In this Issue

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

Incidence of Arthritis in Indigenous North American People

Large prospective observational studies of rheumatoid arthritis (RA) onset in largely white North American populations have suggested an age-adjusted incidence rate of 26.7 cases/100,000 person-years. In this issue, Tanner et al (p. 1494) report on arthritis rates in indigenous North Americans from Alaska and Manitoba, Canada. The authors found that a large proportion of autoantibody-positive indigenous North Americans do not develop inflammatory arthritis but instead revert to an autoantibody-negative state. The reversion is particularly noteworthy since the study involved a cohort of at-risk relatives of individuals with RA, a group that is known to have a high incidence of inflammatory arthritis.

p. 1494

Eighteen of 374 relatives developed inflammatory arthritis during the follow-up, which reflects a transition rate of 9.2 cases/1,000 person-years. Although 30% of those who developed inflammatory arthritis were seronegative at baseline, all were seropositive at the onset of inflammatory arthritis. Moreover, while 30% of anti–citrullinated protein antibodies (ACPAs)/rheumatoid factor (RF) double-seropositive individuals developed inflammatory arthritis, the majority did not.

When the investigators performed multistate modeling, they found a 71% and 68% likelihood of ACPA and RF seropositive state, respectively, reverting to a seronegative states after five years. More than one-third (39%) of patients who were ACPA/RF double seropositive became seronegative after 5 years. Those individuals who did go on to develop inflammatory arthritis experienced an expansion of their ACPA repertoire prior to the development of inflammatory arthritis.

Cluster Analysis to Decipher Systemic Sclerosis Phenotypes

Systemic sclerosis (SSc) is a heterogeneous connective tissue disease that is typically subdivided based upon the extent of skin involvement into diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc). However, this subclassification may not capture the entire variability of clinical phenotypes. Using prospective data from the European Scleroderma Trials and Research database, Sobanski et al (p. 1553) report the results of their cluster analysis study that aimed to distinguish homogeneous groups from a large population of 6,927 SSc patients (from 120 centers). The authors sought to distinguish and characterize homogeneous phenotypes without any a priori assumptions, and to examine survival among the identified clusters.

p. 1553

The clustering analyses provided a first delineation of 2 clusters that showed moderate stability. The researchers note that the presence of these 2 clusters only partially reflects the expected dichotomy between dcSSc and lcSSc. The investigators next performed an analysis to characterize 6 homogeneous groups that differed according to their clinical features, autoantibody profile, and mortality. While some of the groups resembled usual dcSSc or lcSSc prototypes, others exhibited unique features. For example, a majority of lcSSc patients had a high rate of visceral damage and antitopoisomerase antibodies. While prognosis varied among groups, the presence of organ damage markedly affected survival regardless of cutaneous involvement.

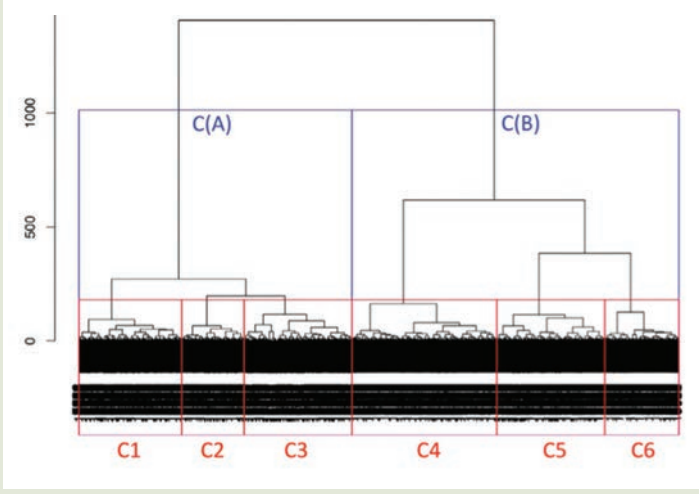

Figure 1. Dendogram of the 6,927 patients with SSc included in the cluster analysis. The length of the vertical lines represents the degree of similarity among patients. Patients were divided into 2 clusters (cluster A and B) and into 6 clusters (clusters 1–6).

The researchers conclude that the practice of restricting subsets of SSc patients to those based only on cutaneous involvement may not capture the complete heterogeneity of the disease, and they suggest that organ damage and antibody profile be taken into consideration when individuating homogeneous groups of patients with a distinct prognosis.

Risk Factors for Major Adverse Cardiovascular Events in Patients Treated With Tofacitinib

Tofacitinib is an oral JAK inhibitor used for the treatment of rheumatoid arthritis (RA). In this issue, Charles-Schoeman et al (p. 1450) report the results of their evaluation of the risk of major adverse cardiovascular events (MACE) in patients with RA receiving tofacitinib. A post hoc analysis was performed of data from phase III and long-term extension studies of tofacitinib in patients with RA. The findings revealed that 24 weeks of tofacitinib treatment increased high-density lipoprotein (HDL) cholesterol but not low-density lipoprotein (LDL) cholesterol or total cholesterol. Moreover, treatment with tofacitinib appeared to be associated with lower future MACE risk.

p. 1450

Fifty-two MACE were documented in 4,076 patients over 12,873 patient-years of exposure. When the investigators performed univariable analyses of baseline variables, they found that traditional cardiovascular risk factors such as older age, higher body mass index, abnormal blood pressure, and a history of either hypertension or diabetes mellitus were associated with MACE risk, as were elevated baseline triglyceride and apolipoprotein B levels and glucocorticoid and statin use. In contrast, disease activity and inflammation measures were not associated with MACE risk. Subsequent multivariable analyses revealed that baseline age, hypertension, and the total to HDL cholesterol ratio remained significantly associated with risk of MACE.

Treatment with tofacitinib resulted in an increase in HDL cholesterol and a decrease in the ratio of total to HDL cholesterol as well as decreased MACE risk. In contrast, changes in total cholesterol, LDL cholesterol, and disease activity measures were not associated with decreased MACE risk. Increased erythrocyte sedimentation rates trended with increased future MACE risk. The authors conclude with a call for further investigation to better determine the cardiovascular safety of tofacitinib.

Atorvastatin for the Primary Prevention of Cardiovascular Events in Patients With Rheumatoid Arthritis

Although cardiovascular disease is significantly increased in people with rheumatoid arthritis (RA) when compared to the general population, the value of statins in the RA patient population has not yet been established. In this issue, Kitas et al (p. 1437) report on their assessment of whether atorvastatin is superior to placebo for the primary prevention of cardiovascular events (CVEs) in RA patients. Results show that atorvastatin 40 mg once daily is safe for patients with RA and is associated with significantly greater reduction of low-density lipoprotein (LDL) cholesterol level than placebo. Moreover, the authors calculated a 40% adjusted CVE risk reduction associated with atorvastatin treatment. This level of risk reduction is consistent with the Cholesterol Treatment Trialists' Collaboration meta-analysis of statin effects in other populations.

p. 1437

The Trial of Atorvastatin for the Primary Prevention of Cardiovascular Events in Patients with RA (TRACE RA) was designed to assess whether RA patients (n = 3,002) who were not already receiving statin therapy would benefit from atorvastatin for the primary prevention of CVEs. Patients in the trial were randomized without consideration of baseline variables and, therefore, current smoking and nonsteroidal antiinflammatory drugs/cyclooxygenase 2 inhibitor usage was unexpectedly higher in the atorvastatin group. The investigators followed patients for a median of 2.51 years, then terminated the study early due to a lower than expected event rate.

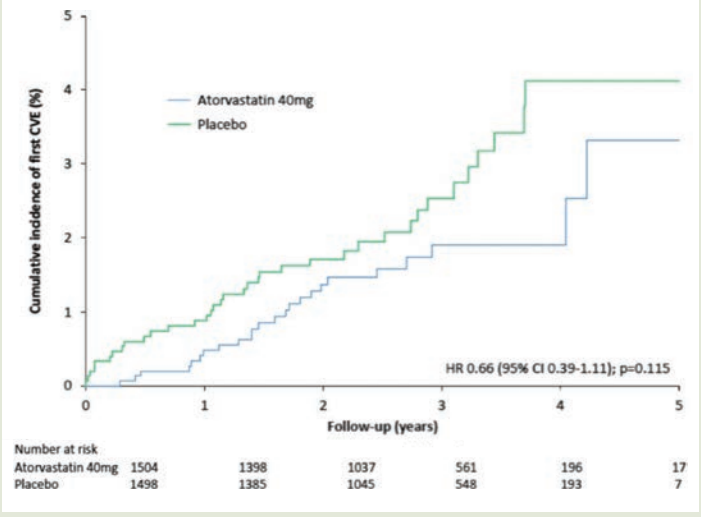

Figure 1. Cumulative incidence of first CVE for patients in the atorvastatin and placebo groups. HR = hazard ratio; 95% CI = 95% confidence interval.

Of the 1,504 patients who received atorvastatin, 1.6% experienced a primary end point compared with 2.4% in the placebo group. At the end of the trial, patients receiving atorvastatin had a mean ± SD LDL cholesterol level of 0.77 ± 0.04 mmoles/liter lower than those receiving placebo. Patients in the atorvastatin group also had lower levels of C-reactive protein than the placebo group. CVE risk reduction per mmoles/liter reduction in LDL cholesterol was 42%. The rates of adverse events were similar between the two groups.

Clinical Connections

Enhanced Programmed Death 1 and Diminished Programmed Death Ligand 1 Up-Regulation Capacity of Post-Activated Lupus B Cells

Stefanski et al, *Arthritis Rheumatol* 2019;71:1539–1544

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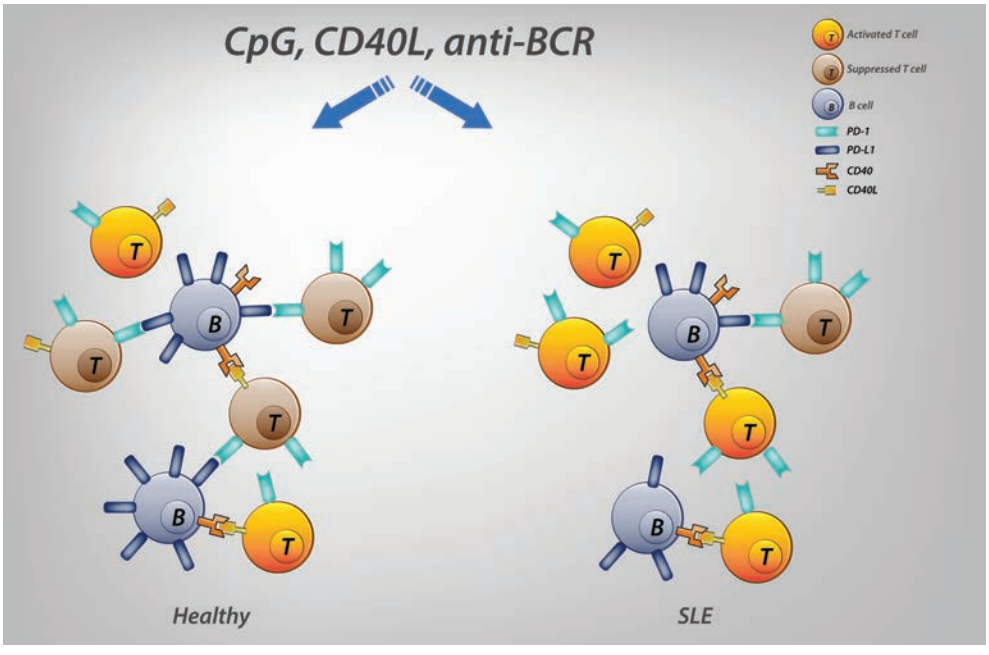

KEY POINTS

- SLE-naive and switched memory B cells are phenotypically characterized by higher PD-1 expression.
- Functionally, the ligand PD-L1, but not PD-L2, shows diminished up-regulation on B cells upon stimulation with anti-BCR, CpG, and CD40L.
- Diminished PD-L1 up-regulation capacity in SLE B cells is inversely correlated with type I IFN and lupus activity.

SUMMARY

Immune checkpoints are vital regulatory pathways that maintain the homeostasis and tolerance of the immune system. Programmed death 1 (PD-1) is a major inhibitory receptor expressed by activated lymphocytes, engaging PD ligand 1 (PD-L1) or PD-L2, and is effectively targeted by cancer immunotherapy. In systemic lupus erythematosus (SLE), PD-1 expression is increased at baseline in naive and switched memory B cells as well as in CD3+ T cells. Notably, the up-regulation of PD-L1 is significantly diminished in SLE B cells upon key B cell stimulation conditions, such as CpG stimulation alone or in combination with anti–B cell receptor (anti-BCR) and CD40L. This finding correlated inversely with type I interferon (IFN) signature as well as lupus disease activity. The data from the study by Stefanski et al support the idea that post-activated hyporesponsive SLE B cells are marked by significantly enhanced PD-1 expression at baseline but show a functionally reduced PD-L1 response upon stimulation. Thus, diminished PD-L1 appears to reflect an impaired immune regulatory function by SLE B cells in suppressing activated T cells under the condition of lupus-related type I IFN inflammation.

Selective Sexual Dimorphisms in Musculoskeletal and Cardiopulmonary Pathologic Manifestations and Mortality Incidence in the Tumor Necrosis Factor–Transgenic Mouse Model of RA

Bell et al, *Arthritis Rheumatol* 2019;71:1512–1523

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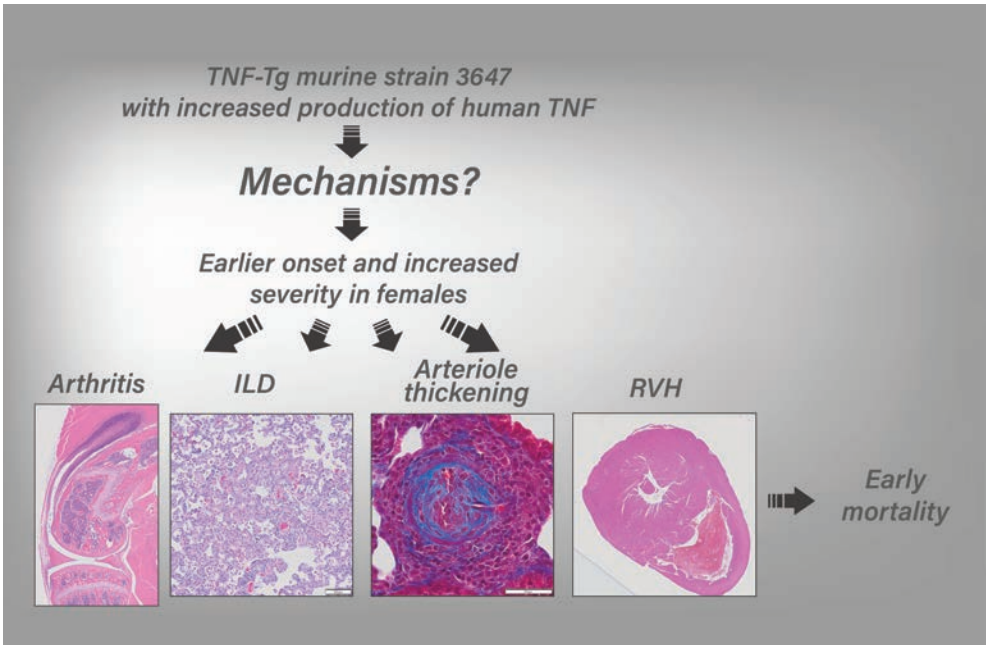

KEY POINTS

- Inflammation of the joints begins earlier in female TNF-Tg mice compared to male TNF-Tg mice, despite similar circulating TNF cytokine levels.
- Compared to male mice, female mice die earlier due to the development of ILD, thickening of the small pulmonary vessels, and subsequent right-sided heart failure.
- TNF-Tg mice have an increased frequency of a dendritic cell subpopulation in their lungs.

SUMMARY

Sexual dimorphism in murine models of autoimmune disease, including inflammatory arthritis, has received little attention to date, which is surprising given the strikingly increased prevalence of autoimmunity in females. The paucity of appropriate preclinical models greatly limits our understanding of the mechanisms that promote sexually dimorphic phenotypes and, most importantly, impedes efforts to develop targeted therapeutics that may differ between males and females. A great deal of attention is now focused on extraarticular manifestations that accompany immune-mediated arthropathies, particularly cardiovascular disease, due to the negative impact on overall function and mortality. Moreover, there is growing evidence that heart and lung complications pose greater threats to females. The 3647 strain of the tumor necrosis factor–transgenic (TNF-Tg) mouse model of arthritis harbors a single copy of the human TNF gene and develops chronic destructive arthritis in a manner that parallels human rheumatoid arthritis. Female mice demonstrate an earlier onset of arthritis and also die at a much younger age than males from complications that stem from cardiopulmonary disease. In this study, Bell et al demonstrate that female mice with the TNF transgene die prematurely from right ventricular hypertrophy (RVH) secondary to pulmonary vascular occlusion and inflammatory lung disease (ILD).

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Cover image: The figure on the cover (from the Rheumatology Image Library, ©American College of Rheumatology) is a photomicrograph demonstrating, by Wright's staining, a characteristic lupus erythematosus cell that was prepared from peripheral blood of a patient with systemic lupus erythematosus. A homogeneous nuclear inclusion is present in a polymorphonuclear leukocyte. This issue of *Arthritis & Rheumatology* features classification criteria for systemic lupus erythematosus, developed as a collaborative initiative of the European League Against Rheumatism and the American College of Rheumatology (page 1400).

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EDITORIAL

Lipids and Cardiovascular Risk Through the Lens of Rheumatoid Arthritis

Katherine P. Liao[1] and Daniel H. Solomon[2]

TR is a 61-year-old woman who has had seropositive rheumatoid arthritis (RA) for 11 years and no history of cardiovascular disease (CVD). She takes methotrexate and sulfasalazine and has low RA disease activity. Her total cholesterol level is 209 mg/dl, her low-density lipoprotein (LDL) cholesterol level is 124 mg/dl, her high-density lipoprotein cholesterol level is 62 mg/dl, and her triglyceride level is 111 mg/dl. Her C-reactive protein (CRP) level is 5 mg/liter. She is a former smoker but has no history of hypertension or diabetes mellitus (DM). Using the 2013 Atherosclerotic Cardiovascular Disease (ASCVD) Risk Estimator (1), her 10-year risk for ASCVD is 3.1%, lower than the threshold for recommended statin initiation.

TR's case highlights a classic predicament when considering CV risk management in RA. She does not have a clear indication for a statin. However, we know that her risk can be 2-fold higher than estimated by a general population CV risk score, such as the Framingham Risk Score (2,3). Furthermore, based on a recent report from the American College of Cardiology and the American Heart Association (4), she has 2 "risk-enhancing" factors, RA and a CRP level of ≥2 mg/dl. Assuming, for the sake of argument, that her risk is 2-fold higher, her 10-year ASCVD risk is now estimated at 6.2%. She now falls into the "borderline risk" category where a discussion regarding moderate-intensity statins should be considered given her risk-enhancing factors. The question at hand is whether TR would benefit from statins for reduction of CV risk.

TR is in fact not a real patient but represents the "average" RA patient in the Trial of Atorvastatin for the Primary Prevention of Cardiovascular Events in Patients with RA (TRACE RA). TRACE RA, presented by Kitas and colleagues in this issue of *Arthritis & Rheumatology* (5), addresses the question raised by the profile of patients such as TR—is statin therapy beneficial in the primary prevention of CVD among RA patients who do not have an indication for statins using population-based CV risk scores? In TRACE RA, a multicenter, randomized, double-blind, placebo-controlled trial in 102 centers in the UK, patients were randomized to receive atorvastatin 40 mg daily or placebo. The inclusion criteria were fulfillment of the American College of Rheumatology 1987 criteria for RA and age >50 years or an RA disease duration of >10 years. All patients received stable RA therapy for at least 3 months prior to inclusion in the study and could not be taking a statin or have an indication for statin therapy, e.g., CVD or DM. These broad inclusion criteria allowed the investigators to test the potential benefits of atorvastatin in a typical RA population.

After enrolling 3,002 RA patients, TRACE RA was prematurely terminated due to lower-than-anticipated CV event (CVE) rates. Patients enrolled in the study were followed up for a median of 2.5 years rather than the planned 5 years. Analyzing the data available, the investigators observed a hazard ratio of 0.66 (95% confidence interval [95% CI] 0.39, 1.11) for the primary composite end point of CV death, myocardial infarction, stroke, transient ischemic attack, or any arterial revascularization. Patients receiving atorvastatin 40 mg had on average a 29.8 mg/dl lower LDL cholesterol level than patients receiving placebo. The risk reduction associated per mmole/liter of LDL cholesterol was 42% (95% CI −14%, 70%) (1 mmole/liter is equivalent to 38.7 mg/dl LDL cholesterol), consistent with the results of statin studies in non-RA populations (6). The adverse event rate was similar between the statin and placebo arms.

Despite the fact that the findings were not statistically significant, TRACE RA provides information about patients similar to TR. First, if one decides to initiate a statin for a patient like TR, statin use does not appear to be associated with increased side effects for patients receiving typical RA treatments. However, while the TRACE RA results are suggestive, the trial was not conclusive regarding whether statin therapy (e.g., atorvastatin 40 mg daily) is beneficial for the primary prevention of CVD. Reasons for this uncertainty likely include insufficient CVEs, as well as noncompliance of the patients randomized to receive atorvastatin—only

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62% of the patients in the atorvastatin arm were still taking the drug after 2 years. The investigators appropriately performed an intent-to-treat analysis, but this may have diluted the potential benefits of atorvastatin because of substantial noncompliance.

We laud the investigators of TRACE RA, as this is the first trial in RA that was designed to study hard CVD end points. While we believe the broad inclusion criteria for TRACE RA were appropriate, the results suggest that we need better methods for identifying the appropriate patient population in RA to target for CV risk reduction strategies.

The study by Giles and colleagues, which also appears elsewhere in this issue of *Arthritis & Rheumatology* (7), may inform the targeting of patients with RA at high risk of CVEs. Their multicenter study examined the association between LDL cholesterol and coronary artery calcium (CAC) scores. CAC scores are associated with an increased risk of coronary heart disease (8) and are being considered as a modality to provide more clarity with regard to CV risk among patients who fall into the category of individuals who should probably be started on statins; these individuals are categorized as "intermediate risk," and have a10-year risk for a CVE ranging from 7.5 to 20%.

Giles et al studied the association between LDL cholesterol and CAC scores using patients from multiple cohorts, and then compared this relationship to that in non-RA patients in the Multi-Ethnic Study of Atherosclerosis (MESA) (8). Their analyses showed that overall CAC scores were higher in RA patients across all levels of LDL cholesterol compared to MESA participants, confirming the known elevated CV risk in RA compared to non-RA populations. Additionally, RA patients with LDL cholesterol concentration <70 mg/dl had adjusted CAC scores similar to those for patients with the highest lipid levels, LDL cholesterol level >160 mg/dl. Moreover, the relationship between LDL cholesterol level and CAC score appeared to differ across patient subgroups. Giles and colleagues' findings support the previously proposed notion that low LDL cholesterol levels in RA patients not taking lipid-lowering therapy may be a red flag for elevated CV risk rather than reassuring (9,10). This unexpected relationship has been termed the lipid paradox—lower LDL cholesterol levels are associated with higher CV risk in RA patients (9), with subsequent studies demonstrating that lower lipid levels are inversely associated with higher levels of inflammation (11,12).

