ, Marquez-Lago TT, Bachus H, Bradley JE, Weinmann AS, et al. Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. Nat Immunol 2017;18:1249–60.
35. Fonseca VR, Agua-Doce A, Maceiras AR, Pierson W, Ribeiro F, Romao VC, et al. Human blood T_{fr} cells are indicators of ongoing humoral activity not fully licensed with suppressive function. Sci Immunol 2017;2.
36. Lu KT, Kanno Y, Cannons JL, Handon R, Bible P, Elkahloun AG, et al. Functional and epigenetic studies reveal multistep differentiation and plasticity of in vitro-generated and in vivo-derived follicular T helper cells. Immunity 2011;35:622–32.
37. Reinhardt RL, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. Nat Immunol 2009;10:385–93.
38. Nakayamada S, Kanno Y, Takahashi H, Jankovic D, Lu KT, Johnson TA, et al. Early Th1 cell differentiation is marked by a Tfh cell-like transition. Immunity 2011;35:919–31.
39. Tsuji M, Komatsu N, Kawamoto S, Suzuki K, Kanagawa O, Honjo T, et al. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. Science 2009;323:1488–92.
40. Hou S, Clement RL, Diallo A, Blazar BR, Rudensky AY, Sharpe AH, et al. FoxP3 and Ezh2 regulate Tfr cell suppressive function and transcriptional program. J Exp Med 2019;216:605–20.
41. Xie MM, Fang S, Chen Q, Liu H, Wan J, Dent AL. Follicular regulatory T cells inhibit the development of granzyme B-expressing follicular helper T cells. JCI Insight 2019;4:e128076.
42. Snook JP, Kim C, Williams MA. TCR signal strength controls the differentiation of $CD4^+$ effector and memory T cells. Sci Immunol 2018;3:eaas9103.
43. Xu L, Huang Q, Wang H, Hao Y, Bai Q, Hu J, et al. The Kinase mTORC1 promotes the generation and suppressive function of follicular regulatory T cells. Immunity 2017;47:538–51.
44. Ross SH, Cantrell DA. Signaling and function of interleukin-2 in T lymphocytes [review]. Annu Rev Immunol 2018;36:411–33.
45. Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, et al. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. J Biol Chem 2007;282:9358–63.
46. Hutchins AP, Diez D, Miranda-Saavedra D. The IL-10/STAT3-mediated antiinflammatory response: recent developments and future challenges. Brief Funct Genomics 2013;12:489–98.
47. Yu B, Zhang K, Milner JJ, Toma C, Chen R, Scott-Browne JP, et al. Epigenetic landscapes reveal transcription factors that regulate CD8+ T cell differentiation. Nat Immunol 2017;18:573–82.
48. Piccirillo AR, Cattley RT, D'Cruz LM, Hawse WF. Histone acetyltransferase CBP is critical for conventional effector and memory T-cell differentiation in mice. J Biol Chem 2019;294:2397–406.
49. Walker SR, Nelson EA, Frank DA. STAT5 represses BCL6 expression by binding to a regulatory region frequently mutated in lymphomas. Oncogene 2007;26:224–33.
50. McDonald PW, Read KA, Baker CE, Anderson AE, Powell MD, Ballesteros-Tato A, et al. IL-7 signalling represses Bcl-6 and the TFH gene program. Nat Commun 2016;7:10285.
51. Wakamatsu E, Omori H, Kawano A, Ogawa S, Abe R. Strong TCR stimulation promotes the stabilization of Foxp3 expression in regulatory T cells induced in vitro through increasing the demethylation of Foxp3 CNS2. Biochem Biophys Res Commun 2018;503:2597–602.
52. Noris M, Casiraghi F, Todeschini M, Cravedi P, Cugini D, Monteferrante G, et al. Regulatory T cells and T cell depletion: role of immunosuppressive drugs. J Am Soc Nephrol 2007;18:1007–18.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 143–150
DOI 10.1002/art.41489

American College of Rheumatology
Empowering Rheumatology Professionals

Improvement of Severe Fatigue Following Nuclease Therapy in Patients With Primary Sjögren's Syndrome: A Randomized Clinical Trial

James Posada,[1] Saba Valadkhan,[2] Daniel Burge,[1] Kristen Davies,[3] Jessica Tarn,[3] John Casement,[3] Kerry Jobling,[4] Peter Gallagher,[3] Douglas Wilson,[5] Francesca Barone,[6] Benjamin A. Fisher,[7] and Wan-Fai Ng[3]

Objective. To assess the safety and efficacy of RSLV-132, an RNase Fc fusion protein, in a phase II randomized, double-blind, placebo-controlled clinical trial in patients with primary Sjögren's syndrome (SS).

Methods. Thirty patients with primary SS were randomized to receive treatment with RSLV-132 or placebo intravenously once per week for 2 weeks, and then every 2 weeks for 12 weeks. Eight patients received placebo and 20 patients received RSLV-132 at a dose of 10 mg/kg. Clinical efficacy measures included the European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index, EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI), Functional Assessment of Chronic Illness Therapy–Fatigue (FACIT-F), Profile of Fatigue (ProF), and the Digit Symbol Substitution Test (DSST).

Results. Patients randomized to receive RSLV-132 experienced clinically meaningful improvements in the ESSPRI score ($P = 0.27$), FACIT-F score ($P = 0.05$), ProF score ($P = 0.07$), and DSST ($P = 0.02$) from baseline to day 99, whereas patients who received placebo showed no changes in any of these clinical efficacy measures. This improvement was significantly correlated with increased expression of selected interferon-inducible genes (Pearson's correlations, each $P < 0.05$).

Conclusion. Administration of RSLV-132 improved severe fatigue, as determined by 4 independent patient-reported measures of fatigue, in patients with primary SS.

INTRODUCTION

Primary Sjögren's syndrome (SS) is a common chronic autoimmune disease that affects primarily women in the middle decades of life (1,2). In the majority of patients with primary SS, the disease is either mild or moderate, and common symptoms include profound fatigue, joint pain, and ocular and/or oral dryness. An estimated 70% of patients with primary SS report profound, debilitating fatigue as the single symptom that has the greatest negative impact on their quality of life. The biochemical basis of profound fatigue associated with primary SS continues to be an area of intense research effort, with many investigations focusing on the role of cytokines (3).

Chronic inflammation accompanied by activation of interferon (IFN)–inducible genes, mimicking the immune response to a viral infection, are common biochemical findings in patients with primary SS (4,5). One of the most fundamental roles of the immune system is to detect and respond to infection by retroviruses. Toll-like receptors and other pattern-recognition receptors are exquisitely sensitive to the presence of circulating RNA and respond with a robust activation of the innate immune system (6,7). In the case of autoimmune disease, such as primary SS and systemic lupus erythematosus (SLE), the presence of circulating autoantibodies that present RNA autoantigens to the immune system mimics retroviral infection and drives chronic inflammation. Approximately 80% of patients with primary SS

Clinicaltrials.gov identifier: NCT03247686.
Supported by Resolve Therapeutics, LLC.
[1]James Posada, PhD, Daniel Burge, MD: Resolve Therapeutics, St. Petersburg, Florida; [2]Saba Valadkhan, PhD: Case Western Reserve University, Cleveland, Ohio; [3]Kristen Davies, MBChB, Jessica Tarn, PhD, John Casement, PhD, Peter Gallagher, PhD, Wan-Fai Ng, PhD: Newcastle University, Newcastle upon Tyne, UK; [4]Kerry Jobling, MBChB: Newcastle Biomedical Research Centre and Newcastle upon Tyne Hospital NHS Foundation Trust, Newcastle upon Tyne, UK; [5]Douglas Wilson, PhD: Q2 Solutions, Morrisville, North Carolina; [6]Francesca Barone, PhD: University of Birmingham, Birmingham, UK; [7]Benjamin A. Fisher, MD: NIHR Birmingham Biomedical Research Centre, University Hospitals Birmingham NHS Foundation Trust, and University of Birmingham, Birmingham, UK.

Dr. Posada owns stock or stock options in Resolve Therapeutics. Dr. Fisher has received consulting fees from Novartis, Roche, Bristol Myers Squibb, and Servier (less than $10,000 each). No other disclosures relevant to this article were reported.

Address correspondence to James Posada, PhD, 721 First Avenue North, St. Petersburg, FL 33701. Email: jp@resolvebio.com.

Submitted for publication March 17, 2020; accepted in revised form August 6, 2020.

have anti-Ro/SSA autoantibodies, which bind to autoantigens containing small noncoding RNA molecules (8–10). These immune complexes are known to trigger inflammatory cytokine production in vitro (11). Furthermore, a large observational study in SLE patients demonstrated a correlation between the presence of RNA-containing immune complexes, chronic IFN pathway activation, and disease activity (12). In addition to RNA autoantigens, many noncoding RNAs are found in the circulation, and many of these noncoding RNAs possess inflammatory gene regulatory functions (13).

RSLV-132 is a biologic drug composed of a full-length, catalytically active human RNase moiety fused to the amino-terminus of an engineered human IgG1 Fc domain. The drug is engineered to remain in the circulation and will not enter cells bearing Fc receptors. The RNase portion of RSLV-132 maintains full enzymatic activity as compared to wild-type human RNase, and has a serum half-life of ~19 days (14). Given the body of evidence implicating circulating RNAs in patients with primary SS, the present study sought to evaluate the biologic and clinical impact of treating the symptoms of primary SS with RSLV-132 nuclease therapy, which leads to significantly increased RNA digestion activity in the whole blood.

PATIENTS AND METHODS

Patients. For the study, we enrolled participants (ages 18–85 years) who were diagnosed as having primary SS according to the American–European Consensus Group 2002 classification criteria (15) and who had elevated serum levels of anti-Ro52/60 autoantibodies, based on the results of central laboratory testing, and a positive IFN signature at screening. A positive IFN signature was defined as expression levels of HERC5, CMPK2, and EPSTI1 that were 2 SD above the values in healthy volunteers (12). Study subjects were required to have been receiving stable concomitant medications for ≥30 days prior to the baseline visit. Patients were excluded on the basis of prior use of any of the following medications: hydroxychloroquine and glucocorticoids within 30 days of baseline; belimumab, abatacept, or tumor necrosis factor inhibitors within 90 days of baseline; or cyclophosphamide or rituximab within 180 days of baseline. Patients were also excluded if they had previously received head and neck radiation therapy or had lymphoma, graft-versus-host disease, or IgG4-related disease.

Study design. Study subjects were randomized to receive the study treatment beginning on January 12, 2017, and the last subject follow-up visit occurred on March 14, 2020. Randomization was conducted by computer algorithm and transmitted to an unblinded pharmacist at each of the 2 clinical evaluation sites. Subjects were randomized 3:1 (RSLV-132:placebo) to receive 10 mg/kg RSLV-132 or placebo on days 1, 8, 15, 29, 43, 53, 71, and 85 by intravenous infusion. The efficacy end points were measured on day 99, and safety follow-ups were conducted on days 141, 176, and 211. The study was conducted in accordance with the principles of the Declaration of Helsinki and the International Council for Harmonisation Guidelines for Good Clinical Practice. Ethics committee and institutional review board approval were obtained, and all patients provided written informed consent.

Efficacy and safety evaluations. The primary biochemical evaluations in this randomized clinical trial were the expression patterns of IFN-inducible genes contained in 3 modules, M1.2, M3.4, and M5.12, as previously described by Chiche et al (16). Clinical efficacy measures included the European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (ESSDAI) (scale 0–123) (17), the EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI) (scale 0–10) (18), the Functional Assessment of Chronic Illness Therapy–Fatigue (FACIT-F) (scale 0–52) (19), and the Profile of Fatigue (ProF) (scale 0–6) (20). A subset of 16 subjects completed the Digit Symbol Substitution Test (DSST), a neuropsychological evaluation of concentration and attention that measures the time required for the correct translation of numbers into symbols (with results herein expressed as the mean time to complete the test) (21). Exploratory analyses included anti-Ro52/60 autoantibody levels, erythrocyte sedimentation rate (ESR) levels, complement C3 and C4 levels, and total immunoglobulin levels. In addition, adverse events were recorded at each visit.

Analysis of gene expression. RNA sequencing of whole blood samples was performed in the laboratory at Q2 Solutions/EA Genomics in Morrisville, North Carolina. Whole blood was collected in PAXGene collection tubes on days 1 and 99 prior to study treatment. RNA was extracted and quantitated by spectrophotometry using a ThermoFisher NanoDrop 8000 instrument, and RNA integrity was assessed using an RNA 6000 Nano Assay on a Bioanalyzer 2100.

Fifty-basepair stranded and paired-end sequencing libraries were generated using an Illumina TruSeq Stranded Total RNA protocol with RiboZero Magnetic Gold depletion of ribosomal RNA. Libraries were sequenced on an Illumina HiSeq 2500 to a target depth of 50 million reads. Prior to gene mapping, adapter trimming, homopolymer filtering, and low-quality read filtering were performed. Preprocessed reads were mapped to the hg38 assembly of the human genome using kallisto version 0.45.0 (22) with the use of Gencode release 31 of human reference gene annotations. For analysis of the IFN-inducible genes, reads were mapped to Gencode release 33 (GRCh38) transcript sequences, using Salmon version 1.1.0 to obtain transcript level quantification estimates. These transcript level estimates were aggregated to gene-level counts using Bioconductor tximport version 1.14.0. Differential gene expression analysis was carried out using Bioconductor DESeq2 version 1.26.0.

Statistical analysis. Formal hypothesis testing was not conducted in this study. To analyze the ESSDAI, ESSPRI, FACIT-F, ProF, and DSST data, the mean values for change from baseline for each group were analyzed using separate one-way analysis of variance models for each visit, each testing the null hypothesis that the true mean difference between treatment groups was 0, with an unadjusted significance level of $\alpha = 0.05$. To analyze the rate of response to treatment in the RSLV-132 group compared to the placebo group, a Fisher's exact test was used for testing the null hypothesis for each clinical instrument. For analysis of gene expression data, DESeq2 estimates of the fold change in IFN-inducible gene expression between experimental conditions using a negative binomial generalized linear regression model with a logarithmic link function were used. The fold change values and *P* values were derived by carrying out Wald tests on the resulting likelihood functions. In the implementation of the Wald test used by DESeq2, the maximum likelihood estimate for the fold change is divided by its standard error to obtain a test statistic, which is then compared to a standard normal distribution.

To analyze the correlation between the expression patterns of the individual genes contained in the 3 modules of IFN-inducible genes and the scores from the patient-reported instruments (mental fatigue, somatic fatigue, and ESSPRI), module scores for each patient were calculated as the mean normalized gene expression across all genes in each module. The normalization method used was the median of ratios method, as used by DESeq2. Pearson's correlation coefficients were calculated to assess the correlations between changes in the mean normalized gene expression for each module and changes in the mean scores for mental fatigue, somatic fatigue, and ESSPRI.

RESULTS

Clinical efficacy outcomes. Thirty-two subjects were screened for entry into the study, and 2 subjects not meeting the autoantibody criteria were excluded. The remaining 30 subjects were randomized into the study at 2 academic medical centers in the UK. Two subjects in the RSLV-132 treatment group withdrew

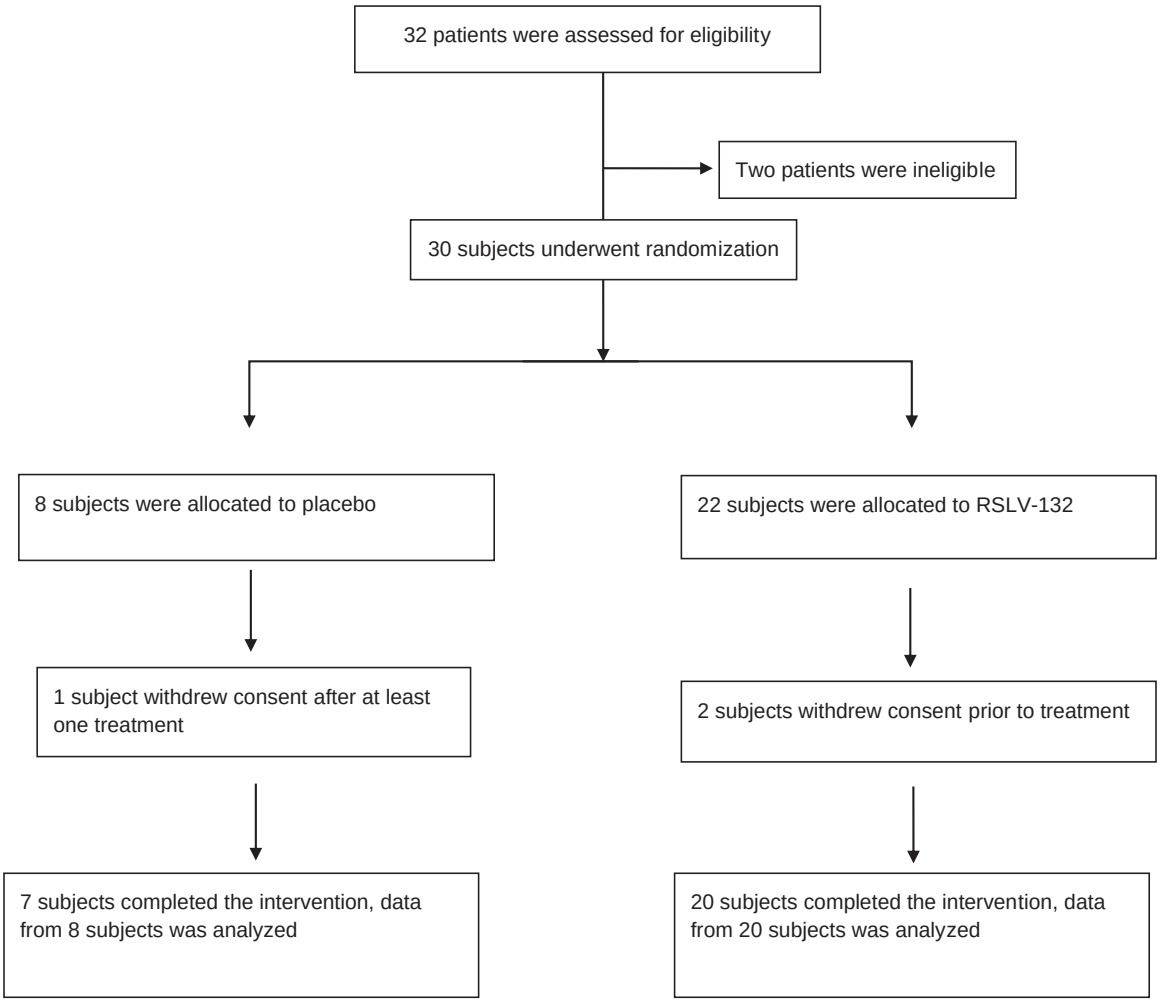


Figure 1. Distribution of patients with primary Sjögren's syndrome to the randomized treatment groups, with follow-up. Two subjects did not meet the eligibility criterion requiring serum positivity for anti-Ro autoantibodies. Eligible patients were randomized to receive RSLV-132 (10 mg/kg) or placebo, once weekly for 2 weeks and then every 2 weeks for 12 weeks.

consent prior to receiving study treatment at the baseline visit. One subject in the placebo group withdrew consent after receiving at least one dose of study treatment. Twenty subjects in the RSLV-132 group and 7 subjects in the placebo group completed the study. The modified intent-to-treat analysis set consisted of 28 subjects who received at least one infusion of study drug. Data from 8 subjects in the placebo group and 20 in the RSLV-132 group were analyzed (Figure 1).

Baseline demographics, disease characteristics, and biochemical data were similar between the treatment groups. The study population had mild-to-moderate disease activity as determined by the ESSDAI, but had high symptom burden according to the ESSPRI. Study subjects also reported experiencing profound fatigue, as indicated by scores on the FACIT-F and ProF instruments. ESSDAI and ESSPRI scores in the placebo group were modestly higher than those in the RSLV-132 group. Levels of C3 and C4 complement and IgG in the serum and ESR levels were similar between the 2 groups (Table 1).

The primary end point in the study was analysis of IFN-inducible gene expression. The IFN-inducible genes contained in the 3 modules (M1.2, M3.4, and M5.12) were analyzed for changes between study day 1 and study day 99 (Figures 2A and B; for individual genes, see Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41489/abstract). Comparison of the mean expression of the genes within these modules revealed increased expression of selected IFN-inducible genes in the RSLV-132–treated patients as compared to the placebo-treated patients. For example, in module M1.2, the mean $\log_2$ fold change in gene expression between day 1 and day 99 was –0.03 in the placebo group and +0.13 in the RSLV-132 group. For module M3.4, the mean $\log_2$ fold change in gene expression between day 1 and day 99 was –0.03 in the placebo group and +0.08 in the RSLV-132 group. For module M5.12, the mean $\log_2$ fold change in gene expression between day 1 and day 99 was –0.06 in the placebo group and +0.03 in the RSLV-132 group.

Table 1. Baseline demographic and clinical characteristics of the study patients in each randomized group*

	Placebo (n = 8)	RSLV-132 (n = 20)
Age, years	59.6 ± 8.8	56.5 ± 12.9
Sex, %		
Female	100	100
Male	0	0
Race, %		
White	87.5	95
Asian	12.5	5
Ethnicity, %		
Not Hispanic or Latino	100	95
Hispanic or Latino	0	5
Height, cm	165.13 ± 4.97	163.22 ± 8.05
Weight, kg	81.4 ± 22.71	70.66 ± 13.95
BMI, kg/m^2	29.79 ± 8.20	26.52 ± 4.56
Complement C3, mg/dl	125.3 ± 33.1	134.1 ± 24.0
Complement C4, mg/dl	19.0 ± 6.2	19.6 ± 8.2
IgG, mg/dl	1,686 ± 563	1,683 ± 810
ESR, mm/hour	23.3 ± 12.1	33.2 ± 33.9
ESSDAI score	5.4 ± 4.1	5.0 ± 4.6
Score ≤4, no. (%)	4 (50)	12 (60)
Score ≥5, no. (%)	4 (50)	8 (40)
ESSPRI score	6.42 ± 2.48	5.97 ± 1.57
FACIT-F score	23.9 ± 11.41	29.6 ± 12.09
ProF score	4.0 ± 1.9	3.5 ± 1.2
Prednisone, no. (%)†	2 (25)	1 (5)

* Except where indicated otherwise, values are the mean ± SD. BMI = body mass index; ESR = erythrocyte sedimentation rate; ESSDAI = European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (scale 0–123); ESSPRI = EULAR Sjögren's Syndrome Patient Reported Index (scale 0–10); FACIT-F = Functional Assessment of Chronic Illness Therapy–Fatigue (scale 0–52); ProF = Profile of Fatigue (scale 0–6).
† Administered as a concomitant immunomodulatory medication.

IFN-inducible gene expression in the subgroup of RSLV-132–treated patients who experienced a clinical response (designated responders; defined as subjects who achieved minimal clinically important improvement in 2 of 3 measures [ESSPRI, FACIT-F, or ProF scores]) was compared to that in RSLV-132–treated patients who did not experience a clinical response (designated nonresponders). The results revealed that IFN-inducible gene up-regulation was higher in the RSLV-132 group compared to the placebo group. In module M1.2, the mean $\log_2$ fold change in IFN-inducible gene expression between day 1 and day 99 was +0.07 in nonresponders and +0.25 in responders. For module M3.4, the mean $\log_2$ fold change in IFN-inducible gene expression was +0.04 in nonresponders and +0.16 in responders. For module M5.12, the mean $\log_2$ fold change in IFN-inducible gene expression was +0.02 in nonresponders and +0.04 in responders (Figure 2B).

The results of the Wald test analyzing the change in expression of each individual gene in each of the 3 modules failed to identify any genes that had a statistically significant difference in expression between the placebo and RSLV-132 groups or between the responder and nonresponder subgroups over the course of the study. Further analysis of the correlation of changes in expression of these IFN-inducible genes and performance on 3 patient-reported outcome measures (ESSPRI, mental fatigue, and somatic fatigue scores) were conducted by analyzing the changes between day 1 and day 99 of the study. Most IFN-inducible genes demonstrated a very weak or no correlation with the changes in the patient-reported outcome measures, with the exception of a strong, statistically significant correlation between the mental fatigue scores and the expression of IFN-inducible genes contained in module M1.2 among patients in the RSLV-132–treated group (P = 0.014) (see Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41489/abstract).