The studies by Kitas et al (5) and by Giles et al (7) both highlight the need to identify RA-specific variables as a screening tool to assess CV risk. Investigators working on future CV prevention studies in RA might even consider selecting for patients with an LDL cholesterol level of <70 mg/dl who are not taking lipid-lowering therapy; although this approach may sound counterintuitive, it likely selects for a high-risk subgroup of RA patients. RA-specific risk scores exist and, if they are found to be valid across populations, they could also be considered for use in targeting patients at high CV risk (13,14). Low LDL cholesterol could be considered in future versions of the risk scores. If the risk scores use routinely available clinical information (versus specialized imaging), then they might have clinical value beyond targeting patients for trials. The two studies published in this issue of *Arthritis & Rheumatology* help fill the evidence base for managing CV risk in RA for research and ultimately in the clinic, and likely provide guidance for considering CV risk across other rheumatic diseases.

AUTHOR CONTRIBUTIONS

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

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EDITORIAL

New Relationships for Old Autoantibodies in Rheumatoid Arthritis

Miriam A. Shelef

In rheumatoid arthritis, there are 2 dominant categories of autoantibodies: anti–citrullinated protein antibodies (ACPAs) and rheumatoid factor (RF). ACPAs target many different citrullinated proteins and underlie diagnostic anti–cyclic citrullinated peptide antibody (anti-CCP) tests. RF, which is also used diagnostically, is an antibody of any isotype that binds to the Fc portion of IgG. Patients with rheumatoid arthritis also produce autoantibodies that bind to homocitrullinated (1), malondialdehyde-acetaldehyde–adducted (2), and acetylated proteins (3), suggesting a propensity to generate antibodies against posttranslationally modified proteins in general. However, the reason that individuals with rheumatoid arthritis generate autoantibodies targeting a variety of posttranslationally modified proteins as well as RF remains a mystery. Moreover, despite their distinct reactivities, ACPAs and RF often coexist in rheumatoid arthritis patients, suggesting a common thread to their development or an interaction between their developmental pathways.

Not only do RF and ACPAs often coexist, they also are associated with similar genetic and environmental risk factors. For example, smoking and the shared epitope, a 5–amino acid sequence motif present in some major histocompatibility complex (MHC) class II molecules, have been strongly linked to ACPAs and RF (4–6). Interestingly, the link between smoking and ACPAs is primarily present in rheumatoid arthritis patients with the shared epitope (7). MHC class II molecules with the shared epitope bind citrullinated peptides more efficiently than native peptides (8), thereby providing a mechanistic rationale for the correlation between ACPAs and the shared epitope. In contrast, smoking can induce RF in mice (9), which do not have the shared epitope. Thus, although smoking and the shared epitope are correlated with the presence of RF and ACPAs, there have been hints that these risk factors may promote RF and ACPAs independently. Teasing apart the factors that uniquely drive ACPAs or RF has been challenging, due to their common coexistence, a frequently used study design that does not evaluate ACPAs or RF separately, and the fact that studies of RF were performed prior to the discovery of ACPAs.

In this issue of *Arthritis & Rheumatology*, Hedström and colleagues carefully dissect the links between smoking and the shared epitope to RF and ACPAs by analyzing 4 subsets of rheumatoid arthritis: CCP–RF–, CCP+RF–, CCP–RF+, and CCP+RF+ disease (10). They demonstrate that nonsmokers with the shared epitope have no significant increased risk of developing CCP–RF+ rheumatoid arthritis but do have an increased risk of developing CCP+RF– rheumatoid arthritis. In contrast, smokers without the shared epitope have an increased risk of CCP–RF+ rheumatoid arthritis, but not CCP+RF– rheumatoid arthritis. However, a connection between smoking and the shared epitope appears to exist, since smoking in homozygotes for the shared epitope is associated with increased risk of CCP+RF– disease. Furthermore, there is a much greater risk of developing CCP+RF+ rheumatoid arthritis in smokers with the shared epitope than in individuals with either risk factor alone.

Taken together, these data suggest that smoking may primarily drive the development of RF, and the shared epitope may be the dominant driver of ACPAs. This conclusion shifts our view of rheumatoid arthritis pathogenesis from multiple risk factors triggering autoantibodies to individual risk factors differentially inducing distinct autoantibodies. However, the story is more complex given the coexistence of RF and ACPAs and the additional risk of ACPA development conferred by smoking in shared epitope–homozygous individuals. Thus, a connection appears to exist between the smoking–RF and shared epitope–ACPA pathways, although we are left uncertain as to what this connection may be.

The authors hypothesized that these 2 pathways converge to accelerate the development of both ACPAs and RF. In a convergent pathways model (Figure 1A), 1 of the 2 pathways that converge is initiated by smoking, inducing IgM-RF (IgM that binds the Fc portion of IgG) in a T cell–independent manner, similar

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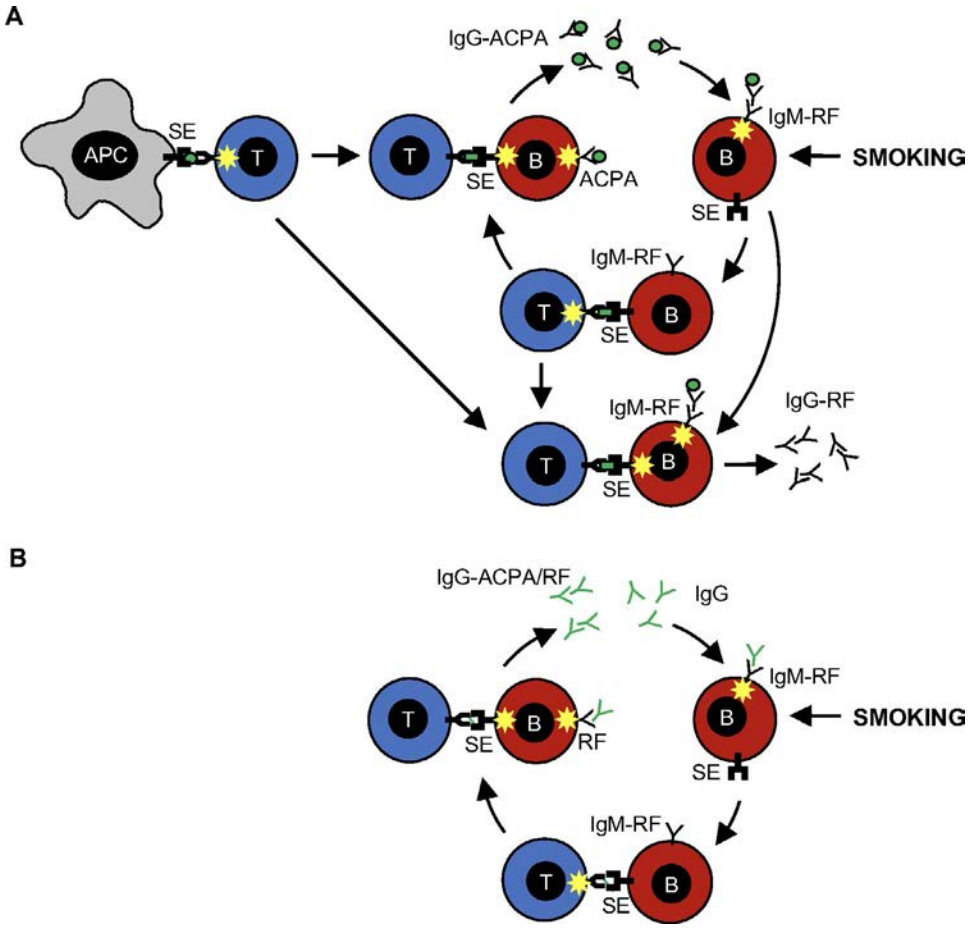

Figure 1. Two models depicting interactions between the shared epitope (SE), smoking, anti–citrullinated protein antibodies (ACPAs), and rheumatoid factor (RF). **A**, In the convergent pathways model, 1 of 2 converging pathways consists of antigen-presenting cells (APCs) presenting citrullinated peptides (green) on SE-containing major histocompatibility complex (MHC) class II molecules to activate T cells. Those T cells then provide help to citrulline-reactive B cells to generate ACPAs of the IgG isotype. In the second of 2 converging pathways, smoking drives IgM-RF–producing B cells. These pathways converge with RF+ B cells binding ACPA immune complexes to present citrullinated antigen via MHC class II to activate more citrulline-reactive T cells. Those T cells activate more citrulline-reactive B cells, leading to more ACPAs. Additionally, activated citrulline-reactive T cells can provide help to IgM-RF–producing B cells that bind ACPA immune complexes to generate IgG-RF. **B**, In the common antigen model, citrullinated IgG (green) is bound by IgM-RF–producing B cells, which then present citrullinated IgG peptides via SE-containing MHC class II to citrulline-reactive T cells. Those T cells then provide help to citrullinated IgG–binding B cells, leading to IgG that targets citrullinated IgG and could be called both an ACPA and an RF.

to the observed rise in the levels of IgM-RF in smokers without rheumatoid arthritis (11). Consistent with this T cell–independent mechanism, RF+ B cells tend to have nonswitched Ig isotypes and low somatic hypermutation rates in rheumatoid arthritis (12).

In the second of the 2 pathways that converge, shared epitope–carrying individuals generate ACPAs, typically with class-switched isotypes and extensive somatic hypermutation (12), suggesting a T cell–, and thus MHC class II–, dependent process. The enhanced binding of shared epitope–containing MHC class II molecules to citrulline-containing peptides likely contributes to this pathology (8). Convergence of the 2 pathways in shared epitope–carrying smokers occurs via binding, internalizing, and processing of ACPA immune complexes by RF+ B cells, followed by presentation of citrullinated peptides to T cells via the B cell's shared epitope–containing MHC class II molecules. An analogous process has been reported with RF+ B cells that are able to stimulate tetanus toxoid–reactive T cells in the presence of tetanus toxoid immune complexes (13). In this manner, RF+ B cells could enlarge the pool of citrulline-reactive T cells, enhancing ACPA production. Additionally, levels of RF, primarily IgM-RF, rise with secondary exposure to an antigen in healthy individuals, possibly due to the activation of RF+ B cells by binding immune complexes (14). In this manner, recurrent exposure to citrullinated proteins, potentially in ACPA immune complexes, could enhance the development of RF. Thus, the smoking–RF and shared epitope–ACPA pathways could converge to enhance each other's production.

As with most models, a convergent pathways model alone does not explain all of the observations. For example, how does smoking enhance the development of CCP+RF– disease in shared epitope–carrying individuals, since RF is not present? One

possibility is that RF was elevated only during the preclinical phase of disease, contributed to ACPA development, and later fell below the levels of detection. Alternatively, since increased citrullination is seen in the lungs of smokers (15,16), perhaps smoking expands the amount and variety of citrullinated antigens presented by shared epitope–containing MHC class II molecules, leading to increased production of ACPAs. In this manner, smoking could drive RF through T cell–independent mechanisms, and ACPAs through T cell–dependent mechanisms.

Additional explanations for the coexistence of ACPAs and RF in rheumatoid arthritis could also be considered. Distinguishing between IgM-RF and IgG-RF would be helpful in this endeavor. Smoking induces IgM-RF, not IgG-RF, in healthy individuals (11). In contrast, IgG-RF is relatively common in individuals with rheumatoid arthritis (17). Furthermore, although RF+ B cells in rheumatoid arthritis have relatively low rates of somatic hypermutation (12), RF in rheumatoid arthritis patients displays increased affinity maturation compared to RF in immunized healthy controls (18). Thus, IgG-RF in rheumatoid arthritis appears to require a break in tolerance and T cell help. However, unlike in the B cell compartment, there is normally tolerance in the T cell compartment against IgG (19). Therefore, questions still remain as to how and why tolerance against both citrullinated proteins and IgG is broken in rheumatoid arthritis to generate both ACPAs and IgG-RF.

One possible mechanism for the development of IgG-RF in CCP+RF+ rheumatoid arthritis fits nicely with the convergent pathways model (Figure 1A). Smoking-induced IgM-RF+ B cells bind ACPA immune complexes and display citrullinated peptides from those immune complexes via shared epitope–containing MHC class II molecules. Citrulline-reactive T cells then provide help to RF+ B cells via an immune synapse containing the T cell receptor bound to a citrullinated peptide that is presented by the B cell's MHC class II molecule. In this manner, a citrulline-reactive T cell would provide help to an IgG-reactive B cell, leading to an affinity-matured, class-switched IgG-RF. A similar epitope mismatch is seen in systemic lupus erythematosus, with histone-reactive T cells providing help to DNA-reactive B cells to generate anti-DNA IgG (20).

Alternatively, an explanation for the break in tolerance against IgG as well as the coexistence of RF and ACPAs could lie in the notion of citrullinated IgG acting as a common antigen for some ACPAs and some RF (21,22). In a common antigen model (Figure 1B), smoking drives IgM-RF in the same manner as described above. IgM-RF–producing B cells bind citrullinated IgG and then present citrullinated IgG peptides via their shared epitope–containing MHC class II molecules to activate citrulline-reactive T cells, the same break in tolerance that drives ACPAs in general. Activated T cells that recognize citrullinated IgG peptides then provide help to RF+ B cells that bind citrullinated IgG, thereby stimulating affinity maturation and class-switch recombination to generate IgG with high affinity for citrullinated IgG. Such antibodies could be classified as both ACPAs and RF, providing a missing link between these 2 autoantibody subsets as well as additional rationale for their common coexistence.

Taking this model further, autoantibodies against citrullinated IgG could trigger the development of the entire ACPA repertoire, due to epitope spreading and the extensive cross-reactivity and general citrulline reactivity of ACPAs (23,24). In this scenario, citrullinated IgG would be "antigen zero" of ACPA development. However, additional versions of a common antigen model are also possible. For example, an ACPA could develop independently of citrullinated IgG, and yet still bind citrullinated IgG in a cross-reactive manner. This mechanism would create an autoantibody that is both an ACPA and an RF, but would not rely on citrullinated IgG as "antigen zero." Of note, it is unlikely that all ACPAs react with IgG, given the presence of CCP+RF– disease.

The simplicity of a common antigen model is appealing, but it likely complements the convergent pathways model, as opposed to replacing it. These 2 models could coexist as well as intersect with the binding of classic ACPA immune complexes, IgG anticitrullinated IgG complexes, or classic ACPA immune complexes bound by RF (targeting native or citrullinated IgG) by B cells that can bind, process, and present citrullinated antigen, native IgG, or citrullinated IgG to activate T cells reactive to any of those antigens. Moreover, while the convergent pathways and common antigen models provide rationale for the coexistence of ACPAs and RF, their roles in CCP+RF– and CCP–RF+ disease, which may be driven preferentially by the shared epitope and smoking, respectively, likely would be minimal. Finally, neither model incorporates the emerging role of the microbiome and mucosal inflammation in rheumatoid arthritis, despite reported connections with smoking, the shared epitope, RF, and ACPAs. For example, periodontitis and its associated microbes have links to smoking, the shared epitope, citrullinated proteins targeted by ACPAs, autoantibodies against native antigens, RF, and rheumatoid arthritis (25–28). Eventually, comprehensive models will need to account for all of the factors linked to rheumatoid arthritis.

So, how do we get closer to finding a more definitive answer as to why 2 different autoantibody subsets exist in rheumatoid arthritis, and what drives their development? One direction of investigation should determine whether autoantibodies that specifically target citrullinated IgG exist, and if the targeting of IgG by RF is different in CCP+ rheumatoid arthritis, CCP– rheumatoid arthritis, and other diseases with RF. Correlates to these studies would include determining the conditions involved with the citrullination of IgG. Is IgG always citrullinated, functionally regulated by citrullination like other posttranslational modifications of IgG, citrullinated during antigen processing, pathologically citrullinated by smoking, and/or modified in other ways that trigger autoantibody reactivity? Additionally, a determination of which RF isotypes are present in CCP+RF+ and CCP–RF+ disease, and if those different isotypes are associated with smoking and/or the shared epitope, would be informative. Finally, further studies are needed to clarify the intersection of the microbiome and mucosal inflammation with

ACPAs and RF. Thus, the study by Hedström and colleagues (10) brings us one step closer to understanding the pathophysiology of rheumatoid arthritis and opens the door for many more exciting discoveries.

AUTHOR CONTRIBUTIONS

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

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SPECIAL ARTICLE

2019 European League Against Rheumatism/American College of Rheumatology Classification Criteria for Systemic Lupus Erythematosus

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This criteria set has been approved by the European League Against Rheumatism (EULAR) Executive Committee and the American College of Rheumatology (ACR) Board of Directors. This signifies that the criteria set has been quantitatively validated using patient data, and it has undergone validation based on an independent data set. All EULAR/ACR-approved criteria sets are expected to undergo intermittent updates.

The ACR is an independent, professional, medical and scientific society that does not guarantee, warrant, or endorse any commercial product or service.

Objective. To develop new classification criteria for systemic lupus erythematosus (SLE) jointly supported by the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR).

Methods. This international initiative had four phases. 1) Evaluation of antinuclear antibody (ANA) as an entry criterion through systematic review and meta-regression of the literature and criteria generation through an international Delphi exercise, an early patient cohort, and a patient survey. 2) Criteria reduction by Delphi and nominal group technique exercises. 3) Criteria definition and weighting based on criterion performance and on results of a multi-criteria decision analysis. 4) Refinement of weights and threshold scores in a new derivation cohort of 1,001 subjects and validation compared with previous criteria in a new validation cohort of 1,270 subjects.

Results. The 2019 EULAR/ACR classification criteria for SLE include positive ANA at least once as obligatory entry criterion; followed by additive weighted criteria grouped in 7 clinical (constitutional, hematologic, neuropsychiatric, mucocutaneous, serosal, musculoskeletal, renal) and 3 immunologic (antiphospholipid antibodies, complement proteins, SLE-specific antibodies) domains, and weighted from 2 to 10. Patients accumulating ≥10 points are classified. In the validation cohort, the new criteria had a sensitivity of 96.1% and specificity of 93.4%, compared with 82.8% sensitivity and 93.4% specificity of the ACR 1997 and 96.7% sensitivity and 83.7% specificity of the Systemic Lupus International Collaborating Clinics 2012 criteria.

Conclusion. These new classification criteria were developed using rigorous methodology with multidisciplinary and international input, and have excellent sensitivity and specificity. Use of ANA entry criterion, hierarchically clustered, and weighted criteria reflects current thinking about SLE and provides an improved foundation for SLE research.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex autoimmune disease with variable clinical features (1,2). SLE manifestations are associated with multiple autoantibodies, ensuing immune complex formation and deposition, and other immune processes (2,3). This complex clinical presentation and pathogenesis makes SLE a difficult disease to grasp and define. Classification criteria are essential for the identification of relatively homogeneous groups of patients for inclusion in research studies and trials (4,5). The 1982 revised American College of Rheumatology (ACR) SLE classification criteria (6) and their 1997 revision (7) have been used worldwide. Since then, our understanding of the disease has advanced. Additional specific skin manifestations were described, some clinical symptoms were better understood, and immunologic tests, such as diminished levels of serum complement components C3 and C4 or testing for anti–β_2-glycoprotein I antibodies, entered routine clinical practice. Better understanding of organ system involvement, such as mucocutaneous abnormalities, led to questions about whether some of the independently counted criteria were in fact manifestations of the same phenomenon (8).

The 2012 Systemic Lupus International Collaborating Clinics (SLICC) classification criteria addressed many of these issues (9). Mucocutaneous and neuropsychiatric manifestations were added, as were hypocomplementemia and new antiphospholipid antibody tests; and criteria definitions were refined. The SLICC criteria emphasized that SLE is primarily an autoantibody disease, requiring at least one immunologic criterion to be present, and

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categorized histology-proven nephritis compatible with SLE as sufficient for classification, if antinuclear antibodies (ANAs) or antibodies to double-stranded DNA (dsDNA) were present. While achieving their goal of increasing sensitivity, the SLICC criteria have lower specificity than the 1997 ACR criteria (9,10).

Existing SLE classification criteria perform better in patients with longstanding disease than in new-onset SLE (11), and there is an increasing recognition and demand that subjects with early SLE should be included in clinical studies and trials. We therefore attempted to enrich our sample populations for early SLE in several phases of the project.

In parallel with improved understanding of SLE, the field of classification criteria development has also seen advances (4,12–14). In order to minimize investigator bias, it is now recommended that the cohorts in which the criteria are tested are from independent centers (4). Other methodologic recommendations include a balanced use of both expert-based and data-driven methods, and inclusion of the patient perspective (13,14). The approach chosen for these 2019 European League Against Rheumatism (EULAR)/ACR SLE classification criteria was specifically designed to maintain this balance and to uphold rigorous methodology.

METHODS

Methodologic overview. Using a methodologic approach based on measurement science the criteria were developed in four phases (10): 1) criteria generation, 2) criteria reduction, 3) criteria definition and weighting, and 4) refinement and validation (Figure 1). The whole initiative was overseen by a 12-member steering committee (MA, KHC, DID, MM, RR-G, JSS, DW, DTB, DLK, DJ, TD, and SRJ) nominated by EULAR and the ACR in equal numbers, based on SLE and/or methodologic experience and previous involvement in international projects.

The current project, jointly supported by the EULAR and the ACR, was originally based on two key concepts. One, we hypothesized that the presence of ANA would be better employed as an entry criterion than as a classification criterion (10). Such an approach was thought to reflect underlying SLE pathogenesis, and take into account ANA test characteristics of high sensitivity and limited specificity. Two, we expected individual criteria would not be of equal utility (weight) for the classification of SLE (15), for example, mucosal ulcers versus biopsy-proven lupus nephritis. Accordingly, the validity of using positive ANA as an entry criterion was explicitly addressed in phase I of the current activity (16). Likewise, methodologic strategies to develop weighted criteria were used.

Phase I: Criteria generation. The purpose of phase I was to test ANA as a potential entry criterion and identify candidate criteria that should be considered for SLE classification using both data-based and expert-based methods, including the patient perspective. *Phase Ia* comprised a systematic literature review of Medline, Embase, and the Cochrane databases with meta-regression to evaluate the operating characteristics of ANA testing for consideration as an entry criterion (16). *Phase Ib* consisted of a Delphi exercise of international SLE experts from the Americas, Europe, and Asia (17). These experts included

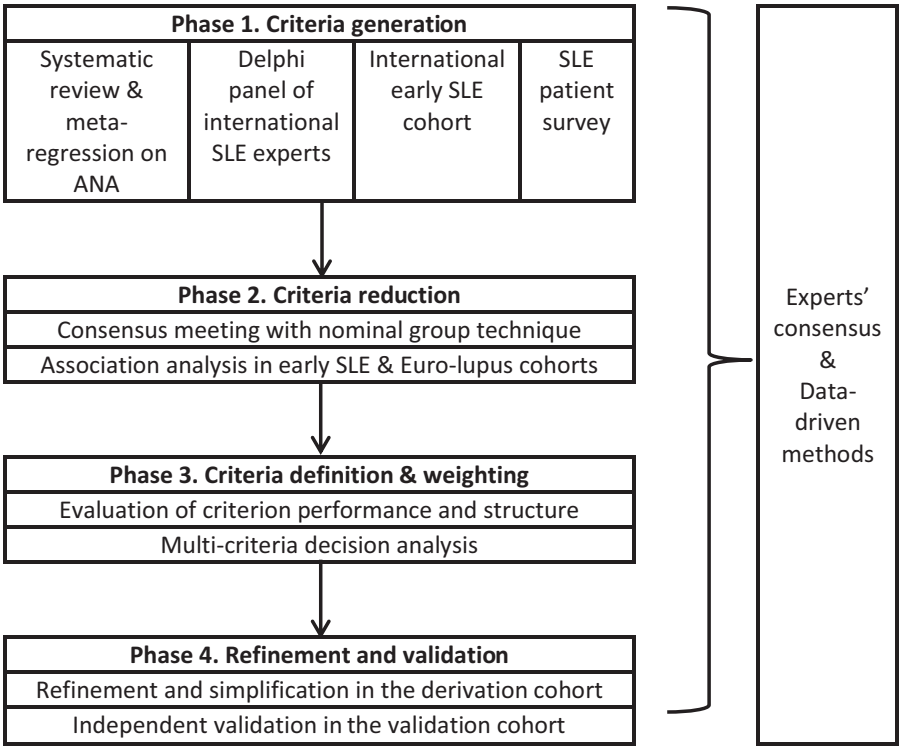

Figure 1. Development and validation of systemic lupus erythematosus (SLE) classification criteria. ANA = antinuclear antibody.

rheumatologists, dermatologists, nephrologists, pediatricians, and non-clinical SLE researchers, providing a broad perspective. The Delphi participants were asked to nominate a broad set of items potentially useful in the classification of SLE (17). In rounds 2 and 3, participants rated the items from 1 (not at all appropriate) to 9 (completely appropriate) for classification of SLE. Criteria were retained if they reached a median rating of ≥6.5; that is, at least 50% of the ratings in the high range (7, 8, or 9). Participants were also asked about the importance of ANA and histopathology for classification of SLE. *Phase Ic* established an international cohort of patients with early SLE or conditions mimicking SLE to identify criteria that may discriminate subjects with early (less than 12 months) disease (18). *Phase Id* comprised a cross-sectional survey of SLE patients, administered via the quarterly journal of the German SLE patient organization, which asked about symptoms within 1 year before and after the patient's diagnosis of SLE (19). While at a risk of recall bias and not necessarily representative of other regions worldwide, this survey was done to explicitly take a patient standpoint into account.

For phases II and III, additional renowned European and North American SLE experts were nominated by the steering committee and invited to participate.

Phase II: Criteria reduction. *Phase IIa.* The objective of this phase was to select a set of criteria from phase I that maximized the likelihood of accurate classification of SLE, particularly of early disease. An independent panel of seven of the international SLE experts (RC, NC-C, DDG, BHH, FH, EM, and JS-G) ranked the candidate criteria from phase I. A consensus meeting of 19 international SLE experts (n = 7 nominal group technique [NGT] experts + steering committee + DK [moderator]) using NGT was conducted to reduce the list of criteria (20). Data for each candidate criterion were reviewed and discussed until consensus was achieved. The NGT experts voted on items to be retained.

Phase IIb. NGT participants pointed out that some criteria could be correlated. With the idea of potentially clustering criteria into domains, associations between candidate criteria were evaluated separately in two cohorts, the phase Ic early SLE and the Euro-lupus cohorts (21).

Phase III: Criteria definition and weighting. *Phase IIIa.* The operating characteristics of the retained candidate criteria were evaluated by literature review. Candidate criteria were hierarchically organized into clinical and immunologic domains, and definitions for the candidate criteria were iteratively refined. SLE patient advocates participated in the review of data and the steering committee discussions (22).

Phase IIIb. One hundred sixty-four case vignettes reflecting broad SLE clinical presentation were sampled from SLE centers across several countries. A panel of six of the international experts not involved in earlier phases of the project (BD, SJ, WJM, GR-I, MS, and MBU) and 11 members of the steering committee assessed and ranked a representative sample of the cases. Subsequently, at a face-to-face meeting, this panel of 17 international SLE experts iteratively compared pairs of criteria, using multicriteria decision analysis facilitated by 1000Minds software (23). The panel unanimously agreed to further reduce the list of criteria. Based on the results, provisional criteria weights were assigned and a provisional threshold score for classification was determined as the lowest score at which the expert panel had achieved consensus on classifying a case vignette as SLE (24).

Phase IV: Refinement and validation. International SLE experts not involved in phase II or phase III panels were asked to contribute cases diagnosed as SLE and controls with conditions mimicking SLE sampled from patients evaluated at their centers. Each center was asked to contribute up to 100 cases and an equal number of controls, preferentially sampling those with early disease, and regardless of their specific clinical or immunologic manifestations. Pseudonymized data on the criteria were collected using a standardized data collection form. Ethics committee approval and informed consent were obtained as per local requirements. The status ("SLE" or not) of each case underwent independent adjudication by three of four SLE experts (GB, BFH, NL, and CT) from different centers. Queries were sent back to the submitting investigator for clarification. Of this cohort, 501 SLE and 500 control subjects were randomly selected to comprise the derivation cohort, while the remaining 696 SLE and 574 control subjects formed the validation cohort.

Refinement. The performance of the draft criteria set was iteratively tested in the derivation cohort. A data-driven threshold for classification was determined by receiver operating characteristics (ROC) analysis and compared with the provisional expert-based consensus threshold. The data of SLE subjects below the threshold (misclassified) were reviewed for groups of patients with unequivocal SLE who still missed classification, and criteria weights adjusted slightly, while preserving the weighting hierarchy (details below in Results, Phase IV section). Sensitivity and specificity were tested against the ACR 1997 and the SLICC 2012 criteria. In addition, ANA as an entry criterion was tested against not having an entry criterion. Finally, the criteria weights were simplified to whole numbers. Refinements to the criteria set were presented to the steering committee and phase III expert panel, and unanimously endorsed.

Validation. The sensitivity and specificity of the final criteria were tested in the validation cohort and compared with previous SLE criteria sets.

Statistical analysis. Descriptive statistics were used to summarize the data. Confidence intervals (CIs) were calculated using the bias-corrected and accelerated bootstrap method (BCa

method) with B = 2000 bootstrap samples. The BCa method resamples the input data B times (with replacement) and calculates the required statistics (sensitivity, specificity, AUC). Based on the B bootstraps samples, the bias-correction is applied and the associated 95% CIs for the statistics are estimated. The BCa method has proven to yield very accurate coverage of estimated CIs (25). The number B of bootstrap resamples is recommended to be at least B = 1,000. We have chosen B = 2,000 and additionally checked if B = 5,000 bootstraps changed the estimated confidence bounds, which was not the case. Statistical analyses were performed using R, v.3.4.0 (The R Foundation of Statistical Computing).

RESULTS

Phase I: Criteria generation. *Phase Ia: ANA as an entry criterion.* A systematic review of Medline, Embase, and the Cochrane database identified 13,080 patients from 64 studies reporting ANA by immunofluorescence on HEp-2 cells. Meta-regression of the operating characteristics of ANA found a sensitivity of 97.8% (95% CI 96.8–98.5%) for ANA of ≥1:80, supporting use of ANA as an entry criterion (16). Since some SLE centers do not have access to HEp-2 ANA, and in view of ongoing work on the standardization of serology and potential future advances in the field, the steering committee and additional autoantibody consultants (MJF and PLM) recommended the provision "or an equivalent positive ANA test. Testing by immunofluorescence on HEp-2 cells or a solid-phase ANA screening immunoassay with at least equivalent performance is highly recommended."

Phase Ib: Delphi exercise. One hundred forty-seven international SLE experts nominated 145 candidate criteria (17). By rating the appropriateness for SLE classification, the participants in the second and third Delphi rounds reduced the list to 40 candidate criteria (Supplementary Table 1, on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40930/abstract).

Phase Ic: International early SLE cohort. The cohort comprised 616 subjects who had been referred for possible SLE with a disease duration of less than 1 year (n = 389 early SLE and n = 227 mimicking diseases) from North America, Europe, Asia, and South America (18). In addition to supporting many of the 40 candidate criteria derived from the Delphi exercise, the comparison between early SLE and non-SLE patients showed that fever occurred more frequently (34.5% versus 13.7%; $P < 0.001$) in SLE, while SLE patients less commonly suffered from arthralgias (20.3% versus 42.7%; $P = 0.001$) and fatigue (28.3% versus 37%; $P = 0.02$).

Phase Id: Patient survey. Three hundred thirty-nine SLE patients (>99% Caucasian, 93% female) responded to the survey (19). More than half of these patients reported mucocutaneous findings in the first year of their disease (Supplementary Table 1, http://onlinelibrary.wiley.com/doi/10.1002/art.40930/abstract), but also fatigue (89%), joint pain (87%), and fever (54%) (19). Given that these items were highlighted both in the early SLE cohort and the patient survey, fever, fatigue, and arthralgias were forwarded to the next phase in addition to the 40 Delphi items. Accordingly, phases Ia–Id resulted in a total of 43 candidate criteria for consideration (Supplementary Table 1).

Phase II: Criteria reduction. *Phase IIa.* The expert panel NGT exercise reduced the candidate criteria from 43 to 21 (26). The panel distinguished potential "entry criteria," which would be required for classification, from potential "additive criteria." They endorsed "positive ANA (≥1:80 by HEp-2 immunofluorescence)" as an entry criterion. The 20 remaining additive criteria included: lupus nephritis by renal biopsy, autoantibodies, cytopenias, fever, arthritis, serositis, mucocutaneous and neuropsychiatric manifestations (Supplementary Table 1).

Phase IIb. Associations between the candidate criteria were evaluated in 389 subjects in the early SLE cohort and the 1,000 SLE subjects of the Euro-lupus cohort. Modest statistically significant correlations were limited to the mucocutaneous (r = 0.22–0.30), neurologic (r = 0.22), and immunologic (r = 0.33) domains in the early SLE cohort, and this modest correlation was replicated in the Euro-lupus cohort (21). Given these associations, criteria were clustered within domains, so that only 1 criterion within each domain would be counted.

Phase III: Criteria definition and weighting. *Phase IIIa.* Based on the literature, definitions of the 20 candidate additive criteria were refined, using a data-driven evaluation of operating characteristics (22), retaining only feasible items with a prevalence of at least 1% according to literature. Literature review led to the consensus decision to evaluate 5 different candidate criteria within the neuropsychiatric domain (delirium, psychosis, seizure, mononeuropathy, cranial neuropathy) and potential separation of acute pericarditis from pleural or pericardial effusions and between diminished C3 or C4 versus diminished C3 and C4. The resulting 23 candidate criteria (Supplementary Table 1, on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40930/abstract) were organized into 7 clinical and 3 immunologic domains, with hierarchical clustering (22). Only the highest-ranking item in each domain was to be counted. Instead of devising exclusion definitions for each criterion, the decision was made to attribute any item to SLE only if no more likely explanation was present. For leukopenia and joint involvement, it was decided to formally test alternative definitions in the derivation cohort. Given the importance of testing for antibodies, particularly for anti-dsDNA, for which tests of relatively low specificity are in use, great care was taken to precisely define testing (Table 1).

Table 1. Definitions of SLE classification criteria*

Criteria	Definition
Antinuclear antibodies (ANA)	ANA at a titer of ≥1:80 on HEp-2 cells or an equivalent positive test at least once. Testing by immunofluorescence on HEp-2 cells or a solid-phase ANA screening immunoassay with at least equivalent performance is highly recommended
Fever	Temperature >38.3°C
Leukopenia	White blood cell count <4,000/mm³
Thrombocytopenia	Platelet count <100,000/mm³
Autoimmune hemolysis	Evidence of hemolysis, such as reticulocytosis, low haptoglobin, elevated indirect bilirubin, elevated LDH, AND positive Coombs' (direct antiglobulin) test
Delirium	Characterized by 1) change in consciousness or level of arousal with reduced ability to focus, 2) symptom development over hours to <2 days, 3) symptom fluctuation throughout the day, 4) either 4a) acute/subacute change in cognition (e.g., memory deficit or disorientation), or 4b) change in behavior, mood, or affect (e.g., restlessness, reversal of sleep/wake cycle)
Psychosis	Characterized by 1) delusions and/or hallucinations without insight and 2) absence of delirium
Seizure	Primary generalized seizure or partial/focal seizure
Non-scarring alopecia	Non-scarring alopecia observed by a clinician†
Oral ulcers	Oral ulcers observed by a clinician†
Subacute cutaneous OR discoid lupus	Subacute cutaneous lupus erythematosus observed by a clinician:† Annular or papulosquamous (psoriasiform) cutaneous eruption, usually photodistributed If skin biopsy is performed, typical changes must be present (interface vacuolar dermatitis consisting of a perivascular lymphohistiocytic infiltrate, often with dermal mucin noted). OR Discoid lupus erythematosus observed by a clinician:† Erythematous-violaceous cutaneous lesions with secondary changes of atrophic scarring, dyspigmentation, often follicular hyperkeratosis/plugging (scalp), leading to scarring alopecia on the scalp If skin biopsy is performed, typical changes must be present (interface vacuolar dermatitis consisting of a perivascular and/or periappendageal lymphohistiocytic infiltrate. In the scalp, follicular keratin plugs may be seen. In longstanding lesions, mucin deposition may be noted)
Acute cutaneous lupus	Malar rash or generalized maculopapular rash observed by a clinician† If skin biopsy is performed, typical changes must be present (interface vacuolar dermatitis consisting of a perivascular lymphohistiocytic infiltrate, often with dermal mucin noted. Perivascular neutrophilic infiltrate may be present early in the course)
Pleural or pericardial effusion	Imaging evidence (such as ultrasound, x-ray, CT scan, MRI) of pleural or pericardial effusion, or both
Acute pericarditis	≥2 of 1) pericardial chest pain (typically sharp, worse with inspiration, improved by leaning forward), 2) pericardial rub, 3) EKG with new widespread ST elevation or PR depression, 4) new or worsened pericardial effusion on imaging (such as ultrasound, x-ray, CT scan, MRI)
Joint involvement	EITHER 1) synovitis involving 2 or more joints characterized by swelling or effusion OR 2) tenderness in 2 or more joints and at least 30 minutes of morning stiffness
Proteinuria >0.5 g/24 hours	Proteinuria >0.5 g/24 hours by 24-hour urine or equivalent spot urine protein-to-creatinine ratio
Class II or V lupus nephritis on renal biopsy according to ISN/RPS 2003 classification	Class II: Mesangial proliferative lupus nephritis: purely mesangial hypercellularity of any degree or mesangial matrix expansion by light microscopy, with mesangial immune deposit. A few isolated subepithelial or subendothelial deposits may be visible by immunofluorescence or electron microscopy, but not by light microscopy Class V: Membranous lupus nephritis: global or segmental subepithelial immune deposits or their morphologic sequelae by light microscopy and by immunofluorescence or electron microscopy, with or without mesangial alterations

(Continued)

Table 1. *(Cont'd)*

Criteria	Definition
Class III or IV lupus nephritis on renal biopsy according to ISN/RPS 2003 classification	Class III: Focal lupus nephritis: active or inactive focal, segmental, or global endocapillary or extracapillary glomerulonephritis involving <50% of all glomeruli, typically with focal subendothelial immune deposits, with or without mesangial alterations Class IV: Diffuse lupus nephritis: active or inactive diffuse, segmental, or global endocapillary or extracapillary glomerulonephritis involving ≥50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations. This class includes cases with diffuse wire loop deposits but with little or no glomerular proliferation
Positive antiphospholipid antibodies	Anticardiolipin antibodies (IgA, IgG, or IgM) at medium or high titer (>40 APL, GPL, or MPL, or >the 99th percentile) or positive anti-β_2GPI antibodies (IgA, IgG, or IgM) or positive lupus anticoagulant
Low C3 OR low C4	C3 OR C4 below the lower limit of normal
Low C3 AND low C4	Both C3 AND C4 below their lower limits of normal
Anti-dsDNA antibodies OR anti-Sm antibodies	Anti-dsDNA antibodies in an immunoassay with demonstrated ≥90% specificity for SLE against relevant disease controls OR anti-Sm antibodies

* SLE = systemic lupus erythematosus; LDH = lactate dehydrogenase; CT = computed tomography; MRI = magnetic resonance imaging; EKG = electrocardiography; ISN = International Society of Nephrology; RPS = Renal Pathology Society; anti-β_2GPI = anti–β_2-glycoprotein I; anti-dsDNA = anti–double-stranded DNA.
† This may include physical examination or review of a photograph.

Phase IIIb. The 1.5-day in-person consensus meeting using multicriteria decision analysis involved 74 decisions between pairs of criteria. Criteria weights were calculated by 1000Minds software based on these decisions (Table 2). International Society of Nephrology/Renal Pathology Society class III or IV nephritis consistently attained higher weight than class II or V nephritis, so lupus nephritis by histology was separated into 2 different criteria. Class VI lupus nephritis as an end-stage manifestation was unanimously eliminated. Likewise, the experts unanimously voted to not retain mononeuropathy and cranial neuropathy, which had been included into the set of potential neuropsychiatric items in phase IIIa but turned out to add little to SLE classification. The use of weighted criteria led to a sum score that is a measure of the relative probability of a subject having SLE, with higher scores indicating higher likelihood. Experts reached full consensus on a classification of SLE at a provisional threshold score of >83 of a theoretical maximum of 305 (24).

Phase IV: Refinement and validation. Twenty-one centers from the US, Canada, Mexico, Austria, Croatia, France, Germany, Greece, Hungary, Italy, Portugal, Spain, the UK, Turkey, Hong Kong, and Japan submitted a total of 2,339 cases from their cohorts; 1,197 SLE and 1,074 non-SLE diagnoses (Table 3) were verified by 3 adjudicators blinded to the proposed classification criteria system. Due to lack of consensus during adjudication, 68 subjects (2.9%) were excluded from the analysis.

Derivation cohort. Of the 2,271 triple-adjudicated cases, 501 SLE and 500 non-SLE cases were randomly assigned to the derivation cohort. The provisional weighting system derived from phase III was tested in the derivation cohort. ROC analysis suggested a data-driven threshold of ≥70 (of a maximum of 305), with a sensitivity of 95.4% and a specificity of 95.2%, which was superior to the consensus-derived provisional threshold of >83 that had high specificity (98.8%), but lower sensitivity (81.6%). Review of subjects below the threshold of 70 identified a subgroup of SLE subjects with joint involvement and/or leukopenia. Thus, weights for leukopenia and joint involvement were each adjusted (Table 2) to reduce misclassification. When alternative definitions for leukopenia and joint involvement were tested, leukopenia defined as a white blood cell count (WBC) <4000/mm^3 at least once (9) also had a slightly higher sensitivity + specificity (1.944 versus 1.942) than leukopenia defined as WBC <4,000/mm^3 on 2 or more occasions (6,26). Joint involvement defined as EITHER "synovitis involving 2 or more joints, characterized by swelling or effusion," OR "tenderness in 2 or more joints and at least 30 minutes of morning stiffness" (9) had a higher combined sensitivity and specificity than arthritis defined simply as synovitis of 2 or more joints (1.944 versus 1.900). When retested, the revised criteria had increased sensitivity, and maintained sensitivity + specificity. Evaluating ANA as an entry criterion, the criteria with the ANA entry criterion had better performance than without (sensitivity + specificity 1.944 versus 1.930). Next, the weights were simplified by division to whole numbers to achieve a threshold of 10 (Table 2). In the derivation cohort, the sensitivity and specificity of the final criteria set (Figure 2) were reaching the performance benchmarks set for this project (Table 4).

Validation. The validation cohort, that is, the full cohort minus the derivation cohort, comprised 1,270 triple-adjudicated subjects (n = 696 SLE, n = 574 controls). The criteria, with

Table 2. Relative weights of the additive classification criteria items*

Domain	Item	Original	Modification	Revised	Simplified
Constitutional	Fever	13		13	2
Hematologic	Leukopenia	12	+7	19	3
	Thrombocytopenia	26		26	4
	Autoimmune hemolysis	28		28	4
Neuropsychiatric	Delirium	12		12	2
	Psychosis	20		20	3
	Seizure	34		34	5
Mucocutaneous	Alopecia	13		13	2
	Oral ulcers	14		14	2
	SCLE/DLE	29		29	4
	ACLE	38		38	6
Serosal	Effusion	34		34	5
	Acute pericarditis	38		38	6
Musculoskeletal	Joint involvement	34	+4	38	6
Renal	Proteinuria	27		27	4
	Class II/V	55		55	8
	Class III/IV	74		74	10
Antiphospholipid antibodies	Antiphospholipid	13		13	2
Complements	C3 or C4 low	19		19	3
	C3 and C4 low	27		27	4
SLE-specific antibodies	Anti-Sm	40		40	6
	Anti-dsDNA	38		38	6

* Weights derived from the phase III consensus meeting with multicriteria decisions analysis (original), added points for leukopenia and joint involvement (modification), the resulting weights (revised), and the final simplified weights (simplified). SCLE = subacute cutaneous lupus erythematosus; DLE = discoid lupus erythematosus; ACLE = acute cutaneous lupus erythematosus; SLE = systemic lupus erythematosus; anti-dsDNA = anti–double-stranded DNA.

positive ANA as an entry criterion, weighted criteria in 7 clinical domains (constitutional, hematologic, neuropsychiatric, mucocutaneous, serosal, musculoskeletal, renal) and 3 immunologic domains (antiphospholipid antibodies, low complements, anti-Sm and anti-dsDNA as SLE-specific antibodies), and a classification threshold score of ≥10 (out of a theoretical maximum of 51) (Figure 2), had a sensitivity of 96.1% and a specificity of 93.4% (Table 4). It demonstrated improved performance compared with the ACR 1997 and SLICC 2012 criteria.

DISCUSSION

New SLE classification criteria were developed with support by both the ACR and EULAR. Through a four-phase, iterative process, we have defined an additive, weighted multicriteria system that produces a measure of the relative probability that an individual can be classified as SLE. The system defines a threshold above which experts would classify cases as SLE for the purpose of research studies. We have carefully defined the criteria to improve reliability and precision, and have grouped the criteria into 10 hierarchical domains. We have validated the criteria against a large number of cases, including many patients with manifestations that resemble SLE but who do not have SLE. This approach, as well as the resulting criteria system, represents a paradigm shift for the classification of SLE.

We have defined positive ANA at any time as required entry criterion. There were three possible ways to deal with ANA testing. The previous criteria sets have treated ANA the same as the much more specific antibodies against Sm and dsDNA, which we considered suboptimal given important differences in sensitivity and specificity. We could have excluded ANA completely in classifying lupus, but we still consider ANA a useful test and concept. We therefore decided to test ANA as an entry criterion, which reflects the use of ANA as a highly sensitive screening test.