The secondary end point in the study was the ESSDAI, a measure of disease activity. Mean ESSDAI scores in the RSLV-132 group remained constant, with a mean score of 5 throughout the study, whereas the mean ESSDAI score in the placebo

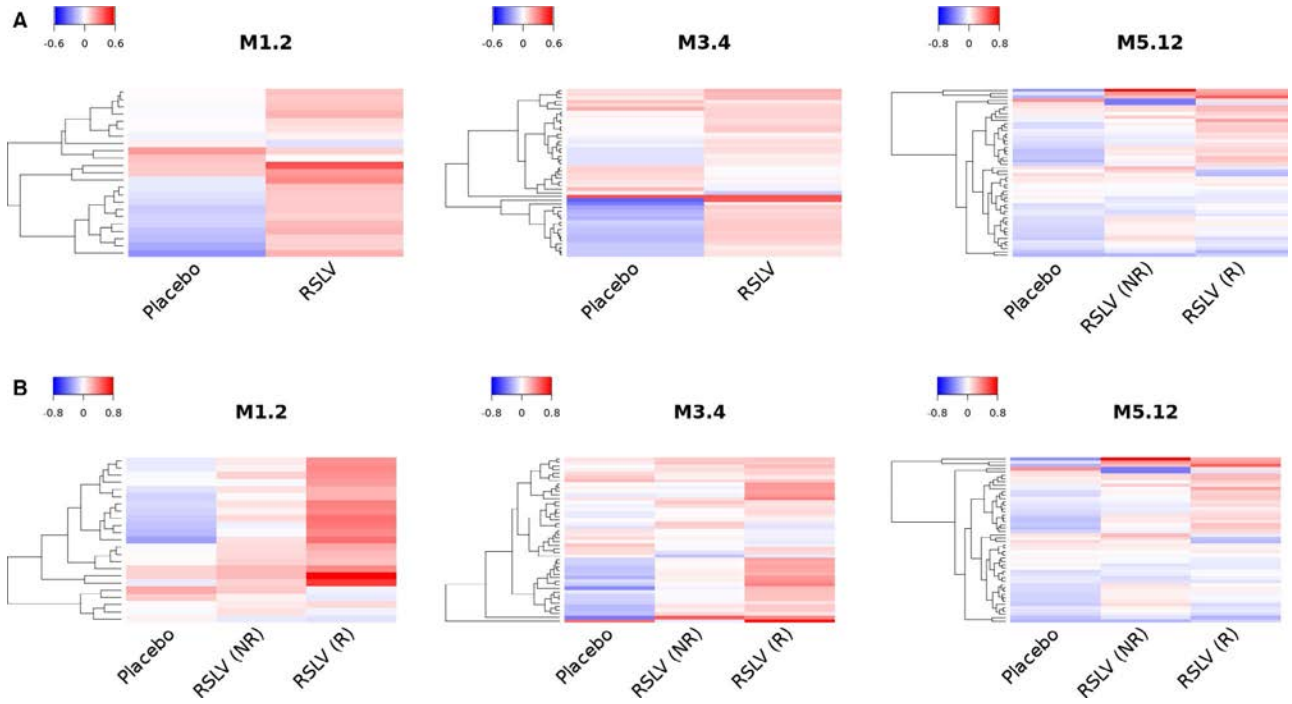


Figure 2. Heatmaps showing changes in expression of interferon (IFN)–inducible genes from day 1 to day 99. The $\log_2$ fold change in expression of 3 modules (M1.2, M3.4, and M5.12) of IFN-inducible genes was assessed in whole blood samples from the placebo group (n = 7) compared to the RSLV-132 group as a whole (n = 20) (**A**) or the subgroups of RSLV-132–treated patients who either achieved a clinical response (R) (n = 13) or did not achieve a clinical response (NR) (n = 7) (**B**) over the follow-up.

group declined from a mean of 5 at baseline to a mean of 2.9 by day 99. This reduction was based largely on the change in ESSDAI score in 2 outlier placebo subjects who had peripheral nervous system and glandular symptoms at baseline that had resolved by day 29.

Several patient-reported outcome instruments were used in the study to measure changes in fatigue, ocular and oral dryness, and joint pain. Subjects in the RSLV-132 group experienced improvement in the mean ESSPRI score from baseline to day 99 (mean change from baseline –1.2 points), while subjects in the

Table 2. Clinical efficacy measures*

	Placebo (n = 8)	RSLV-132 (n = 20)	*P* between groups on day 99
Mean on day 99 (mean change from baseline)			
ESSDAI score	2.9 (−2.50)	5.0 (0.00)	0.28†
ESSPRI score	5.88 (−0.54)	4.75 (−1.22)	0.27†
ESSPRI fatigue subscale score	6.30 (0.00)	4.60 (−1.40)	0.19†
FACIT-F score	25.00 (1.13)	35.50 (5.90)	0.05†
ProF score	3.98 (−0.02)	2.54 (−1.04)	0.07†
Somatic component	4.17 (0.00)	2.87 (−0.80)	0.13†
Mental component	3.75 (0.06)	2.13 (−1.53)	0.04†
DSST time to complete, seconds	(+2.8)	(−16.4)	0.02†
Responders on day 99, no. (%)			
≥3-point decrease in ESSDAI	3 (37.5)	4 (20)	0.75‡
≥1-point decrease in ESSPRI	1 (12.5)	12 (60)	0.06‡
≥6-point increase in FACIT-F	2 (25)	9 (45)	0.002‡
≥1-point decrease in ProF somatic	2 (25)	10 (50)	0.37‡
≥1-point decrease in ProF mental	2 (25)	11 (55)	0.19‡

* ESSDAI = European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index; ESSPRI = EULAR Sjögren's Syndrome Patient Reported Index; FACIT-F = Functional Assessment of Chronic Illness Therapy–Fatigue; ProF = Profile of Fatigue; DSST = Digit Symbol Substitution Test.
† Mean values and change from baseline were compared between groups using separate one-way analyses of variance for each visit, with each testing the null hypothesis that the true mean difference between treatment groups was 0 (unadjusted $\alpha = 0.05$).
‡ Differences in the rates of response between treatment groups were analyzed using Fisher's exact tests, with each testing the null hypothesis for each clinical instrument.

placebo group showed a mean improvement of –0.54 points (Table 2).

Additional clinical end points included change from baseline in the FACIT-F score, ProF score, and DSST (measured as time to complete the test). The mean ESSPRI fatigue score was reduced in the RSLV-132 group at day 99 (mean change from baseline –1.4 points) whereas the mean change from baseline was 0 in the placebo group (Figure 3A). Subject-level data revealed that 25% of subjects in the placebo group and 55% of RSLV-132–treated subjects achieved minimal clinically important improvement in the ESSPRI score by day 99 (Table 2).

The impact of RSLV-132 on fatigue in patients with primary SS was further evaluated using 2 additional patient-reported outcome measures, the FACIT-F and the ProF mental fatigue scores. Mean FACIT-F scores increased from baseline by a mean 1.13 points in the placebo group compared to a mean increase of 5.90 points in the RSLV-132 group (Figure 3B). Subject-level data revealed that 25% of subjects in the placebo group and 45% of the RSLV-132–treated subjects achieved minimal clinically important improvement in the FACIT-F score by day 99.

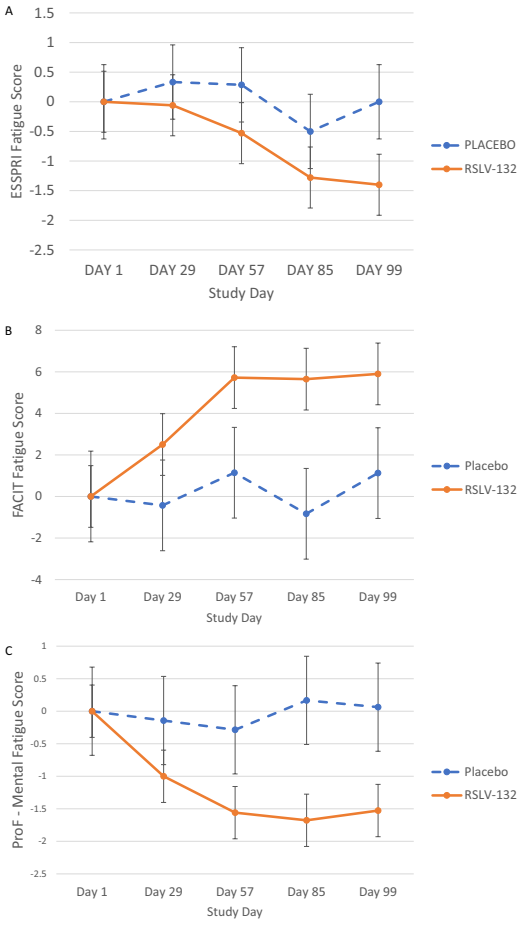


Figure 3. Secondary end point efficacy measures. Clinical efficacy was assessed as the mean change from baseline in the fatigue component of the European League Against Rheumatism Sjögren's Syndrome Patient Reported Index (ESSPRI) (**A**), Functional Assessment of Chronic Illness Therapy–Fatigue (FACIT-F) (**B**), and mental fatigue component of the Profile of Fatigue (ProF) (**C**) in the RSLV-132 and placebo treatment groups. Groups were compared using separate one-way analysis of variance models for each visit, each testing the null hypothesis that the true mean difference between treatment groups was 0 (unadjusted $\alpha = 0.05$). Between-group differences were as follows: $P = 0.136$ in **A**, $P = 0.092$ in **B**, and $P = 0.046$ in **C**. Results at each time point are the mean ± SEM.

With respect to the mean change from baseline in the ProF mental fatigue score, the placebo group experienced a mean reduction of 0.02 points, while the RSLV-132–treated group experienced a mean reduction of 1.04 points. The ProF can be subdivided into somatic and mental components. The placebo group did not experience an improvement in the somatic fatigue score, whereas the RSLV-132 group had a mean decrease in the somatic fatigue score of 0.8 points. The largest change was observed in the mental fatigue component, in which the placebo group experienced a mean decrease in the mental fatigue score of 0.06 points, while the RSLV-132 group experienced a mean decrease in the mental fatigue score of 1.53 points (Figure 3C).

Among the subset of 16 patients who were administered the DSST, patients in the placebo group were observed to have a slight worsening in the mean time to complete the DSST (mean change from baseline +2.8 seconds), whereas patients in the RSLV-132 group showed improvement in the mean time to complete the DSST (mean change from baseline –16.4 seconds) (Table 2).

Exploratory end points included anti-Ro/SSA levels, immunoglobulin levels, and ESR. There were no significant changes in autoantibody, immunoglobulin, or ESR levels in either treatment group during the study (data not shown). Ocular and oral dryness were measured using the Schirmer's test (for eye dryness) and stimulated and unstimulated salivary flow tests (for mouth dryness). No meaningful differences between the RSLV-132 and placebo groups were observed for any of these measures (data not shown).

Safety and tolerability. The incidence of treatment-emergent adverse events, serious adverse events, and drug-related adverse events were comparable between the RSLV-132 and placebo treatment groups (Table 3). No deaths occurred during the study. There were no serious infections or infusion reactions observed in either treatment group during the study. No patients discontinued the study drug due to an adverse event. One patient in the RSLV-132 group experienced a serious adverse event and was hospitalized for parotitis 88 days after receiving the last dose of study drug.

Pharmacokinetics and immunogenicity. RSLV-132 protein concentrations in the serum were measured using a validated electrochemiluminescence-based assay. In addition, RSLV-132 catalytic RNase activity was measured using an RNase enzyme assay. We found that the 2 measurements were highly correlated. The median RSLV-132 serum concentration, as determined by enzyme-linked immunosorbent assay in the serum at

Table 3. Treatment-emergent adverse events (TEAEs) in the safety analysis set*

	Placebo (n = 8)	RSLV-132 (n = 20)
At least 1 TEAE	8 (100)	20 (100)
At least 1 drug-related TEAE	5 (62.5)	13 (65)
At least 1 serious AE	0	1 (5)†
At least 1 drug-related serious TEAE	0	0
Infections	6 (75)	16 (80)
Deaths	0	0
Most common AEs		
Fatigue	1 (12.5)	6 (30)
URTI	2 (25)	5 (25)
Arthralgia	0	5 (25)
Viral URI	1 (13)	4 (20)
Conjunctivitis	1 (13)	3 (15)
Headache	1 (13)	3 (15)
LRTI	3 (38)	1 (5)
Worsening of Sjögren's syndrome	2 (25)	0

* Values are the number (%) of patients. URTI = upper respiratory tract infection; URI = upper respiratory infection; LRTI = lower respiratory tract infection.
† Hospitalized for parotitis 88 days after receiving the last dose of study drug; the event was unrelated to the study drug.

steady state, was 4 μg/ml (1.3–11.0 μg/ml). The RNase catalytic enzyme activity measurement of serum drug levels was 4.6 μg/ml (2.8–12 μg/ml) at steady state on day 57 (data not shown). A clear correlation between serum RSLV-132 levels and clinical responses was not observed in this study. Serum was assayed at multiple time points for anti–RSLV-132 antibodies using an assay validated in accordance with US Food and Drug Administration guidance. None of the subjects in the study were positive for anti–RSLV-132 antibodies (data not shown).

DISCUSSION

In the present study, RSLV-132–induced up-regulation of selected IFN-inducible genes was observed, a finding that was unexpected based on the mechanism of action of RSLV-132. Although increased expression of IFN-inducible genes has historically been thought to be associated with higher disease activity, recent studies have highlighted a counterintuitive relationship between cytokines and fatigue in patients with primary SS. For example, in an observational study of 159 patients with primary SS, the serum levels of several cytokines were observed to increase as fatigue decreased (23,24). In another observational study of 2 European cohorts of patients with primary SS, fatigue was observed to decrease as systemic IFN activity increased (25). In a third large observational study from the UK, conducted in 608 patients with primary SS, patients with the lowest symptom burden had the highest expression of IFN-inducible genes (26). In a recent clinical study of hydroxychloroquine for the treatment of primary SS, it was observed that hydroxychloroquine treatment resulted in a significant decrease in IFN-inducible gene expression, but no clinical improvement (27).

Furthermore, in a phase III clinical trial involving treatment of patients with SLE with anifrolumab, an anti–IFN receptor antibody, improvement in the British Isles Lupus Assessment Group–based Composite Lupus Assessment score at week 52 was greater in the active treatment group as compared to the placebo group (47.8% versus 31.5%) (28). It is unclear what impact anifrolumab might have on fatigue and other patient-reported outcome measures, as these data await further study. Interestingly, at a biochemical level, the majority of anifrolumab-treated patients in that previous study had a significant reduction in IFN-inducible gene expression, although only a small subset of those subjects experienced clinical benefit incremental to placebo treatment (28). The cumulative data on the role of the IFN signature in patients with primary SS suggest that increased activation of this pathway may be a homeostatic compensatory mechanism to overcome the disease, since increased activation of the pathway is correlated with improved symptoms in primary SS.

RSLV-132 contains a catalytically active RNase enzyme moiety, which in the context of primary SS was hypothesized to digest RNA associated with immune complexes that are inducing IFN expression from immune system cells. This would be expected to decrease IFN-inducible gene activation. However, in the present study, increased RNase activity in the circulation resulted in an increase, not decrease, in the expression of IFN-inducible genes in module M1.2. Since our analysis did not measure IFNα directly, but rather analyzed the expression of genes under the transcriptional regulation of IFNα as a proxy for its increase, we cannot rule out the possibility that RNA molecules in the circulation that have a negative regulatory effect on these IFN-inducible genes were removed or decreased by RSLV-132 treatment, thereby resulting in the increased expression of selected IFN-inducible genes.

The presence of circulating microRNAs and their sensitivity to RNase digestion is well established, as is the regulation of various Toll-like receptor pathways by these RNAs (6,29). The RSLV-132–induced increase in selected IFN-inducible genes correlated with decreased fatigue based on several different patient-reported outcome measures. Broad-based improvements in the RSLV-132 treatment group were noted in the ESSPRI scores, FACIT-F scores, ProF scores, and time to complete the DSST test. Of note, one limitation of this study was the relatively small number of patients included in the analyses.

Notwithstanding the derivative nature and complexity of the biomarker data, the present study presents the first compelling evidence from an interventional randomized clinical trial to show that the profound fatigue experienced by patients with primary SS can be improved by pharmacologic intervention with nuclease therapy such as RSLV-132. The data support continued development of RSLV-132 as a treatment strategy in patients with primary SS, with future testing in additional, larger randomized clinical trials.

ACKNOWLEDGMENT

We thank the patients who participated in the study and who agreed to make the data available to the scientific community.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Posada had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Posada, Burge, Fisher, Ng.
Acquisition of data. Posada, Valadkhan, Burge, Davies, Tarn, Casement, Jobling, Gallagher, Wilson, Barone, Fisher, Ng.
Analysis and interpretation of data. Posada, Burge, Fisher, Ng.

ROLE OF THE STUDY SPONSOR

Resolve Therapeutics, LLC funded the study. Authors Posada and Burge are employees of Resolve Therapeutics, LLC and were involved in the study design, the collection, analysis, and interpretation of the data, the writing of the manuscript, and the decision to submit the manuscript for publication. Publication of this article was not contingent upon approval by Resolve Therapeutics, LLC.

ADDITIONAL DISCLOSURE

Author Wilson is an employee of Q2 Solutions.

REFERENCES

1. Haldorsen K, Bjelland I, Bolstad AI, Jonsson R, Brun JG. A five-year prospective study of fatigue in primary Sjögren's syndrome. Arthritis Res Ther 2011;13:R167.
2. Overman CL, Kool MB, da Silva JA, Geenen R. The prevalence of severe fatigue in rheumatic diseases: an international study. Clin Rheumatol 2016;35:409–15.
3. Bodewes IL, van der Spek PJ, Leon LG, Wijkhuijs AJ, van Helden-Meeuwsen CG, Tas L, et al. Fatigue in Sjögren's syndrome: a search for biomarkers and treatment targets. Front Immunol 2019;10:312.
4. Nezos A, Gravani F, Tassidou A, Kapsogeorgou EK, Voulgarelis M, Koutsilieris M, et al. Type I and II interferon signatures in Sjögren's syndrome pathogenesis: contributions in distinct clinical phenotypes and Sjögren's related lymphomagenesis. J Autoimmun 2015;63:47–58.
5. Hall JC, Baer AN, Shah AA, Criswell LA, Shiboski CH, Rosen A, et al. Molecular subsetting of interferon pathways in Sjögren's syndrome. Arthritis Rheumatol 2015;67:2437–46.
6. Driedonks TA, Hoen EN. Circulating Y-RNAs in extracellular vesicles and ribonucleoprotein complexes: implications for the immune system [review]. Front Immunol 2019;9:3164.
7. Katze MG, Fornek JL, Palermo RE, Walters KA, Korth MJ. Innate immune modulation by RNA viruses: emerging insights from functional genomics [review]. Nat Rev Immunol 2008;8:644–54.
8. Moutsopoulos HM, Zerva LV. Anti-Ro (SSA)/La (SSB) antibodies and Sjögren's syndrome. Clin Rheumatol 1990;9:123–30.
9. Kattah NH, Kattah MG, Utz PJ. The U1-snRNP complex: structural properties relating to autoimmune pathogenesis in rheumatic diseases [review]. Immunol Rev 2010;233:126–45.
10. Noll F, Behnke J, Leiting S, Troidl K, Alves GT, Müller-Redetzky H, et al. Self-extracellular RNA acts in synergy with exogenous danger signals to promote inflammation. PLoS One 2017;12:e0190002.
11. Eloranta ML, Lövgren T, Finke D, Mathsson L, Rönnelid J, Kastner B, et al. Regulation of the interferon-α production induced by RNA-containing immune complexes in plasmacytoid dendritic cells. Arthritis Rheum 2009;60:2418–27.
12. Doedens JR, Jones WD, Hill K, Mason MJ, Gersuk VH, Mease PJ, et al. Blood-borne RNA correlates with disease activity and IFN-stimulated gene expression in systemic lupus erythematosus. J Immunol 2016;197:2854–63.
13. Hur K, Kim SH, Kim JM. Potential implications of long noncoding RNAs in autoimmune diseases [review]. Immune Netw 2019;19:e4.
14. Burge DJ, Eisenman J, Byrnes-Blake K, Smolak P, Lau K, Cohen SB, et al. Safety, pharmacokinetics, and pharmacodynamics of RSLV-132, an RNase-Fc fusion protein in systemic lupus erythematosus: a randomized, double-blind, placebo-controlled study. Lupus 2017;26:825–34.
15. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al, and the European Study Group on Classification Criteria for Sjögren's Syndrome. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002;61:554–8.
16. Chiche L, Jourde-Chiche N, Whalen E, Presnell S, Gersuk V, Dang K, et al. Modular transcriptional repertoire analyses of adults with systemic lupus erythematosus reveal distinct type I and type II interferon signatures. Arthritis Rheumatol 2014;66:1583–95.
17. Seror R, Ravaud P, Bowman SJ, Baron G, Tzioufas A, Theander E, et al, on behalf of the EULAR Sjögren's Task Force. EULAR Sjögren's Syndrome Disease Activity Index: development of a consensus systemic disease activity index for primary Sjögren's syndrome. Ann Rheum Dis 2010;69:1103–9.
18. Seror R, Ravaud P, Mariette X, Bootsma H, Theander E, Hansen A, et al, on behalf of the EULAR Sjögren's Task Force. EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI): development of a consensus patient index for primary Sjögren's syndrome. Ann Rheum Dis 2011;70:968–72.
19. Cella D, Yount S, Sorensen M, Chartash E, Sengupta N, Grober J. Validation of the Functional Assessment of Chronic Illness Therapy Fatigue Scale relative to other instrumentation in patients with rheumatoid arthritis. J Rheumatol 2005;32:811–9.
20. Bowman SJ, Booth DA, Platts RG, UK Sjögren's Interest Group. Measurement of fatigue and discomfort in primary Sjögren's syndrome using a new questionnaire tool. Rheumatology (Oxford) 2004;43:758–64.
21. Wechsler D. Wechsler Adult Intelligence Scale-revised. San Antonio, TX: The Psychological Corporation; 1991.
22. Bray NL, Pimentel H, Melsted P, Pachter L. Near-optimal probabilistic RNA-seq quantification. Nat Biotechnol 2016;34:525–7.
23. Tripp HN, Tarn J, Natasari A, Gillespie C, Mitchell S, Hackett KL, et al. Fatigue in primary Sjögren's syndrome is associated with lower levels of proinflammatory cytokines. RMD Open 2016;2:e000282.
24. Davies K, Mirza K, Tarn J, Howard-Tripp N, Bowman SJ, Lendrem D, et al. Fatigue in primary Sjögren's syndrome (pSS) is associated with lower levels of proinflammatory cytokines: a validation study. Rheumatol Int 2019;39:1867–73.
25. Bodewes IL, Al-Ali S, van Helden-Meeuwsen CG, Maria NI, Tarn J, Lendrem DW, et al. Systemic interferon type I and type II signatures in primary Sjögren's syndrome reveal differences in biological disease activity. Rheumatology (Oxford) 2018;57:921–30.
26. Tarn JR, Howard-Tripp N, Lendrem DW, Mariette X, Saraux A, Devauchelle-Pensec V, et al. Symptom-based stratification of patients with primary Sjögren's syndrome: multi-dimensional characterisation of international observational cohorts and reanalyses of randomised clinical trials. Lancet Rheumatol 2019;1:e85–94.
27. Bodewes IL, Gottenberg JE, van Helden-Meeuwsen CG, Mariette X, Versnel MA. Hydroxychloroquine treatment downregulates systemic interferon activation in primary Sjögren's syndrome in the JOQUER randomized trial. Rheumatology (Oxford) 2020;59:107–11.
28. Morand EF, Furie R, Tanaka Y, Bruce IN, Askanase AD, Richez C, et al. Trial of anifrolumab in active systemic lupus erythematosus. N Engl J Med 2020;382:211–21.
29. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Givson DF, et al. Argonaut-2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A 2011;108:5003–8.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 151–161
DOI 10.1002/art.41481

American College of Rheumatology
Empowering Rheumatology Professionals

Phase II Open-Label Study of Anakinra in Intravenous Immunoglobulin–Resistant Kawasaki Disease

Isabelle Koné-Paut,[1] Stéphanie Tellier,[2] Alexandre Belot,[3] Karine Brochard,[2] Corinne Guitton,[1] Isabelle Marie,[1] Ulrich Meinzer,[4] Bilade Cherqaoui,[5] Caroline Galeotti,[1] Nadja Boukhedouni,[6] Helene Agostini,[6] Moshe Arditi,[7] Virginie Lambert,[8] and Céline Piedvache[6]

Objective. Anakinra has been shown to be successful in preventing and treating cardiovascular lesions both in experimental murine models of Kawasaki disease (KD) and in several studies of intravenous immunoglobulin (IVIG)– and steroid-resistant patients with KD. This study was undertaken to determine the safety of blocking interleukin-1 in patients with IVIG-resistant KD.

Methods. Sixteen patients were included in the present study. Patients with KD who were not responsive to 1 or more courses of 2 mg/kg of IVIG received anakinra by subcutaneous daily injections. The starting dose was 2 mg/kg of anakinra (4 mg/kg in patients who were age <8 months and who weighed ≥5 kilograms), and the dose was increased up to 6 mg/kg every 24 hours if the patient's body temperature remained >38°C, indicative of a fever. Treatment duration was 14 days. The last visit was on day 45. The primary outcome was abatement of fever. Secondary outcome measures included disease activity, coronary artery Z score, and C-reactive protein (CRP) levels.

Results. Seventy-five percent of the patients in the intent-to-treat group and 87.5% in the per-protocol group became afebrile within 48 hours of the last escalation dose of anakinra. Reduction of disease activity by 50% was indicated on 93.3% of physician evaluations (95% confidence interval [95% CI] 68.1–99.8%) and on 100% of parent evaluations (95% CI 73.5–100%) of parent evaluations. CRP values normalized by day 30. At the initial screening, 12 of 16 patients had a maximum coronary artery Z score of >2, and 10 of 16 patients had a maximum Z score of >2.5. On day 45, 5 of 10 patients (50% [95% CI 18.7–81.3%]) and 6 of 12 patients (50% [95% CI 21.1–78.9%]) had achieved coronary artery Z scores of <2.5 and <2, respectively. Five serious adverse events were observed in 3 patients, but no serious infections or deaths occurred.