Criteria using ANA as entry criterion had better performance. During the phase I Delphi exercise, 58% of SLE experts did not feel comfortable and an additional 19% were uncertain about classifying a patient with SLE in the absence of ever having a

Table 3. Demographic characteristics of the derivation and validation cohorts*

	Derivation cohort		Validation cohort	
	SLE	Non-SLE	SLE	Non-SLE
n	501	500	696	574
Female/male	447/54	421/79	608/88	490/84
Age, mean ± SD years	45 ± 14	54 ± 16	45 ± 14	56 ± 16
Disease duration, mean ± SD years	11 ± 8	9 ± 8	11 ± 8	9 ± 8
Ethnicity				
Black	29	10	56	12
East Asian	36	29	53	34
Hispanic	59	48	73	51
South/Southeast Asian	16	6	21	11
White	355	404	480	461
Other	6	3	13	5
SLE	501		696	
Non-SLE		500		574
Adult-onset Still's disease		2		11
Autoimmune thyroiditis		6		5
Behçet's disease		7		9
Cancer		2		3
Inflammatory myositis		37		27
Fibromyalgia		6		3
Membranous nephritis		11		14
Mixed connective tissue disease		9		15
Osteoarthritis		2		0
Primary antiphospholipid antibody syndrome		45		48
Psoriatic arthritis		12		9
Rheumatoid arthritis		94		110
Sarcoidosis		2		2
Sjögren's syndrome		112		124
Spondyloarthritis		5		5
Systemic sclerosis		99		120
Tuberculosis		0		2
Undifferentiated connective tissue disease		16		20
Vasculitis		9		13
Viral infection		5		5
Other		19		29

* Inflammatory myositis includes dermatomyositis, polymyositis, and juvenile dermatomyositis. SLE = systemic lupus erythematosus.

positive ANA (17). The systematic literature review and meta-regression of data on 13,080 subjects demonstrated ANA ≥1:80 to have a sensitivity of 98% with a lower limit of the 95% CI at 97% (16). In the phase I early SLE cohort, 99.5% of the 389 SLE patients were ANA positive (18). The frequencies of ANA-positive SLE patients in the derivation and validation cohorts (99.6% and 99.3%, respectively) were in the same range. Since both in the early SLE cohort and in the derivation and validation cohorts, patients were included in many centers worldwide independent of ANA positivity, the latter data provide additional support for ANA as an entry criterion.

Using ANA as entry criterion means the new criteria cannot classify SLE among patients who are persistently ANA negative. While possibly also distinguished by lower cytokine levels (27) and lower efficacy of immunomodulatory treatment (28), such a subgroup of patients exists. Although small, it may vary in size

Entry criterion

Antinuclear antibodies (ANA) at a titer of ≥1:80 on HEp-2 cells or an equivalent positive test (ever)

↓

If absent, do not classify as SLE
If present, apply additive criteria

↓

Additive criteria

Do not count a criterion if there is a more likely explanation than SLE.
Occurrence of a criterion on at least one occasion is sufficient.
SLE classification requires at least one clinical criterion and ≥10 points.
Criteria need not occur simultaneously.
Within each domain, only the highest weighted criterion is counted toward the total score§.

Clinical domains and criteria	Weight	Immunology domains and criteria	Weight
Constitutional		***Antiphospholipid antibodies***	
Fever	2	Anti-cardiolipin antibodies OR	
Hematologic		Anti-β2GP1 antibodies OR	
Leukopenia	3	Lupus anticoagulant	2
Thrombocytopenia	4	***Complement proteins***	
Autoimmune hemolysis	4	Low C3 OR low C4	3
Neuropsychiatric		Low C3 AND low C4	4
Delirium	2	***SLE-specific antibodies***	
Psychosis	3	Anti-dsDNA antibody* OR	
Seizure	5	Anti-Smith antibody	6
Mucocutaneous			
Non-scarring alopecia	2		
Oral ulcers	2		
Subacute cutaneous OR discoid lupus	4		
Acute cutaneous lupus	6		
Serosal			
Pleural or pericardial effusion	5		
Acute pericarditis	6		
Musculoskeletal			
Joint involvement	6		
Renal			
Proteinuria >0.5g/24h	4		
Renal biopsy Class II or V lupus nephritis	8		
Renal biopsy Class III or IV lupus nephritis	10		

Total score:

↓

Classify as Systemic Lupus Erythematosus with a score of 10 or more if entry criterion fulfilled.

Figure 2. Classification criteria for systemic lupus erythematosus (SLE). § = additional criteria within the same domain will not be counted; * = in an assay with 90% specificity against relevant disease controls. Anti-β_2GPI = anti–β_2-glycoprotein I; anti-dsDNA = anti–double-stranded DNA.

in different populations (16). This patient subset needs to be put high on the scientific agenda for further investigation. Additional characterization of this phenomenon may lead to an alternative entry criterion for this small group of patients. For the moment, we still think it is acceptable to exclude ANA-negative patients from clinical trials.

Molecular classification criteria were also considered during the development of these criteria (29). Many novel biomarkers were

Table 4. Operating characteristics of the new classification criteria compared with the ACR 1997 and SLICC 2012 classification criteria in the derivation and the validation cohorts*

	ACR 1997 criteria	SLICC 2012 criteria	EULAR/ACR 2019 criteria
Derivation			
Sensitivity (95% CI)	0.85 (0.81–0.88)	0.97 (0.95–0.98)	0.98 (0.97–0.99)
Specificity (95% CI)	0.95 (0.93–0.97)	0.90 (0.87–0.92)	0.96 (0.95–0.98)
Combined (95% CI)	1.80 (1.76–1.83)	1.87 (1.84–1.90)	1.94 (1.92–1.96)
Validation			
Sensitivity (95% CI)	0.83 (0.80–0.85)	0.97 (0.95–0.98)	0.96 (0.95–0.98)
Specificity (95% CI)	0.93 (0.91–0.95)	0.84 (0.80–0.87)	0.93 (0.91–0.95)
Combined (95% CI)	1.76 (1.73–1.80)	1.80 (1.77–1.84)	1.90 (1.87–1.92)

* ACR = American College of Rheumatology; SLICC = Systemic Lupus International Collaborating Clinics; EULAR = European League Against Rheumatism; 95% CI = 95% confidence interval.

nominated, such as increased circulating B lymphocyte stimulator (BLyS), interferon-γ (IFNγ)–induced protein 10 kd (IP-10), monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor α (TNFα), type I IFN signature, or increased Th17 and plasma cell populations. They were all voted out in the expert Delphi exercise, largely because of limited availability in the clinical setting and/or insufficient evidence (5). However, inclusion of novel biomarkers, beyond autoantibodies, may ultimately further improve the specificity of SLE classification, increase alignment of classification with underlying disease pathogenesis, and improve the performance and information content of clinical trials. Thus, testing of biomarkers against these criteria is an important area for future research.

A new clinical criterion, unexplained fever, turned out to be common and remarkably characteristic for SLE. However, since infections are a major cause of death in SLE, it is of utmost importance to stress that fever, like all other criteria manifestations, should only be counted if no better explanation exists, and that infections have to be suspected first in any patient with (potential) SLE, particularly when C-reactive protein is elevated (30). The concept that all criteria are only to be counted if SLE is thought to be the most likely cause of the manifestation (i.e., no other more likely cause exists) is central to these new EULAR/ACR criteria, and is explicitly stated as an overarching principle. Some criteria, such as delirium, psychosis, and acute pericarditis, were in part redefined based on existing scientific definitions (22). Where alternative definitions were used, the performance of the alternative definitions was comparatively evaluated in the derivation cohort.

The differential weighting of criteria better represents their relative contribution to an individual's classification of SLE. For SLE, renal biopsy with class III or IV lupus nephritis carries the most weight and in the presence of a positive ANA is enough to classify a patient as SLE. This further develops a concept of the SLICC criteria (9) and reflects the current thinking of SLE experts; in the Delphi exercise, 85% would classify SLE on renal pathology alone (17). Renal biopsy with class II or V lupus nephritis still carries a large weight (8 points) but is not by itself sufficient for the classification of SLE.

The numerical goal of this project was to keep the specificity similar to the specificity of the ACR 1997 criteria, but increase the sensitivity to the high sensitivity level of the SLICC criteria, if possible. The validation cohort data suggest that this goal has been achieved. From our data, it appears that the SLICC criteria increase in sensitivity was to a significant degree founded in accepting renal histology and adding subacute cutaneous lupus and low complement levels. These three advances are mirrored in the current criteria. Many of the other additional symptoms of the SLICC criteria were of very low frequency. Specificity was increased by weighting of criteria, by the NGT expert panel decision to not allow lymphopenia to go forward, and, importantly, by the decision that no criterion be counted if better explained by another condition.

The new criteria provide a simple, directed, and highly accurate method for classifying SLE. An electronic "app" is in preparation, which will assist in the use of these criteria. However, it is important to stress that classification criteria are not designed for diagnosis or treatment decisions (5). They should never be used to exclude patients who do not fully meet these criteria from receiving appropriate therapies. This is also pertinent to patients with ANA-negative SLE discussed above. Diagnosis of SLE remains the purview of an appropriately trained physician evaluating an individual patient (5).

The new SLE classification system also provides new research opportunities. With much interest in early or latent SLE (31,32), the additive point system and the relative probability of classification it produces allows for systematic study of individuals who fall below the classification threshold. This will facilitate studies of disease evolution and early intervention. Furthermore, the use of an additive scoring system will allow for studying the idea of "ominousity," that is, the potential implications of having very high scores on disease severity and subsequent prognosis. This work would need to reconsider the

relative contribution of individual criteria (weights) and consider additional criteria that potentially contribute to ominousity.

It is anticipated that other groups will test these criteria, which will constitute important external validation. This will be particularly important for pediatric SLE and those with organ-dominant, for example, skin-dominant, disease, since it is a limitation of this criteria project that the patient cohorts do not represent these subgroups. Similar limitations also pertain to several racial/ethnic groups (for example, African American/Black, Hispanic, and Asian patients) and to men with SLE, each only included in lower numbers (Table 3). It is important to independently test the EULAR/ACR criteria in these subgroups. Leukocyte counts, for example, are more frequently below 4,000/mm^3 in African Americans (33), which may have an influence on criteria performance. It is also possible that the academic center patient populations included differ from patients in community practice clinics. Investigators testing the new criteria in different populations are reminded about the critical importance of the correct attribution of each criterion. Criteria can only be counted when not better explained by another condition (see Supplementary Table 2, on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40930/abstract). The attribution process requires diligence and clinical experience.

In summary, our multiphase methodologic approach and ensuing classification system using ANA as an entry criterion and weighted, hierarchically clustered criteria constitute a paradigm shift in the classification of SLE. These criteria have excellent performance characteristics and face validity, as the structure and weighting were designed to reflect current thinking about SLE. The inclusion of fever assists with the classification of early SLE. The separation of renal biopsy findings reflects their differential impact on the probability of SLE classification. These criteria have strong operating characteristics, with excellent sensitivity and specificity. This classification system was built using rigorous methodology that was both data-driven and expert-based. With the inclusion of over 200 SLE experts from multiple countries and medical disciplines, methodologists, patient advocates, and over 4,000 subjects, this work is the largest international, collaborative SLE classification effort to date.

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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. Drs. Aringer and Johnson had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Aringer, Costenbader, Daikh, Brinks, Mosca, Ramsey-Goldman, Smolen, Wofsy, Boumpas, Kamen, Jayne, Naden, Dörner, Johnson.

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REVIEW

Cognitive Dysfunction in Systemic Lupus Erythematosus: A Case for Initiating Trials

Nina Kello, Erik Anderson, and Betty Diamond

Cognitive dysfunction (CD) is an insidious and underdiagnosed manifestation of systemic lupus erythematosus (SLE) that has a considerable impact on quality of life, which can be devastating. Given the inconsistencies in the modes of assessment and the difficulties in attribution to SLE, the reported prevalence of CD ranges from 5% to 80%. Although clinical studies of SLE-related CD have been hampered by heterogeneous subject populations and a lack of sensitive and standardized cognitive tests or other validated objective biomarkers for CD, there are, nonetheless, strong data from mouse models and from the clinical arena that show CD is related to known disease mechanisms. Several cytokines, inflammatory molecules, and antibodies have been associated with CD. Proposed mechanisms for antibody- and cytokine-mediated neuronal injury include the abrogation of blood–brain barrier integrity with direct access of soluble molecules in the circulation to the brain and ensuing neurotoxicity and microglial activation. No treatments for SLE-mediated CD exist, but potential candidates include agents that inhibit microglial activation, such as angiotensin-converting enzyme inhibitors, or that protect blood–brain barrier integrity, such as C5a receptor blockers. Structural and functional neuroimaging data have shown a range of regional abnormalities in metabolism and white matter microstructural integrity in SLE patients that correlate with CD and could in the future become diagnostic tools and outcome measures in clinical trials aimed at preserving cognitive function in SLE.

Introduction

Neuropsychiatric systemic lupus erythematosus (NPSLE) encompasses a range of neurologic, psychiatric, and cognitive disorders that collectively affect up to 40% of SLE patients at the time of diagnosis and a majority of SLE patients throughout the course of their disease (1). NPSLE is associated with worse quality of life independent of SLE activity and medications (1), high unemployment and disability rates (2), high damage accrual (1), and a 3–9-fold increase in mortality (3). The 1999 American College of Rheumatology (ACR) nomenclature organized the heterogeneous NPSLE conditions into 19 standardized "case definitions" (4). These can also be classified as central, peripheral, and vascular manifestations or, alternatively, as diffuse and focal manifestations. We will focus this review on cognitive dysfunction (CD), a common diffuse central nervous system (CNS) manifestation of NPSLE.

CD can be slowly progressive, and its presence or progression does not necessarily correlate with disease activity. Because the assessments are not standardized and the attribution to SLE is difficult, the reported prevalence of CD is highly variable at 6–81% (5). SLE patients identify CD as one of their most distressing symptoms (6) that detracts from quality of life; however, with poor screening and diagnostic metrics, CD is still grossly underrecognized by rheumatologists. Its pathogenesis is poorly understood and no treatments are available.

The ACR nomenclature defines CD as a significant deficit in any or all of the following cognitive domains: simple or complex attention, reasoning, executive skills, memory, visual-spatial processing, language, and psychomotor speed (4). Previous studies have revealed attention, memory, and language to be among the most commonly affected domains in SLE (7). Two major obstacles to our understanding of the contribution of SLE to CD are the potential confounders in diagnosis and a lack of understanding of pathogenesis. Neurotoxic medications such as glucocorticoids and cyclophosphamide, as well as infection, metabolic disorders, and hypertension, can all cause symptoms that overlap with CD (1). Furthermore, it is important to recognize that other CNS manifestations of SLE, such as seizures, stroke, and mood disorders,

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may also contribute to CD. Another prevalent problem with the current approach to studying CD in SLE is that studies often include patients with both focal (such as ischemic stroke) and diffuse manifestations within a single cohort, although pathogenesis is likely to differ in these 2 groups.

An additional obstacle to studying CD is that a wide variety of cognitive tests have been used across cohorts; some of these may not be sensitive to a particular cognitive deficit (7). Studies that explore pathogenic mechanisms and the contribution of autoantibodies, cytokines, or other mediators to CD may require specifically designed cognitive assessments and patient selection, as CD in different domains may result from different pathogenic mechanisms.

Detection of CD

The ascertainment of CD involves both clinical history, i.e., impaired functioning supported by patient-reported outcomes, and neuropsychological testing.

Neurocognitive testing is the gold standard in the diagnosis of CD in NPSLE. The most frequently used battery of tests to assess cognition in SLE, according to a recent review and meta-analysis (7), are comprehensive traditional assessments that are often administered by a psychologist or trained psychometrist (e.g., the Rey-Osterrieth Complex Figure Test, the Trail Making Test, or the Automated Neuropsychological Assessment Metric [ANAM]). Other tests used less frequently include the Modified Mini-Mental State Examination (MMSE), the Montreal Cognitive Assessment (MoCA), the Controlled Oral Word Association Test, and the Hopkins Verbal Learning Test-Revised, as well as various additional instruments. Importantly, the meta-analysis reported a wide prevalence of CD ranging between 3% and 81%. Several factors may have contributed to this wide range, including patient heterogeneity (SLE patients with and without predetermined NPSLE), the use of different assessments, including those that may not be sensitive to a particular cognitive deficit, and the lack of a standardized definition for CD despite the ACR guideline. Despite these complexities, the overwhelming evidence supports an increased frequency of CD in SLE patients compared to the general population.

While many studies have compared cognitive tests in SLE, many have included patients with neurologic or psychiatric disease (7), which makes interpretation difficult. Several studies have compared tests in those without known neuropsychiatric disease. One study compared the ANAM to a set of traditional measures, including those recommended by the ACR, and found that the ANAM subtests, particularly those testing learning and memory, correlated with the traditional measures (8). In regard to the search for an appropriate screening tool for CD in SLE, one study compared the MMSE, the MoCA, and the Cognitive Symptom Inventory, and showed the MoCA to be the most sensitive/specific and with results that highly correlated with those obtained using the ACR-recommended assessments (9).

Neuroimaging in SLE CD

Neuroimaging has the potential to be a valuable tool for understanding the pathogenesis of CD in SLE and for monitoring treatment response (Figure 1). We focus here on neuroimaging studies in SLE patients who lacked confounding CNS manifestations in order to summarize the associations of structural or functional lesions with CD.

Magnetic resonance imaging (MRI) studies, both conventional and functional, demonstrate abnormalities in SLE and in SLE patients with CD (10). Conventional MRI studies reveal decreased hippocampal volumes in SLE patients with CD compared to those without CD (10). One study demonstrated decreased activation of the hippocampus/parahippocampal gyrus on functional MRI during a spatial working memory task in SLE patients (11), and another study showed abnormal regional activity in the parahippocampal gyrus on functional MRI during the resting state (12). This is of interest, as multiple lines of evidence in rodent models indicate that hippocampal integrity is critical for spatial memory (13). Similarly, diffusion tensor imaging (DTI), an advanced MRI technique that assesses white matter integrity, demonstrates SLE-related abnormalities (14). White matter integrity in DTI is often measured by fractional anisotropy (FA), which describes the directionality of water diffusion in tissue. A low FA finding indicates isotropic diffusion (directionless or random) and represents damaged white matter, which may be due to decreased axonal density, number, diameter, or myelination. Although studies in SLE patients reveal white matter abnormalities throughout the brain, two independent studies demonstrate an association of decreased FA in the external capsule in SLE patients with CD (15,16).

CD is also correlated with abnormalities in the ratio of choline to creatine on MR spectroscopy (17,18). This ratio is used as an index of white matter integrity; choline is essential to neuronal membranes and myelin, while creatinine is a stored phosphate used as a reference. An elevated choline-to-creatinine ratio is interpreted as representing increased membrane turnover due to demyelination, ischemia, and/or gliosis. Additionally, single-photon emission computed tomography displayed a focal area of hypoperfusion in the right precuneus (parietal lobe) in SLE patients with memory impairment compared to those without (19). Hypoperfusion in the parietal lobe was demonstrated in two other studies (20,21).

In vitro studies reveal microglial activation following exposure to SLE serum (22), and studies of murine models of SLE demonstrated that type I interferon (IFN)–mediated microglial activation contributes to CNS damage and possibly to CD (23). Recent advances in neuroimaging enhance our ability to assess microglial activity in humans; the most utilized positron emission tomography (PET) target is the 18-kd translocator protein (TSPO). PET tracers targeting TSPO have shown that it is expressed on the outer mitochondrial membrane of microglia and is markedly up-regulated in response to brain injury and inflammation. In several neurodegenerative diseases, including

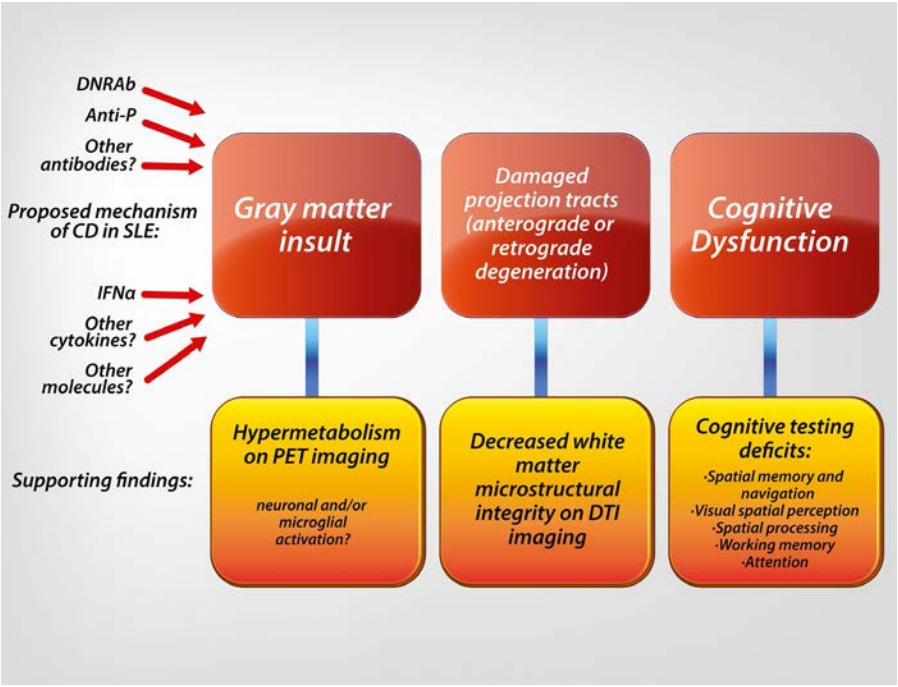

Figure 1. Proposed mechanism of systemic lupus erythematosus (SLE)–mediated cognitive dysfunction (CD). Neuroimaging studies support the notion that CD begins with hippocampal injury and altered microstructural integrity in the parahippocampus, leading to decreased integrity of white matter outflow tracts and resulting in impaired cognition. DNRAb = DNA-reactive antibodies; anti-P = anti–ribosomal protein P antibody; IFNα = interferon-α; PET = positron emission tomography; DTI = diffusion tensor imaging.

Alzheimer's disease (AD), there is compelling evidence for an association between TSPO overexpression in disease-specific brain regions and poor cognitive performance (24). In SLE, only one neuroimaging study using the TSPO ligand has been performed, revealing higher TPSO expression in the cerebellum and hippocampus in those with CD compared to those without (25). Of note, TSPO overexpression in the hippocampus is found in AD and Parkinson's disease, and has been found in the cerebellum in AD (24).

Overall, these studies suggest that CD can be assessed through neuroimaging modalities, but better definition of cohorts will be needed to correlate specific abnormalities with impairment in specific cognitive domains.

Potential molecular mediators of CD in SLE

A variety of mechanisms have been proposed as potential mediators of CD in SLE (Table 1). We highlight several of them below.

Cytokines and chemokines. Several cytokines, chemokines, and other proteins are associated with NPSLE. These associations have usually been studied in patients with a variety of NPSLE manifestations (diffuse and focal), which limits the ability to link a particular protein to a specific manifestation.

IFNα is the cytokine with the best described relationship with CD. In a recent study, mice with IFNα-mediated autoimmunity displayed CD that was diminished by an anti-IFNα receptor antibody (23). Moreover, wild-type mice injected peripherally with IFNα demonstrated CNS microglial activation with increased engulfment of neuronal synapses (synaptic pruning) and reduced synaptic density in the frontal cortex. The potential importance of IFNα in CD is corroborated by the observation of IFNα gene transcription in activated microglia in SLE brain tissue. These results are in accordance with clinical observations in patients with hepatitis C and liver cancer receiving exogenous IFNα therapy, which is associated with CD, including a spatial memory deficit independent of depression (26) and a lupus-like illness (27).