Conclusion. Anakinra was well tolerated in the study patients and may have some efficacy in reducing fever, markers of systemic inflammation, and coronary artery dilatation in individuals with IVIG-refractory KD.

INTRODUCTION

Kawasaki disease (KD) is a condition characterized by systemic vasculitis and myocarditis and is the leading cause of acquired heart disease in children in developed countries (1). Left untreated, KD leads to coronary artery abnormalities, including aneurysms in <30% of patients (2). First-line treatment, which includes a single high dose of intravenous immunoglobulin (IVIG) and aspirin,

ClinicalTrials.gov identifier: NCT02390596. EudraCT database no. 2014-002715-41.

Supported by the French Ministry of Health (Programme Hospitalier de Recherche Clinique, AP-HP, 2013).

[1]Isabelle Koné-Paut, MD, Corinne Guitton, MD, Isabelle Marie, Caroline Galeotti, MD, PhD: CeRéMAIA, AP-HP, Bicêtre Hospital, University of Paris Sud Saclay, Paris, France; [2]Stéphanie Tellier, MD, Karine Brochard, MD: University of Toulouse, Toulouse, France; [3]Alexandre Belot, MD, PhD: University of Lyon, INSERM U1111, ENS, Lyon, France; [4]Ulrich Meinzer, MD, PhD: Robert Debre University Hospital, Paris, France; [5]Bilade Cherqaoui, MD: CeRéMAIA, Bicêtre Hospital, AP-HP, University of Paris Sud Saclay, INSERM U1173, Paris, France; [6]Nadja Boukhedouni, Helene Agostini, MD, Céline Piedvache: AP-HP, University of Paris Saclay, Bicêtre Hospital, Paris, France; [7]Moshe Arditi, MD, PhD: Cedars-Sinai Medical Center, Los Angeles, California; [8]Virginie Lambert, MD: Institut Mutualiste Montsouris and Bicêtre Hospital, AP-HP, University of Paris Sud Saclay, Paris, France.

Dr. Koné-Paut has received consulting fees from AbbVie, Pfizer, Roche, Chugai, Novartis, Sobi, and Novimmune (less than $10,000 each). Dr. Belot has received research support paid to his institution from AbbVie, Novartis, Pfizer, Roche Chugai, and Merck Serono. Dr. Galeotti has received consulting fees from Novartis (less than $10,000). No other disclosures relevant to this article were reported.

Deidentified and aggregated participant data (including data dictionaries) as well as study protocols, the statistical analysis plan, and the informed consent form are available if requested up until 5 years following publication of this article. Requests for access to data are restricted to institutional partners and require evidence of ethics approval of a methodologically sound proposal for use, and a data sharing agreement must be made available. Requests should be addressed to the corresponding author, Dr. Isabelle Koné-Paut, at isabelle.kone-paut@aphp.fr.

Address correspondence to Isabelle Koné-Paut, MD, Bicêtre Hospital, AP-HP, Division of Pediatric Rheumatology and CeRéMAIA, 78 Rue du Général Leclerc, 94270 Le Kremlin-Bicêtre, France. Email: isabelle.kone-paut@aphp.fr.

Submitted for publication March 23, 2020; accepted in revised form August 4, 2020.

reduces the risk of developing a coronary artery aneurysm (CAA) or coronary dilation from 25–30% to 5–7% (3–6). However, 10–20% of patients with KD develop recrudescence of fever or have persistent fever at least 36 hours after the IVIG infusion is completed, and these IVIG-resistant patients are at a 3-fold increased risk of developing CAA (1,6–8). In addition, patients younger than 1 year of age have an elevated risk of developing coronary dilatations and CAA, which may occur during the first days of fever, limiting the protective effect of IVIG treatment even when it is given early (9,10).

Intensification of initial treatment, in which glucocorticoids are added to a regimen of standard IVIG plus aspirin, has been studied in at least 6 randomized clinical trials (11–16). A meta-analysis of these trials concluded that combination treatment with steroids and IVIG resulted in fewer CAAs than treatment with IVIG alone (17). Five of these 6 studies excluded patients with KD and coronary artery dilatation or aneurysm at baseline, and 2 studies used the Japanese scoring system for predicting the risk of IVIG resistance in patients with KD (17,18). However, the Japanese scoring system has not successfully identified children in the US at higher risk of having IVIG resistance (6,17,18), severely limiting the application of these treatment protocols to Japanese and European populations. Furthermore, early steroid therapy may increase the risk of thrombosis in patients with giant CAA (19).

Given the observations of increased serum concentrations of tumor necrosis factor (TNF) in acute KD (20,21), therapeutic interventions targeting TNF have been evaluated for either primary KD treatment or treatment of IVIG-resistant patients (22,23). Antagonism of TNF with infliximab, a chimeric anti-TNF monoclonal antibody (mAb), or etanercept, a soluble TNF receptor fusion protein, is safe and well-tolerated (22,23). However, phase III randomized controlled clinical trials using infliximab and etanercept as intensifiers of primary IVIG therapy or in IVIG-resistant patients were underpowered to show impact on rates of IVIG resistance or CAA development (22–24). For these reasons, an essential unmet clinical need remains for an adjunctive therapy in addition to IVIG.

Abundant evidence from human patients, genetic studies, and experimental KD mouse models supports the critical involvement of NLRP3 inflammasome activation and interleukin-1β (IL-1β) production in innate immune cells in the pathogenesis of acute KD and the development of related cardiovascular lesions and myocarditis. For example, in vitro cultured peripheral blood mononuclear cells (PBMCs) isolated from KD patients spontaneously release IL-1β in the supernatant—a process that is significantly reduced following IVIG treatment (25). Circulating concentrations of both IL-1β and IL-18 are elevated in the sera of children with acute KD compared with febrile controls and significantly decreased during the convalescent phase of the disease (26). Similarly, IL-1 and NLRP3-related gene transcripts are up-regulated in PBMCs from patients with KD in the acute phase and decreased during the convalescent phase of the disease (26), and an *IL1B*-related gene signature is associated with the acute phase of KD and with IVIG resistance (27). This suggests that IL-1 is the main mediator of inflammation in KD, as is true in other polyfactorial autoinflammatory diseases (28). Furthermore, in a *Lactobacillus casei* cell wall extract murine model of vasculitis in KD, caspase 1/IL-1α and IL-1β pathways were shown to be instrumental in the development of coronary arteritis, aneurysms, myocarditis, and abdominal aorta aneurysms (29–31), and treatment with IL-1 receptor antagonist (IL-1Ra) (anakinra) prevented the development of cardiovascular complications in this model (29,32).

IL-1 is an endogenous pyrogen, and IL-1 blockade is a powerful antipyretic (33).Therefore, blocking the IL-1 pathway may be a valid therapeutic option for IVIG-resistant KD patients. IL-1 blockade with anakinra has been successfully used to treat patients with diseases caused by *NLRP3* mutations and excessive IL-1β production, such as cryopyrin-associated periodic syndrome (CAPS) (34) and systemic-onset juvenile idiopathic arthritis (JIA) (35). Furthermore, multiple case reports now outline the successful clinical use of anakinra in treating KD patients with refractory IVIG resistance, many of whom had severe cardiac complications (36–43).

We hypothesized that IL-1 blockade could have a rapid and sustained effect on symptoms and coronary vasculitis in patients with KD. To test this hypothesis, we designed and implemented the KAWAKINRA study, an exploratory phase IIa, open-label, dose-finding clinical trial aimed at treating patients with KD who do not respond to standard treatment with IVIG (remaining febrile 48 hours after receiving IVIG). The aim of the study was to determine the safety of blocking IL-1 signaling in patients with acute KD who are unresponsive to treatment with IVIG.

PATIENTS AND METHODS

Study design and population. The KAWAKINRA study was a 45-day phase IIa, multicenter, open-label, proof-of-concept study with a single treatment arm that assessed the efficacy and safety of anakinra in KD patients who were unresponsive to IVIG treatment. Active anakinra, a recombinant, selective IL-1Ra which blocks the action of both IL-1α and IL-1β, was given via subcutaneous daily injections to IVIG-refractory KD patients who had not responded to 1 or more courses of 2 gm/kg of IVIG and were within 14 days of the onset of fever.

Institutional review boards at each participating center approved the study, which was conducted between February 5, 2016 and February 18, 2019. A national institutional review board and the French National Agency for Medicines and Health Products Safety approved the present study protocol. Written informed consent was provided by each study participant or their parent/legal guardian, as appropriate. The study was designed by Dr. Koné-Paut with agreement with regard to its direction by the Recherche Clinique of the Assistance Publique Hôpitaux de Paris (AP-HP). The study was funded by the national research call from the French Ministry of Health (PHRC 2013). AP-HP was

responsible for all data gathering, processing, and management, statistical analysis, and reporting of the results. A Data and Safety Monitoring Board reviewed all serious adverse events (SAEs).

Study participants were enrolled from 4 medical centers in France and included children who were ages 3 months to 18 years, weighed ≥5 kilograms, and were diagnosed as having KD according to the American Heart Association (AHA) definition for complete or incomplete KD (1). In August 2017, the eligibility criteria, initially limited to patients who were at least 8 months old and weighed ≥10 kilograms, were extended to include patients who were age <8 months and who weighed ≥5 kilograms in order to increase the cohort size. Eligibility for study enrollment required persistence or recrudescence of a body temperature of ≥38°C (measured orally or rectally) or an axillary temperature of ≥37.5°C 48 hours after the last infusion of 2 gm/kg of IVIG. Overt bacterial infection, any type of immunodeficiency, and risk for tuberculosis were exclusion criteria, as was the use of glucocorticoids or other immunosuppressive medications prior to enrollment. As long as the patients received the study medication (anakinra), they could not receive preventative antipyretics (i.e., acetaminophen or nonsteroidal antiinflammatory drugs [NSAIDs] other than aspirin). However, patients could receive glucocorticoids while receiving anakinra treatment based on the principal investigator's judgment.

Ten visits occurred over the study period, from the time of initial screening (visit 1) to day 45 (visit 10), as outlined in Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41481/abstract. The study design is summarized in Figure 1. The inclusion visit on day 0 (visit 2) occurred a maximum of 14 days after the onset of fever. Patients received a starting dose of 2 mg/kg of anakinra, with patients who weighed <10 kilograms and who were age <8 months receiving a starting dose of 4 mg/kg of anakinra. Higher doses were administered to patients who were <8 months old, due to the inherent higher risk of CAA and consistent with previous experience in treating very young patients who had CAPS (44). If patients had persistent fever or recrudescence of fever (a body temperature of ≥38°C) after the first administration of anakinra, they received 4 mg/kg of anakinra after 24 hours at visit 3 (day 1), with 6 mg/kg of anakinra administered in patients who were <8 months old and weighed <10 kilograms. If patients did not respond to the 4 mg/kg dose at visit 3 within 24 hours, they received 6 mg/kg of anakinra at visit 4 (day 2), with 8 mg/kg of anakinra administered in patients who were <8 months old and weighed <10 kilograms.

If a patient remained afebrile following a dose of anakinra, the same dose was administered in that patient until day 14. When a patient became febrile again after a 24-hour interval of anakinra treatment, the investigator could increase the dose to a maximum of 6 mg/kg (or 8 mg/kg in patients who were <8 months old and weighed <10 kilograms) after thorough examination and after ruling out another cause of fever, particularly infection. Total study treatment duration was 14 days, including the escalation dose period, if any. Follow-up of enrolled patients included 2 visits on days 30 and 45.

Outcome measures. The primary objective was to assess the effect of anakinra on fever, i.e., the patient had to achieve a (tympanic or oral) body temperature of <38°C or an axillary temperature of 37.5°C within 48 hours of anakinra treatment (after the last escalation dose, if necessary). Secondary objectives included a >50% decrease in disease activity scores on a 10-point scale (physician's and parent's global assessments of disease activity) between initial screening and day 14, resolution of coronary abnormalities (if present) as determined by echocardiography (Z scores) on day 45, and achievement of normal levels of the inflammation marker C-reactive protein (CRP) between baseline and days 14 and 30.

Data obtained from physical evaluation, adverse events, injection tolerability, vital signs, tuberculosis risk, laboratory evaluations, and echocardiograms formed the basis for the assessment of safety and tolerability as well as efficacy of anakinra. All echocardiograms were reviewed for coronary artery dimensions for the left main coronary artery, left anterior descending artery, and the circumflex and right coronary artery, and aneurysms were identified by the reference pediatric cardiologist in each center. Subsequently, all data were collected by the primary investigator, and assessed using the Z score calculation both manually and with the Cardio Z application, consistent with international recommendations (1). These methods were concordant.

We also assessed changes in Z scores between baseline (screening visit), day 14, and day 45 as well as the evolution of individual symptoms of KD from baseline to day 30. CRP as a marker of inflammation was measured at screening before anakinra dosing began and then on days 4, 5, 6, 7, 14, and 30. Of note, we did not consider cutaneous desquamation and peeling as a sign of active KD. Version 17.1 of the Medical Dictionary for Regulatory Activities reporting criteria for AEs and SAEs in clinical trials was used in the assessment of the safety and side effect profile upon treatment with anakinra.

Statistical analysis. The principal hypothesis of the present study was that anakinra is safe and effective in patients with IVIG-resistant KD. All efficacy analyses were performed in the intent-to-treat (ITT) population, which included any patient who received ≥1 dose of the study drug. We also analyzed the primary end point and the secondary end points in the per-protocol (PP) population, which included all patients from the ITT population who did not have major protocol deviations that might have affected the results of the main analysis. Of note, patients who received steroids after the primary end point (48 hours after starting anakinra treatment) were analyzed in the PP group. Safety analyses included all patients who received ≥1 dose of anakinra. Results are expressed as the mean ± SD or median (minimum–maximum) for summarized continuous variables, and as the

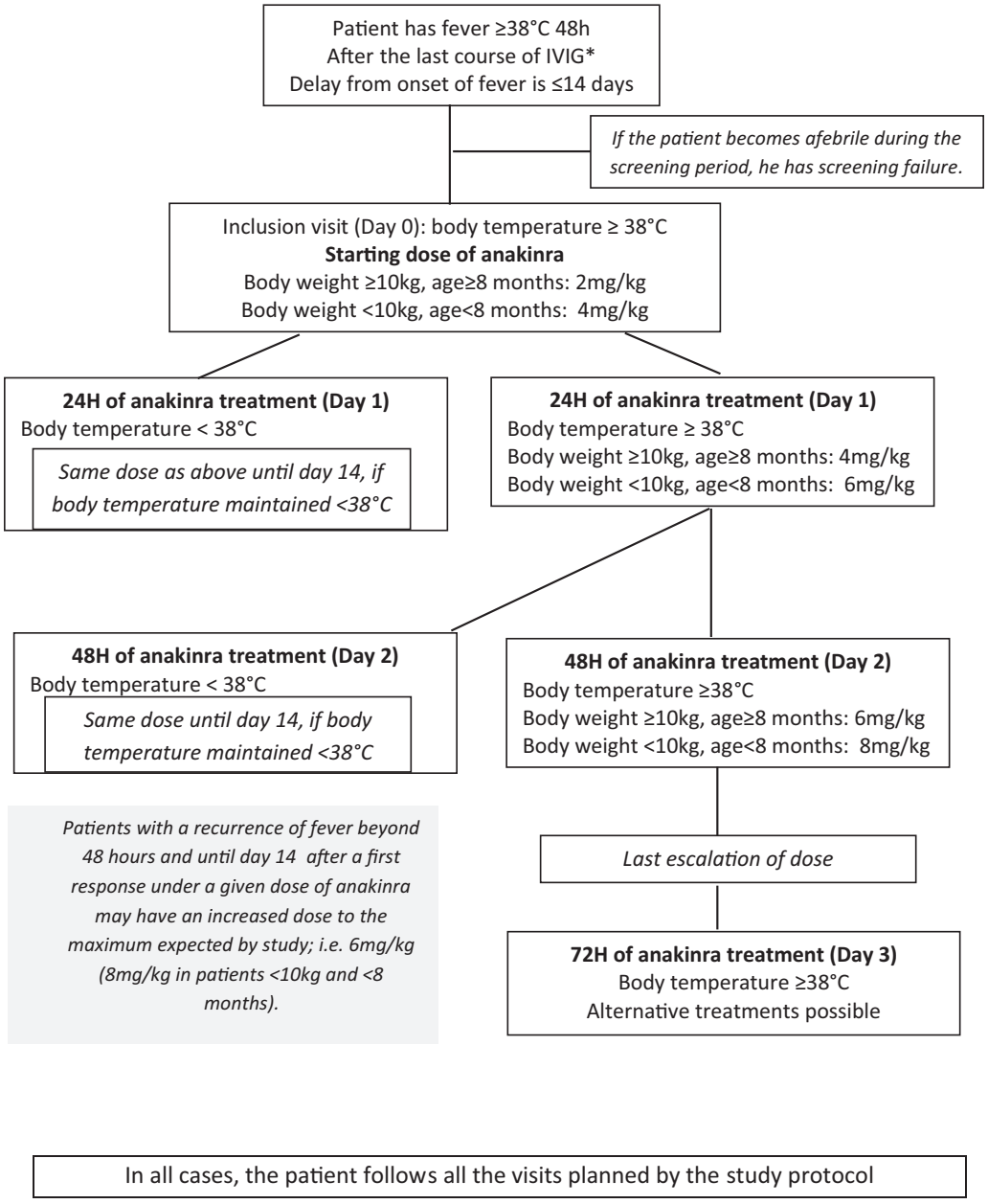


Figure 1. KAWAKINRA study design and procedure for anakinra dose escalation. IVIG = intravenous immunoglobulin.

frequency for categorical variables. In the case of a missing result, we calculated the total percentage of patients who received at least 1 dose of the study drug (the ITT population) for the efficacy analyses but excluded missing data from other analyses.

The primary and secondary end points are reported in terms of percentages and 95% confidence intervals (95% CIs), with the Clopper–Pearson interval used to calculate 95% CIs (45). The Dallaire formula was applied for Z score measurement of the right coronary artery, left coronary artery, left anterior descending artery, and circumflex artery (46). The Pettersen formula was used for measuring the ascendant aorta (47). We defined CAA as an individual having maximal Z scores of ≥2.5, and moderate coronary dilation as an individual having a Z score of 2–2.5. Missing values for the primary end point were imputed as follows: for premature termination of treatment, the maximal temperature within 48 hours of the last injection of anakinra was used. If the body temperature was only available for within 24 hours of the last dose escalation, that temperature was reported for the 48-hour time point. Missing values for the secondary end point were imputed as follows: for missing Z scores, coronary measurement was considered to be within the normal range. Indeed, it is quite likely that if we could not obtain an echocardiogram, clinical concern about the patient was low (i.e., no CAA present). In the case of a missing CRP value at the last visit, the last observation carried forward

(LOCF) method was used, or imputation based on the mean value for the recorded CRP was utilized. SAS software version 9.4 (SAS Institute) was used to make the statistical calculations.

RESULTS

Study population and KD characteristics at baseline. During a 38-month recruitment period, 18 consecutive patients with IVIG-refractory KD who did not meet exclusion criteria were screened. Two patients became afebrile before the inclusion visit. Sixteen patients were included in the final cohort, of whom 14 were male and 2 were female and the median age was 31 months (range 3–83 months). Of the 16 subjects, 15 met the AHA criteria for complete KD and 1 for incomplete KD (fever plus 3 criteria plus coronary dilatation). Demographic characteristics of the patients are listed in Supplementary Table 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41481/abstract. The ITT group included 16 patients who received at least 1 dose of anakinra (Figure 2). Among these patients, the median time between the first day of fever and first IVIG infusion was 4.5 days (range 3–7 days). Median time between the first day of fever and first injection of anakinra was 9.5 days (range 5–12 days). Median body temperature at baseline was 39.2°C (range 38–40.1°C) with a mean ± SD body temperature of 38.68 ± 0.55. All patients presented with eye redness, 93.7% had diffuse skin rash and redness of the lips and oral mucosa, 87.5% exhibited redness, edema, and swelling of the extremities, and 81% were irritable (Supplementary Table 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41481/abstract).

At the screening visit, the median CRP value was 135 mg/liter (range 24–403 mg/liter) with a mean ± SD of 155.81 ± 114.4 mg/liter, and the median neutrophil count was 10,375/mm^3 (range 3,600–28,530 mm^3) with a mean ± SD of 11,062.69 ± 6,484 mm^3 (Supplementary Table 4, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41481/abstract). Based on interpretation of the echocardiograms, 75% of the patients had a maximum coronary Z score (or "worst" Z score) of >2, and 62.5% had a Z score of >2.5 (Table 1). In a post hoc analysis, 1 patient was considered to have been improperly included as he had received glucocorticoids (hydrocortisone hemisuccinate) together with IVIG prior to study enrollment. In total, PP analysis of the study population excluded 8 of 16 patients from the ITT group for major protocol violations or discrepancies (Figure 2).

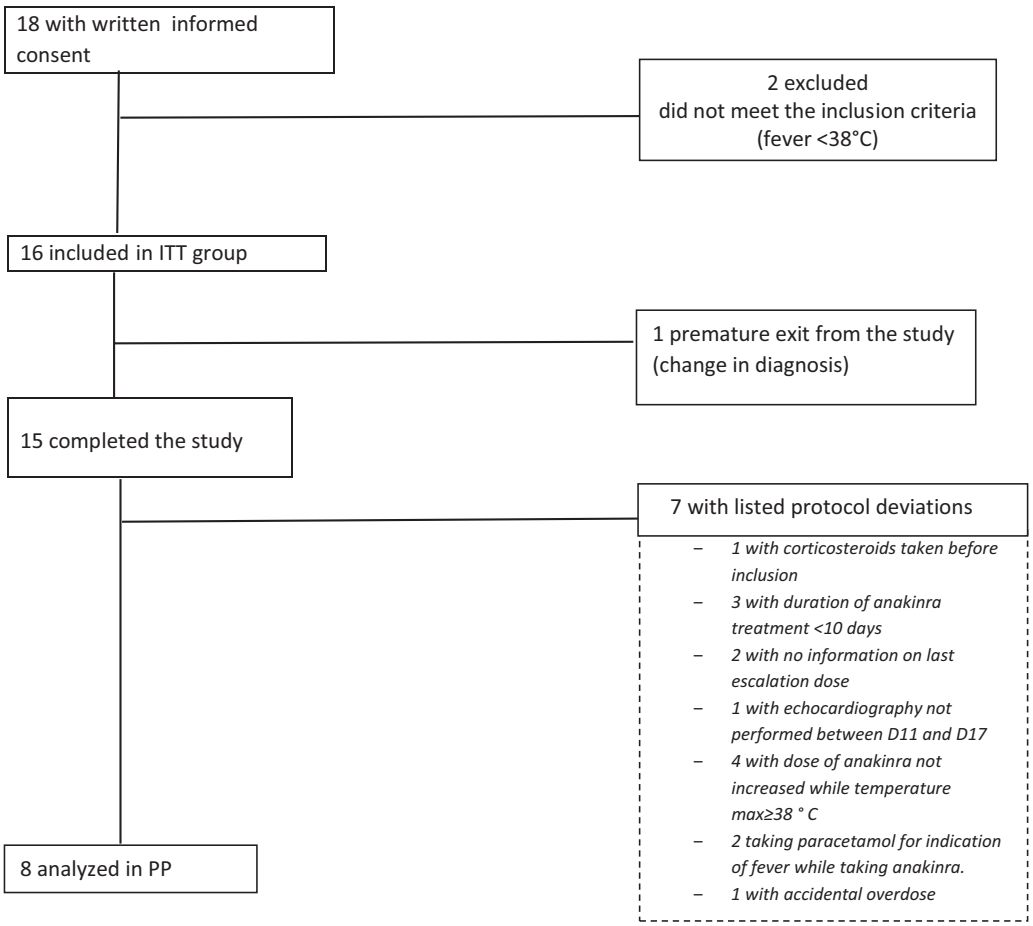


Figure 2. Patient disposition in the KAWAKINRA study. ITT = intent-to-treat population; PP = per-protocol population.

Table 1. Maximal coronary Z scores from screening visit to day 45 in patients from the intent-to-treat group in the KAWAKINRA study

Patient	Maximum dose of anakinra (mg/kg)	Glucocorticoid use	Maximal Z score			Δ maximal Z score	
			At screening visit	On day 14	On day 45	Screening visit to day 14	Screening visit to day 45
1	6	Yes	1.19	–	–	–	–
2*	6	No	3.43	0.77	−0.28	−2.66	−3.71
3	6	Yes	3.22	20.93	13.52	17.71	10.3
4*	2	No	3.48	6.76	5.62	3.27	2.14
5*	4	No	6.17	2.94	1.63	−3.23	−4.54
6*	6	No	4.69	1.09	0.46	−3.6	−4.23
7*	6	Yes	9.85	18.72	23.83	8.87	13.99
8	10	Yes	3.74	3.89	2.67	0.15	−1.07
9	4	No	3.05	0.3	0.04	−2.76	−3.01
10	2	No	0.48	0.84	0.87	0.36	0.4
11*	6	No	0.44	−0.1	−0.08	−0.53	−0.52
12	2	No	−0.05	0.8	−0.24	0.86	−0.18
13*	4	No	4.76	0.84	1.25	−3.92	−3.52
14	4	No	2.41	0.18	−1.17	−2.23	−3.58
15	4	Yes	4.42	2.24	4.11	−2.17	−0.31
16*	2	No	2.12	1.91	2.05	−0.21	−0.07

* Included in the per-protocol population.