The source of IFNα in NPSLE can be systemic or central. Intrathecal immune complexes may play a key role in FNα production in the brain in NPSLE, and act as powerful amplifiers of brain inflammation (28). Santer et al (28) showed that cerebral spinal fluid (CSF) in NPSLE patients contains high levels of immune complexes that form as a result of autoantibodies that either traverse the blood–brain barrier or are locally produced by infiltrating B cells. These antibodies bind to cellular antigens that are released by damaged neurons. They further demonstrated ex vivo that these immune complexes bind Fc receptors on microglia and lead to the production of high levels of IFNα as well as other proinflammatory mediators, including 10-kd IFNγ-inducible protein, interleukin-8 (IL-8), and monocyte chemotactic protein 1.

Table 1. Mechanisms of cognitive dysfunction in systemic lupus erythematosus*

Mechanism	Authors, year (ref.)
Disruption of blood–brain barrier	Schwartz et al, 2019 (5); Hirohata et al, 2014 (60); Diamond et al, 2009 (62); Yoshio et al, 2006 (63); Mahajan et al, 2016 (64); Mahajan et al, 2015 (83)
Mononuclear cell infiltration	Jeltsch-David et al, 2014 (51); Kier, 1990 (55); Bracci-Laudierro 1999 (56)
Antibody-mediated injury	Jeltsch-David and Muller, 2014 (43)
Cytokine-mediated injury	Jeltsch-David and Muller, 2014 (43)
Microglial synaptic pruning	Bialas et al, 2017 (23)
Antibodies	
DNRAb	Tay et al, 2017 (59); Hirohata et al, 2014 (60); Chang et al, 2015 (66)
Anti-P/NSPA	Segovia-Miranda et al, 2015 (45); Massardo et al, 2015 (84)
Antiphospholipid antibody	Yelnik et al, 2016 (48)
Anti-α internexin	Lu et al, 2010 (41)
Cells	
Neuronal injury	Kowal et al, 2004 (13); Bialas et al, 2017 (23)
Microglial activation and pruning	Bialas et al, 2017 (23); Nestor et al, 2018 (42); Chang et al, 2015 (66)
Cytokines	
IFNα	Bialas et al, 2017 (23)
TWEAK	Stock et al, 2013 (36)
IL-6	Kwieciński et al, 2009 (35)
IL-8	Kwieciński et al, 2009 (35)
Chemokines	
MCP-1/CCL2	Duarte-García et al, 2018 (37)
Excitotoxic mediators	
HMGB-1	Nestor et al, 2018 (42)
Angiotensin II	Nestor et al, 2018 (42)
Quinolinic acid	Vogelgesang et al, 1996 (34)
MMP-9	Ainiala et al, 2004 (39)
Myelin-associated neurite outgrowth inhibitor	Lei et al, 2017 (38)
Lipocalin	Mike et al, 2018 (40)

* DNRAb = DNA-reactive antibodies; anti-P = anti–ribosomal protein P antibody; NSPA = anti-neuronal surface P antigen; IFNα = interferon-α; IL-6 = interleukin-6; MCP-1 = monocyte chemotactic protein 1; HMGB-1 = high mobility group box chromosomal protein 1; MMP-9 = matrix metalloproteinase 9.

One candidate mechanism for IFNα-induced CD relates to its activation of indoleamine 2,3-dioxygenase (IDO) in the kynurenine/tryptophan metabolic pathway. IFNα stimulates IDO, catalyzing the breakdown of tryptophan into kynurenine, which is further metabolized to quinolinic acid (QA) or kynurenic acid (KA). QA is an *N*-methyl-D-aspartate receptor (NMDAR) agonist, and can cause excessive glutamate excitotoxicity to neurons (29). QA is synthesized by microglia (29), and neurons cultured in supernatant from IFNγ-stimulated microglia exhibit reduced neurite outgrowth and complexity, which can be prevented by pretreatment of microglia with an IDO inhibitor or an NMDAR inhibitor (30). Notably, KA is an NMDAR antagonist (29), which can protect neurons from excitotoxic damage. An imbalance between QA and KA contributes to spatial memory deficits and functional and structural changes in the brain in animal models of neuroinflammation (31). In humans with SLE, an increased kynurenine-to-tryptophan ratio in blood has been reported (32) and correlates with IFNα gene expression (33). Additionally, levels of QA in CSF are higher in SLE patients with NPSLE syndromes (not limited to CD) than in those with CNS dysfunction not related to SLE or in healthy controls (34).

Among the multiple other cytokines associated with NPSLE, IL-6 and IL-8 have the most plausible association with neuronal damage, given their presence in CSF in association with proteins that are indicative of neuronal and astrocytic damage (35). Other inflammatory mediators, such as TWEAK (36), CCL2 (37), myelin-associated neurite outgrowth inhibitor (38), matrix metalloproteinase 9 (39), lipocalin 2 (40), anti–α-internexin (41), and the renin–angiotensin system (42), are also associated with CD in SLE. TWEAK, a cytokine in the tumor necrosis factor (TNF) family, may be implicated in CD, as memory impairment is ameliorated in TWEAK-deficient MRL/*lpr* mice (36).

The contribution of cytokines, chemokines, and other proteins and molecules to specific molecular mechanisms in NPSLE is, for the most part, unclear. Cytokines and chemokines are known to recruit immune cells to the CNS, promote intrathecal antibody production by infiltrating B cells, and modulate neurotransmitter release (43). In CD, however, the effects may largely result from direct stimulation of neurons and microglia, as evidence for cellular infiltration into the brain is limited.

Serology. To date, some antibody specificities have been associated with CD in mice and in patients. Undoubtedly, more await discovery.

Anti–ribosomal protein P antibody (anti-P) or anti–neuronal surface P antigen (anti-NSPA). Anti-P antibodies are associated with psychosis and CD in SLE patients, and studies in mice reveal a plausible pathogenic mechanism behind this association (44). NSPA is an integral plasma membrane protein that is bound by anti-P. NSPA engagement by anti-P antibodies induces calcium influx and glutamatergic transmission in neurons (45). By activating both AMPA receptors and NMDARs, anti-P–induced glutamatergic overactivation leads to suppression of long-term potentiation, which provides a mechanism for anti-P–mediated pathogenic alterations in the brain. In addition, glutamatergic dysfunction also mediates psychotic symptoms, as in NMDAR encephalitis (46). Although an association of anti-P with depression has not been confirmed in humans, mice injected intracerebroventricularly with anti-P displayed depression-like behavior (47). Anti-NSPA antibodies, induced in rabbits by immunization with NSPA, trigger calcium influx, enhance glutamatergic transmission, and induce memory impairment, mimicking the effect of anti-P antibodies (45).

Antiphospholipid antibody (aPL). In serum and CSF, aPLs, specifically IgG anticardiolipin antibody (aCL) and lupus anticoagulant (LAC), correlate with CD in several studies (48), with up to a 3-fold increase of CD in SLE patients with positive aPLs. While aPLs are well-established in mediating a prothrombotic vasculopathy leading to stroke and multiinfarct dementia (48), experimental models of antiphospholipid syndrome suggest that nonthrombotic mechanisms may also be responsible for aPL-mediated CD, such as direct toxic effects of aPL on neurons and glia (48,49). For aPLs to directly bind cells in the brain, they need to traverse the blood–brain barrier; aPLs may affect blood–brain barrier permeability through endothelial cell dysfunction (50).

DNA-reactive antibody (DNRAb). These antibodies represent a subset of anti-DNA antibodies that cross-react with the NMDAR and are discussed in detail below.

Pathogenesis

Mouse models of CD. The limitations in studying pathogenic mechanisms of CD in SLE patients, such as the paucity of brain tissue samples, their procurement postmortem, and the heterogeneity of neuropsychiatric manifestations, have made experimental mouse models fundamental.

The most common and best-studied mouse strain is the MRL/*lpr* strain of mice. MRL/*lpr* mice display depression, anxiety, and CD by age 8 weeks that is positively correlated with serum anti–double-stranded DNA (anti-dsDNA) antibody titers and proinflammatory cytokines and can precede the onset of renal disease (51). These mice exhibit a notable decrease in midbrain and limbic brain volumes by age 5–8 weeks. Several mechanisms are likely involved in the neuropsychiatric manifestations, including autoantibodies, cytokines, mononuclear cell infiltration, and disruption of the blood–brain barrier (51). In addition to anti-dsDNA antibodies, other autoantibodies such as anti-P, aCL, and DNRAb are often present in MRL/*lpr* mice and can lead to CNS disease. An early onset of neuropsychiatric disease may be explained by intrauterine exposure of the fetal brain to maternal autoantibodies or to high cytokine levels (52) and a dysfunctional Fas/Fas receptor signaling pathway, leading to abnormal hippocampal neurogenesis and postnatal brain development (51,53). Bialas et al (23) demonstrated that type I IFN–mediated microglial activation leads to dendritic pruning in MRL/*lpr* mice, which is likely related to the CD observed in this strain.

Several other mouse models have been used to study CD. Another commonly studied lupus-prone strain of mice, NZB/NZW F1 mice, display learning difficulties and mood-related disorders that occur later in the course of disease (54), but these findings can be confounded by the high prevalence of brain anomalies in the non–lupus-prone NZB parental strain. The mechanisms of disease include mononuclear cell infiltration of different brain regions, most notably the hippocampus and cortex, as well as disturbances in neuropeptides in the affected areas (55,56). BXSB male mice demonstrate impaired spatial and nonspatial learning. Like NZB mice, the BXSB male mice demonstrate congenital structural abnormalities (51). Genetically engineered lupus-prone mouse strains, such as the 564Igi strain, a B cell receptor knock-in model with IFNα receptor 1–dependent pathogenesis (23), and the bicongenic strain, Sle1/Sle3 (40), also exhibit spatial and object memory impairment, as well as other behavioral abnormalities.

DNRAb as a mechanism for CD in SLE. We have been studying DNRAb and their contribution to CD in SLE. In 2001, our group identified a subset of anti-dsDNA antibodies, DNRAb, also known as anti-NR2 antibodies, which bind DNA and cross-react with the GluN2A and GluN2B subunits of the NMDAR, the brain's main excitatory receptor (57). NMDARs are found in the highest numbers in the hippocampus and are integral to learning and memory. DNRAb enhance the excitatory activation of NMDARs, and excessive activation leads to excitotoxic cell death. DNRAb isolated from the serum and CSF of an SLE patient with progressive cognitive decline and injected directly into a mouse brain caused neuronal cell death (57), confirming their ability to mediate

brain pathology once present in brain tissue. Serum DNRAb are found in 30–50% of SLE patients (58), and pooled data from a recent meta-analysis reveal that SLE patients with NPSLE were more likely to have elevated serum/plasma DNRAb (mean serum level 0.4 mg/ml in patients with NPSLE compared to 0.2 mg/ml in patients without NPSLE) (59). Although some studies have not shown an association between serum DNRAb positivity and CD, the presence of DNRAb in CSF is associated with diffuse NPSLE, including CD in several studies (mean DNRAb level in CSF 0.61 units/ml for NPSLE and 0.31 units/ml for non-NPSLE SLE) (59,60). Additionally, since a blood–brain barrier breach is needed to result in CNS disease, serum titers may not accurately reflect CNS disease.

DNRAb should not be confused with the anti-NMDAR antibodies found in autoimmune encephalitis, which bind to the GluN1 subunit of the NMDAR. These antibodies result in internalization of the receptor and subsequently lead to a reversible decrease in NMDAR surface density and thus synaptic dysfunction, without significant neuronal cell death or loss of dendritic tree or spine complexity (61). Clinical manifestations, which include severe neurologic, psychiatric, and behavioral symptoms, tend to be transient and positively correlated with CSF antibody titers, as opposed to the manifestations associated with DNRAb, which are persistent.

In SLE it is believed that abrogation of blood–brain barrier integrity and direct access of antibodies to the CNS is needed for antibody-mediated damage, since existing evidence suggests autoantibody is not produced within the CNS in SLE (60). This is based on findings of elevated albumin (normally only found in serum) in the CSF of lupus patients with NPSLE compared to those without (60). Several conditions compromise blood–brain barrier integrity, including viral and bacterial infection, systemic inflammation, stress (epinephrine), ischemia, aging, hypertension, nicotine, alcohol, and certain inflammatory cytokines, such as TNFα, IL-1β, IL-6, and IL-8 (62). DNRAb also directly affect blood–brain barrier integrity by activating endothelial cells and leading to the production of proinflammatory cytokines such as TNFα, IL-6, and IL-8 (63). The complement activation product C5a, present in SLE patients with active disease, alters blood–brain barrier integrity in MRL/*lpr* mice through endothelial cell apoptosis (64). In SLE, it is likely that complement peripheral cytokines, and autoantibodies, as well as non–disease-related mechanisms, all compromise blood–brain barrier integrity.

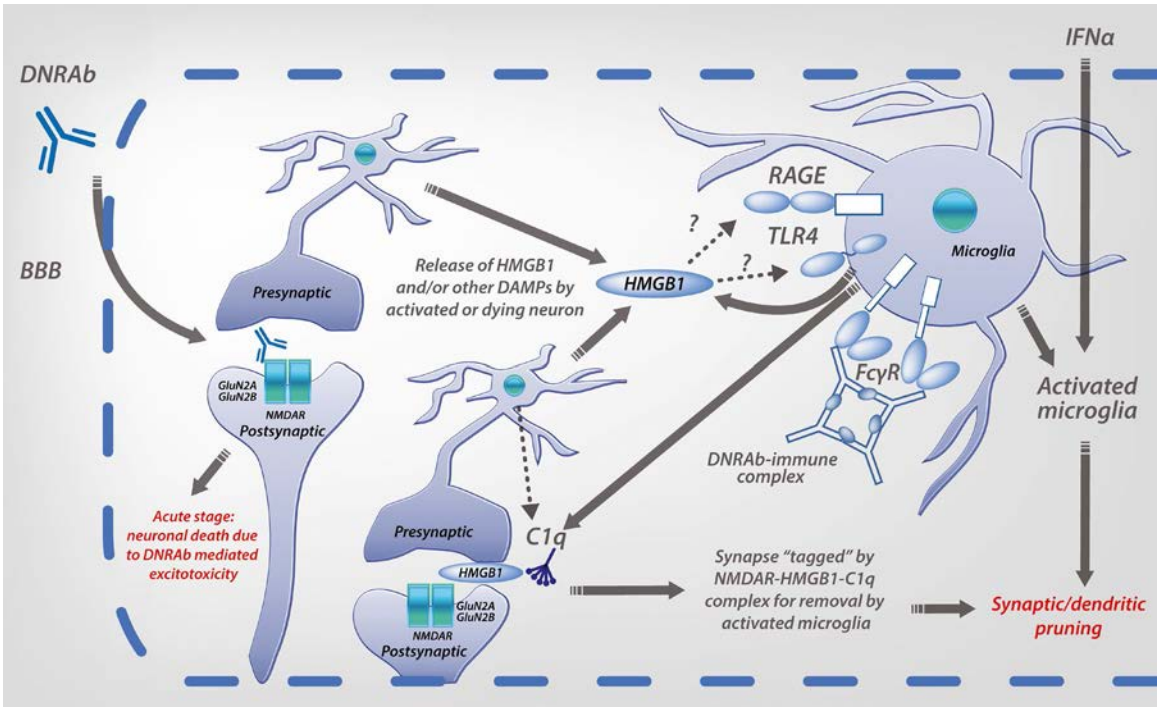

Figure 2. Proposed 2-stage model for DNA-reactive antibody (DNRAb)–mediated neurotoxicity and the contribution of interferon-α (IFNα) to neurotoxicity. Exposure to DNRAb mediates immediate excitotoxic death of some neurons (acute stage). The surviving neurons experience strong *N*-methyl-D-aspartate receptor (NMDAR) stimulation that induces high mobility group box chromosomal protein 1 (HMGB-1) secretion. Microglia are activated following DNRAb penetration of the blood–brain barrier (BBB). There are at least 3 possible mechanisms for microglial activation in the DNRAb model: binding of secreted HMGB-1 to receptor for advanced glycation end products (RAGE) or Toll-like receptor 4 (TLR-4), engagement of activating Fc receptors (FcγR) by DNRAb–immune complexes, and/or exposure to damage-associated molecular patterns (DAMPs) from apoptotic neurons. Activated microglia contribute to the loss of dendrites and synapses, which are "tagged" for destruction by an NMDAR–HMGB-1–C1q complex (chronic stage). IFNα penetrates the blood–brain barrier, or is produced centrally, and activates microglia, resulting in the loss of neuronal dendrites and synapses.

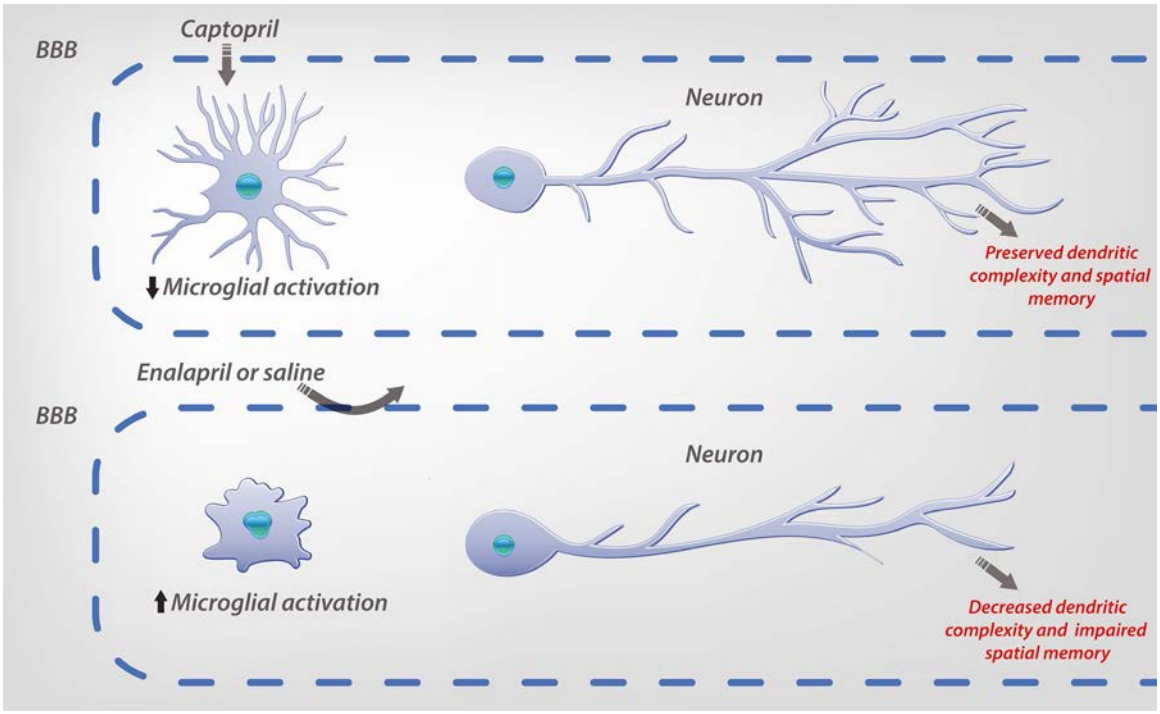

Figure 3. Proposed mechanism of angiotensin-converting enzyme (ACE) inhibitor treatment of systemic lupus erythematosus–mediated cognitive dysfunction. Treatment with a blood–brain barrier (BBB)–permeable ACE inhibitor (captopril), but not with a blood–brain barrier–impermeable ACE inhibitor (enalapril) or saline, suppresses microglial activation and preserves dendritic complexity and spatial memory in DNA-reactive antibody–positive mice. Importantly, treatment with captopril after the onset of microglial activation can restore dendritic complexity, suggesting that damaged neurons can recover following treatment.

The mechanism of blood–brain barrier insult determines the anatomic site of the breach, which dictates the location of antibody-mediated damage. In this way, the same antibody can cause more than one neuropsychiatric manifestation, depending on the affected brain region. For example, administration of lipopolysaccharide (LPS) to DNRAb-positive mice leads to hippocampal damage, whereas epinephrine administration causes damage to the amygdala (65). Of note, other mechanisms for antibody and leukocyte entry into the brain have been proposed, including through the choroid plexus, the meningeal–arachnoid barrier, and glymphatic system (5).

The fact that DNRAb are commonly present in SLE patients and can mediate neurotoxicity led us to develop a nonspontaneously autoimmune mouse model to study the effects of DNRAb and eliminate confounding variables such as cytokines and other brain-reactive autoantibodies, which are present in spontaneously autoimmune lupus mouse strains (13). In this model, mice are injected with a consensus sequence contained within the GluN2A and GluN2B subunits that is bound by DNRAb, leading to the production of DNRAb. In order to provide DNRAb with access to brain parenchyma, the blood–brain barrier is breached with systemic LPS administration. Within 1 week, in the absence of an inflammatory infiltrate, a 20–25% hippocampal neuronal loss is observed (13), followed by loss of dendritic complexity and spine density with an associated spatial memory impairment that occurs after DNRAb is no longer detectable in the brain (66) (Figure 2).

Microglia have emerged as central players in human neuropathologies and are increasingly being associated with neuropsychiatric symptoms in murine lupus models as well (23,42). In DNRAb-mediated CD, microglia may be activated via several mechanisms (Figure 2). This activation also is only detected after DNRAb is no longer detectable in the brain. Activated microglia can phagocytose (or prune) dendritic synapses with a resultant loss in dendritic complexity and spine density. This mechanism is associated with CD in several murine lupus models, including DNRAb+ mice, MRL/*lpr* mice, and NZB/NZW mice (23,66). DNRAb+ mice develop a selective spatial memory impairment. Microglial depletion in DNRAb+ mice given LPS to allow antibody to penetrate brain parenchyma results in preserved neuronal dendritic architecture (42). The role of complement, notably C1q, in microglial-mediated synaptic pruning is critical. C1q is produced by both neurons and microglia and can "tag" synapses for removal. In DNRAb+ mice, an NMDAR–high mobility group box chromosomal protein 1–C1q complex forms at synapses on neuronal dendrites, targeting them for destruction. C1q knockout DNRAb+ mice maintain normal dendritic complexity and spine density following LPS administration (42), confirming a critical contribution of C1q in DNRAb-mediated pathology.

Table 2. Assessments of spatial cognition in the DNRAb+ mouse model*

Approach	Cognitive feature tested	Description of test	Behavior in mice with hippocampal dysfunction
OPM task in tandem with NOR task	OPM, spatial memory NOR, nonspatial/ recognition memory (short-term visual memory)	Mouse is placed in a chamber and allowed to explore 2 objects. Then 1 of the 2 following procedures is performed: 1) OPM task; afterwards, the location of one of the training objects is moved to a new location within the chamber and the mouse is placed back in the chamber. Because mice have an innate preference for novelty, if the mouse recognizes that the object has been moved, it will spend more of its time at the moved object; or 2) NOR task; afterwards, one of the training objects is replaced with a novel object and the mouse is placed back in the chamber. Because mice have an innate preference for novelty, if the mouse recognizes the familiar object, it will spend most of its time at the novel object.	Behavior in the DNRAb mouse model, in which DNRAb+ mice display enlarged place field size on recordings from CA1 hippocampal neurons, indicating a spatial map with less resolution as follows: OPM task: DNRAb− mice preferentially explore the moved object, while DNRAb+ mice do not (66), and NOR task: both DNRAb+ and DNRAb− mice display a robust response in exploring the novel object. This result, together with the OPM result, indicates a selective spatial memory deficit caused by DNRAb (65,66).
T or Y maze	Spatial working memory	The mouse is placed in a T- or Y-shaped chamber with one arm of the chamber blocked off. Afterwards, the barrier is removed and the mouse is again allowed to explore. Because mice have an innate preference for novelty, the mouse will spend more of its time in the unexplored arm. This requires that mice recognize which arm of the maze they had previously explored.	DNRAb+ mice spent less time alternating between the 2 arms of a T maze than DNRAb− mice (13,65).
Morris water maze	Spatial reference memory	The mouse is placed in a pool of water with a hidden platform located just below the surface. Mice learn to escape from water by swimming to the platform over repeated sessions. This is followed by a trial in which the platform is removed. Mice that memorize the position of the platform preferentially swim in that area (trained sector). Since mice may find swimming stressful, leading to difficulties in interpreting results, the paddling pool maze was designed to overcome this problem (see below).	DNRAb+ mice display reduced exploration of the trained sector compared to DNRAb− mice (13).
Training to criterion task	Spatial flexibility	Mice are required to find 5 consecutive locations of the platform in the Morris water maze.	DNRAb+ mice have poor ability to learn a given location compared to DNRAb− mice (13,65).
Shallow water paddling pool maze	Spatial memory, spatial flexibility	Mice are placed in a large, bright circular arena with transparent sides containing shallow water. Around the perimeter are 12 potential exits, one of which is connected to an escape tunnel (target). The target is in a fixed position until the mouse finds it, then a new target position is selected. The mice are trained to find sequential targets in the maze.	DNRAb+ mice need more trials to reach a moved target relative to DNRAb− mice (85).

* DNRAb = DNA-reactive antibody; OPM = object place memory; NOR = novel object recognition.

We have shown that angiotensin-converting enzyme (ACE) inhibitors, through their centrally acting effects, reduce microglial activation, prevent loss of dendritic arborization, and prevent spatial memory impairment in DNRAb+ mice (42). Treatment with captopril after onset of microglial activation also restored dendritic arborization and spine density (Figure 3), suggesting that surviving neurons in this model do not experience irreversible damage.

Multiple behavioral studies have revealed the impact of DNRAb on spatial memory in the mouse model (Table 2). Although it may be difficult to extrapolate results from mice to humans, the mouse model informed our choice of applying tasks related to spatial memory in humans with SLE. Using a 2 × 2 array of objects that assessed both object recognition and memory for spatial relations, we found that DNRAb is associated with a

spatial memory deficit in humans with SLE (66,67). Additionally, we used a desktop, 3-dimensional spatial navigation task that may be more clinically relevant than the 2 × 2 array and found that DNRAb-positive SLE patients performed poorly compared to DNRAb-negative SLE patients, who performed similarly to healthy controls (68). This work demonstrates that mouse models provide structural information related to pathogenic mechanisms in the brain so that appropriate cognitive tasks may be applied.

Our group is also investigating the impact of DNRAb on brain structure and function in patients with SLE with stable disease activity and without CNS disease. We found that these SLE patients demonstrate hypermetabolism on ^{18}F-fluorodeoxyglucose (FDG)–PET in the hippocampus, among other brain regions, and hippocampal hypermetabolism correlates with poor working memory (69), demonstrating that SLE patients with no other NPSLE symptoms may exhibit CD that correlates with clear abnormalities in brain function. Further, DNRAb antibody positivity was shown to correlate with hippocampal hypermetabolism (69) and decreased white matter microstructural integrity in the parahippocampal gyrus on DTI (67). This decreased microstructural integrity correlated with increased serum DNRAb and poor spatial memory performance. DTI findings did not correlate with deficits in other cognitive domains. FDG-PET studies performed concurrently with DTI revealed hypermetabolism in gray matter areas, such as the hippocampus, adjacent to areas with decreased white matter microstructural integrity, suggesting that changes in regional metabolism may indicate a pathophysiologic process leading to structural changes (Figure 1). Hypermetabolism and reduced white matter microstructural integrity were stable over a mean of 15 months. These findings suggest that metabolic activity in these regions may be a marker for SLE that is potentially responsive to targeted therapies, and possibly useful as an outcome measure in clinical trials.

Implications for treatment of CD in SLE

There are no treatments for CD in SLE, and there are limited data on the use of immunosuppressive therapy in CD. The insidious nature of CD and its occurrence independent of systemic disease activity have shifted the risk-benefit assessment in favor of using less aggressive, less immunosuppressive options, despite emerging evidence of immune-mediated mechanisms. In one small, prospective, double-blind placebo-controlled study, a trial of glucocorticoid therapy (prednisone 0.5 mg/kg) led to clinical improvement in 5 of 8 patients with mild SLE and CD who completed the trial (70). The duration of therapy varied from 2 to 19 months, and relapse of CD after treatment taper was not reported.

Given its moderate success in slowing cognitive decline in AD (71), memantine, an NMDAR antagonist, was tested in SLE patients with mild self-reported baseline CD, but did not exhibit significant improvement in cognitive performance in SLE patients compared to placebo (72). The study was not powered to test for an effect in patients who were positive for DNRAb (of which there were only 5), although mice positive for DNRAb who were treated with memantine prior to breaching of the blood–brain barrier demonstrated no evidence of antibody-mediated neuronal death (65). That said, long-term attenuation of the NMDAR has deleterious impacts on brain function, and it is therefore an unfavorable therapeutic option (73).

Although no studies have assessed the benefit of anticoagulation or antiplatelet therapy in SLE patients with CD without thromboembolic phenomena, antiplatelet therapy such as low-dose aspirin or antimalarials may be considered in SLE patients with CD who are positive for aPLs. In a 3-year prospective observational study assessing predictors of CD in SLE patients, regular use of low-dose aspirin improved cognitive function in SLE patients with or without aPLs compared to those not taking aspirin (74).

One potential therapeutic strategy is to protect and enhance blood–brain barrier integrity. C5a receptor blockade ameliorates blood–brain barrier disruption and attenuates behavioral abnormalities in MRL/*lpr* mice (75), revealing a potential therapeutic target for CD. While sphingosine 1-phosphate receptor modulation with FTY720 stabilizes the blood–brain barrier in MRL/*lpr* mice and mitigates CD (76), its use in SLE will be limited by its known toxicity.

Another therapeutic strategy is to block microglial activation. The renin-angiotensin system, best known for maintaining hemodynamic and mineralocorticoid homeostasis, consists of multiple neuroactive peptides that when unbalanced play a significant role in the neuroinflammatory processes central to CD (77). The most potent component of this complex system is angiotensin II, which activates microglia to assume a proinflammatory phenotype, and when overexpressed is directly neurotoxic, resulting in neuronal injury and cell death. Another proinflammatory mechanism of the renin-angiotensin system is the ACE-mediated inactivation of bradykinin, which has antiinflammatory effects, suppresses microglial activation, and diminishes type 1 IFN responses in normal and lupus-prone mice (78). Genome-wide association studies identify an ACE allele as a risk factor in SLE, with the risk allele leading to increased serum ACE levels. In a randomized trial (79) and several observational studies (80), ACE inhibition slowed cognitive decline in AD. These data, together with the findings from murine studies, support the potential use of ACE inhibitors as novel neuroprotective therapeutics for CD in SLE. Angiotensin receptor blockers may also be a useful treatment alternative. Minocycline has also emerged as a potent inhibitor of microglial activation with benefits in several neurologic conditions (81); however, its toxicity profile and potential risk of drug-induced lupus may limit its usefulness.

The current approach of broad immunosuppression or no treatment for CD in SLE, with the inherent dangers of immunosuppression or increasing impairment, respectively, illustrates that clinical trials are greatly needed. Several potential treatment strategies appear promising; however, much work is needed to confirm suitable biomarkers and end points for use in clinical trials. When trials begin, they need to be performed in well-defined SLE populations.

Considerations for potential trials of neuroprotection in SLE CD

A key question that arises related to potential clinical trials in SLE CD is whether CD may be decelerated or reversed, with or without therapy. Several longitudinal studies have examined CD in SLE (7); however, only two studies included patients without any history of neuropsychiatric disease, and in one of these studies CD was not detected, which limits the interpretation of results. Our study examining ACE inhibitors and microglia supports the idea that cognition may be "retrieved" rather than only prevented to decline, as ACE inhibitors given after the onset of microglial activation restored dendritic arborization and spine density (Kain J, et al: unpublished observations).

Another key question related to potential trials is what design and outcome measures should be used? Clinical trials in neurodegenerative diseases that affect cognition, such as in AD, lend some insight into potential trials in SLE CD, but mostly with respect to therapeutic targets rather than cognitive testing as an outcome measure. This is because the broad cognitive tests that are utilized in AD, such as the MMSE, lack sensitivity to mild CD (82), which is often encountered in SLE. Given this problem, we have chosen to use more sensitive tests such as the ANAM, and specific tests related to a known pathogenic mechanism (e.g., DNRAb and a spatial memory deficit). However, the use of cognitive testing as an outcome measure has several limitations in potential trials. Even with a relatively sensitive test, such as the ANAM, many patients are needed to ensure adequate power to detect a significant change in performance over the limited time of a trial. Furthermore, the clinically meaningful change in cognitive tests is unclear. Therefore, clinical trials that employ imaging as an outcome measure based on a plausible pathogenic mechanism, such as DNRAb-mediated neurotoxicity and microglial activation, may be advantageous before moving onto larger clinical trials with cognitive testing. Several longitudinal studies of TSPO-PET in AD reveal increased microglial activation over time, and in one study it correlated with worsening CD (24). These results suggest that TSPO-PET imaging may apply as a biomarker of CD in SLE.

Importantly, future clinical trials in SLE CD will require specifically defined patient samples. Current clinical trials may include SLE patients with unrelated neurologic or psychiatric diseases, which can potentially confound the interpretation of results.

Conclusions

CD in SLE, although insidious and sometimes difficult to diagnose, can be devastating, with a considerable impact on quality of life. The pathogenesis of CD in SLE is poorly understood, which translates into a lack of biomarkers that can aid in diagnosis. However, dismissing CD as merely a confounding symptom in SLE is shortsighted and does a disservice to patients. While clinical studies to date have been hampered by heterogeneous subject populations and a lack of sensitive and standardized cognitive assessments that test for an association of a specific cognitive deficit with a defined pathogenic mechanism, there are strong data from the clinical arena and from mouse models that show CD is present in many patients and is related to known disease mechanisms. Future clinical studies that utilize sensitive and specific tests for cognitive deficits related to known pathogenic mechanisms (e.g., spatial memory deficits related to DNRAb) are necessary and will provide the information needed to design treatments and interventions that will preserve cognitive function and improve quality of life for patients.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and approved the final version to be submitted for publication.

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Exploring the Lipid Paradox Theory in Rheumatoid Arthritis: Associations of Low Circulating Low-Density Lipoprotein Concentration With Subclinical Coronary Atherosclerosis

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Objective. Rheumatoid arthritis (RA) patients with the lowest circulating low-density lipoprotein (LDL) concentrations are at heightened risk of cardiovascular events. However, the atherosclerosis burden within this subgroup is unknown.

Methods. RA patients pooled from 4 cohort studies of cardiovascular disease (CVD; n = 546) were compared with non-RA controls from the Multi-Ethnic Study of Atherosclerosis (n = 5,279). Those taking lipid-lowering medications were excluded. Differences in cardiac computed tomography–derived Agatston coronary artery calcium (CAC) scores between the RA and control groups were compared across strata of LDL concentration.

Results. Among those with low LDL concentrations (<70 mg/dl), mean adjusted CAC scores were >4-fold higher for RA patients than for controls (18.6 versus 4.6 Agatston units, respectively; $P < 0.001$), a difference significantly greater than that in any other LDL concentration stratum except LDL concentration ≥160 mg/dl. Similarly, 32% of the RA patients with low LDL concentration had a CAC score of ≥100 Agatston units compared with only 7% of controls in the same LDL concentration stratum (odds ratio 5.97; $P < 0.001$), a difference significantly greater than that in all of the other LDL concentration strata. Low LDL concentration was most strongly associated with higher CAC score among RA patients who were white, had ever smoked, or were not obese. Other than a higher frequency of current smokers, RA patients with low LDL concentrations did not have more CVD risk factors or higher measures of RA disease activity or severity than RA patients with higher LDL concentrations.

Conclusion. RA patients with low LDL concentration may represent a group for whom heightened screening and prevention of atherosclerotic CVD is appropriate.

INTRODUCTION

Among individuals with rheumatoid arthritis (RA), rates of myocardial infarction and overall cardiovascular disease (CVD) mortality are 50% higher than in non-RA controls (1,2), rates that are comparable to those in individuals with diabetes mellitus (3). Accordingly, RA patients have a greater burden of atherosclerosis, with coronary artery calcium (CAC) scores markedly higher than those in non-RA controls (4–7). Although the majority of prior studies of accelerated atherogenesis in RA have focused on the contribution of chronic systemic inflammation, traditional CVD risk factors are also important, but may differ from those in the non-RA population (8). In particular, several observational studies have identified RA patients with the lowest circulating low-density

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lipoprotein (LDL) concentrations (i.e., LDL <70 mg/dl) as having an unexpectedly high risk of cardiovascular events (CVEs), with risk comparable to or exceeding that observed in RA patients with the highest LDL levels (9,10). The etiologic mechanism underlying this observation, now commonly referred to as the "lipid paradox," is unclear, although inflammation-induced reduction in lipid levels has been postulated. In contrast, high levels of high-density lipoprotein (HDL) and low levels of triglycerides appear to be associated with a decreased risk of CVEs in both RA and non-RA populations. Whether RA patients with very low LDL concentrations have a greater burden of atherosclerosis is unknown, but clarification of the role of LDL concentration has important implications for CVD prevention strategies.

In the present study, we compared CAC scores for RA patients to those for non-RA controls within and between strata of circulating fasting lipid concentrations. We hypothesized that RA patients with very low LDL concentrations who were not treated with lipid-lowering therapy would demonstrate subclinical CAC scores higher than those for non-RA controls. Further, we postulated that the subgroup of RA patients with very low LDL concentrations would have more severe and active RA disease as a potential mediator of higher CAC scores in this subgroup.

PATIENTS AND METHODS

Participants and recruitment. RA patients were pooled from 4 cohort studies of CVD in RA in which cardiac computed tomography (CT) was performed (4–6,11). The 4 cohort studies enrolled patients from and around Nashville, Tennessee (n = 169); Pittsburgh, Pennsylvania (n = 195); Baltimore, Maryland (n = 197); and New York, New York (n = 101). Detailed methods and the findings of each study have been published previously (4–6,11). RA patients were enrolled between 2001 and 2005 in the Nashville cohort, between 2000 and 2004 in the Pittsburgh cohort, between 2004 and 2006 in the Baltimore cohort, and between 2011 and 2015 in the New York City cohort. Each of the cohorts included patients who fulfilled the American College of Rheumatology 1987 classification criteria (12). The RA sample for the analyses reported here was restricted to those without prior CVEs or cardiovascular procedures and those who were not treated with lipid-lowering medications, for a final cohort total of 546 RA patients (n = 137 from the Nashville cohort, n = 165 from the Pittsburgh cohort, n = 161 from the Baltimore cohort, and n = 83 from the New York cohort). Each study was approved by the institutional review board (IRB) of the associated university, and all subjects provided written informed consent prior to enrollment. The IRB of Columbia University Medical Center approved the pooled analyses.

Non-RA controls were enrollees in the Multi-Ethnic Study of Atherosclerosis (MESA). A description of the MESA study design and methods has been published previously (13), and a full list of participating MESA investigators and institutions can be found online at http://www.mesa-nhlbi.org. Briefly, MESA enrolled a multi-ethnic cohort of 6,814 participants from 6 US communities between 2000 and 2002, all of whom had a cardiac CT performed at baseline for quantification of CAC according to the Agatston method. MESA participants with RA and those treated with lipid-lowering medications were excluded from the present study (14). In total, 1,535 controls were excluded, leaving 5,279 MESA controls.

Assessments. RA patients in the Baltimore and New York cohorts underwent 64-slice cardiac multidetector row CT (MDCT). RA patients in the Nashville and Pittsburgh cohorts underwent cardiac electron beam CT (EBCT). In MESA, both MDCT and EBCT were used. The comparability of both methods has been validated (15). CAC was quantified using the Agatston method (16) in each cohort. In all cohorts, demographic characteristics, smoking history, and current medications were assessed by participant self-report. Resting blood pressure and anthropometrics were assessed similarly for all cohorts, and a fasting blood sample was stored from which circulating lipid concentrations and glucose were measured. For the pooled analyses, RA cases and controls were classified as having hypertension or diabetes mellitus based on the same definitions. Hypertension was defined by systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or use of antihypertensive medication. Diabetes mellitus was defined as a fasting serum glucose level ≥126 mg/dl or use of antidiabetic medications. Circulating C-reactive protein (CRP) level was measured in all RA cases and controls except for enrollees in the Pittsburgh cohort.

The duration of RA from diagnosis and the duration of morning stiffness were assessed by self-report. Joints were examined for swelling and tenderness in the Nashville, Baltimore, and New York City cohorts, but not the Pittsburgh cohort.

Statistical analysis. Participant characteristics were compared between the RA and control groups using *t*-tests for normally distributed continuous variables, the Kruskal-Wallis test for non-normally distributed continuous variables, and the chi-square goodness-of-fit test or Fisher's exact test, as appropriate, for categorical variables. Due to demographic imbalances between the RA and control groups, we additionally compared nondemographic characteristics using linear or binomial logistic regression, according to the characteristic, in models that included variables for RA status, age, sex, and race. Non-normally distributed continuous variables were transformed as required. Demographically adjusted means and percentages and their 95% confidence intervals (95% CIs) were calculated, and transformed variables were back-transformed for ease of interpretation.

Next, we compared CAC scores between the RA and control groups by strata of LDL concentration, defined as LDL concentration <70 mg/dl, 70–99 mg/dl, 100–129 mg/dl, 130–159 mg/dl, and ≥160 mg/dl, using linear regression with CAC score, transformed as log(CAC+1) to meet the normality requirements

for linear regression, modeled as the dependent variable and RA × LDL concentration strata modeled as an interaction term. Back-transformed mean CAC scores and their corresponding 95% CIs were calculated and plotted for the RA and control groups within each stratum. Differences between LDL concentration strata in the magnitude of the within-stratum RA versus control difference in CAC score were compared by calculating *P* for the multiplicative interaction terms for each LDL concentration stratum referent to the stratum of LDL concentration <70 mg/dl. Additional models included adjustment for relevant shared characteristics unbalanced by RA status and associated with CAC score in univariate models at the $P < 0.20$ level (age, sex, race, waist circumference, ever and current smoker, diabetes mellitus, hypertension, HDL concentration, and aspirin use).

Binomial logistic regression was used to model CAC score ≥100 and ≥300 units with covariates modeled as described above

Table 1. Demographic and clinical characteristics of the RA patients and controls*

	Controls (n = 5,279)	RA patients (n = 546)	*P*	Demographically adjusted†		
				Controls	RA patients	*P*
Age, mean ± SD years	61 ± 10	56 ± 11	<0.001	–	–	–
Male, no. (%)	2,514 (48)	112 (21)	<0.001	–	–	–
White, no. (%)	2,010 (38)	443 (81)	<0.001	–	–	–
BMI, mean ± SD kg/m^2	28.1 ± 5.5	28.3 ± 5.9	0.58	28.1 (28.0–28.3)	28.2 (27.7–28.7)	0.72
BMI <18.50 kg/m^2, no. (%)	54 (1)	10 (2)	0.082	0.9 (0.7–0.1)	2.1 (1.1–3.9)	0.030
BMI 18.50–24.99 kg/m^2, no. (%)	1,575 (30)	171 (31)	0.44	30 (29–31)	28 (25–32)	0.42
BMI 25.00–29.99 kg/m^2, no. (%)	2,036 (39)	177 (33)	0.006	38 (37–39)	35 (31–40)	0.27
BMI ≥30.00 kg/m^2, no. (%)	1,614 (31)	186 (34)	0.082	30 (29–31)	33 (29–38)	0.14
Waist circumference, mean ± SD	97 ± 14	93 ± 16	<0.001	97 (97–98)	94 (93–95)	<0.001
Diabetes mellitus, no. (%)	557 (11)	27 (5)	<0.001	8 (8–9)	8 (6–12)	0.85
Ever smoker, no. (%)	2,589 (49)	261 (48)	0.58	49 (48–50)	49 (44–53)	0.91
Current smoker, no. (%)	709 (13)	74 (14)	0.94	13 (12–14)	12 (10–16)	0.90
Hypertension, no. (%)	2,144 (41)	249 (46)	0.024	38 (37–40)	56 (51–61)	<0.001
SBP, mean ± SD mm Hg	126 ± 21	126 ± 19	0.35	125 (125–126)	131 (130–133)	<0.001
DBP, mean ± SD mm Hg	72 ± 10	75 ± 10	<0.001	72 (71–72)	77 (76–78)	<0.001
Antihypertensive use, no. (%)	1,695 (32)	184 (34)	0.43	30 (29–31)	41 (36–45)	<0.001
Current NSAIDs, no. (%)	1,164 (22)	301 (55)	<0.001	22 (21–23)	45 (41–50)	<0.001
COX-2 inhibitors, no. (%)‡	299 (6)	129 (28)	<0.001	5 (4–6)	27 (22–32)	<0.001
Current aspirin use, no. (%)	1,157 (22)	70 (13)	<0.001	20 (19–21)	11 (9–14)	<0.001
Total cholesterol, mean ± SD mg/dl	196 ± 34	199 ± 39	0.057	196 (195–197)	195 (192–198)	0.47
LDL, mean ± SD mg/dl	120 ± 31	118 ± 33	0.21	120 (119–121)	117 (114–120)	0.070
LDL <70 mg/dl, no. (%)	244 (5)	47 (9)	<0.001	6 (6–7)	12 (9–15)	<0.001
LDL ≥130 mg/dl, no. (%)	1,859 (35)	195 (36)	0.82	35 (34–37)	34 (30–38)	0.60
HDL, mean ± SD mg/dl	51 ± 15	56 ± 17	<0.001	52 (51–52)	53 (52–54)	0.041
Triglycerides, median (IQR) mg/dl§	111 (78–161)	112 (79–153)	0.76	110 (108–111)	115 (109–121)	0.13
Non-HDL, mean ± SD mg/dl	145 ± 35	143 ± 38	0.26	145 (144–146)	142 (139–145)	0.13
CRP, median (IQR) mg/liter¶	1.9 (0.8–4.3)	4.0 (1.4–9.7)	<0.001	1.9 (1.9–2.0)	3.7 (3.3–4.2)	<0.001
CAC score, median (IQR)	0 (0–65)	0 (0–90)	0.35	7 (7–7)	14 (12–17)	<0.001
CAC score ≥1 unit, no. (%)	2,443 (46)	266 (49)	0.28	44 (43–46)	61 (56–66)	<0.001
CAC score ≥100 units, no. (%)	1,097 (21)	132 (24)	0.064	14 (13–15)	27 (23–31)	<0.001
CAC score ≥300 units, no. (%)	556 (11)	66 (12)	0.26	6 (5–6)	12 (9–15)	<0.001

* BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure; NSAIDs = nonsteroidal antiinflammatory drugs; COX-2 = cyclooxygenase 2; LDL = low-density lipoprotein; HDL = high-density lipoprotein; IQR = interquartile range; CRP = C-reactive protein; CAC = coronary artery calcium.