Study treatment disposition throughout the KAWAKINRA study. The initial dose of anakinra was 2 mg/kg/day for 13 of 16 patients, and 4 mg/kg/day for 3 of 16 patients. Maximum daily doses of anakinra were calculated in 15 of 16 patients as 1 patient received an initial dose of 10 mg/kg of anakinra and was thus excluded from analysis. Four patients received a maximum dose of 2 mg/kg/day, 5 received 4 mg/kg/day, and 6 received 6 mg/kg/day. The median duration of anakinra treatment was 15 days, with 10 patients receiving anakinra for 15 days. Two patients were treated with anakinra for 14 days, 2 were treated for 5 days, 2 were treated for 4 days, and 2 were treated for 1 day.

Concomitant treatments. *Prior IVIG treatment.* Thirteen (81.25%) of 16 patients had received a single IVIG infusion before anakinra treatment, whereas 2 patients (12.5%) had received 2 infusions, and 1 (6.25%) had received 3 infusions.

Glucocorticoid use. Patients who had received glucocorticoids prior to study enrollment were not included in the KAWAKINRA trial. However, 1 patient had received glucocorticoids in combination with IVIG prior to receiving anakinra and was therefore excluded as a protocol deviation. Two patients had received glucocorticoids while receiving anakinra, and 2 patients had received glucocorticoids after stopping anakinra. Among these 4 patients with glucocorticoid use, indications included macrophage activation syndrome (MAS) and polyarthritis in 1 patient, and persistent clinical signs of KD activity and rapid increase of coronary artery dilatation among the other patients.

Other treatments. All patients received aspirin together with anakinra. Two patients received 1 dose of acetaminophen on day 2 of anakinra treatment and were excluded from the PP group. Ten patients received other treatments that we did not consider as modifying the efficacy of anakinra (see Supplementary Table 5 for a list of concomitant therapies, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41481/abstract).

Treatment response. When assessing treatment response for the primary objective of reducing fever, analysis of the ITT population (with imputation of the missing data) demonstrated that 12 (75%) of 16 patients had reached a body temperature of <38°C within 48 hours of the last escalation dose of anakinra. Analysis of the PP population indicated that 7 (87.5%) of the patients had reached a body temperature of <38°C within 48 hours of the last escalation dose of anakinra.

Physician's and parent's global assessments of disease activity and CRP levels in the patient cohort. *ITT population.* As a secondary objective, global assessments of disease activity were also performed. In the ITT population (with imputation of missing data), we evaluated the physician's global disease activity scores in 15 of the 16 patients on the day of initial screening and on day 14, and we evaluated the parent's global disease activity scores in 12 of the 16 patients on the initial screening day and on day 14. Anakinra treatment resulted in a noteworthy reduction in the disease activity score from a mean of 7 (range 4–10) and 7.75 (range 4–10) at baseline to a mean of 1.25 (range 0–8) and 1 (range 0–3) after treatment, for physicians' and parents' observations, respectively. Disease activity scores decreased by ≥50% in 93.3% of the patients according to the physician's assessment (95% CI 68.1–99.8%), and 100% of the patients achieved a ≥50% reduction in parent's global disease activity scores (95% CI 73.5–100%).

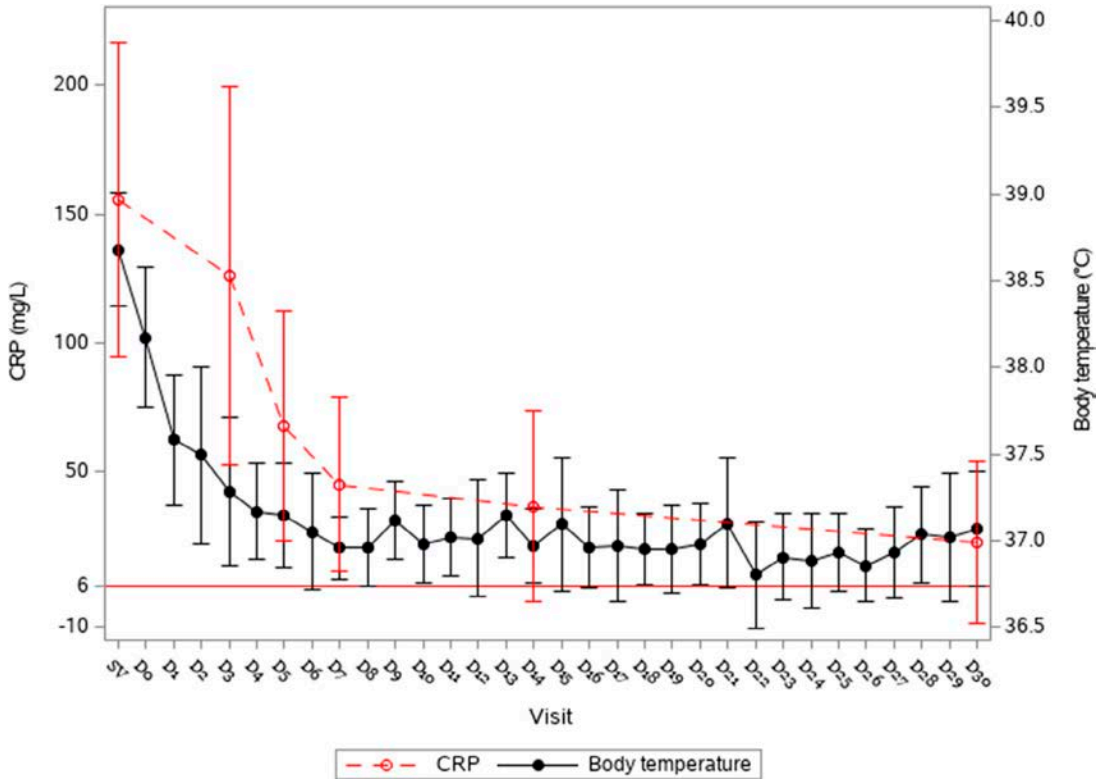


Figure 3. Changes in daily body temperature and C-reactive protein (CRP) levels from screening visit (SV) to day 30 (D30) in the intent-to-treat group. Temperature was measured at the time of injection on days 0–14, and maximum daily values are shown for days 15–30. Bars show the mean ± SD.

We also evaluated the inflammatory response as measured by serial CRP values, which decreased progressively from the time of screening to day 14 and day 30 (Figure 3 and Supplementary Table 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41481/abstract). All patients had a CRP value >10 mg/liter at the time of screening. On day 14, 7 of 16 patients (43.75% [95% CI 19.8–70.1%]) had CRP values of <10 mg/liter. On day 30, 13 of 16 patients (81.25% [95% CI 54.3–95.9%]) had CRP levels of <10 mg/liter (Supplementary Table 4).

Analysis of the coronary arteries at the screening visit showed a maximum Z score of >2 in 12 patients, and a maximum Z score of >2.5 in 10 patients (Table 1). On day 45, 5 of 10 patients (50% [95% CI 18.7–81.3%]) and 6 of 12 patients (50% [95% CI 21.1–78.9%]) had achieved Z scores of <2.5 and <2, respectively (Table 1 and Figure 4). Six patients had a maximum Z score of <2.5 at screening, and all 5 patients who were able to complete follow-up had maximum Z scores of <2.5.

PP population. Physicians evaluated global disease activity in all 8 patients in the PP cohort, both at screening and on day 14, and parents evaluated global disease activity in 7 of the 8 patients. Both the physician's and parent's global disease activity scores were reduced by at least 50% on day 14 (mean reductions of 63.1% and 59.0% in the physician's and parent's evaluations, respectively) (Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41481/abstract). All patients had CRP levels of >10 mg/liter at screening. On day 14, 2 of the 7 these patients who were evaluated (28.57% [95% CI 3.7–71.0%]) had CRP levels of <10 mg/liter. On day 30, all 8 patients

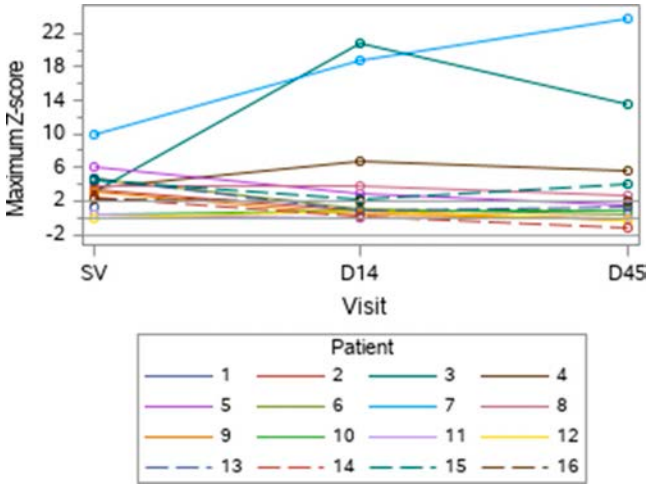


Figure 4. Coronary artery Z scores before and after treatment with anakinra. Evolution of the worst Z scores from screening visit (SV) to day 45 (D45) was examined in the intent-to-treat group (n = 16). Missing values on days 14 and 45 were imputed if the patient exited the study early.

(100% [95% CI 63.1–100%]) had a CRP level of <10 mg/liter (Figure 3). Seven of the 8 patients had a maximum Z score of >2 at screening, with 6 having a maximum Z score of >2.5. On day 45, 4 of these 7 patients (57.14% [95% CI 18.4–90.1%]) and 4 of these 6 patients (66.67% [95% CI 22.3–95.7%]) had achieved Z scores of <2 and <2.5, respectively (Figure 5 and Table 1).

Other assessments. Symptoms of active KD vasculitis disappeared in all but 1 patient treated with anakinra by day 14 (Supplementary Table 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41481/abstract), at which point almost all biologic markers had returned to normal levels (Supplementary Table 4). We calculated the variation of coronary artery Z scores first between the initial screening and day 14, and then between the initial screening and day 45, for each patient. The median change in Z score between initial screening and day 14 in 16 patients (taking into consideration that the Z score was imputed for 1 patient) was –0.4 (minimum, maximum –3.92, 17.71). The median variation in Z score between initial screening and day 45 was –0.4 (minimum, maximum –4.54, 13.99) (Table 1). In addition, we evaluated the worst Z scores depending on whether or not patients received steroids. As shown in Supplementary Figures 1 and 2, the worst Z scores tended to be higher in patients who received steroids together with anakinra (2 patients on day 3) and in patients who took steroids after stopping anakinra (2 patients on day 3).

Safety. Three patients experienced 5 SAEs during the study period, which included the following: an episode of increased coronary dilatation with pericarditis in 1 patient, occurrence of MAS and polyarthritis in 1 other patient, and a higher dose of anakinra than that administered in the escalation doses with relapse of KD symptoms (while stopping anakinra and receiving glucocorticoid treatment) in the last patient. All patients who experienced SAEs required prolonged hospitalization and interruption of anakinra treatment. At the last evaluation, all events had resolved. Other nonserious AEs are summarized in Supplementary Table 7, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41481/abstract.

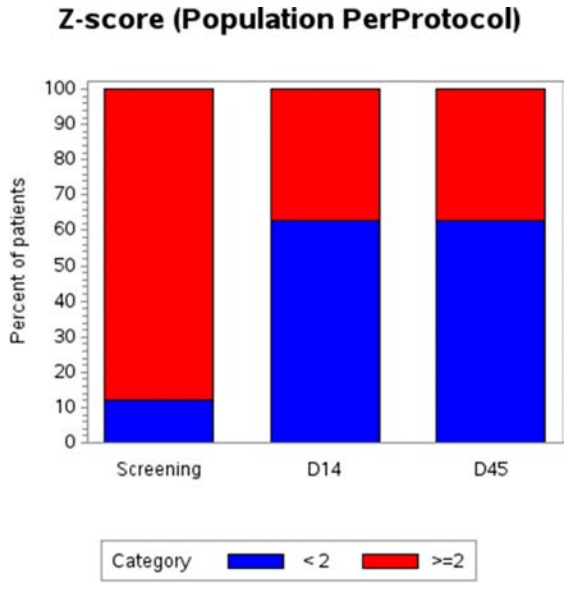


Figure 5. Distribution of coronary artery Z scores before and after treatment with anakinra in the per-protocol group (n = 8). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41481/abstract.

DISCUSSION

We have performed the first investigative trial of IL-1 signaling blockade in KD, with open-label use of the IL-1Ra anakinra in patients who do not respond to IVIG treatment. Anakinra promptly resolved fever in 12 (75%) of the 16 patients in the ITT group and 7 (87.5%) of the 8 patients in the PP group and reduced disease activity and KD symptoms in almost all patients after 14 days. In addition, in this cohort of patients, anakinra showed efficacy in both treating and preventing coronary involvement. Indeed, 10 (62.5%) of 16 patients had a coronary Z score of >2.5 at the initial screening, which decreased to 5 (31%) of 16 patients at the end of therapy. The median required dose of anakinra for attainment of the primary objective was 4 mg/kg. We did not identify any significant differences in the primary and secondary end points, nor did we observe any differences in the doses of anakinra used, between the whole cohort and the subgroup of patients (19%) who were younger than 1 year of age (data not shown). We acknowledge that a certain percentage of small aneurysms may also have regressed independently, which will need to be fully addressed in a randomized phase III clinical study.

The primary objective of reduction in fever (achieving a body temperature of <38°C) within 48 hours of anakinra treatment is a common measure used in clinical practice. To limit the chance of spontaneous regression of fever, we required that the first injection of anakinra be administered within the first 14 days after detection of fever. Among secondary outcome measures, we assessed the normalization of CRP values on day 14. However, despite resolution of clinical symptoms by day 14, CRP values remained between 10 and 15 mg/liter in most patients, both in the ITT and PP populations (Supplementary Table 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41481/abstract). Of the 2 patients with highest CRP values, 1 patient experienced relapse of KD symptoms, and the other developed MAS and polyarthritis, resulting in a final diagnosis of systemic JIA.

Overall, the safety and tolerability of anakinra was very good in this study. Excluding the accidental overdose of anakinra in 1 patient, other SAEs were mostly due to persistent KD activity or associated diseases. We observed only 1 patient in whom treatment had to be discontinued due to edema and itching at the injection site (Supplementary Table 7, available on the *Arthritis*

& Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41481/abstract). We recorded no deaths and no opportunistic infections.

The rapid increase in the incidence of IVIG resistance coupled with the high frequency of cardiac complications in children younger than 1 year of age together create an urgent need to find therapeutic alternatives to IVIG. A meta-analysis of controlled trials that investigated the combination of glucocorticoids and IVIG therapy showed a moderate benefit among Asian patients with high risk scores (20). To date, comparative studies using infliximab have failed to demonstrate efficacy in terms of reducing the incidence of coronary dilatation or aneurysm (48,49). Importantly, the findings of these trials may not be applicable to all KD patients, and each study requires further investigation.

We chose to target IL-1 signaling in KD due to strong observational and experimental data indicating that the pathogenesis of KD vasculitis is IL-1–driven (27,29,30). In particular, the well-characterized actions of IL-1β on innate and adaptive immunity support this approach. For example, IL-1β promotes CD8+ T cell differentiation and migration into tissues (50), which is relevant to KD as CD8+ T cells infiltrating the coronary artery wall contribute to aneurysm formation in KD (51). IL-1β also promotes matrix metalloproteinase (MMP)–driven proliferation of vascular smooth muscle cells (VSMCs) and myofibroblast formation (52–54). MMP-3 and MMP-9 are implicated in KD (55–57), and the proliferation of VSMCs and presence of myofibroblasts are hallmarks of arterial pathology in KD (58,59).

Notably, it is likely that both IL-1α and IL-1β play a role in the pathogenesis of KD vasculitis. The endothelium of the human vasculature expresses the IL-1α precursor, which can induce neutrophil activation, among other inflammatory responses (60). In addition, the *Lactobacillus casei* cell wall extract model of KD vasculitis has recently been shown to be mediated by both IL-1α and IL-1β (29–31) and is successfully ameliorated by treatment with anakinra (29,32), which blocks both IL-1α and IL-1β signaling by targeting the IL-1 receptor. Taken together, these data provided the powerful rationale for the current phase I/II clinical trials investigating blockade of the IL-1 pathway using anakinra in patients with KD.

Due to the very young age of the study population and the difficulty of recruiting patients in the context of this rare acute illness that is potentially life-threatening, we observed several protocol deviations, which may limit the power of our results and are potential weaknesses of the present study. In addition, and consistent with what has been reported in patients with IVIG resistance, our study population had a high rate of coronary abnormalities (12 of 16 patients), which may have influenced the interpretation of the results, and in some cases, led the investigator to administer steroids. However, in 4 of the 5 patients who received steroids in addition to anakinra, steroids were administered 48 hours after anakinra was started, and therefore, steroid treatment did not interfere with results pertaining to the primary objective. Of note, patients who received steroids had the worst maximum Z score on days 14 and 45, reflecting a possibly higher level of inflammation and disease severity than that observed in patients who experienced a decrease in Z scores with anakinra alone. Frequency of the protocol deviations justifies the priority we give to the ITT analyses in the present study. Furthermore, our study design was not comparative, was limited to a small number of patients who were IVIG-resistant and was designed as a phase IIa clinical study to establish safety, tolerability, and efficacy.

Our data show that anakinra is safe, well-tolerated, and may have efficacy, and together with the accumulated clinical, genetic, and experimental mouse models, suggest that anakinra may be useful in IVIG-resistant KD patients. Our results lay the foundation for a future randomized, placebo-controlled trial of anakinra for intensification therapy with IVIG, which remains a highly effective first-line treatment (36–43). In addition, our work supports the pursuit of controlled clinical trials to evaluate the efficacy of anakinra as a first-line treatment for KD.

ACKNOWLEDGMENTS

We thank our collaborators Gregory Allain, Jordi Anton, Marco Catallini, Rolando Cimaz, Perrine Dusser, Vincent Gajdos, Behrouz Kassai, Elise Launay, Etienne Merlin, Linda Rossi-Semerano, and Andrea Taddio.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Koné-Paut had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Koné-Paut.

Acquisition of data. Koné-Paut, Tellier, Belot, Brochard, Guitton, Marie, Meinzer, Cherqaoui, Galeotti.

Analysis and interpretation of data. Koné-Paut, Boukhedouni, Agostini, Arditi, Piedvache.

ADDITIONAL DISCLOSURES

Swedish Orphan Biovitrum (Sobi) provided the study drug, but members of Sobi had no part in influencing or writing the manuscript submitted for publication.

REFERENCES

1. McCrindle BW, Rowley AH, Newburger JW, Burns JC, Bolger AF, Gewitz M, et al. Diagnosis, treatment, and long-term management of Kawasaki disease: a scientific statement for health professionals from the American Heart Association. Circulation 2017;135: e927–99.
2. Burns JC. Kawasaki disease update. Indian J Pediatr 2009;76:71–6.
3. Burns JC, Capparelli EV, Brown JA, Newburger JW, Glode MP, on behalf of the US/Canadian Kawasaki Syndrome Study Group. Intravenous gamma globulin treatment and retreatment in Kawasaki disease. Pediatr Infect Dis J 1998;17:1144–8.
4. Newburger JW, Takahashi M, Burns JC, Beiser AS, Chung KJ, Duffy CE, et al. The treatment of Kawasaki syndrome with intravenous gamma globulin. N Engl J Med 1986;315:341–7.
5. Sundel RP, Burns JC, Baker A, Beiser AS, Newburger JW. Gamma globulin retreatment in Kawasaki disease. J Pediatr 1993;123: 657–9.

6. Tremoulet AH, Best BM, Song S, Wang S, Corinaldesi E, Eichenfield JR, et al. Resistance to intravenous immunoglobulin in children with Kawasaki disease. J Pediatr 2008;153:117–21.
7. Hong YM, Jin HS, Park IS, Hong SJ. Association of the matrix metalloproteinase-3 (–439C/G) promoter polymorphism with Kawasaki disease in Korean children. Heart Vessels 2008;23:341–7.
8. Newburger JW, Takahashi M, Burns JC. Kawasaki disease. J Am Coll Cardiol 2016;67:1738–49.
9. Fernandez-Cooke E, Tascón AB, Sánchez-Manubens J, Antón J, Lozano CD, Santos JA, et al, on behalf of the KAWA-RACE Study Group. Epidemiological and clinical features of Kawasaki disease in Spain over 5 years and risk factors for aneurysm development. (2011–2016). PLoS One 2019;14:e0215665.
10. Tulloh RM, Mayon-White R, Harnden A, Ramanan AV, Tizard EJ, Shingadia D, et al. Kawasaki disease: a prospective population survey in the UK and Ireland from 2013 to 2015. Arch Dis Child 2019;104:640–6.
11. Inoue Y, Okada Y, Shinohara M, Kobayashi T, Kobayashi T, Tomomasa T, et al. A multicenter prospective randomized trial of corticosteroids in primary therapy for Kawasaki disease: clinical course and coronary artery outcome. J Pediatr 2006;149:336–41.
12. Kobayashi T, Saji T, Otani T, Takeuchi K, Nakamura T, Arakawa H, et al. Efficacy of immunoglobulin plus prednisolone for prevention of coronary artery abnormalities in severe Kawasaki disease (RAISE study): a randomised, open-label, blinded-endpoints trial. Lancet 2012;379:1613–20.
13. Newburger JW, Sleeper LA, McCrindle BW, Minich LL, Gersony W, Vetter VL, et al. Randomized trial of pulsed corticosteroid therapy for primary treatment of Kawasaki disease. N Engl J Med 2007;356:663–75.
14. Ogata S, Shimizu C, Franco A, Touma R, Kanegaye JT, Choudhury BP, et al. Treatment response in Kawasaki disease is associated with sialylation levels of endogenous but not therapeutic intravenous immunoglobulin G. PLoS One 2013;8:e81448.
15. Okada Y, Shinohara M, Kobayashi T, Inoue Y, Tomomasa T, Kobayashi T, et al. Effect of corticosteroids in addition to intravenous gamma globulin therapy on serum cytokine levels in the acute phase of Kawasaki disease in children. J Pediatr 2003;143:363–7.
16. Sundel RP, Baker AL, Fulton DR, Newburger JW. Corticosteroids in the initial treatment of Kawasaki disease: report of a randomized trial. J Pediatr 2003;142:611–6.
17. Chen S, Dong Y, Yin Y, Krucoff MW. Intravenous immunoglobulin plus corticosteroid to prevent coronary artery abnormalities in Kawasaki disease: a meta-analysis. Heart 2013;99:76–82.
18. Sleeper LA, Minich LL, McCrindle BM, Li JS, Mason W, Colan SD, et al. Evaluation of Kawasaki disease risk-scoring systems for intravenous immunoglobulin resistance. J Pediatr 2011;158:831–5.
19. Fukazawa R, Kobayashi T, Mikami M, Saji T, Hamaoka K, Kato H, et al. Nationwide survey of patients with giant coronary aneurysm secondary to Kawasaki disease 1999–2010 in Japan. Circ J 2017;82:239–46.
20. Furukawa S, Matsubara T, Jujoh K, Yone K, Sugawara T, Sasai K, et al. Peripheral blood monocyte/macrophages and serum tumor necrosis factor in Kawasaki disease. Clin Immunol Immunopathol 1988;48:247–51.
21. Matsubara T, Furukawa S, Yabuta K. Serum levels of tumor necrosis factor, interleukin 2 receptor, and interferon-γ in Kawasaki disease involved coronary artery lesions. Clin Immunol Immunopathol 1990;56:29–36.
22. Portman MA, Dahdah NS, Slee A, Olson AK, Choueiter NF, Soriano BD, et al. Etanercept with IVIg for acute Kawasaki disease: a randomized controlled trial. Pediatrics 2019;143:e20183675.
23. Tremoulet AH, Jain S, Jaggi P, Jimenez-Fernandez S, Pancheri JM, Sun X, et al. Infliximab for intensification of primary therapy for Kawasaki disease: a phase 3 randomised, double-blind, placebo-controlled trial. Lancet 2014;383:1731–8.
24. Burgner DP, Newburger JW. Etanercept as adjunctive primary therapy in Kawasaki disease. Pediatrics 2019;143:e20190912.
25. Leung DY, Geha RS, Newburger JW, Burns JC, Fiers W, Lapierre LA, et al. Two monokines, interleukin 1 and tumor necrosis factor, render cultured vascular endothelial cells susceptible to lysis by antibodies circulating during Kawasaki syndrome. J Exp Med 1986;164:1958–72.
26. Alphonse MP, Duong TT, Shumitzu C, Hoang TL, McCrindle BW, Franco A, et al. Inositol-triphosphate 3-kinase C mediates inflammasome activation and treatment response in Kawasaki disease. J Immunol 2016;197:3481–9.
27. Fury W, Tremoulet AH, Watson VE, Best BM, Shimizu C, Hamilton J, et al. Transcript abundance patterns in Kawasaki disease patients with intravenous immunoglobulin resistance. Hum Immunol 2010;71:865–73.
28. Berda-Haddad Y, Robert S, Salers P, Zekraoui L, Farnarier C, Dinarello CA, et al. Sterile inflammation of endothelial cell-derived apoptotic bodies is mediated by interleukin-1α. Proc Natl Acad Sci U S A 2011;108:20684–9.
29. Lee Y, Schulte DJ, Shimada K, Chen S, Crother TR, Chiba N, et al. Interleukin-1β is crucial for the induction of coronary artery inflammation in a mouse model of Kawasaki disease. Circulation 2012;125:1542–50.
30. Lee Y, Wakita D, Dagvadorj J, Shimada K, Chen S, Huang G, et al. IL-1 signaling is critically required in stromal cells in Kawasaki disease vasculitis mouse model: role of both IL-1α and IL-1β. Arterioscler Thromb Vasc Biol 2015;35:2605–16.
31. Wakita D, Kurashima Y, Crother TR, Rivas MN, Lee Y, Chen S, et al. Role of interleukin-1 signaling in a mouse model of Kawasaki disease-associated abdominal aortic aneurysm. Arterioscler Thromb Vasc Biol 2016;36:886–97.
32. Gorelik M, Lee Y, Abe M, Andrews T, Davis L, Patterson J, et al. IL-1 receptor antagonist, anakinra, prevents myocardial dysfunction in a mouse model of Kawasaki disease vasculitis and myocarditis. Clin Exp Immunol 2019;198:101–10.
33. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family [review]. Annu Rev Immunol 2009;27:519–50.
34. Haar NT, Lachmann H, Özen S, Woo P, Uziel Y, Modesto C, et al. Treatment of autoinflammatory diseases: results from the Eurofever Registry and a literature review. Ann Rheum Dis 2013;72:678–85.
35. Pascual V, Allantaz F, Arce E, Punaro M, Banchereau J. Role of interleukin-1 (IL-1) in the pathogenesis of systemic onset juvenile idiopathic arthritis and clinical response to IL-1 blockade. J Exp Med 2005;201:1479–86.
36. Shafferman A, Birmingham JD, Cron RQ. High dose anakinra for treatment of severe neonatal Kawasaki disease: a case report. Pediatr Rheumatol Online J 2014;12:26.
37. Sánchez-Manubens J, Gelman A, Franch N, Teodoro S, Palacios JR, Rudi N, et al. A child with resistant Kawasaki disease successfully treated with anakinra: a case report. BMC Pediatr 2017;17:102.
38. Miettunen PM, Narendran A, Jayanthan A, Behrens EM, Cron RQ. Successful treatment of severe paediatric rheumatic disease-associated macrophage activation syndrome with interleukin-1 inhibition following conventional immunosuppressive therapy: case series with 12 patients. Rheumatology (Oxford) 2011;50:417–9.
39. Lind-Holst M, Hartling UB, Christensen AE. High-dose anakinra as treatment for macrophage activation syndrome caused by refractory Kawasaki disease in an infant. BMJ Case Rep 2019;12:e229708.
40. Kone-Paut I, Cimaz R, Herberg J, Bates O, Carbasse A, Saulnier JP, et al. The use of interleukin 1 receptor antagonist (anakinra) in

Kawasaki disease: a retrospective cases series [review]. Autoimmun Rev 2018;17:768–74.

41. Guillaume MP, Reumaux H, Dubos F. Usefulness and safety of anakinra in refractory Kawasaki disease complicated by coronary artery aneurysm. Cardiol Young 2018;28:739–42.
42. Cohen S, Tacke CE, Straver B, Meijer N, Kuipers IM, Kuijpers TW. A child with severe relapsing Kawasaki disease rescued by IL-1 receptor blockade and extracorporeal membrane oxygenation. Ann Rheum Dis 2012;71:2059–61.
43. Blonz G, Lacroix S, Benbrik N, Warin-Fresse K, Masseau A, Trewick D, et al. Severe late-onset Kawasaki disease successfully treated with anakinra [letter]. J Clin Rheumatol 2018;26:e42–3.
44. Koné-Paut I, Galeotti C. Anakinra for cryopyrin-associated periodic syndrome. Expert Rev Clin Immunol 2014;10:7–18.
45. Clopper CJ, Pearson ES. The use of confidence or fiducial limits illustrated in the case of the binomial. Biometrika 1934;26:404–13.
46. Dallaire F, Dahdah N. New equations and a critical appraisal of coronary artery Z scores in healthy children. J Am Soc Echocardiogr 2011;24:60–74.
47. Pettersen MD, Du W, Skeens ME, Humes RA. Regression equations for calculation of Z scores of cardiac structures in a large cohort of healthy infants, children, and adolescents: an echocardiographic study. J Am Soc Echocardiogr 2008;21:922–34.
48. Burns JC, Best BM, Mejias A, Mahony L, Fixler DE, Jafri HS, et al. Infliximab treatment of intravenous immunoglobulin-resistant Kawasaki disease. J Pediatr 2008;153:833–8.
49. Armaroli G, Verweyen E, Pretzer C, Kessel K, Hirono K, Ichida F, et al. Monocyte-derived interleukin-1β as the driver of S100A12-induced sterile inflammatory activation of human coronary artery endothelial cells: implications for the pathogenesis of Kawasaki disease. Arthritis Rheumatol 2019;71:792–804.
50. Ben-Sasson SZ, Hogg A, Hu-Li J, Wingfield P, Chen X, Crank M, et al. IL-1 enhances expansion, effector function, tissue localization, and memory response of antigen-specific CD8 T cells. J Exp Med 2013;210:491–502.
51. Guzman-Cottrill JA, Garcia FL, Shulman ST, Rowley AH. CD8 T lymphocytes do not express cytotoxic proteins in coronary artery aneurysms in acute Kawasaki disease. Pediatr Infect Dis J 2005;24:382–4.
52. Johnson JL, Dwivedi A, Somerville M, George SJ, Newby AC. Matrix metalloproteinase (MMP)-3 activates MMP-9 mediated vascular smooth muscle cell migration and neointima formation in mice. Arterioscler Thromb Vasc Biol 2011;31:e35–44.
53. Bonin PD, Fici GJ, Singh JP. Interleukin-1 promotes proliferation of vascular smooth muscle cells in coordination with PDGF or a monocyte derived growth factor. Exp Cell Res 1989;181:475–82.
54. Alexander MR, Moehle CW, Johnson JL, Yang Z, Lee JK, Jackson CL, et al. Genetic inactivation of IL-1 signaling enhances atherosclerotic plaque instability and reduces outward vessel remodeling in advanced atherosclerosis in mice. J Clin Invest 2012;122:70–9.
55. Popper SJ, Shimizu C, Shike H, Kanegaye JT, Newburger JW, Sundel RP, et al. Gene-expression patterns reveal underlying biological processes in Kawasaki disease. Genome Biol 2007;8:R261.
56. Matsuyama T. Tissue inhibitor of metalloproteinases-1 and matrix metalloproteinase-3 in Japanese healthy children and in Kawasaki disease and their clinical usefulness in juvenile rheumatoid arthritis. Pediatr Int 1999;41:239–45.
57. Lau AC, Duong TT, Ito S, Yeung RS. Matrix metalloproteinase 9 activity leads to elastin breakdown in an animal model of Kawasaki disease. Arthritis Rheum 2008;58:854–63.
58. Shulman ST, Rowley AH. Kawasaki disease: insights into pathogenesis and approaches to treatment. Nat Rev Rheumatol 2015;11:475–82.
59. Orenstein JM, Shulman ST, Fox LM, Baker SC, Takahashi M, Bhatti TR, et al. Three linked vasculopathic processes characterize Kawasaki disease: a light and transmission electron microscopic study. PLoS One 2012;7:e38998.
60. Brunn GJ, Saadi S, Platt JL. Constitutive repression of interleukin-1α in endothelial cells. Circ Res 2008;102:823–30.

American College of Rheumatology
Empowering Rheumatology Professionals

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 162–167
DOI 10.1002/art.41490

Representation of Women as Authors of Rheumatology Research Articles

Ekta Bagga, Sarah Stewart, Gregory D. Gamble, Janine Hill, Andrew Grey, and Nicola Dalbeth

Objective. In academic medicine, journal article authorship is central to career advancement and promotion. This study aimed to examine the contemporary representation of women as first and senior authors of rheumatology original research articles.

Methods. The gender of the first and senior author, disease category, research design, and funding source were extracted from rheumatology original research articles published in high-impact rheumatology and general medical journals between 2015 and 2019.

Results. The analysis included 7,651 original research articles. In total, 51.5% of the articles had women first authors (95% confidence interval [95% CI] 50.4–52.6%) and 35.3% had women senior authors (95% CI 34.2–36.4%). Women were significantly less likely to be first and senior authors of articles reporting randomized controlled trials compared with other clinical research designs ($P < 0.001$), and of articles reporting industry-funded/industry-initiated studies compared with studies not funded by industry ($P \leq 0.01$). Of the articles reporting industry-funded/industry-initiated randomized controlled trials, women were first authors in 18.5% (95% CI 13.8–24.0%) and senior authors in 23.9% (95% CI 18.6–29.8%).

Conclusion. In rheumatology research articles, there is gender parity for first authorship, but women are underrepresented in senior authorship positions. Underrepresentation of women in authorship is particularly apparent in articles reporting randomized controlled trials, and especially those that are initiated by industry.

INTRODUCTION

Globally, there have been some advances in gender equity within the medical workforce, with an increase in women physicians in recent decades (1,2). According to American College of Rheumatology (ACR) workforce surveys, 30% of rheumatologists in the US were women in 2005 (3), improving to 41% in 2015 (4). By 2030, it is anticipated that women will make up 57% of the US rheumatology workforce (4). Women represented 47% of the rheumatology consultant workforce in the UK in 2018 (5), and ~50% of rheumatology specialists in Australia and New Zealand in 2019 (6). In 2015, although 41% of US academic rheumatology faculty were women, women were less likely to be associate or full professors (7).

Publication of research articles is central to academic promotion (8–10). Gender bias in authorship of scientific articles is well-described. Overall, men have a higher publication rate than women across multiple scientific disciplines (1,11,12), and women authors receive fewer citations (13,14). Women authors are also underrepresented in first and senior authorship positions in articles published in medical journals, even in disciplines such as family medicine which are enriched for women practitioners (1,12,15). Even in articles in which first and second authors of different gender contribute equally, men are more likely to be listed first (16).

In academic medicine, clinical trial leadership is important for career advancement, prominence in the field, and future funding opportunities. In oncology clinical trials, women are underrepresented as lead investigators in industry-funded studies (17). Furthermore, in industry-funded collaborative cancer trials, women are underrepresented as first and senior authors, compared with trials not funded by industry (18). It is unknown whether funding source influences authorship gender for other specialties.

The aim of this study was to examine the contemporary representation of women as first and senior authors of rheumatology original research articles.

Ms Bagga's work was supported by a University of Auckland Summer Studentship award.

Ekta Bagga, Sarah Stewart, PhD, Gregory D. Gamble, MSc, Janine Hill, MSc, Andrew Grey, MD, Nicola Dalbeth, MD: University of Auckland, Auckland, New Zealand.

Dr. Dalbeth has received consulting fees, speaking fees, and/or honoraria from AstraZeneca, Horizon, Amgen, Selecta, Arthrosi, Dyve Biosciences, Hengrui, AbbVie, and Janssen (less than $10,000 each). No other disclosures relevant to this article were reported.

Address correspondence to Nicola Dalbeth, MD, University of Auckland, Department of Medicine, Faculty of Medical and Health Sciences, 85 Park Road, Grafton, Auckland 1010, New Zealand. Email: n.dalbeth@auckland.ac.nz.

Submitted for publication May 12, 2020; accepted in revised form August 11, 2020.

MATERIALS AND METHODS

Identification of journals and articles for inclusion. All original articles published in general rheumatology journals with a 2016 Thomson Reuters impact factor >3.0 (*Annals of the Rheumatic Diseases, Arthritis & Rheumatology, Rheumatology, Seminars in Arthritis and Rheumatism, Arthritis Research & Therapy, Joint Bone Spine, Arthritis Care & Research,* and *The Journal of Rheumatology*) were considered for inclusion. All original research articles describing rheumatic diseases published in general medical journals with a 2016 Thomson Reuters impact factor >15.0 (*The New England Journal of Medicine, The Lancet, Journal of the American Medical Association, The British Medical Journal, JAMA Internal Medicine,* and *Annals of Internal Medicine*) were also considered for inclusion.

All original research articles published over a 5-year period between January 2015 and December 2019 were included in the analysis. Included articles were full or concise reports of clinical or basic research or systematic literature reviews and meta-analyses. Articles were excluded if they were narrative review articles, recommendations, guidelines, letters, or meeting proceedings.

Data extraction. All data were extracted into a Microsoft Access database. For each article, the journal, year, issue, gender of the first and last (senior) authors, research design (randomized controlled trial, other clinical, systematic review/meta-analysis, or basic research), funding source, industry initiation, and region of affiliation of the first author (categorized as North America, Europe, or other) was recorded. In addition, the disease category (ankylosing spondylitis and other spondyloarthritides, crystal arthritis, osteoarthritis, miscellaneous rheumatic disease, pediatric rheumatology, pain syndromes, psoriatic arthritis, rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis/scleroderma, other connective tissue disorders, vasculitis, not disease-specific) was also extracted using a previously established set of rules to ensure standardization in categorizing (19).

When the author's gender was uncertain on initial inspection of their first name, or in cases where only an initial of their first name was provided, an internet search using the author's name and institutional affiliation was used to identify individual web pages or online profiles that included a photograph of the individual. If the gender remained unclear, the author's first name was entered into genderize.io (https://api.genderize.io/?name=) which returns the gender and probability of certainty. Probabilities <0.5 were labeled as "unknown."

The source of funding for each study was categorized as industry-funded or not industry-funded based on funding declaration statements appearing in the article (i.e., under "funding" or "acknowledgments" sections). Articles that did not declare industry funding were assumed to be not industry-funded. Industry-funded studies were further separated into investigator-initiated or industry-initiated studies, based on declarations in the manuscript. For the purpose of analysis, articles were categorized as "industry-funded/industry-initiated," "industry-funded/investigator-initiated," and "not industry-funded".

Prior to data extraction, 2 researchers (EB and SS) independently extracted data from 5 randomly selected issues to ensure standardization. A total of 65 articles were reviewed, with kappa scores of 1.00 for first author gender, last author gender, geographic region, and funding source, and 0.98 (95% confidence interval [95% CI] 92.7–99.9%) for disease category (98.5% agreement). All data were then extracted by one of the 2 researchers (EB or SS).

Data analysis. Descriptive statistics were used to report the proportion of articles with women and men as first and last authors, as well as the proportion of articles authored by women according to geographic region, disease category, research design, and funding source for each gender. The percentage and 95% CIs for the proportion of articles with first and senior authors who were

Table 1. Characteristics of the 7,651 articles included in the analysis*

Journal type	
General rheumatology journal	7,554 (98.7)
General medical journal	97 (1.3)
First author gender	
Woman	3,939 (51.5)
Man	3,712 (48.5)
Senior author gender	
Woman	2,699 (35.3)
Man	4,952 (64.7)
Geographic region	
Europe	3,852 (50.3)
North America	2,410 (31.5)
Other	1,389 (18.2)
Disease category	
Ankylosing spondylitis	558 (7.3)
Crystal arthritis	347 (4.5)
Miscellaneous	294 (3.8)
Not disease-specific	491 (6.4)
Osteoarthritis	857 (11.2)
Other connective tissue diseases	370 (4.8)
Pain syndromes	154 (2.0)
Pediatric rheumatology	442 (5.8)
Psoriatic arthritis	287 (3.8)
Rheumatoid arthritis	2,159 (28.2)
Systemic lupus erythematosus	762 (10.0)
Systemic sclerosis	538 (7.0)
Vasculitis	392 (5.1)
Research design	
Basic science	1,801 (23.5)
Randomized controlled trial	603 (7.9)
Systematic literature review/meta-analysis	449 (5.9)
Other clinical	4,798 (62.7)
Funding source	
Industry-funded/industry-initiated	724 (9.5)
Industry-funded/investigator-initiated	734 (9.6)
Not industry-funded	6,193 (80.9)

* Values are the number (%).

Table 2. Proportion of articles according to author gender*

	First author gender, woman		Senior author gender, woman	
	No. of articles	% (95% CI)	No. of articles	% (95% CI)
All (n = 7,651)	3,939	51.5 (50.4–52.6%)	2,699	35.3 (34.2–36.4)
Geographic region				
Europe (n = 3,852)	2,092	54.3 (52.7–55.9)	1,350	35.0 (33.6–36.6)
North America (n = 2,410)	1,254	52.0 (50.0–54.0)	899	37.3 (35.4–39.3)
Other (n = 1,389)	593	42.7 (40.1–45.3)	450	32.4 (30.0–34.9)
Disease category				
Ankylosing spondylitis (n = 558)	294	52.7 (48.5–56.8)	206	36.9 (33.0–41.0)
Crystal arthritis (n = 347)	188	54.2 (48.9–59.4)	115	33.1 (28.3–38.2)
Miscellaneous (n = 294)	135	45.9 (40.3–51.6)	94	32.0 (26.8–37.5)
Not disease-specific (n = 491)	267	54.4 (50.0–58.8)	165	33.6 (29.5–37.9)
Osteoarthritis (n = 857)	419	48.9 (45.6–52.2)	326	38.0 (34.8–41.3)
Other connective tissue diseases (n = 370)	176	47.6 (42.5–52.7)	119	32.2 (27.6–37.1)
Pain syndromes (n = 154)	86	55.8 (47.9–63.5)	62	40.3 (32.7–48.2)
Pediatric rheumatology (n = 442)	258	58.4 (53.7–62.9)	187	42.3 (37.8–47.0)
Psoriatic arthritis (n = 287)	147	51.2 (45.4–57.0)	112	39.0 (33.5–44.8)
Rheumatoid arthritis (n = 2,159)	1,098	50.9 (48.8–53.0)	770	35.7 (33.7–37.7)
Systemic lupus erythematosus (n = 762)	417	54.7 (51.2–58.2)	274	36.0 (32.6–39.4)
Systemic sclerosis (n = 538)	283	52.6 (48.4–56.8)	173	32.2 (28.3–36.2)
Vasculitis (n = 392)	171	43.6 (38.8–48.6)	96	24.5 (20.4–28.9)
Research design				
Basic science (n = 1,801)	931	51.7 (49.4–54.0)	542	30.1 (28.0–32.2)
Randomized controlled trial (n = 603)	201	33.3 (29.7–37.2)	159	26.4 (23.0–30.0)
Systematic literature review/meta-analysis (n = 449)	227	50.6 (45.9–55.2)	178	39.6 (35.2–44.2)
Other clinical (n = 4,798)	2,580	53.7 (52.4–55.2)	1,820	37.9 (36.6–39.3)
Funding source				
Industry-funded/industry-initiated (n = 724)	284	39.2 (35.7–42.8)	224	30.9 (27.7–34.3)
Industry-funded/investigator-initiated (n = 734)	369	50.3 (46.7–53.9)	261	35.6 (32.2–39.1)
Not industry-funded (n = 6,193)	3,286	53.1 (51.8–54.3)	2,214	35.8 (34.6–37.0)

* 95% CI = 95% confidence interval.

women was calculated using openepi.com (20). Since relatively few articles (18.2%) were from regions outside Europe and North America, 3 geographic region categories were analyzed (Europe, North America, and other). Data were plotted against a hypothetical gender parity (50%) and the percentage of women in the 2015 US academic rheumatology workforce (41%) (7).

To compare differences between groups, odds ratios (ORs) and their 95% CIs were computed. Linear-by-linear association tests (Cochran-Armitage trend tests) were used to analyze trends in authorship gender between 2015 and 2019 using SPSS version 26.0 (IBM). All tests were 2-tailed. *P* values less than 0.05 were considered significant, and no adjustments for multiplicity were made.

RESULTS

Articles. Data were extracted from a total of 7,699 articles, including 7,602 articles from general rheumatology journals. From the general medical journals, there were 4,588 original research articles published between 2015 and 2019, of which 97 articles reporting rheumatology research were included. Gender could not be determined for the first author in 14 articles (0.2%), the senior author in 31 articles (0.4%), and both the first and senior authors in 3 articles (0.04%); these articles were excluded from further analysis. In total, 7,651 articles were analyzed. The characteristics of these articles are shown in Table 1.

Of the articles included, 51.5% had women first authors (95% CI 50.4–52.6%), and 35.3% had women senior authors (95% CI 34.2–36.4%) (Table 2). Articles from geographic regions other than Europe and North America had a lower proportion of first

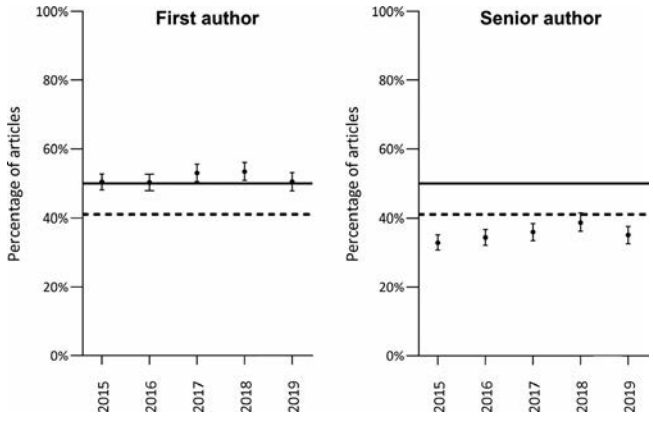


Figure 1. Percentage of articles with women first authors and women senior authors over the study period. Solid line indicates gender parity (50%); broken line shows the percentage of women in the 2015 US academic rheumatology workforce. Values are the percent and 95% confidence interval.

authors who were women (42.7% [95% CI 40.1–45.3%]) (Table 2). The proportion of women senior authors was <40% for articles from all geographic regions.

Similar patterns of authorship gender were observed for articles related to different rheumatic diseases (Table 2). Pediatric rheumatology articles had the highest proportion of women first and senior authors, and vasculitis articles had the lowest proportion of women first and senior authors.

There was no significant change in gender patterns for first authors between 2015 and 2019 (P for trend = 0.30). However, there was a small increase in women senior authors over this period (P for trend = 0.019) (Figure 1 and Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41490/abstract).

Analysis of the gender of first author and senior author pairs demonstrated that women had higher odds than men of being a first author on an article with a woman senior author (OR 1.91 [95% CI 1.73, 2.10]), $P < 0.001$) (Table 3).

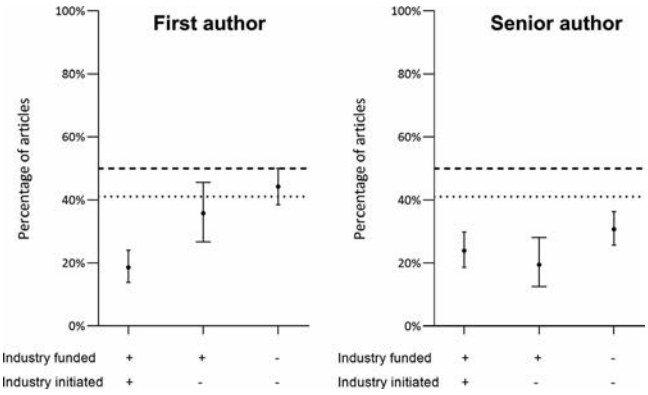


Figure 2. Percentage of articles reporting randomized controlled trials with women first authors and women senior authors according to funding source. Dashed line indicates gender parity (50%); dotted line shows the percentage of women in the 2015 US academic rheumatology workforce. Values are the percent and 95% confidence interval.

Research design. Similar proportions of women first authors were observed for articles reporting basic science (51.7% [95% CI 49.4–54.0%]), systematic literature reviews/meta-analyses (50.6% [95% CI 45.9–55.2%]), and other clinical research (53.7% [95% CI 52.4–55.2%]), but only 33.3% of articles that reported randomized controlled trials had women first authors (95% CI 29.7–37.2%) (Table 2). Women had significantly lower odds of being first authors of articles reporting randomized controlled trials compared with articles reporting basic science research (OR 0.47 [95% CI 0.39–0.57], $P < 0.001$), systematic literature reviews/meta-analyses (OR 0.49 [95% CI 0.38–0.63], $P < 0.001$), and other clinical research (OR 0.43 [95% CI 0.36–0.51], $P < 0.001$).

The highest proportions of women senior authors were for articles reporting systematic literature reviews/meta-analyses and other clinical research (39.6% [95% CI 35.2–44.2%] and 37.9% [95% CI 36.6–39.3%], respectively). The lowest proportions of women senior authors were observed for articles reporting basic science (30.1% [95% CI 28.0–32.2%]) and randomized controlled trials (26.4% [95% CI 23.0–30.0%]) (Table 2). Women had significantly lower odds of being senior authors on articles reporting randomized controlled trials compared with articles reporting other clinical research (OR 0.59 [95% CI 0.48–0.71], $P < 0.001$) and systematic literature reviews/meta-analyses (OR 0.55 [95% CI 0.42–0.71], $P < 0.001$).