† Demographically adjusted values are the mean (95% confidence interval) and are derived from linear or logistic regression, as appropriate to the characteristic of interest, in models adjusted for age, sex, and race/ethnicity. Characteristics that required normal transformation for modeling were back-transformed.

‡ Data were available for all controls and 463 rheumatoid arthritis (RA) patients.

§ Data were available for all controls and 439 RA patients.

¶ Data were available for 5,251 controls and 436 RA patients.

for linear regression. Similar models were constructed for non-HDL concentration (using published cut points from current guidelines [17]) and HDL concentration (modeled in quintiles). Sensitivity analyses explored differences in the patterns of association of LDL concentration strata with CAC score restricted to strata of patient characteristics (age >60 years, sex, white versus nonwhite race, ever smoker, hypertension, diabetes mellitus, and body mass index [BMI] >30 kg/m^2 or <30 kg/m^2) and by RA cohort.

Finally, we compared patient characteristics according to LDL concentration (<70 mg/dl versus >70 mg/dl) using the univariate tests described above for the RA and control groups separately. Differences in the associations of characteristics with LDL concentration <70 mg/dl between the RA and control groups were compared by modeling LDL concentration <70 mg/dl as the dependent variable in binomial logistic regression models that included RA × characteristic interaction terms as the primary covariates of interest. Throughout, a 2-tailed alpha of 0.05 was used. Stata SE version 14 (StataCorp) was used for all analyses.

RESULTS

Characteristics of the patients and controls. Characteristics of the 546 RA patients and 5,279 controls are summarized in Table 1. Compared with controls, RA patients were significantly younger and more likely to be female and white. After adjustment for these demographic characteristics, a significantly higher proportion of RA patients were underweight as defined by a BMI of <18.5 kg/m^2, and the RA patient group had a sig-

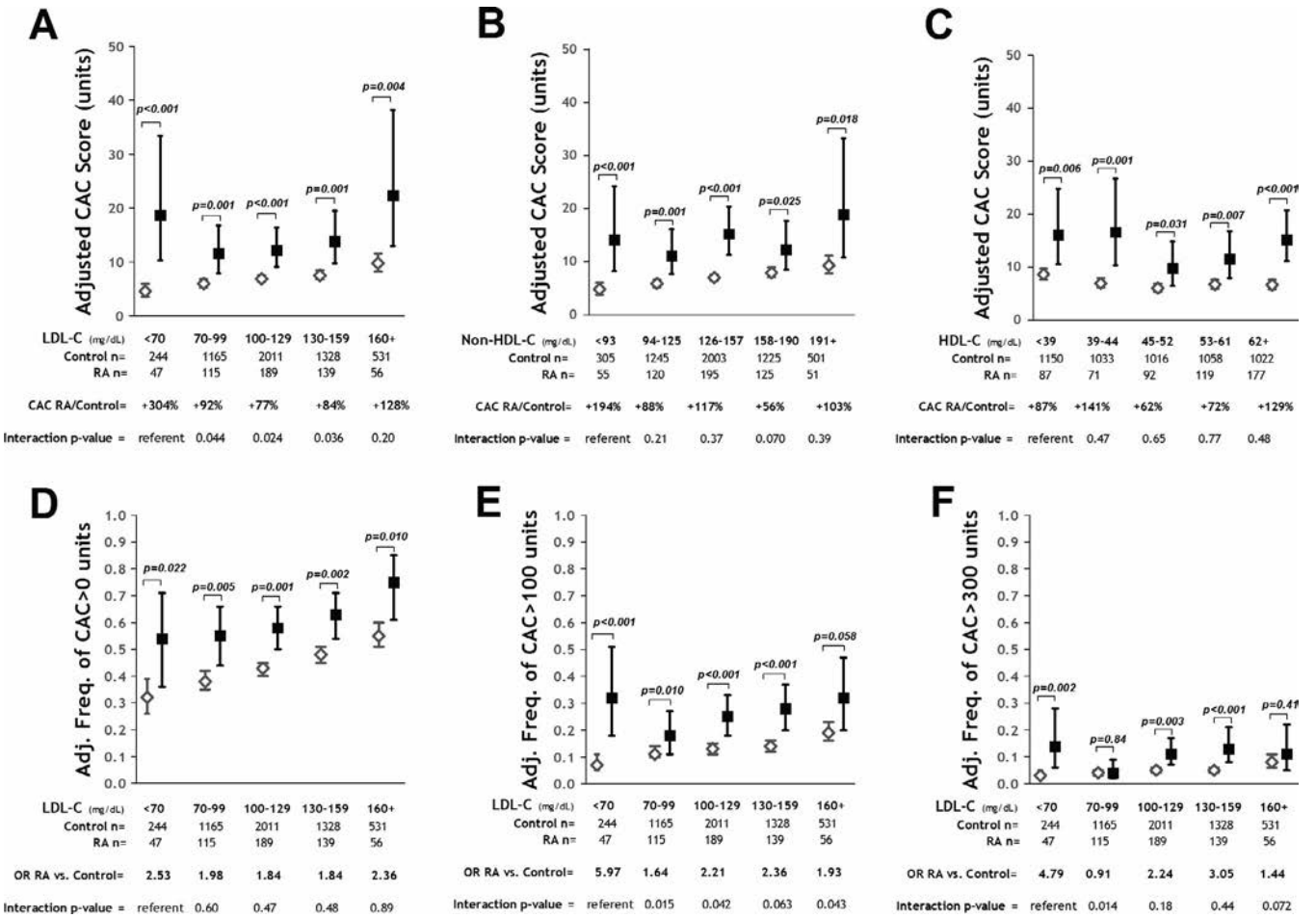

Figure 1. Adjusted coronary artery calcium (CAC) levels in controls and rheumatoid arthritis (RA) patients. **A–C**, Adjusted CAC scores in controls (open symbols) and RA patients (solid symbols) according to low-density lipoprotein concentration (LDL-C) (**A**), non–high-density lipoprotein concentration (non–HDL-C) (**B**), and high-density lipoprotein concentration (HDL-C) (**C**). Relative differences in CAC scores for the RA versus control groups are indicated for each stratum. Interaction *P* values compare the relative difference in CAC score between the RA and control groups for the given stratum versus the lowest (referent) stratum. **D–F**, Adjusted (adj) frequencies (freq) of any CAC score (CAC score >0 units) (**D**), CAC score ≥100 units (**E**), and CAC score ≥300 units (**F**) in controls (open symbols) and RA patients (solid symbols) according to LDL concentration. Odds ratios (ORs) for the RA versus control groups are shown for each LDL concentration stratum. Interaction *P* values compare the magnitude of the OR between the RA and control groups for the given stratum versus the lowest (referent) stratum. Models were adjusted for age, sex, race, waist circumference, smoking status, diabetes mellitus, hypertension, HDL concentration (where appropriate), and aspirin use. Values are the mean and 95% confidence interval.

nificantly lower waist circumference and a higher prevalence of hypertension, which included higher mean adjusted systolic blood pressure and diastolic blood pressure, and more frequent use of antihypertensive agents, compared with controls. A greater percentage of RA patients were taking nonsteroidal antiinflammatory drugs, including cyclooxygenase 2 inhibitors, and a lower percentage were taking aspirin, compared with controls. While total cholesterol levels did not differ significantly between the groups, RA patients had a lower demographically adjusted mean LDL concentration than controls, and the demographically adjusted frequency of LDL concentration <70 mg/dl in the RA group was double that in the control group (12% versus 6%, respectively). As expected, the mean adjusted CRP level was higher in the RA group than in the control group. As previously established (4–6), the average demographically adjusted CAC score was twice as high in the RA group than in the control group, as were the frequencies of CAC score ≥100 units and CAC score ≥300 units.

Association of LDL concentration <70 mg/dl with a markedly higher CAC score in RA patients compared with non-RA controls. Adjusted CAC scores according to strata of LDL concentration, non-HDL concentration, and HDL concentration are depicted in Figures 1A–C. Mean CAC scores were significantly higher for the RA versus control groups across all LDL concentration strata after adjustment for demographic characteristics and relevant CVD risk factors (Figure 1A) and demonstrated a U-shaped pattern in the RA group compared with a linear increase, on average, in

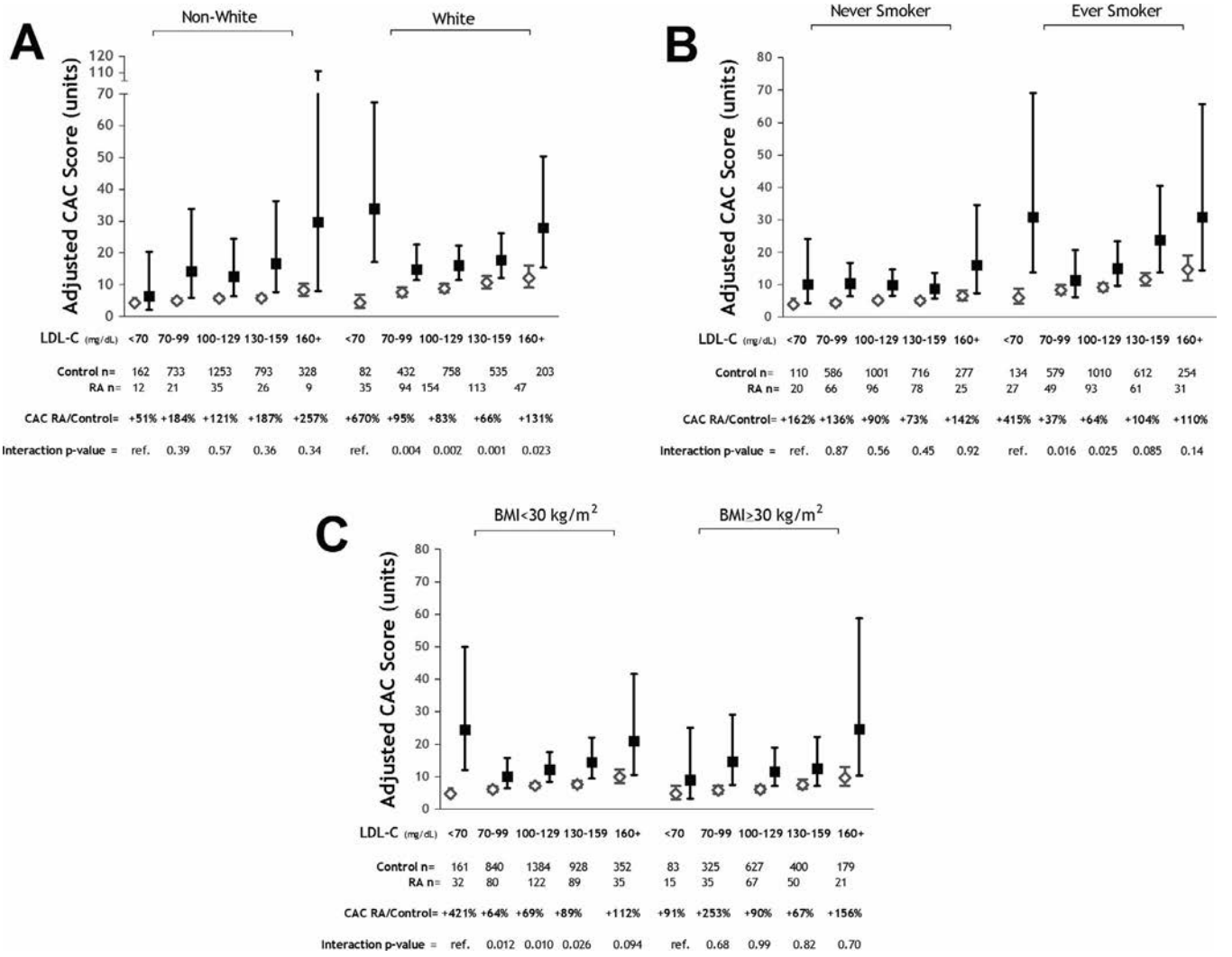

Figure 2. Adjusted CAC levels in controls (open symbols) and RA patients (solid symbols) according to LDL concentration stratum stratified by race, smoking status, and body mass index (BMI). Adjusted CAC scores are shown for **A**, nonwhite and white subjects, **B**, subjects who had never smoked and those who had ever smoked, and **C**, subjects with a BMI of <30 kg/m^2 and those with a BMI of >30 kg/m^2, stratified by LDL concentration. Relative differences in CAC scores for the RA versus control groups are indicated per stratum. Interaction *P* values compare the relative difference in CAC score between the RA and control groups for the given stratum versus the lowest (referent) stratum. Models were adjusted for age, sex, race (where appropriate), waist circumference, smoking status (where appropriate), diabetes mellitus, hypertension, HDL concentration, and aspirin use. Values are the mean and 95% confidence interval. See Figure 1 for other definitions.

the control group. The greatest difference in mean adjusted CAC scores between the RA and control groups was observed in those with an LDL concentration of <70 mg/dl, for whom the mean adjusted CAC score was >3-fold higher in the RA group than in the control group (18.6 versus 4.6 Agatston units, respectively; $P < 0.001$). This magnitude of difference in adjusted CAC scores between the RA and control groups was significantly larger for those with an LDL concentration of <70 mg/dl than for those in the next 3 highest LDL concentration strata (i.e., P for interaction < 0.05 for all). Mean adjusted CAC scores were also higher for the RA group than the control group across all strata of non-HDL concentration (Figure 1B) and HDL concentration (Figure 1C); however, the magnitude of the difference in mean adjusted CAC scores between the RA and control groups within the lowest stratum did not differ significantly from the difference in scores in the other strata. Similar patterns were observed across each of the 4 RA cohorts (data not shown).

Adjusted frequencies of any CAC score (i.e., CAC score >0), CAC score ≥100 units, and CAC score ≥300 units according to LDL concentration stratum are depicted in Figures 1D–F. For any CAC score (Figure 1D), the greatest relative difference in the adjusted frequency between RA patients and controls was observed in the lowest LDL concentration stratum; however, the magnitude of difference between RA patients and controls did not differ significantly from that within the other strata. For CAC score ≥100 units (Figure 1E), the adjusted odds ratio (OR) was nearly 6-fold higher for RA patients with an LDL concentration of <70 mg/dl than for controls in the same stratum. The magnitude of the difference in this stratum was significantly larger than the differences between RA patients and controls in the other strata (i.e., interaction $P < 0.05$ for all). A similar pattern was observed for CAC score ≥300 units (Figure 1F), although the magnitude of difference between RA patients and controls in the lowest LDL concentration stratum was only significantly different from that in the stratum of LDL concentration of 70–99 mg/dl. These patterns were similar across each of the 4 RA cohorts (data not shown).

Association of low LDL concentration with higher CAC scores among RA patients who were white, ever smoked, or were not obese. We explored whether the association of low LDL concentration with higher CAC scores differed according to patient characteristic. There were 3 subgroups of RA patients—nonwhite patients, never smokers, and those with a BMI of >30 kg/m^2—for whom the difference in mean adjusted CAC score between the RA and control groups was not the greatest in the LDL concentration <70 mg/dl stratum relative to other LDL concentration strata (Figure 2). Accordingly, the associations of low LDL concentration with CAC score were stronger when the white, ever smoker, and BMI <30 kg/m^2 subgroups were analyzed separately (Figure 2). The pattern was similar for both former and current smokers, so ever smokers were modeled as a single group (data not shown). Similarly, patterns were similar for the normal BMI and overweight BMI groups, which were combined and modeled together.

In analyses restricted to the subgroup of white ever smokers, RA patients with an LDL concentration of <70 mg/dl had a mean adjusted CAC score >10-fold higher than controls in the same stratum (61.2 versus 5.7 units, respectively; $P < 0.001$), a difference that was significantly greater than the differences between the RA and control groups within each of the other LDL concentration strata (Figure 3A). A similar pattern was observed for CAC score ≥100 units, with more than two-thirds of the white, ever-smoker RA patients with an LDL concentration of <70 mg/dl demonstrating CAC scores ≥100 units compared with only 8% of the similar controls, after adjustment (OR 23.85; $P < 0.001$) (Figure 3C). This difference was, as for adjusted CAC score, significantly greater than the differences observed in the other LDL concentration strata. Differences between RA patients and controls within the lowest LDL concentration stratum were even greater when the analysis was restricted to white participants who were ever smokers and had a BMI of <30 kg/m^2 (Figures 3B and D).

CVD risk factors and RA disease activity/severity measures were not highly prevalent among RA patients with low LDL concentrations. We explored whether RA patients with low LDL concentrations demonstrated a risk factor profile that could explain their markedly higher CAC scores (Table 2). With the exception of a significantly higher prevalence of current smokers, the frequencies of CVD risk factors were not higher among RA patients with low LDL concentrations than among RA patients with higher LDL concentrations. Several CVD risk factors (BMI, waist circumference, and triglyceride levels) were lower in RA patients with low LDL concentrations than in those with higher LDL concentrations. Importantly, the lower average BMI in the RA patients with low LDL concentrations was not driven by a higher proportion of those in the underweight category (i.e., BMI <18.5 kg/m^2). Likewise, RA disease and treatment characteristics were not higher in those with low LDL concentrations, and the presence of shared epitope alleles was significantly lower among those with low LDL concentrations. Associations of demographic characteristics, lifestyle characteristics, and CVD risk factors with low LDL concentrations were generally similar for non-RA controls and RA patients, with the exceptions of BMI and waist circumference, which were not lower in the controls with low LDL concentrations compared with the controls with high LDL concentrations.

DISCUSSION

In this study, which is the first to explore the coronary atherosclerosis burden among RA patients with very low

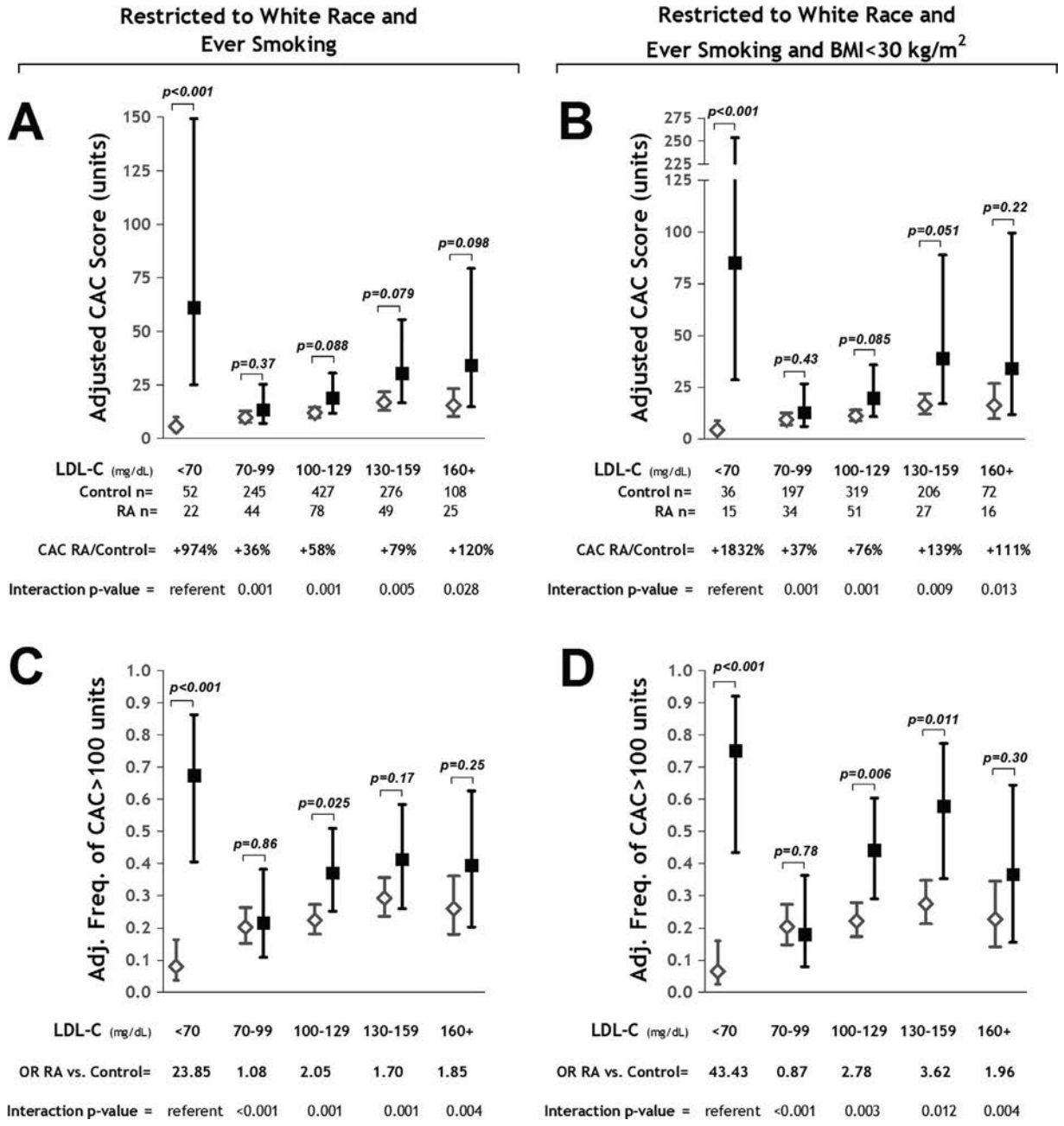

Figure 3. Adjusted CAC levels in controls (open symbols) and RA patients (solid symbols) according to LDL concentration stratum, restricted to high impact subgroups. **A** and **B**, Adjusted CAC score according to LDL concentration stratum for white controls and RA patients who had ever smoked (**A**) and for white controls and RA patients who had ever smoked and were not obese (had a body mass index [BMI] of <30 kg/m^2) (**B**). Relative differences in CAC scores for the RA versus control groups are indicated per stratum. Interaction *P* values compare the relative difference in CAC score between the RA and control groups for the given stratum versus the lowest (referent) stratum. **C** and **D**, Adjusted frequency of CAC score ≥100 units according to LDL concentration stratum for white controls and RA patients who had ever smoked (**C**) and for white controls and RA patients who had ever smoked and were not obese (**D**). Interaction *P* values compare the magnitude of the OR between the RA and control groups for the given stratum versus the lowest (referent) stratum. Models were adjusted for age, sex, race, waist circumference, diabetes mellitus, hypertension, HDL concentration, and aspirin use. Values are the mean and 95% confidence interval. See Figure 1 for other definitions.

LDL concentrations who were not treated with lipid-lowering medications, we observed a U-shaped association of LDL concentrations with CAC score among RA patients that was not present in non-RA controls. The largest relative difference in CAC score between the RA and control groups was observed for those with an LDL concentration of <70 mg/dl. The magnitude of this association was larger among those who were white, those who ever smoked, and those who were not obese.