Table 3. Gender of first and senior author pairs in the 7,651 articles included in the analysis*

	First author gender, woman	First author gender, man
Senior author gender, woman		
No. of articles	1,667	1,032
% (95% CI)	21.8 (21–23)	13.5 (13–14)
Senior author gender, man		
No. of articles	2,272	2,680
% (95% CI)	29.7 (29–31)	35.0 (34–36)

* 95% CI = 95% confidence interval.

Funding sources. Of the articles reporting industry-funded/industry-initiated studies, women were first authors of 39.2% (95% CI 35.7–42.8%) and senior authors of 30.9% (95% CI 27.7–34.3%). Women were less likely to be first authors of articles reporting industry-funded/industry-initiated research than of articles reporting industry-funded/investigator-initiated studies (OR 0.64 [95% CI 0.52–0.79], $P < 0.001$) or of research not funded by industry (OR 0.57 [95% CI 0.49–0.67], $P < 0.001$) (Table 2).

Similarly, women were less likely to be senior authors of articles reporting industry-funded/industry-initiated research compared with articles reporting research not funded by industry (OR 0.81 [95% CI 0.68–0.95], $P = 0.010$), with a similar trend for comparison with articles reporting industry-funded/investigator-initiated research (OR 0.81 [95% CI 0.65–1.01], $P = 0.067$). Of the articles reporting industry-funded/industry-initiated randomized controlled trials, only 18.5% had women first authors (95% CI 13.8–24.0%) and 23.9% had women senior authors (95% CI 18.6–29.8%) (Figure 2 and Supplementary Table 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41490/abstract).

DISCUSSION

This study demonstrated that women are underrepresented in senior authorship of rheumatology research articles, compared with both hypothetical gender parity (50%) and the percentage of women in the 2015 US academic rheumatology workforce

(41%). There is underrepresentation of women in both first and senior authorship positions in articles reporting rheumatology randomized controlled trials, especially those that are initiated by industry.

For the entire data set, the proportion of women first authors was consistent with hypothetical gender parity and higher than the academic rheumatology workforce proportion. The pattern of more women first authors compared with senior authors is consistent with studies in other fields, including gastroenterology (21), oncology research (21), pharmacy (22), and pediatrics (23). The proportion of women in the rheumatology workforce has grown rapidly over the past decade, and it is predicted that women will be the majority of the rheumatology workforce in the next 10 years (4). As first authors of most studies tend to be those who are more junior in experience (24), the higher proportion of women first authors is consistent with the changing gender distribution of the rheumatology workforce. The gender differences between first and senior author may also signify challenges to career progression for women entering academic rheumatology (25). The genders of the first and last authors were associated, with women more likely than men to be first authors of rheumatology articles with women senior authors. This authorship pattern is also described in other disciplines (26,27) and may be due to the tendency for women in senior positions to select and mentor women; prior research has shown that male senior authors are less likely to mentor junior women in medical academia (28). Early in their career, women may also seek women mentors due to shared social identity (29).

In contrast to first author position, we observed fewer women senior authors, below both hypothetical gender parity and US academic rheumatology workforce levels. The most striking gender disparities were observed for randomized controlled trials, with low proportions of both women first authors and women senior authors. This finding is consistent with a recent analysis of biomedical and internal medicine journals in which women were less likely than men to author articles reporting clinical trials (7% versus 13%, respectively) (30). Our findings may be due to the low number of women in rheumatology academic leadership positions, with women less likely to be full or associate professors compared to men (7,31). Given that randomized controlled trials are widely regarded as the highest quality of research and have a large impact on clinical practice (32), these findings highlight a potential barrier in career advancement for women rheumatologists.

Consistent with findings in oncology (18), women were less likely to be first or senior authors of rheumatology articles that were funded and initiated by industry. Women physicians and academics receive significantly fewer industry-sponsored research grants compared to men (33,34). Gender differences in financial relationships are also apparent for speaker and consulting relationships with industry (33,35). Our analysis does not allow interrogation of the causes of these gender differences, but our results may reflect industry selection of men with higher perceived "authority" status (13). Women may also be less willing to or have less interest in work with industry.

A potential limitation of this analysis was that individual author names were not analyzed, and it is possible that multiple articles were authored by the same person. Given the relatively low number of women in academic rheumatology leadership positions (7,31), our method of analysis may have overrepresented the number of women authors of rheumatology publications, particularly in senior positions. A further limitation is that our analysis of industry funding was dependent on author disclosures, which may have been incomplete (36).

In conclusion, this analysis of rheumatology publications has identified some gender disparities in the authorship of original research articles. Women are underrepresented as senior authors, and as authors of clinical trials, particularly those funded and initiated by industry. These findings highlight the need for institutional and industry leaders to take steps to ensure that women are represented equally as the gender gap in the rheumatology workforce narrows in the future.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Dalbeth had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Stewart, Gamble, Grey, Dalbeth.
Acquisition of data. Bagga, Stewart, Gamble, Hill.
Analysis and interpretation of data. Bagga, Stewart, Gamble, Grey, Dalbeth.

REFERENCES

1. Filardo G, da Graca B, Sass DM, Pollock BD, Smith EB, Martinez MA. Trends and comparison of female first authorship in high impact medical journals: observational study (1994–2014). BMJ 2016;352:i847.
2. Allen I. Women doctors and their careers: what now? BMJ 2005; 331:569–72.
3. Hogan PF, Boucherry E, on behalf of the Lewin Group. Workforce study of rheumatologists: final report. American College of Rheumatology. May 2006. URL: https://www.rheumatology.org/Portals/0/Files/LewinReport.pdf.
4. Bolster MB, Bass AR, Hausmann JS, Deal C, Ditmyer M, Greene KL, et al. 2015 American College of Rheumatology Workforce Study: the role of graduate medical education in adult rheumatology. Arthritis Rheumatol 2018;70:817–25.
5. Royal College of Physicians. Focus on physicians: 2018–19 census (UK consultants and higher specialty trainees). October 2019. URL: https://www.rcplondon.ac.uk/projects/outputs/focus-physicians-2018-19-census-uk-consultants-and-higher-specialty-trainees.
6. RACP Member Statistics and Insights. Sydney, Australia: Royal Australasian College of Physicians; 2020. URL: https://www.racp.edu.au//docs/default-source/secured-documents-library/racp-member-statistics-and-insights-report-2020.pdf?sfvrsn=1183f41a_6.
7. Jorge A, Fu X, Blumenthal D, Gross N, Bolster M, Wallace Z. The association between physician sex and faculty rank among academic rheumatologists in the United States [abstract]. Arthritis Rheumatol

2019;71 Suppl 10. URL: https://acrabstracts.org/abstract/the-association-between-physician-sex-and-faculty-rank-among-academic-rheumatologists-in-the-united-states/.

8. Rexrode KM. The gender gap in first authorship of research papers. BMJ 2016;352:i1130.
9. Bavdekar SB, Tullu MS. Research publications for academic career advancement: an idea whose time has come. But is this the right way? [editorial]. J Postgrad Med 2016;62:1–3.
10. Post RE, Weese TJ, Mainous AG III, Weiss BD. Publication productivity by family medicine faculty: 1999 to 2009. Fam Med 2012;44:312–7.
11. Lariviere V, Ni C, Gingras Y, Cronin B, Sugimoto CR. Bibliometrics: global gender disparities in science. Nature 2013;504:211–3.
12. Jagsi R, Guancial EA, Worobey CC, Henault LE, Chang Y, Starr R, et al. The "gender gap" in authorship of academic medical literature: a 35-year perspective. N Engl J Med 2006;355:281–7.
13. Knobloch-Westerwick S, Glynn CJ. The Matilda effect—role congruity effects on scholarly communication: a citation analysis of Communication Research and Journal of Communication articles. Commun Res 2011;40:3–26.
14. Peñas CS, Willett P. Brief communication: gender differences in publication and citation counts in librarianship and information science research. J Inf Sci 2006;32:480–5.
15. Schrager S, Bouwkamp C, Mundt M. Gender and first authorship of papers in family medicine journals 2006–2008. Fam Med 2011;43:155–9.
16. Broderick NA, Casadevall A. Gender inequalities among authors who contributed equally. Elife 2019;8:e36399.
17. Ludmir EB, Mainwaring W, Miller AB, Lin TA, Jethanandani A, Espinoza AF, et al. Women's representation among lead investigators of clinical trials in oncology [letter]. JAMA Oncol 2019;5:1501–2.
18. Sun GH, Moloci NM, Schmidt K, MacEachern MP, Jagsi R. Representation of women as authors of collaborative cancer clinical trials [letter]. JAMA Intern Med 2014;174:806–8.
19. Stewart S, Gamble G, Grey A, Dalbeth N. Article placement order in rheumatology journals: a content analysis. BMJ Open 2020; 10:e034550.
20. Dean AG, Sullivan KM, Soe MM. OpenEpi: a web-based epidemiologic and statistical calculator for public health. Public Health Rep 2009;124:471–4.
21. Long MT, Leszczynski A, Thompson KD, Wasan SK, Calderwood AH. Female authorship in major academic gastroenterology journals: a look over 20 years. Gastrointest Endosc 2015;81:1440–7.
22. Dotson B. Women as authors in the pharmacy literature: 1989–2009. Am J Health Syst Pharm 2011;68:1736–9.
23. Fishman M, Williams WA II, Goodman DM, Ross LF. Gender differences in the authorship of original research in pediatric journals, 2001–2016. J Pediatr 2017;191:244–9.
24. Bhattacharya S. Authorship issue explained [letter]. Indian J Plast Surg 2010;43:233–4.
25. Bates C, Gordon L, Travis E, Chatterjee A, Chaudron L, Fivush B, et al. Striving for gender equity in academic medicine careers: a call to action. Acad Med 2016;91:1050–2.
26. Dworkin JD, Linn KA, Teich EG, Zurn P, Shinohara RT, Bassett DS. The extent and drivers of gender imbalance in neuroscience reference lists. Nat Neurosci 2020;23:918–26.
27. Silver JK, Poorman JA, Reilly JM, Spector ND, Goldstein R, Zafonte RD. Assessment of women physicians among authors of perspective-type articles published in high-impact pediatric journals. JAMA Network Open 2018;1:e180802.
28. DeCastro R, Griffith KA, Ubel PA, Stewart A, Jagsi R. Mentoring and the career satisfaction of male and female academic medical faculty. Acad Med 2014;89:301–11.
29. Sosik JJ, Godshalk VM. The role of gender in mentoring: implications for diversified and homogenous mentoring relationships. J Vocational Behav 2000;57:102–22.
30. Sebo P, Maisonneuve H, Fournier JP. Gender gap in research: a bibliometric study of published articles in primary health care and general internal medicine. Fam Pract 2020;37:325–31.
31. Lundberg IE, Ozen S, Gunes-Ayata A, Kaplan MJ. Women in academic rheumatology. Arthritis Rheum 2005;52:697–706.
32. Guyatt GH, Oxman AD, Kunz R, Vist GE, Falck-Ytter Y, Schünemann HJ. What is "quality of evidence" and why is it important to clinicians? BMJ 2008;336:995–8.
33. Rose SL, Sanghani RM, Schmidt C, Karafa MT, Kodish E, Chisolm GM. Gender differences in physicians' financial ties to industry: a study of national disclosure data. PLoS One 2015;10: e0129197.
34. Waisbren S, Bowles H, Hasan T, Zou KH, Emans SJ, Goldberg C, et al. Gender differences in research grant applications and funding outcomes for medical school faculty. J Womens Health (Larchmt) 2008;17:207–14.
35. Raber I, McCarthy CP, Al Rifai M, Vaduganathan M, Michos ED, Wood MJ, et al. Gender differences in industry payments among cardiologists. Am Heart J 2020;223:123–31.
36. Andreatos N, Zacharioudakis IM, Zervou FN, Muhammed M, Mylonakis E. Discrepancy between financial disclosures of authors of clinical practice guidelines and reports by industry. Medicine (Baltimore) 2017;96:e5711.

American College of Rheumatology
Empowering Rheumatology Professionals

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 168–172
DOI 10.1002/art.41492

BRIEF REPORT

The Association Between Physician Gender and Career Advancement Among Academic Rheumatologists in the United States

April Jorge,[1] Marcy Bolster,[1] Xiaoqing Fu,[1] Daniel M. Blumenthal,[1] Nate Gross,[2] Kimberly G. Blumenthal,[1] and Zachary Wallace[1]

Objective. To determine the potential association between physician gender and academic advancement among US rheumatologists.

Methods. We performed a nationwide, cross-sectional study of all rheumatologists practicing in the US in 2014 using a comprehensive database of all licensed physicians. Among academic rheumatologists, we estimated gender differences in faculty rank, adjusting for differences in physician age, years since residency graduation, publications, National Institutes of Health (NIH) grants, registered clinical trials, and appointment at a top 20 medical school using a multivariate logistic regression model. We also estimated gender differences in leadership positions (i.e., division director and fellowship program director).

Results. Among 6,125 total practicing rheumatologists, 941 (15%) had academic faculty appointments in 2014. Women academic rheumatologists (41.4%) were younger and had completed residency more recently than men. Women had fewer total publications, publications on which they were the first or last author, and NIH grants. In fully adjusted analyses, women were less likely to be full or associate professors than men, with an adjusted odds ratio (OR) of 0.78 (95% confidence interval [95% CI] 0.62–0.99]). Women in rheumatology had similar odds as men of being a fellowship program director or division director (adjusted OR 0.99 [95% CI 0.69–1.43] and adjusted OR 0.96 [95% CI 0.66–1.41], respectively).

Conclusion. Among academic rheumatologists, women are less likely than men to be full or associate professors but have similar odds of being fellowship program directors or division directors, when adjusting for several factors known to influence faculty promotion. These differences suggest barriers to academic promotion despite representation in leadership positions within rheumatology divisions.

INTRODUCTION

The number of women in medicine continues to increase, with current medical school graduating classes comprising ~50% women (1,2). In comparison, 50 years ago, women made up <10% of medical school graduating classes and academic faculty (3). Rheumatology has seen a dramatic increase in the number of women in the specialty, with the 2015 American College of Rheumatology (ACR) Workforce Study reporting that women currently represent 41% of the rheumatology workforce and 66% of rheumatology fellows (4,5). Projections suggest that women will comprise the majority of the rheumatology workforce by 2025 (4). Since rheumatology is a specialty comprising men and women equally, it is of interest to determine the gender equity among

Presented in part at the 83rd Annual Scientific Meeting of the American College of Rheumatology, Atlanta, GA, November 2019.

Dr. Jorge is recipient of a Scientist Development award from the Rheumatology Research Foundation. Dr. Wallace's work was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH (grants K23-AR-073334 and L30-AR-070520).

[1]April Jorge, MD, Marcy Bolster, MD, Xiaoqing Fu, MS, Daniel M. Blumenthal, MD, MBA, Kimberly G. Blumenthal, MD, MSc, Zachary Wallace, MD, MSc: Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts; [2]Nate Gross, MD, MBA: Doximity, San Francisco, California.

Dr. Bolster has received consulting fees from Gilead (less than $10,000), research support from Genentech, and owns stock or stock options in Johnson & Johnson. Dr. Gross owns stock or stock options in Doximity. No other disclosures relevant to this article were reported.

Address correspondence to April Jorge, MD, Mongan Institute, Massachusetts General Hospital, 100 Cambridge Street, 1667F, Boston, MA 02114. Email: AMJorge@mgh.harvard.edu.

Submitted for publication February 20, 2020; accepted in revised form August 11, 2020.

faculty and leadership positions as well as the opportunity for men and women to achieve professional growth.

It is well documented that a gender gap exists between men and women with regard to academic rank, leadership roles, and remuneration across US academic medical centers (6–8). In a study of the US academic physician workforce, women were less likely than men to achieve academic ranks of associate or full professor, even after adjustment for age, experience, specialty, and productivity (1). Similar studies in internal medicine have found variation across subspecialties with regard to the likelihood of women achieving faculty promotion. For instance, women in cardiology are less likely to achieve higher academic ranks than men, whereas no differences were observed between men and women with regard to faculty rank in allergy/immunology (9,10). Differences in academic promotion and leadership positions among women and men in rheumatology have not been previously evaluated. Defining these benchmarks is important to facilitate actions that will improve parity in the rheumatology workforce, help maintain women in academic rheumatology, and ensure that the distribution of men and women in academic rheumatology reflects the demographics of the specialty. In this study, we sought to evaluate differences in academic advancement by gender within rheumatology.

MATERIALS AND METHODS

Data source and study population. We obtained comprehensive, cross-sectional information on physicians in the US from Doximity, a company that provides a free online networking service for physicians. Physicians do not need to register for an account to be included in this database; it includes all physicians with a registered National Provider Identifier (NPI) number as well as physicians without NPI numbers who have self-registered for an account with Doximity. Data captured on US physicians include age, sex, year of medical school graduation, year of residency graduation, appointment at a US medical school, faculty rank, and American Board of Internal Medicine specialty certification. The database also includes total numbers of publications as well as numbers of those designated as first-author and last-author publications (derived from PubMed), the number of National Institutes of Health (NIH) grants with the role of the principal investigator (PI) (derived from NIH Research Portfolio Online Reporting Tools), and the number of clinical trials with the role of PI or subinvestigator (derived from ClinicalTrials.gov). The designation of the top 20 US medical schools was identified by US News and World Report in 2013. Prior studies have used this database to study faculty promotion in academic medicine (1,9–11), and the data validity for faculty rank, NIH grants, and publications has been previously verified (1). We additionally validated the academic rank of 25 randomly selected rheumatologists.

We identified all adult rheumatologists in the US physician database with an academic faculty appointment in 2014 and a listed faculty rank of instructor, assistant professor, associate professor, or professor. We extracted the physician information described above.

We also identified the physicians with leadership positions as academic division directors and fellowship program directors by performing a manual review of all academic rheumatology division websites, which were obtained from the Association of American Medical Colleges (12). These data were collected between May and October, 2019. We subsequently linked these positions with the covariates extracted from Doximity. If data were missing from a website, we contacted members of the rheumatology faculty at those institutions to clarify department leadership.

Statistical analysis. We performed descriptive statistics, comparing physician characteristics among men and women, and performed 2-sided *t*-tests for continuous variables and chi-square tests for categorical variables. We divided physicians by 10-year period of internal medicine residency graduation (1965–1974, 1975–1984, 1985–1994, 1995–2004, and 2005–2014) and determined the proportions of women and men in rheumatology with each academic faculty appointment (e.g., instructor, assistant professor, associate professor, and professor) in 2014 per cohort.

Using the primary outcome of faculty rank as a function of gender, we performed univariate logistic regression to determine the odds ratio (OR) and associated 95% confidence interval (95% CI) of women achieving the rank of associate professor or professor compared to men. We combined associate and professor ranks because these reflect senior faculty positions. As a secondary outcome, we determined the OR of women achieving the rank of professor compared with men. We performed multivariate logistic regression analyses to determine the adjusted ORs of these primary and secondary outcomes as a function of physician gender when adjusting for age, years since internal medicine residency graduation, total publications, total NIH grants, total clinical trials involvement, and faculty at top 20 medical schools. We excluded rheumatologists with the rank of instructor from these analyses.

Next, we compared the unadjusted and adjusted OR of women being division directors or fellowship program directors compared with men. In the multivariable analyses, we adjusted for the same physician characteristics as in the faculty rank analysis.

All analyses were performed using SAS version 9.4 (SAS Institute). *P* values less than or equal to 0.05 (2-tailed) were considered significant. This study was exempted from review by the Partners HealthCare Institutional Review Board.

RESULTS

We identified 6,125 practicing rheumatologists, 941 (15%) of whom had academic faculty appointments in 2014. Among academic rheumatologists, we observed a gradual increase in the proportion of women entering academic rheumatology in each successive decade.

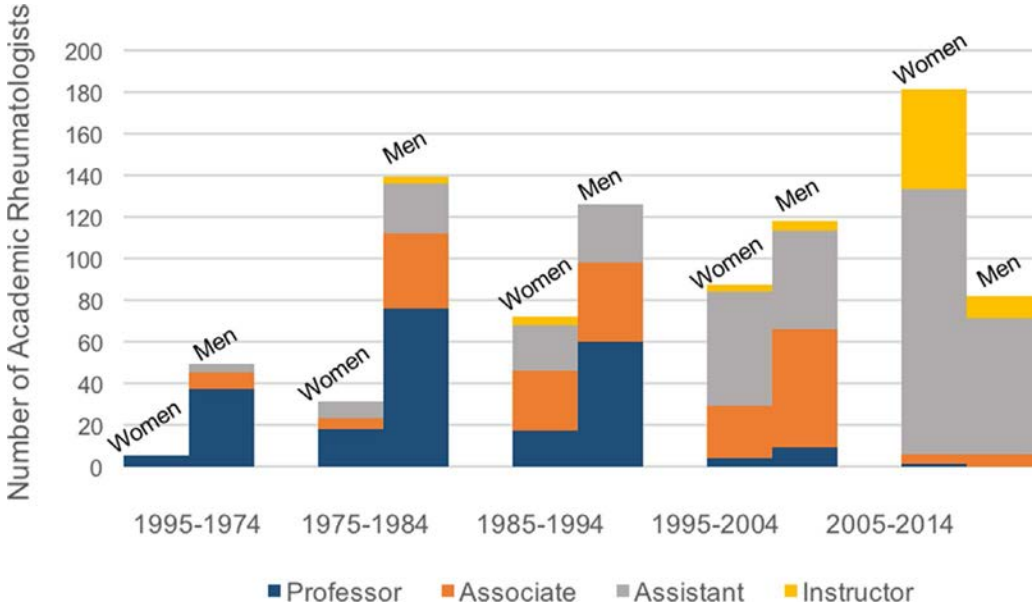


Figure 1. Academic rank of faculty rheumatologists in 2014 by year of residency graduation, according to gender.

Most recently, the number of women entering academic rheumatology exceeded the number of men (Figure 1). We also observed several differences between men and women in academic rheumatology (Table 1). On average, women were younger than men (mean ± SD age 46.0 ± 9.7 years versus 56.8 ± 11.9 years; $P < 0.001$) and had fewer total publications (mean ± SD 12.4 ± 20.8 versus 26.4 ± 39.4; $P < 0.001$) and NIH grants (mean ± SD 0.8 ± 3.0 versus 1.7 ± 4.4; $P < 0.001$). There was no difference in the number of clinical trials for which women and men were listed as the PI (mean ± SD 0.2 ± 0.8 versus 0.2 ± 0.8; $P = 0.7$).

Table 1. Characteristics of academic rheumatologists in the US

	Overall (n = 941)	Men (n = 551)	Women (n = 390)	*P*
Faculty rank, no. (%)				<0.001
Professor	249 (26.7)	200 (36.8)	49 (12.6)	
Associate professor	220 (23.6)	152 (28.0)	68 (17.5)	
Assistant professor	387 (41.5)	171 (31.5)	216 (55.5)	
Instructor	76 (8.2)	20 (3.7)	56 (14.4)	
Faculty at top 20 medical school, no. (%)	320 (38.1)	184 (35.9)	136 (41.6)	0.096
Age, mean ± SD years	52.3 ± 12.3	56.8 ± 11.9	46.0 ± 9.7	<0.001
Age group, no. (%)				<0.001
<40 years	165 (18.4)	41 (7.8)	124 (33.5)	
40–44 years	132 (14.7)	64 (12.1)	68 (18.4)	
45–49 years	96 (10.7)	52 (9.9)	44 (11.9)	
50–54 years	106 (11.8)	54 (10.2)	52 (14.1)	
55–59 years	121 (13.5)	79 (15.0)	42 (11.4)	
60–64 years	127 (14.1)	99 (18.8)	28 (7.6)	
≥65 years	151 (16.8)	139 (26.3)	12 (3.2)	
Years since residency, mean ± SD	22.0 ± 12.9	26.6 ± 13.0	15.7 ± 9.8	<0.001
FACR, no. (%)*	507 (53.9)	300 (54.5)	207 (53.1)	0.678
Publications				
Total no., mean ± SD	20.6 ± 33.7	26.4 ± 39.4	12.4 ± 20.8	<0.001
No. as first or last author, mean ± SD	14.0 ± 28.9	18.4 ± 33.9	7.8 ± 17.9	<0.001
No. (%) with any publication	739 (78.5)	449 (81.5)	290 (74.4)	0.009
National Institutes of Health grants				
No., mean ± SD	1.3 ± 3.9	1.7 ± 4.4	0.8 ± 3.0	<0.001
No. (%) with any grant	164 (17.4)	117 (21.2)	47 (12.1)	<0.001
Clinical trials†				
Total no., mean ± SD	0.2 ± 0.8	0.2 ± 0.8	0.2 ± 0.8	0.676
Any clinical trial, no. (%)	76 (8.1)	50 (9.1)	26 (6.7)	0.182

* FACR = Fellow of the American College of Rheumatology.
† Listed as principal investigator for studies registered on ClinicalTrials.gov.

Table 2. Leadership positions by gender in academic rheumatology*

Academic position	Women	Men	Unadjusted OR (95% CI)	Adjusted OR (95% CI)†
No.	390	551	–	–
Faculty rank				
Associate professor or professor	117 (30.1)	352 (64.8)	0.52 (0.45–0.60)	0.78 (0.62–0.99)
Professor	49 (12.6)	152 (28.0)	0.54 (0.45–0.64)	1.02 (0.77–1.37)
Leadership role				
Division director	34 (8.7)	74 (13.4)	0.94 (0.72–1.21)	0.96 (0.66–1.41)
Program director	53 (13.6)	64 (11.6)	1.13 (0.88–1.46)	0.99 (0.69–1.43)

* Values are the number (%). OR = odds ratio; 95% CI = 95% confidence interval.
† Adjusted for age, years since residency graduation, total number of publications, total number of National Institutes of Health grants, total number of clinical trials, and faculty at top 20 medical schools.

Compared to men, fewer women were professors (12.6% versus 36.8%) or associate professors (17.5% versus 28.0%); however, a greater proportion of women were assistant professors (55.5% versus 31.5%). These differences were observed in the 2 most recent decades of residency graduation for which sufficient follow-up time had accrued to permit academic promotion (Figure 1). In unadjusted analyses, women were less likely than men to be full or associate professors (OR 0.52 [95% CI 0.45–0.60]) (Table 2). These differences persisted in fully adjusted analyses (adjusted OR 0.78 [95% CI 0.62–0.99]). When the odds of being a full professor were examined individually (versus assistant professor or associate professor), we found no difference between women and men in fully adjusted analyses (adjusted OR 1.02 [95% CI 0.77–1.37]). There were only 5 women represented among all practicing academic rheumatologists who graduated from internal medicine residency between 1965 and 1974 (9.3%), and all of them were eventually promoted to the rank of full professor (Figure 1).

Of the 117 academic rheumatology divisions in the US, 108 had an identifiable division director who was a rheumatologist. Of these, 34 programs (31.5%) had women as division directors. Fifty-three programs (45.3%) had women as fellowship program directors. In contrast to differences in academic rank (Table 2), women and men had similar adjusted odds of being rheumatology fellowship program directors (13.6% versus 11.6%; adjusted OR 0.99 [95% CI 0.69–1.43]) and rheumatology division directors (8.7% versus 13.4%; adjusted OR 0.96 [95% CI 0.66–1.41]).

DISCUSSION

We utilized comprehensive cross-sectional information on all licensed US rheumatologists to examine gender differences in academic rank and division-level leadership roles for women in academic rheumatology. We found that women were less likely than men to be associate or full professors, even after accounting for several measures of research and clinical productivity. However, women were as likely as men to hold leadership roles within rheumatology divisions. These findings establish important benchmarks for the rheumatology workforce and identify opportunities to improve equity among men and women in rheumatology.

The reasons remain unclear as to why there is inequity in attaining advanced academic rank in a specialty that comprises nearly equal numbers of women and men. However, we found important differences in the characteristics of women and men in academic rheumatology which may contribute to our observations. A traditional pathway to academic promotion entails scholarly productivity, which can be evaluated using publications and grant funding. We found that women had fewer publications and less grant funding than men, but observed no significant gender differences in the likelihood of being the PI of a clinical trial. The explanation for women having fewer publications and NIH grants is likely multifactorial, including time in the workforce, mentorship, work–life balance, and time spent on parental leave. However, gender differences in academic promotion remained after adjusting for each of these typical promotion criteria, indicating that other unidentified factors also contribute to the gap in promotion for women academic rheumatologists.

Gender differences in academic promotion could be partially explained by differences in the amount of effort devoted to medical education and administrative roles, which may reduce time available for research and may not be valued as highly as research productivity in terms of academic promotion. We were unable to determine primary academic roles (e.g., basic science or clinical investigation, medical education, clinical care), and this information is not publicly available or systematically collected to our knowledge for most academic rheumatologists. We were also unable to account for potential differences in tenure versus non–tenure track academic positions and part-time positions. Additional studies are needed to understand the institutional and societal barriers to academic promotion for women. We could not assess the impact of institutional support, mentorship, or overt and unconscious bias on our findings.

While barriers to academic promotion may negatively impact the opportunities for women in rheumatology to achieve senior faculty positions, we found that women and men were similarly likely to occupy key leadership positions within rheumatology divisions. These differences may reflect how decisions regarding who will be advanced through academic and leadership ranks are based on different factors. While academic promotion tracks typically prioritize stringent productivity requirements in the research

setting, selection for academic leadership roles may be based on other attributes such as interpersonal, mentorship, and leadership skills. Both achievements reflect important successes in one's academic career as well as an individual's impact on his or her profession. Further work is needed to understand how these barriers and opportunities may influence the recruitment and retention of women academic rheumatologists.

As internal medicine residents have grown more interested in pursuing careers in rheumatology, competition for a limited number of spots in rheumatology fellowship training programs has increased; in fact, rheumatology has become nearly as competitive as the historically most competitive medicine subspecialty, cardiology, in terms of the proportion of fellowship applicants who fail to match in a training program (13). However, rheumatology faces major challenges with the projected upcoming workforce gap (5). While the academic workforce comprises only a small proportion (15%) of the entire rheumatology workforce, academic institutions provide the vast majority of rheumatology specialty training (4). It is important to maintain adequate representation of women among leadership positions in these academic rheumatology divisions, and to ensure that there is equity between genders for advancement in an academic rheumatology career. Furthermore, given growth in supply–demand mismatches for rheumatologists, it behooves the specialty of rheumatology to not only increase its numbers of new trainees but also undertake efforts to reduce avoidable attrition from this specialty. Addressing physician job satisfaction, physician wellness, and successful professional growth are important features in sustaining a healthy rheumatology workforce.

Our study has several strengths and limitations. Our data source contained comprehensive cross-sectional information on all US physicians with an NPI number. Therefore, our findings are highly generalizable. However, we are not able to account for physicians who left academic practice. If greater numbers of women than men left the academic rheumatology workforce—for one of many reasons, including that they were not promoted—our findings could underestimate sex differences in academic rank. We also cannot account for how parental leave, which has historically been longer for women than for men, may have impacted our results. In addition, we cannot account for differences in work effort, and we know that women rheumatologists are more likely to work part-time than are their male counterparts (4). We were also unable to assess gender disparities in pay.

In conclusion, we found that women in rheumatology are less likely than men to achieve senior faculty positions in US medical schools but have similar opportunities for attaining leadership opportunities within rheumatology divisions. These discrepancies might indicate differences in the value placed on different roles women and men may have in the academic setting and highlight barriers to the promotion of women faculty. Further work is needed to characterize and address these barriers. As the workforce gender balance continues to shift, equity in the academic advancement of women in rheumatology must be ensured.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Jorge had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Jorge, Bolster, D. M. Blumenthal, Gross, K. G. Blumenthal, Wallace.

Acquisition of data. Gross.

Analysis and interpretation of data. Jorge, Bolster, Fu, K. G. Blumenthal, Wallace.

ADDITIONAL DISCLOSURES

Author Gross is an employee of Doximity.

REFERENCES

1. Jena AB, Khullar D, Ho O, Olenski AR, Blumenthal DM. Sex differences in academic rank in US medical schools in 2014. JAMA 2015;314:1149–58.
2. Applicants, Matriculants, Enrollment, Graduates, MD-PhD, and Residency Applicants Data. AAMC Facts. Association of American Medical Colleges; 2019. URL: https://www.aamc.org/data-reports/students-residents/report/facts.
3. Magrane D, Jolly P. The Changing Representation of Men and Women in Academic Medicine: Analysis in Brief. Association of American Medical Colleges; 2005. URL: https://www.aamc.org/system/files/reports/1/aibvol5no2.pdf.
4. Battafarano DF, Ditmyer M, Bolster MB, Fitzgerald JD, Deal C, Bass AR, et al. 2015 American College of Rheumatology Workforce Study: supply and demand projections of adult rheumatology workforce, 2015–2030. Arthritis Care Res (Hoboken) 2018;70:617–26.
5. Bolster MB, Bass AR, Hausmann JS, Deal C, Ditmyer M, Greene KL, et al. 2015 American College of Rheumatology Workforce Study: the role of graduate medical education in adult rheumatology. Arthritis Rheumatol 2018;70:817–25.
6. Nonnemaker L. Women physicians in academic medicine: new insights from cohort studies. N Engl J Med 2000;342:399–405.
7. Ruzycki SM, Freeman G, Bharwani A, Brown A. Association of physician characteristics with perceptions and experiences of gender equity in an academic internal medicine department. JAMA Netw Open 2019;2:e1915165.
8. Jena AB, Olenski AR, Blumenthal DM. Sex differences in physician salary in US public medical schools. JAMA Intern Med 2016;176:1294–304.
9. Blumenthal DM, Olenski AR, Yeh RW, Yeh DD, Sarma A, Schmidt AC, et al. Sex differences in faculty rank among academic cardiologists in the United States. Circulation 2017;135:506–17.
10. Blumenthal KG, Huebner EM, Banerji A, Long AA, Gross N, Kapoor N, et al. Sex differences in academic rank in allergy/immunology. J Allergy Clin Immunol 2019;144:1697–702.
11. Manne-Goehler J, Kapoor N, Blumenthal DM, Stead W. Sex differences in achievement and faculty rank in academic infectious diseases. Clin Infect Dis 2019;70:290–6.
12. AAMC. Services. URL: https://services.aamc.org/.
13. Charting Outcomes in the match, Specialties Matching Service, Appointment Year 2018: National Resident Matching Program; October 2018. URL: https://www.nrmp.org/new-charting-outcomes-in-the-match-specialties-matching-service-appointment-year-2018/.

Arthritis & Rheumatology
Vol. 73, No. 1, January 2021, pp 173–180

American College of Rheumatology
Empowering Rheumatology Professionals

LETTERS

DOI 10.1002/art.41472

Reality check on antiphospholipid antibodies in COVID-19–associated coagulopathy

To the Editor:

Thromboses are severe complications of coronavirus disease 2019 (COVID-19), caused by the novel severe acute respiratory system coronavirus 2 (SARS–CoV-2). The mechanism of COVID-19–associated thrombophilia is unknown; increasing global reports of positivity for antiphospholipid antibody (aPL) in COVID-19 suggest that the virus may induce antiphospholipid syndrome (APS), a separate autoimmune thrombotic illness (1). Because laboratory criteria used to diagnose APS are neither highly specific nor sensitive, and because clinical circumstances, including anticoagulation therapy, alter the laboratory results, international committees have published strict guidelines for aPL testing (2,3). The hypothesis that SARS–CoV-2 induces APS requires demonstrating that COVID-19 patients fulfill both the clinical and laboratory criteria for APS (4). We reviewed recent publications (see Supplementary Methods, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41472/abstract) in order to assess the likelihood that aPL contributes to thromboses in COVID-19 patients.

As of June 1, 2020, we identified 23 studies, in which 250 COVID-19 patients were tested for aPL; 145 of 250 (58%) were aPL positive (Table 1 and Supplementary Table 1 available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41472/abstract). Lupus anticoagulant (LAC) was present in 64% of tested COVID-19 patients, anticardiolipin antibody (aCL) in 9%, and anti–β_2 glycoprotein I (anti-β_2GPI) antibody in 13%. When aCL isotypes were reported, IgM was the most frequent. Relevant clinical information (whether patients were receiving anticoagulation therapy at the time of LAC testing or had a history of aPL positivity/APS) was rarely provided. In studies with aPL test details, 65% of the patients (135 of 209) had a clinically meaningful aPL profile (LAC and/or moderate-to-high titers of aCL/anti-β_2GPI). No reports of studies included information on confirmatory aPL testing at 12 weeks.

Similar to patients with severe COVID-19–associated coagulopathy, in a subtype of APS, patients develop multiorgan thromboses over very short periods of time (catastrophic APS [CAPS]) (1). In both COVID-19–associated coagulopathy and CAPS, acute inflammatory response, cytokine storm, and highly elevated ferritin levels occur (5,6). In patients with definite CAPS, along with a clinically meaningful aPL profile (7), aPL test results remain positive over long periods of time. Infection-induced aPL, in contrast, may be transient. The persistence of aPL in COVID-19 is unknown.

As seen in CAPS, a subgroup of patients with severe COVID-19 develop retiform purpura and livedoid rashes. Biopsy findings include thrombotic microvascular injury of the skin as well as lungs, with endothelial damage and cytokine reaction, propagated by complement activation and deposition that predisposes to thrombosis (8). Unlike CAPS, thrombocytopenia is relatively uncommon in COVID-19 (9).

The question of whether transient aPL positivity may be pathogenic for the development of incident thromboses and lead to APS, particularly in critically ill patients or during infection, has been poorly understood. Reports to date suggest clinical and laboratory similarities between severe COVID-19 and CAPS; however, the studies suggesting an aPL-related mechanism for thrombosis in COVID-19 are only hypothesis generating. A starting point for future research is to adhere to the criteria for defining clinically important aPL

Table 1. Antiphospholipid antibody profiles in 250 COVID-19 patients included in 23 studies from January 1, 2020 to June 1, 2020*

Assessment	No. of studies (%)	No. of aPL-positive patients (%)
LAC test†	13/23 (57)	134/208 (64)
Patient anticoagulation status reported‡	0/13 (0)	–
Methodology provided§	3/13 (23)	106/147 (72)
aCL test¶	20/23 (87)	18/207 (9)
Cutoff for positivity reported	2/20 (10)	3/25 (12)
Anti-β_2GPI¶	14/23 (61)	17/135 (13)
Cutoff for positivity reported	2/14 (14)	3/25 (12)
LAC, aCL, or anti-β_2GPI tested (positivity for any aPL)	23/23 (100)	145/250 (58)
LAC, aCL, and anti-β_2GPI tested (positivity for any aPL)	6/23 (26)	56/106 (53)
LAC, aCL, and anti-β_2GPI tested (positivity for all 3 aPL)	1/6 (17)	1/106 (1)#
Reported persistence of aPL	0 (0)	–

* COVID-19 = coronavirus disease 2019.
† Defined by dilute Russell's viper venom time and activated partial thromboplastin time screening.
‡ At the time of lupus anticoagulant (LAC) testing.
§ Details of screening and confirmation assays.
¶ In a study (see Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41472/abstract) in which 1 positive anticardiolipin antibody (aCL) test result was reported, we assumed that all 57 patients were tested for aCL. No data were provided for the number of anti–β_2-glycoprotein I (anti-β_2GPI) tests performed.
# In another study (see Supplementary Table 1), antiphospholipid antibody (aPL) test results were reported separately for all 45 patients and therefore, no assumptions could be made regarding double or triple positivity in any given patient. Positivity for LAC (21 of 45) was considered to be driving a clinically meaningful aPL profile.

profiles (see Supplementary Table 2, http://onlinelibrary.wiley.com/doi/10.1002/art.41472/abstract) in COVID-19 patients, including detailed information regarding LAC test results and immunoassays, and serial studies of aPL to demonstrate persistence.

In conclusion, there is an opportunity to discover potentially common mechanisms that will inform our understanding of both COVID-19 and APS. However, this will require correct use and measurement of aPL.

Elena Gkrouzman, MD
Medha Barbhaiya, MD, MPH
Doruk Erkan, MD, MPH
Michael D. Lockshin, MD
Hospital for Special Surgery
Weill Cornell Medicine
New York, NY

1. Garcia D, Erkan D. Diagnosis and management of the antiphospholipid syndrome [review]. N Engl J Med 2018;378:1210–21.
2. Pengo V, Tripodi A, Reber G, Rand JH, Ortel TL, Galli M, et al. Update of the guidelines for lupus anticoagulant detection. Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis. J Thromb Haemost 2009;7:1737–40.
3. Bertolaccini ML, Amengual O, Andreoli L, Atsumi T, Chighizola CB, Forastiero R, et al. 14th International Congress on Antiphospholipid Antibodies Task Force: report on antiphospholipid syndrome laboratory diagnostics and trends [review]. Autoimmun Rev 2014;13:917–30.
4. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006;4:295–306.
5. Henderson LA, Canna SW, Schulert GS, Volpi S, Lee PY, Kernan KF, et al. On the alert for cytokine storm: immunopathology in COVID-19. Arthritis Rheumatol 2020;72:1059–63.
6. Rosário C, Zandman-Goddard G, Meyron-Holtz EG, D'Cruz DP, Shoenfeld Y. The hyperferritinemic syndrome: macrophage activation syndrome, Still's disease, septic shock and catastrophic antiphospholipid syndrome [review]. BMC Med 2013;11:185.
7. Rodríguez-Pintó I, Moitinho M, Santacreu I, Shoenfeld Y, Erkan D, Espinosa G, et al. Catastrophic antiphospholipid syndrome (CAPS): Descriptive analysis of 500 patients from the International CAPS Registry [review]. Autoimmun Rev 2016;15:1120–4.
8. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, et al. Complement associated microvascular injury and thrombosis in the pathogenesis of severe COVID-19 infection: a report of five cases. Transl Res 2020;220:1–13.
9. Connors JM, Levy JH. COVID-19 and its implications for thrombosis and anticoagulation. Blood 2020;135:2033–40.

DOI 10.1002/art.41459

Determinants of morning stiffness in rheumatoid arthritis: comment on the article by Orange et al

To the Editor:

We read with great interest the article by Orange et al on the histologic correlates of morning stiffness in rheumatoid arthritis (RA). We wish to highlight a few important issues that merit consideration (1).

First, although RA is a systemic disease, flares are often patchy in distribution, and different joints may be in different states of activity at a particular cross-sectional assessment. Similarly, morning stiffness in RA may also be variable and involve some joints more predominantly. Orange and colleagues assessed the association of global morning stiffness (using questions from the Rheumatoid Arthritis Disease Activity Index [2] and the Outcome Measures in Rheumatology RA Flare Questionnaire [3]) with synovial histologic features (predominantly in the knee joint). We are curious to know if measurements of knee joint morning stiffness were obtained, such as difficulty walking or folding legs while getting up from bed in the morning. The severity and duration of morning stiffness in the affected joint (preoperatively), rather than global morning stiffness, would be a better parameter to compare with synovial histologic features.

Second, it has been previously shown that synovial histologic features in RA depend, to a large extent, on the presence or absence of joint effusion (local joint activity) and may not correlate well with disease duration or global measures of disease activity, such as levels of C-reactive protein (4). All patients who underwent knee arthroplasty obviously must have had advanced joint damage; however, this does not preclude ongoing RA disease activity in the joints being replaced. Including information on the preoperative activity status of the affected joint, in terms of effusion (presence or absence) and tenderness, and the correlation of these data with synovial histologic features and morning stiffness could result in a more focused and appropriate analysis.

Third, it would be interesting to explore the contribution of NETosis to the interaction of neutrophils with fibrin in the putative pathogenesis of morning stiffness. Neutrophils in RA are more likely to undergo NETosis (5), and DNase (used in the in vitro experiments in the study by Orange et al) are required for disassembly of these neutrophil extracellular traps (NETs). Correlation of NETosis-derived products in synovium with parameters of morning stiffness could be of interest.

Finally, the Disease Activity Score in 28 joints (DAS28) (6) of the overall population was only mildly elevated, with essentially normal age-adjusted levels of inflammation markers. Therefore, it would be prudent to report the number of patients in each of the 3 DAS28 categories of disease activity (low, moderate, and high) when studying the association of duration and severity of morning stiffness with disease activity.

Siddharth Jain, MD
Debashish Mishra, MD
Varun Dhir, DM
Postgraduate Institute of Medical Education and Research
Chandigarh, India

1. Orange DE, Blachere NE, DiCarlo EF, Mirza S, Pannellini T, Jiang CS, et al. Rheumatoid arthritis morning stiffness is associated with synovial fibrin and neutrophils. Arthritis Rheumatol 2020;72:557–64.
2. Stucki G, Liang MH, Stucki S, Brühlmann P, Michel BA. A self-administered rheumatoid arthritis disease activity index (RADAI) for epidemiologic research: psychometric properties and correlation with parameters of disease activity. Arthritis Rheum 1995;38:795–8.
3. Bykerk VP, Bingham CO, Choy EH, Lin D, Alten R, Christensen R, et al, on behalf of the OMERACT RA Flare Group and CATCH Investigators. Identifying flares in rheumatoid arthritis: reliability and construct validation of the OMERACT RA Flare Core Domain Set. RMD Open 2016;2:e000225.
4. Baeten D, Demetter P, Cuvelier C, Van Den Bosch F, Kruithof E, Van Damme N, et al. Comparative study of the synovial histology in rheumatoid arthritis, spondyloarthropathy, and osteoarthritis: influence of disease duration and activity. Ann Rheum Dis 2000;59:945–53.
5. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. Sci Transl Med 2013;5:178ra140.
6. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight–joint counts: development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44–8.

DOI 10.1002/art.41463

Reply

To the Editor:

We are grateful to Dr. Jain and colleagues for their interest in our article. Jain et al raise an important point: not all joints in a patient with RA are affected equally at any given time, making it challenging to associate synovial findings from only 1 joint with assessments of global patient symptoms. We had discovered that morning stiffness duration was associated with the presence of fibrin and neutrophils in synovium; however, neither of the questions used in this analysis focused on any particular joint, and it is likely that joints other than the replaced joint influenced patient responses.

We did also collect Hip disability and Osteoarthritis Outcome Scores (HOOS) (1) and Knee injury and Osteoarthritis Outcome Scores (KOOS) (2) questionnaires in our cohort, which queried patients specifically about hips or knees, the joints for which arthroplasty was performed in our study, and included questions regarding stiffness severity. Just as a large percentage (43%) of patients with <1 hour of morning stiffness reported relatively high general stiffness severity, 58% of patients who reported <1 hour of morning stiffness rated their HOOS/KOOS morning stiffness severity as either severe or extreme, supporting our conclusion that stiffness severity and duration represent different constructs. In an attempt to address the question raised by Jain et al, we evaluated responses to these 2 joint-specific questions in relation to synovial histologic features but did not find any significant associations between any of the synovial histologic features and stiffness severity in the morning or later in the day. This is consistent with our original finding that synovial fibrin and neutrophils were associated with prolonged duration of morning stiffness but not stiffness severity. We look forward to future studies comparing synovial histologic features to morning stiffness duration and physical examination findings from a specific joint.

Jain et al also note that it would be interesting to explore the role of NETosis in the interaction of neutrophils with fibrin in the putative pathogenesis of morning stiffness. Given that neutrophil-derived DNA impedes fibrinolysis and RA synovium contains NETs, we agree that NETs may contribute to prolonged morning stiffness, and this warrants further study.

Finally, there is a question regarding the number of patients in each of the 3 DAS28 categories. Thirty-six percent, 47%, and 15% of patients had DAS28 scores of low, moderate, or high, respectively. Since assessments of synovial neutrophils and fibrin can easily be performed in any clinical research setting, we are eager to learn whether their association with morning stiffness duration is reproducible by independent laboratories and look forward to future investigations to clarify the pathogenesis of this vexing symptom.

Supported by the NIH (National Center for Advancing Translational Sciences grant UL1-TR-001866 and National Institute of Arthritis and Musculoskeletal and Skin Diseases grant 1UH2-AR-067691). Dr. DiCarlo has received consulting fees from Wright Medical Technology (less than $10,000). Dr. Frank has received consulting fees from the Memorial Sloan Kettering Cancer Center (less than $10,000) and from the New York Genome Cancer Center (more than $10,000). Dr. Bykerk has received consulting fees from Bristol Myers Squibb, Gilead Sciences, UCB, Amgen, and Sanofi (less than $10,000 each). Dr. Mackie has received consulting fees from Roche (less than $10,000) and research support from the NIHR, Vasculitis UK, and Sanofi. Dr. Goodman owns stock or stock options in Regenosine. No other disclosures relevant to this letter were reported.

Dana E. Orange, MD, MS
Hospital for Special Surgery and The Rockefeller University
Nathalie E. Blachere, PhD
Mayu O. Frank, NP, PhD
Salina Parveen, MA
The Rockefeller University
Edward F. DiCarlo, MD
Serene Mirza, BS
Tania Pannellini, MD, PhD
Caroline S. Jiang, MS
Mark P. Figgie, MD
Vivian P. Bykerk, MD
Hospital for Special Surgery
New York, NY
Ellen M. Gravallese, MD
Brigham and Women's Hospital
Boston, MA
Ana-Maria Orbai, MD, MHS
Johns Hopkins Medicine
Baltimore, MD

Sarah L. Mackie, BMBCh
University of Leeds
Leeds NIHR Biomedical Research Centre and Leeds Teaching Hospitals NHS Trust
Leeds, UK
Susan M. Goodman, MD
Hospital for Special Surgery
New York, NY

1. Nilsdotter AK, Lohmander LS, Klässbo M, Roos EM. Hip disability and Osteoarthritis Outcome Score (HOOS): validity and responsiveness in total hip replacement. BMC Musculoskelet Disord 2003;4:10.
2. Roos EM, Roos HP, Lohmander LS, Ekdahl C, Beynnon BD. Knee injury and Osteoarthritis Outcome Score (KOOS): development of a self-administered outcome measure. J Orthop Sports Phys Ther 1998;28:88–96.

DOI 10.1002/art.41482

Examining the role of NF-E2–related factor 2 in lupus: comment on the article by Han et al

To the Editor:

Systemic lupus erythematosus (SLE) is an inflammatory autoimmune disease that damages multiple organ systems. To date, the pathogenesis of SLE has not been fully clarified. However, genetic factors, environmental factors, and dysregulated immunity are related to the onset of this complex disease.

In their recent study, Han et al demonstrated an increased expression of inflammatory genes regulated by the transcription factor NF-E2–related factor 2 (Nrf2), including *Gclc*, *Nqo1*, *Sod2*, *Gsr*, *Gpx4*, *Srxn*, and *Prdx1*, in nonclassic macrophages (NCMs) from control mice, whereas there was a reduced expression of these genes in NCMs from mice with pristane-induced lupus (1). Nrf2 activator CDDO-imidazole promoted the development of proresolving NCMs. In contrast, Nrf2 inhibitor brusatol suppressed NCM differentiation (1). In addition, Nrf2 activator inhibitor *Ifnar1* inhibited interferon-stimulated gene (ISG) expression in macrophages and reduced oxidative stress, suggesting that Nrf2 may help to resolve chronic inflammation in lupus and may have potential for treating this disease.

However, these interesting findings are inconsistent with available evidence. Zhao et al found that in mice with lupus, *Nrf2* deficiency promoted early-stage lupus nephritis (2). Th17 cells and interleukin-17 were up-regulated in $Nrf2^{-/-}$ mice with lupus. Naive T cells isolated from $Nrf2^{-/-}$ mice were reported to have elevated Th17 cell differentiation and reduced expression of *Soc3*, indicating that Nrf2 may inhibit Th17 differentiation, delaying the development of lupus (2). Similarly, pathologic analysis of patients with lupus nephritis revealed oxidative damage in the glomeruli, along with an active Nrf2 antioxidant response (3). $Nrf2^{-/-}$ mice with lupus had severe renal damage and increased expression of transforming growth factor β1, fibronectin, and inducible nitric oxide synthase in the kidneys (3). In a study evaluating the association of Nrf2 gene polymorphisms –617 C/A and –653 G/A in SLE patients, it was demonstrated that these 2 polymorphisms were not related to genetic susceptibility to SLE (4). Interestingly, Morito et al found that the lifespans of $Nrf2^{-/-}$ mice with lupus were significantly prolonged, accompanied by an improvement in nephritis compared with $Nrf2^{+/+}$ mice with lupus (5). In $Nrf2^{-/-}$ mice with lupus, immunologic abnormalities and hypergammaglobulinemia were alleviated, relating to inhibited lymphadenopathy due to elevated apoptosis. Glutathione expression was reduced in renal tissue from $Nrf2^{-/-}$ mice with lupus compared with tissue from $Nrf2^{+/+}$ mice with lupus, indicating that Nrf2 deficiency improves autoimmune nephritis (5).

Considering the contradictory results regarding a role of Nrf2 in lupus, it is doubtful that Nrf2 is protective against lupus development or has a proinflammatory role in lupus onset. Further studies using both human subjects and animal models are needed to further elucidate the potential of Nrf2 for treating lupus.

Supported by the National Natural Science Foundation of China grant 81701606.

Wang-Dong Xu, MD
Southwest Medical University
An-Fang Huang, MM
Affiliated Hospital of Southwest Medical University
Luzhou, China

1. Han S, Zhuang H, Lee PY, Li M, Yang L, Nigrovic PA, et al. NF-E2–related factor 2 regulates interferon receptor expression and alters macrophage polarization in lupus. Arthritis Rheumatol 2020;72:1707–20.
2. Zhao M, Chen H, Ding Q, Xu X, Yu B, Huang Z. Nuclear factor erythroid 2-related factor 2 deficiency exacerbates lupus nephritis in B6/lpr mice by regulating Th17 cell function. Sci Rep 2016;6:38619.
3. Jiang T, Tian F, Zheng H, Whitman SA, Lin Y, Zhang Z, et al. Nrf2 suppresses lupus nephritis through inhibition of oxidative injury and the NF-κB-mediated inflammatory response. Kidney Int 2004; 85:333–43.
4. Córdova EJ, Velázquez-Cruz R, Centeno F, Baca V, Orozco L. The NRF2 gene variant, -653G/A, is associated with nephritis in childhood-onset systemic lupus erythematosus. Lupus 2010;19:1237–42.
5. Morito N, You K, Hirayama A, Itoh K, Nose M, Koyama A, et al. Nrf2 deficiency improves autoimmune nephritis caused by the Fas mutation lpr. Kidney Int 2004;65:1703–13.

DOI 10.1002/art.41478

Reply

To the Editor:

We agree with Dr. Xu and Ms Huang that lupus is complex. This is illustrated by lupus-like disease in mice with defective Fas (B6/*lpr* and MRL/*lpr*) versus mice with pristane-induced lupus, which may represent distinct forms of lupus. While type I interferon receptor (IFNAR) signaling ("interferon [IFN] signature") is implicated in pristane-induced lupus, Zhao et al suggest

interleukin-17 (IL-17) plays a role in the pathogenesis of lupus in B6/*lpr* mice lacking Nrf2 (*Nfe2l2*$^{-/-}$) (1). However, lupus in *lpr* mice differs from lupus in pristane-treated mice and in most SLE patients.

B6/*lpr* mice produce antichromatin and anti–single-stranded DNA (anti-ssDNA) autoantibodies, but not anti–double-stranded DNA (anti-dsDNA), anti-Sm, or anti-RNP (2). Anti-DNA antibody levels have been found to be increased in B6/*lpr Nfe2l2*$^{-/-}$ mice (1), although the assay used did not distinguish anti-ssDNA from anti-dsDNA antibodies. In B6 mice, pristane induces anti-Sm and RNP autoantibodies (but generally not anti-dsDNA or chromatin) (2). However, B6/*lpr* mice are resistant to the induction of these autoantibodies by pristane, suggesting that *lpr* generates autoantibodies by a mechanism other than pristane. B6/*lpr* mice also develop little or no nephritis (2,3), as confirmed by Zhao et al (1). In B6/*lpr Nfe2l2*$^{-/-}$ mice, renal immune deposits were shown to be increased, but the levels of proteinuria were not reported. Although high levels of IL-17 were associated with anti-DNA antibodies and renal immune complex deposition (1), it remains to be directly confirmed that IL-17 is pathogenic. In autoimmune-prone MRL mice, *lpr* promotes severe nephritis and anti-Sm and anti-dsDNA antibodies. In contrast to mice with pristane-induced lupus, which have an IFN signature comparable to that observed in SLE patients, an IFN signature is absent in MRL/*lpr* mice (Figure 1). Moreover, pristane-induced lupus is milder in mice lacking IFNAR, whereas autoantibody production and nephritis are exacerbated in IFNAR$^{-/-}$ MRL/*lpr* mice (4). NZB/NZW mice develop a weak IFN signature at disease onset (Figure 1), and disease is greatly exacerbated by treatment with pristane (1) or IFN alfa-5 (5). Thus, lupus-like disease induced by *lpr* may represent an IFN-independent form of lupus, possibly mediated by IL-17 (1).

The human disease may be analogous. Patients with Fas mutations develop autoimmune lymphoproliferative syndrome, in which hematologic autoimmunity is common, but anti-Sm, RNP, dsDNA, and nephritis are unusual (6). In contrast, most patients with idiopathic SLE exhibit the IFN signature. Pristane-induced lupus is a good model for that subset (7), and both autoantibody production and renal disease are greatly attenuated in mice lacking IFNAR (8).

We agree that additional studies are necessary before Nrf2 activators can be recommended for treating lupus. However, just as anifrolumab (anti-IFNAR) is a logical (and effective) treatment for IFN signature–associated lupus (9), Nrf2 activators, which decrease IFNAR expression, may also make sense. Similarly, the Th17 pathway may be pathogenic in a

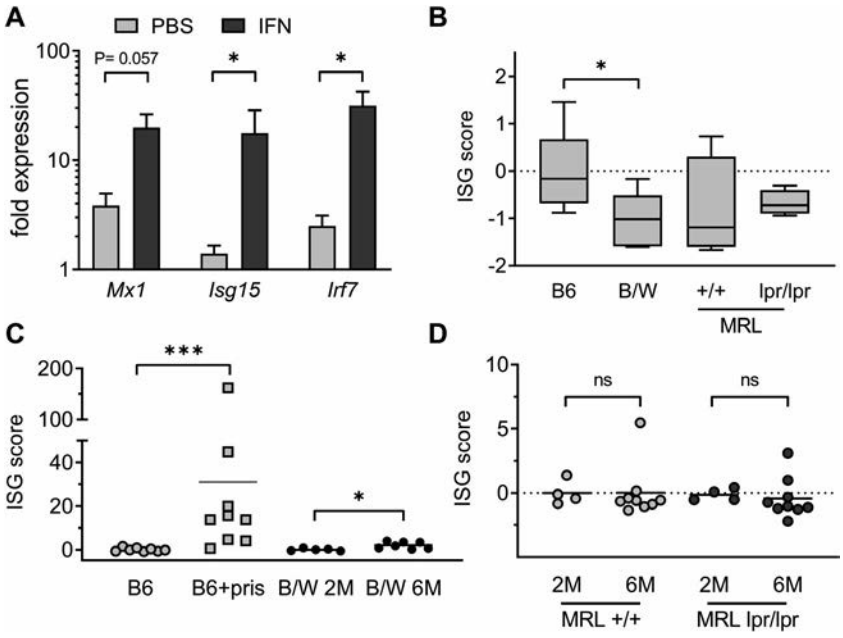


Figure 1. **A**, Expression of the interferon (IFN)–stimulated genes (ISGs) *Mx1*, *Isg15*, and *Irf7* in C57BL/6 (B6) mice (4 mice per group) treated intraperitoneally 24 hours earlier with recombinant IFN alfa-5 (10^4 units) or phosphate buffered saline (PBS) alone. ISG expression in peripheral blood collected into PAXgene tubes was determined by real-time polymerase chain reaction. Values are the mean ± SEM. **B**, ISG scores ([*Mx1* + *Isg15* + *Irf7*] ÷ 3) derived from peripheral blood leukocytes from 2-month-old B6, NZB/NZW (B/W), MRL$^{+/+}$, and MRL-*lpr/lpr* mice (4–8 mice per group). Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. **C**, ISG scores in peripheral blood leukocytes from untreated B6 mice, B6 mice 8 months after pristane (pris) treatment, and non–pristane-treated 2-month-old (2M) and 6-month old (6M) NZB/NZW mice. **D**, ISG scores in peripheral blood leukocytes from untreated MRL$^{+/+}$ and MRL-*lpr/lpr* mice at 2 or 6 months of age. Each symbol represents a single mouse; horizontal lines show the mean. * = $P < 0.05$; *** = $P < 0.001$, by Mann-Whitney test. NS = not significant.

subset of lupus. While there is evidence that IL-17 could play a role in murine models of lupus, there are limited data on treating SLE with IL-17 antagonists.

Supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH (grant K08-AR-074562 to Dr. Lee, grants P30-AR-070253 and R01-AR-065538 to Dr. Nigrovic, and grant R01-AR-44731 to Dr. Reeves) and a Lupus Research Alliance Target Identification in Lupus grant to Dr. Nigrovic.

Shuhong Han, PhD
Haoyang Zhuang, PhD
Mingjia Li
Lijun Yang, MD
University of Florida
Gainesville, FL
Pui Y. Lee, PhD, MD
Boston Children's Hospital
Boston, MA
Peter A. Nigrovic, MD
Boston Children's Hospital
and Brigham and Women's Hospital
Boston, MA
Westley H. Reeves, MD
University of Florida
Gainesville, FL

1. Zhao M, Chen H, Ding Q, Xu X, Yu B, Huang Z. Nuclear factor erythroid 2-related factor 2 deficiency exacerbates lupus nephritis in B6/lpr mice by regulating Th17 cell function. Sci Rep 2016;6:38619.
2. Satoh M, Weintraub JP, Yoshida H, Shaheen VM, Richards HB, Shaw M, et al. Fas and Fas ligand mutations inhibit autoantibody production in pristane-induced lupus. J Immunol 2000;165:1036–43.
3. Cohen PL, Eisenberg RA. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease [review]. Annu Rev Immunol 1991;9:243–69.
4. Hron JD, Peng SL. Type I IFN protects against murine lupus. J Immunol 2004;173:2134–42.
5. Mathian A, Weinberg A, Gallegos M, Banchereau J, Koutouzov S. IFN-α induces early lethal lupus in preautoimmune (New Zealand Black x New Zealand White) F1 but not in BALB/c mice. J Immunol 2005;174:2499–506.
6. Bleesing JJ, Brown MR, Straus SE, Dale JK, Siegel RM, Johnson M, et al. Immunophenotypic profiles in families with autoimmune lymphoproliferative syndrome. Blood 2001;98:2466–73.
7. Reeves WH, Lee PY, Weinstein JS, Satoh M, Lu L. Induction of autoimmunity by pristane and other naturally occurring hydrocarbons [review]. Trends Immunol 2009;30:455–64.
8. Nacionales DC, Kelly-Scumpia KM, Lee PY, Weinstein JS, Lyons R, Sobel E, et al. Deficiency of the type I interferon receptor protects mice from experimental lupus. Arthritis Rheum 2007;56:3770–83.
9. Morand EF, Furie R, Tanaka Y, Bruce IN, Askanase AD, Richez C, et al. Trial of anifrolumab in active systemic lupus erythematosus. N Engl J Med 2020;382:211–21.

DOI 10.1002/art.41470

Dipeptidylpeptidase 4 as a marker of fibrosis in systemic sclerosis: comment on the article by Soare et al

To the Editor:

I read with interest the article by Soare et al describing their study which demonstrated that dipeptidylpeptidase 4 (DPP-4) is a marker of activated fibroblasts that may have implications for the treatment of fibrosis in patients with systemic sclerosis (SSc) (1). Their findings suggest a paradigm shift in the therapy of scleroderma, as well as, perhaps, other fibrosing disorders. There are a couple of issues, however, which I believe require clarification.

First, Soare and colleagues state in their Materials and Methods section under statistical analysis, "All data are presented as the median ± interquartile range," but the legends for every figure indicate, "bars show the mean ± SEM." The latter would be preferable because as Soare et al state, "differences between the groups were tested for their statistical significance using…Mann-Whitney U nonparametric tests for nonrelated samples." It is my understanding that testing for differences in medians using Mann-Whitney U tests requires the assumption that the shape of the distributions of the data from the 2 nonrelated groups (e.g., wild-type [WT] and knockout [KO] mice) is the same (2). It would be beneficial if Soare and colleagues could clarify how the data were displayed and assessed, since that could effect the validity of their interpretation.

It was also concerning that in all of the experiments using DPP4-KO mice where transforming growth factor β (TGFβ) was the actual or implied actor (e.g., in the mouse models of SSc), the resulting measures of fibrosis were frequently higher in the DPP4-KO group than in the controls. I would not expect this to be the case if TGFβ were acting through DPP-4 alone. The same appeared to be true when the DPP-4 inhibitors sitagliptin or vildagliptin were used in either the WT or KO mice.

Indeed, in the bleomycin-induced skin fibrosis model (Figure 4B in ref. 1), Soare and colleagues even indicate that these elevations of fibrotic markers in the DPP4-KO mice on exposure to bleomycin were statistically significantly greater than in the control mice. In the bleomycin-induced lung fibrosis model (Figure 4A in ref. 1), in WT mice with presumably intact DPP4, treatment with sitagliptin produced levels of fibrotic markers that appear to be significantly higher than levels in control mice, as indicated by fibrotic area, Ashcroft scores, myofibroblast counts, and hydroxyproline content. I would be interested in Soare and colleagues' thoughts with regard to these apparent discrepancies. They suggest that TGFβ can employ pathways and actors other than DPP-4 to

induce fibrosis (3). This interpretation has potentially significant implications for the efficacy of DPP-4 inhibitors in scleroderma.

Michael R. Liebling, MD
Oregon Health and Science University
Portland, OR

1. Soare A, Györfi HA, Matei AE, Dees C, Rauber S, Wohlfahrt T, et al. Dipeptidylpeptidase 4 as a marker of activated fibroblasts and a potential target for the treatment of fibrosis in systemic sclerosis. Arthritis Rheumatol 2020;72:137–49.
2. Hart A. Mann-Whitney test is not just a test of medians: differences in spread can be important. BMJ 2001;323:391–3.
3. Sanchez CG, Molinski SV, Gongora R, Sosulski M, Fuselier T, MacKinnon SS, et al. The antiretroviral agent nelfinavir mesylate: a potential therapy for systemic sclerosis. Arthritis Rheumatol 2018;70:115–26.

DOI 10.1002/art.41467

Reply

To the Editor:

Our study demonstrated that expression of DPP4 was increased in the skin of SSc patients in a TGFβ-dependent manner, and DPP4 expression identified a subset of activated fibroblasts with increased expression of myofibroblast markers and release of collagen. Overexpression of DPP4 promoted fibroblast-to-myofibroblast transition, whereas inactivation of DPP4 interfered with TGFβ-induced fibroblast activation. Genetic inactivation or pharmaceutical inhibition of DPP4 ameliorated fibrosis in 4 different mouse models of dermal and pulmonary fibrosis. Thus, in accordance with the results of studies from other groups using other models of fibrosis, our data highlight that DPP-4 amplifies the profibrotic effects of TGFβ (1–3); TGFβ induces the expression of DPP4, which, in turn, is a downstream mediator of the profibrotic effects of TGFβ on fibroblasts.

Dr. Liebling highlights that inhibition or knockdown of DPP4 does not completely abrogate the profibrotic effects of TGFβ and that DPP4-KO mice are not completely protected against fibrotic injury. We fully agree with this interpretation; antifibrotic effects are highly statistically and biologically significant across different fibroblast assays and mouse models but do not entirely protect against TGFβ-induced fibroblast-to-myofibroblast transition and fibrotic injury. Complete prevention of TGFβ-induced fibroblast activation would require that all (profibrotic) intracellular TGFβ signals are transmitted via DPP-4. However, TGFβ signaling is complex and activates various intracellular signaling cascades, which synergize to mediate the profibrotic effects of TGFβ (4–6). Indeed, we demonstrated that DPP-4 modulates only certain intracellular downstream mediators of TGFβ such as ERK signaling, whereas other mediators are not affected. Consistent with the activation of multiple intracellular cascades by TGFβ, we never observed complete abrogation of the profibrotic effects of TGFβ on fibroblasts with any target downstream of the TGFβ receptors. This holds even more true for mouse models, such as bleomycin-induced fibrosis, in which fibrosis is triggered not only by TGFβ-dependent cascades, but also by multiple other profibrotic mediators (7–9).

How to perform statistical analyses in molecular studies with a relatively limited sample size of ≤10 mice per group is a subject of debate. Our results showed pronounced differences between treatment/knockout groups and controls. Due to the frequent use of the *t*-test in biologic studies with a small sample size and strong biologic effects, we ultimately decided to display the data as mean ± SD and to conduct statistical evaluation using the *t*-test. However, we agree that using the Wilcoxon-Mann-Whitney test is the standard approach. We recalculated our results and found that *t*-tests and Wilcoxon-Mann-Whitney tests yielded similar levels of significance throughout all experiments. Thus, alternative statistics did not change the interpretation of the results. However, we apologize for the contradictory information in the figure legends and the Materials and Methods section.

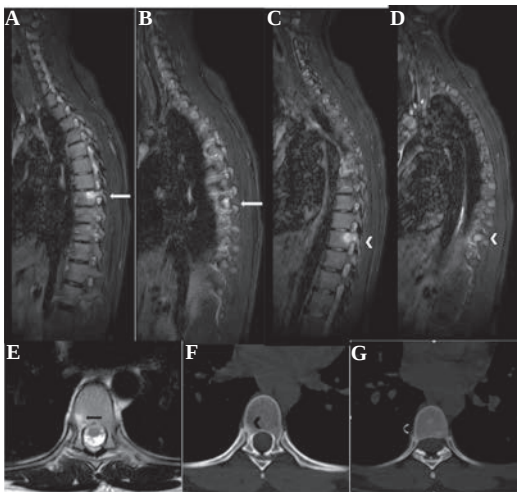
Alina Soare, MD
Jörg H. W. Distler, MD
Friedrich-Alexander University Erlangen-Nuremberg and Universitätsklinikum Erlangen
Erlangen, Germany

1. Suzuki T, Tada Y, Gladson S, Nishimura R, Shimomura I, Karasawa S, et al. Vildagliptin ameliorates pulmonary fibrosis in lipopolysaccharide-induced lung injury by inhibiting endothelial-to-mesenchymal transition. Respir Res 2017;18:177.
2. Kaji K, Yoshiji H, Ikenaka Y, Noguchi R, Aihara Y, Douhara A, et al. Dipeptidyl peptidase-4 inhibitor attenuates hepatic fibrosis via suppression of activated hepatic stellate cell in rats. J Gastroenterol 2014;49:481–91.
3. Wang XM, Holz LE, Chowdhury S, Cordoba SP, Evans KA, Gall MG, et al. The pro-fibrotic role of dipeptidyl peptidase 4 in carbon tetrachloride-induced experimental liver injury. Immunol Cell Biol 2017; 95:443–53.
4. Györfi AH, Matei AE, Distler JH. Targeting TGF-β signaling for the treatment of fibrosis [review]. Matrix Biol 2018;68–69:8–27.
5. Distler JH, Györfi AH, Ramanujam M, Whitfield ML, Königshoff M, Lafyatis R. Shared and distinct mechanisms of fibrosis [review]. Nat Rev Rheumatol 2019;15:705–30.

6. Batlle E, Massagué J. Transforming growth factor-β signaling in immunity and cancer [review]. Immunity 2019;50:924–40.
7. Avouac J, Elhai M, Allanore Y. Experimental models of dermal fibrosis and systemic sclerosis [review]. Joint Bone Spine 2013; 80:23–8.
8. Beyer C, Schett G, Distler O, Distler JH. Animal models of systemic sclerosis: prospects and limitations [review]. Arthritis Rheum 2010;62:2831–44.
9. Soare A, Ramming A, Avouac J, Distler JH. Updates on animal models of systemic sclerosis [review]. J Scleroderma Relat Disord 2016;1:266–76.

DOI 10.1002/art.41514

Clinical Images: Erosive costovertebral joint arthritis—an uncommon manifestation of ankylosing spondylitis



The patient, a 26-year-old HLA–B27–positive woman with ankylosing spondylitis (AS) who was currently not receiving therapy, presented with new-onset mid-back pain. The pain was particularly noticeable during inspiration and yawning. Point tenderness at 2 costovertebral joints on both sides of the spine was noted. Findings of electrocardiography and radiography of the chest were unremarkable. Laboratory studies showed elevated levels of inflammation markers. Magnetic resonance imaging (MRI) of the spine and sacroiliac joints revealed left-sided sacroiliitis. STIR sagittal MRI demonstrated subchondral edema in the right costovertebral joint at T8 (**arrows** in **A** and **B**), and in the left costovertebral joint at T10 (**arrowheads** in **C** and **D**), and axial T2-weighted MRI also showed subchondral edema at T8 (**arrow** in **E**). Axial computed tomography (CT) of the spine at the same level showed subchondral osteitis (**arrowhead** in **F**), cortical irregularity, and joint space narrowing (**arrow** in **G**) with no evidence of discitis, fracture, tumor, or visceral/retroperitoneal abnormalities. Findings were consistent with active early destructive arthropathy of the costovertebral joints without involvement of the adjacent synchondrodial, fibrous capsule–enclosed costotransverse joints. Costovertebral joint involvement is a rare manifestation of AS, characterized by joint space widening, sclerosis, erosions, and joint ankylosis (1). Costovertebral joints at T1, T11, and T12 are usually affected, as they fully articulate with the vertebral body without any contact with the intervertebral discs and are thus thought to be more susceptible to mechanical stress; though in this patient, T8 and T10 were involved. The costovertebral joints at other levels are complex-compound joints divided into 2 separate synovial cavities by an intraarticular ligament, with more protection from arthritis (1,2). Costovertebral joint arthritis may cause severe pain that is worsened by local palpation, deep inspiration, sneezing, coughing, or truncal rotation (3) and can be demonstrated on CT or MRI (1). Accurate diagnosis is imperative for instituting appropriate antirheumatic drug therapy.

1. Pascual E, Castellano JA, López E. Costovertebral joint changes in ankylosing spondylitis with thoracic pain. Br J Rheumatol 1992;31:413–5.
2. Nathan H, Weinberg H, Robin GC, Aviad I. The costovertebral joints: anatomical-clinical observations in arthritis. Arthritis Rheum 1964;7:228–40.
3. Benhamou CL, Roux C, Tourliere D, Gervais T, Viala JF, Amor B. Pseudovisceral pain referred from costovertebral arthropathies: twenty-eight cases. Spine (Phila Pa 1976) 1993;18:790–5.

Tal Gazitt, MD, MSc
Najwan Nassrallah, MD
Carmel Medical Center
Devy Zisman, MD
Carmel Medical Center
and *Technion Israel Institute of Technology*
Haifa, Israel