Table 2. Characteristics of the RA patients and controls according to LDL concentration strata*

	RA patients			Controls			
	LDL <70 mg/dl (n = 47)	LDL >70 mg/dl (n = 499)	*P*	LDL <70 mg/dl (n = 244)	LDL >70 mg/dl (n = 5,035)	*P*	Interaction *P*
Age, mean ± SD years	55 ± 15	57 ± 11	0.26	62 ± 11	61 ± 10	0.67	0.20
Male, no. (%)	13 (28)	99 (20)	0.20	113 (46)	2,401 (48)	0.68	0.17
White, no. (%)	35 (74)	408 (82)	0.22	82 (34)	1,928 (38)	0.14	0.60
BMI, mean ± SD kg/m^2	26.5 ± 5.8	28.4 ± 5.9	0.030	28.2 ± 5.9	28.1 ± 5.5	0.78	0.020
BMI <18.50 kg/m^2, no. (%)	2 (4)	8 (2)	0.21	4 (2)	50 (1)	0.32	0.61
BMI 18.50–24.99 kg/m^2, no. (%)	21 (45)	150 (30)	0.041	77 (32)	1,498 (30)	0.57	0.11
BMI 25.00–29.99 kg/m^2, no. (%)	9 (19)	168 (34)	0.040	80 (33)	1,956 (39)	0.059	0.22
BMI ≥30.00 kg/m^2, no. (%)	15 (32)	171 (34)	0.73	83 (34)	1,531 (30)	0.26	0.43
Waist circumference, mean ± SD	87 ± 16	93 ± 16	0.011	98 ± 16	97 ± 14	0.61	0.005
Diabetes mellitus, no. (%)	3 (6)	24 (5)	0.50	42 (17)	515 (10)	0.001	0.57
Ever smoker, no. (%)	27 (57)	234 (47)	0.17	134 (55)	2,455 (49)	0.060	0.73
Current smoker, no. (%)	11 (23)	63 (13)	0.039	54 (22)	655 (13)	<0.001	0.90
Hypertension, no. (%)	20 (43)	229 (46)	0.66	125 (51)	2,019 (40)	0.001	0.14
SBP, mean ± SD mm Hg	123 ± 21	127 ± 19	0.21	126 ± 21	126 ± 21	0.82	0.44
DBP, mean ± SD mm Hg	75 ± 10	75 ± 10	0.72	71 ± 11	72 ± 10	0.38	0.68
Antihypertensive use, no. (%)	17 (37)	167 (33)	0.63	113 (46)	1582 (31)	<0.001	0.23
Total cholesterol, mean ± SD mg/dl	139 ± 24	205 ± 35	<0.001	136 ± 22	199 ± 32	<0.001	0.77
HDL, mean ± SD mg/dl	56 ± 21	56 ± 17	0.83	52 ± 20	51 ± 15	0.22	0.88
Triglycerides, median (IQR) mg/dl†	87 (67–127)	112 (81–152)	0.005	97 (63–174)	109 (77–156)	0.075	0.092
CRP, median (IQR) mg/liter‡	3.4 (0.5–8.0)	4.0 (1.4–10.0)	0.23	2.2 (0.8–5.1)	1.9 (0.8–4.2)	0.20	0.10
CRP ≥5 mg/liter, no. (%)	15 (41)	161 (47)	0.48	62 (25)	998 (20)	0.037	0.14
Current NSAIDs, no. (%)	27 (57)	274 (55)	0.74	57 (23)	1,107 (22)	0.61	0.82
COX-2 inhibitors, no. (%)§	7 (20)	122 (28)	0.28	17 (7)	282 (6)	0.37	0.17
Current aspirin use, no. (%)	8 (17)	62 (12)	0.37	53 (22)	1,104 (22)	0.94	0.37
RA duration, median (IQR) years	10 (3–20)	9 (3–19)	0.76	–	–	–	–
RF seropositive, no. (%)	31 (66)	363 (73)	0.18	–	–	–	–
DAS28-CRP, median (IQR)‡	3.6 (2.5–4.7)	3.7 (2.8–4.5)	0.42	–	–	–	–
Morning stiffness, median (IQR) minutes	20 (5–60)	20 (5–60)	0.77	–	–	–	–
Any shared epitope alleles, no. (%)†	15 (52)	256 (70)	0.039	–	–	–	–
Any nonbiologic agents, no. (%)	39 (83)	425 (85)	0.66	–	–	–	–
Current methotrexate, no. (%)	29 (62)	319 (64)	0.76	–	–	–	–
Current hydroxychloroquine, no. (%)	14 (30)	103 (21)	0.14	–	–	–	–
Current biologic agents, no. (%)	15 (32)	166 (33)	0.84	–	–	–	–
Current prednisone, no. (%)	21 (45)	215 (43)	0.83	–	–	–	–

* RF = rheumatoid factor; DAS28-CRP = Disease Activity Score in 28 joints using the C-reactive protein level (see Table 1 for other definitions).
† Data were available for 385 RA patients.
‡ Data were available for 382 RA patients.
§ Data were available for 463 RA patients.

However, other than a higher proportion of current smokers among those with very low LDL concentrations, traditional CVD risk factors and RA characteristics did not account for the findings. The study also confirmed, in the largest sample to date, higher overall CAC scores in RA patients than controls across the entire range of LDL concentrations.

Observational studies showing lower levels of circulating total cholesterol and LDL among RA patients compared with non-RA controls date back decades (18). Recognition that the magnitude of association of LDL concentration with CVEs is lower among RA patients than in the general population is derived from more recent studies (8). However, RA

patients with very low LDL concentrations were identified as being at heightened risk of CVEs only in 2010 in a study by Myasoedova et al, who named the association the "lipid paradox"(9). Since then, the association has been demonstrated in additional cohorts (10); however, whether the association truly differs between RA and non-RA populations has been questioned (19).

Our findings support the notion of a heightened CVD risk for RA patients with very low LDL concentrations, particularly since >30% of the RA patients in this group had a CAC score of ≥100 units, an established threshold predictive of future atherosclerotic CVEs (20). Moreover, 75% of those with very low LDL concentrations in the group that we identified as having the highest risk of CVD (white patients who had ever smoked and were not obese) had a CAC score of ≥100 units. Such individuals would not be considered at high risk of CVD based on risk algorithms validated in the general population that are weighted heavily toward CVD risk driven by hyperlipidemia, such as the current American College of Cardiology/American Heart Association guidelines (21). These algorithms have consistently been shown to underperform in RA patients (22,23), suggesting that additional predictive factors for RA patients should be identified. However, efforts to improve prediction by factoring in systemic markers of inflammation have been unsuccessful (22,23).

In RA, systemic markers of inflammation vary with time and treatment, and current levels are likely not reflective of past levels that may have contributed to atherogenesis. Low LDL concentrations in patients not receiving lipid-lowering medications may represent a more consistent and stable marker of an RA-driven atherogenic propensity, and more aggressive CVD screening and primary prevention measures, including targeting of non-lipid risk factors, may be appropriate for RA patients with this phenotype. Using cardiac CT for secondary screening for atherosclerosis is already advocated for those at uncertain or intermediate risk in the general population (21); however, the utility of such a strategy has not been evaluated in RA patients with very low LDL concentrations. Our data indicate that studies evaluating the utility of secondary screening with an imaging assessment of atherosclerosis among RA patients with very low LDL concentrations are warranted.

Mechanistically, it is unclear what factor(s) may mediate the disconnect between circulating LDL concentration and atherogenesis in this subgroup of RA patients. Inflammatory cytokines associated with RA, such as interleukin-6, up-regulate LDL receptors and scavenger receptors for modified LDL particles on hepatocytes and macrophages, potentially leading to lower circulating LDL levels while also being proatherogenic (24,25). However, we did not observe an association of higher CRP levels or Disease Activity Scores with low LDL concentration, making these unlikely to be mediators of the associations we observed. Another mechanism potentially leading to reduced circulating LDL levels is oxidation, since oxidized LDL particles are more readily taken up by macrophages and removed from circulation (26). RA patients, on average, have higher levels of oxidized LDL (27). HDL protects against such LDL oxidation, largely through the activity of its paraoxonase cargo. In RA, HDL particles are deficient of paraoxonase (28) and paraoxonase function is diminished (29), an effect that is potentially reversible with treatment (30). However, whether these or other mechanisms mediate the low LDL phenotype to be proatherogenic warrants further investigation. Very low LDL concentrations, along with lower HDL concentrations and triglyceride levels, have also been linked to higher mortality in patients with moderate-to-severe heart failure (31). Whether this phenomenon is due to an increase in atherosclerosis, consistent with our findings, or is a consequence of malnutrition and/or the cachectic hypermetabolic state of advanced heart failure is unclear.

The fact that a larger effect of very low LDL concentration on CAC was seen among white RA patients who had ever smoked was consistent with the expected contribution of these risk factors; however, the protective effect of higher BMI was unexpected. Interestingly, higher BMI has also been associated with lower all-cause and CVD mortality in RA patients (32,33). It has been postulated that this association is due to the presence of sarcopenia and frailty induced by prolonged disease activity and severity that characterizes RA patients with lower BMIs; however, Escalante et al (32) showed that the protective effect of BMI on all-cause mortality was incremental, even when moving from the normal weight to overweight to obese BMI categories. Nevertheless, because patients with low BMI and very low LDL concentration make up a relatively small subset of the RA population, it seems unlikely that this is the primary mechanism whereby BMI appears to be protective against all-cause and CVD mortality in RA.

Our study has notable strengths and limitations. Among its strengths, the RA patient sample included participants in 4 of the largest North American cohort studies of CAC in RA and was sufficiently large to explore associations within subsets of patients. Likewise, the ability to leverage the size of the MESA cohort for non-RA controls allowed additional precision to detect differences within subsets. Among the limitations of the study, there were differences in the 4 RA cohorts in inclusion/exclusion criteria, dates of enrollment, data captured, and geographic location. However, the primary exposures and outcomes were collected in a similar manner in the 4 cohorts and, in sensitivity analyses, there were no differences between the cohorts with regard to the associations of very low LDL concentration with CAC score.

There were also differences in demographic characteristics between the pooled RA sample and the MESA control group. However, we chose not to sacrifice precision by attempting to match or restrict inclusion based on demographic variables; rather, we used multivariable regression to adjust for demographic differences and we conducted sensitivity analyses restricted to age group, sex, and race, with notable differences observed only for race, as discussed above. Because of the smaller sample size of the subgroup analyses and the inherent

reduction in statistical power, the magnitude and significance of the associations should be interpreted as less reliable than that of the main effects identified in the full cohort. However, these subgroup dichotomies are potentially hypothesis-generating and warrant validation in subsequent studies. Finally, our comparisons are cross-sectional only, with no ability to determine temporality in the associations. In particular, future studies exploring atherosclerosis progression according to LDL concentration stratum are warranted. We did not find an interaction of age or RA duration with the association between very low LDL concentration and CAC score, suggesting that the association is not related solely to the cumulative effects of RA disease.

In summary, RA patients not treated with lipid-lowering medications who had the lowest circulating LDL concentrations (i.e., <70 mg/dl) had markedly higher CAC scores relative to non-RA controls, including a high frequency of CAC scores potentially associated with CVEs (i.e., CAC score ≥100 units), even after adjustment for relevant confounders. The association was not observed for HDL concentration or non-HDL concentration, suggesting an effect specific to LDL concentration. The association was stronger in some subsets of RA patients, particularly those who were white, those who had ever smoked, and those who were not obese. However, the high risk of CVD observed for these patients did not appear to be based on either their traditional CVD risk profile or RA disease or treatment characteristics. Our data support the notion of the so-called "lipid paradox," in which RA patients with similarly low LDL concentrations have been noted to be at unexpectedly high risk of CVEs, and suggest that there is a susceptible subgroup of RA patients that may benefit from additional CVD screening and/or preemptory aggressive primary prevention efforts targeting non-lipid risk factors.

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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. Giles 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. Giles, Wasko, Chung, Szklo, Blumenthal, Kao, Bokhari, Zartoshti, Stein, Bathon.

Acquisition of data. Giles, Wasko, Chung, Bokhari, Zartoshti, Stein, Bathon.

Analysis and interpretation of data. Giles, Wasko, Chung, Szklo, Blumenthal, Kao, Bokhari, Zartoshti, Stein, Bathon.

ADDITIONAL DISCLOSURES

Author Kao is an employee of EMD Serono Research & Development Institute.

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A Multicenter, Randomized, Placebo-Controlled Trial of Atorvastatin for the Primary Prevention of Cardiovascular Events in Patients With Rheumatoid Arthritis

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Objective. Rheumatoid arthritis (RA) is associated with increased cardiovascular event (CVE) risk. The impact of statins in RA is not established. We assessed whether atorvastatin is superior to placebo for the primary prevention of CVEs in RA patients.

Methods. A randomized, double-blind, placebo-controlled trial was designed to detect a 32% CVE risk reduction based on an estimated 1.6% per annum event rate with 80% power at $P < 0.05$. RA patients age >50 years or with a disease duration of >10 years who did not have clinical atherosclerosis, diabetes, or myopathy received atorvastatin 40 mg daily or matching placebo. The primary end point was a composite of cardiovascular death, myocardial infarction, stroke, transient ischemic attack, or any arterial revascularization. Secondary and tertiary end points included plasma lipids and safety.

Results. A total of 3,002 patients (mean age 61 years; 74% female) were followed up for a median of 2.51 years (interquartile range [IQR] 1.90, 3.49 years) (7,827 patient-years). The study was terminated early due to a lower than expected event rate (0.70% per annum). Of the 1,504 patients receiving atorvastatin, 24 (1.6%) experienced a primary end point, compared with 36 (2.4%) of the 1,498 receiving placebo (hazard ratio [HR] 0.66 [95% confidence interval (95% CI) 0.39, 1.11]; $P = 0.115$ and adjusted HR 0.60 [95% CI 0.32, 1.15]; $P = 0.127$). At trial end, patients receiving atorvastatin had a mean ± SD low-density lipoprotein (LDL) cholesterol level 0.77 ± 0.04 mmoles/liter lower than those receiving placebo ($P < 0.0001$). C-reactive protein level was also significantly lower in the atorvastatin group than the placebo group (median 2.59 mg/liter [IQR 0.94, 6.08] versus 3.60 mg/liter [IQR 1.47, 7.49]; $P < 0.0001$). CVE risk reduction per mmole/liter reduction in LDL cholesterol was 42% (95% CI –14%, 70%). The rates of adverse events in the atorvastatin group (n = 298 [19.8%]) and placebo group (n = 292 [19.5%]) were similar.

Conclusion. Atorvastatin 40 mg daily is safe and results in a significantly greater reduction of LDL cholesterol level than placebo in patients with RA. The 34% CVE risk reduction is consistent with the Cholesterol Treatment Trialists' Collaboration meta-analysis of statin effects in other populations.

INTRODUCTION

Despite major advances in therapy over the last two decades, rheumatoid arthritis (RA) continues to be associated with reduced life expectancy compared to the general population (1). Almost half of all deaths in RA (~35–40% of the excess deaths) are attributed to cardiovascular disease (CVD) (2). There are many mechanisms that may underlie the increased CVD morbidity and

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mortality in RA, but their cross-talk and relative contributions are not yet fully elucidated. CVD risk factors, including smoking, hypertension, dyslipidemia, increased adiposity, and reduced physical activity, are highly prevalent in RA (3) but do not fully account for the excess CVD (4). A significant part is attributed to "novel" CVD risk factors, such as "high-grade" inflammation promoting atherothrombotic cardiovascular events (CVEs) (4,5). Risk algorithms developed for the general population may underestimate CVE risk in patients with RA (6–8), even when multipliers are applied, as in recently updated European League Against Rheumatism recommendations (9). This makes identification of RA patients who would benefit from primary prevention therapy less precise, leads to significant underuse of statins even in patients who fulfill general population thresholds for statin treatment (10), and has led some to suggest universal prescription of statins in RA (11), as practiced in diabetes mellitus (DM).

The efficacy of statins in the primary and secondary prevention of CVEs has been demonstrated in large-scale trials and meta-analyses (12). CVE reduction is related to the degree of reduction of low-density lipoprotein (LDL) cholesterol levels. Each millimole per liter reduction in LDL cholesterol is associated with a 20–22% reduction in the risk of myocardial infarction (MI), revascularization, and stroke (12). In RA, high-grade inflammation is associated with suppression of total cholesterol, LDL cholesterol, and high-density lipoprotein (HDL) cholesterol levels, as well as changes in lipid structure and function, promoting atherosclerosis (13,14). The potential pleiotropic antiinflammatory/immunomodulatory effects of statins (15) may therefore be more relevant in RA than in the general population. In the Trial of Atorvastatin in Rheumatoid Arthritis (TARA), atorvastatin 40 mg daily, as an adjunct to disease-modifying antirheumatic drug (DMARD) therapy, provided a modest additional benefit for control of inflammation in RA, at least in a subgroup of patients (16), while the Tayside controlled study of rosuvastatin in RA suggested a potentially beneficial effect on C-reactive protein (CRP) levels (17). The extent to which statins affect lipid levels and reduce CVEs in RA remains uncertain, due to the small number of RA patients included in general population trials (18).

The lack of robust primary prevention data, coupled with the multifaceted pharmacologic potential of statins in RA suspected at that time, prompted the Trial of Atorvastatin for the Primary Prevention of Cardiovascular Events in Patients with Rheumatoid Arthritis (TRACE RA), the only statin trial with hard CVE end points in this population.

PATIENTS AND METHODS

Study design. TRACE RA was a multicenter, randomized, double-blind, placebo-controlled trial comparing atorvastatin 40 mg once daily (supplied by Pfizer UK) with placebo (dummy atorvastatin) for the primary prevention of CVEs in patients with RA. The trial was conducted in 102 rheumatology units in the UK, approved by the Southampton and South West Hampshire Multicentre Research Ethics Committee (Ref. No 06/Q1704/171), and registered with International Standard Randomised Controlled Trial Number 41829447. The final protocol is available at https://www.staffnet.manchester.ac.uk/rbe/ethics-integrity/clinical-trials/portfolio/tracera/ and in Supplementary Methods 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40892/abstract. See Appendix A for study centers and members of the TRACE RA Consortium.

Participants. Patients were eligible if they fulfilled the American College of Rheumatology 1987 criteria for RA (19), were >50 years of age or had an RA disease duration of >10 years, and gave informed consent. Patients taking statins and those with known CVD requiring statins, DM, myopathy, or other contraindications to statins were excluded (see Supplementary Methods 1). Recruiting centers continued their routine practice for screening (or not) for cardiovascular risk. There were no restrictions with regard to RA treatment prior to or during the trial period, other than the requirement that patients receive stable doses of antirheumatic medication for the 3 months prior to inclusion in the study. Potentially eligible patients were identified during routine clinical visits, given the patient information sheet, and invited to contact the local trial team if they were interested in participating. A screening visit was then arranged. All patients recruited provided written informed consent.

Randomization and masking. Trial medication was provided by Pfizer UK, bottled by an independent pharmaceutical company (Catalent Pharma Solutions UK) to good manufacturing practice standards, and dispensed by the local study pharmacist. The randomization process was incorporated into the drug labeling. Center was the only stratifying variable. Catalent performed the randomization, labeled each bottle with a unique number, and supplied the packaged drugs to hospital pharmacies with scratch cards to allow a patient's treatment allocation to be revealed, if necessary. On entering the trial, each patient was given a filled and labeled bottle, coded with a unique study number, which was used for all future supplies for that patient. Study treatment remained double-blind for patients, investigators, and study personnel throughout.

Procedures. The trial comprised 3 stages: 1) a screening visit to confirm patient eligibility, secure consent, counsel (verbally and with a leaflet) patients on modifiable cardiovascular risk factors, and randomize patients (there was no run-in period); 2) a 3-month visit to check drug tolerability and safety (by liver function tests and creatine kinase [CK] level); and 3) an intended minimum 5-year treatment period. At the screening visit, baseline information on demographic characteristics, medical history, family history of premature CVD, smoking status, and concomitant medication was obtained through interview and case note review. The presence of hypertension was assessed by the case report form question, "Is the patient known to have hypertension?"

Height, weight, and waist circumference were measured. RA disease activity, severity (physical function), and quality of life were assessed using the Disease Activity Score in 28 joints (DAS28) (20), the UK version of the Health Assessment Questionnaire (HAQ) (21) disability index (DI), and the EuroQol 5-domain (EQ-5D) instrument (22), respectively. Blood samples were collected for routine measurements of hematologic features, biochemical features, erythrocyte sedimentation rate, CRP, rheumatoid factor, and/or anti–cyclic citrullinated peptide antibodies.

The protocol did not require measurement of lipid levels at baseline. The results of any lipid measurements that had been requested routinely in primary or secondary care over the previous 12 months were recorded in the clinical trial record. If there was more than 1 lipid measurement, the most recent was used. General practitioners (GPs) were informed if patients were found to have hypertension, DM, or an existing indication for statins (e.g., known hyperlipidemia, DM, or previously known high CVD risk [according to standard guidelines] requiring a statin for primary or secondary prevention).

Randomized patients were followed up at 3 and 6 months and every 6 months thereafter in person or by telephone. Information on trial efficacy and safety end points, disease activity, severity, and concomitant medication was collected at each visit. Patients were asked if they had taken "most," "some," or "none" of their tablets. Patients were considered compliant if they reported taking "most" of their study tablets since their last visit. Study drug administration could be paused, if necessary, for up to 4 weeks without violating the protocol. Patients who experienced a validated primary end point had no further trial visits but were followed up for mortality via linkage with national death registers. Secondary prevention in these patients was decided by the GP and/or treating physician. Patients who were withdrawn from the study for reasons other than a primary end point continued to attend follow-up visits to facilitate adverse event and clinical end point data collection. Patients who developed a clinical need for a statin, other than a primary end point, after randomization could be prescribed up to 40 mg of atorvastatin in addition to the randomized trial medication and remain in the trial.

Outcome measures. The prespecified primary end point was "major vascular events," defined as nonfatal MI, nonfatal presumed ischemic stroke, transient ischemic attack (TIA), any coronary or non-coronary revascularization, or cardiovascular death, excluding both confirmed cerebral hemorrhage (International Statistical Classification of Diseases and Related Health Problems, Tenth Revision [ICD-10] codes I64–I99) (23) and non-coronary cardiac death (ICD-10 codes I00–I15 and I26–I52), occurring during the scheduled treatment period. Secondary end points were the separate components of the primary end point. Tertiary end points included total and cause-specific mortality (coronary, other vascular, and nonvascular death separately); hospitalizations; statin safety-related outcomes (persistent elevation of alanine transaminase [ALT] or aspartate transaminase [AST] or myopathy [muscle symptoms plus CK >10× the upper limit of normal (ULN)]; and between-group differences at study end in lipid levels and health-related outcomes (physical function and quality of life).

Additional information about all potential primary end points was collected from medical records, death certificates, and post-mortem examinations (where available). An independent trial end points committee reviewed such information on all potential CVEs and deaths and classified them according to prespecified criteria (see Supplementary Methods 1). Information about hospital admissions was ascertained via linkage, using each patient's unique National Health Service number, with the Health and Social Care Information Centre (HSCIC) for England and Wales and the Scottish Office's Information and Statistics Division (ISD), and the local hospital medical records departments. Information on mortality and cause of death was obtained via linkage with the HSCIC and ISD. Patients were asked at each visit about adverse events including muscle pain, and ALT, AST, and CK were measured at 3 months. Liver function tests were also performed regularly (usually every 2–3 months) as part of routine DMARD monitoring. At the final study visit, patients were asked to provide a blood sample for lipid and CRP analysis. These samples were shipped to a single laboratory and measured, blinded with regard to treatment group, on an automated validated platform (c311; Roche Diagnostics) using the manufacturer's calibrators and quality control material. Between-run coefficients of variation were all <5.2%. LDL cholesterol level was estimated using the Friedewald equation (24).

Statistical analysis. The original protocol (Supplementary Methods 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40892/abstract) anticipated that a trial of 3,800 patients followed up for 5 years would have sufficient statistical power to detect plausible risk reduction with atorvastatin. However, a lower than expected event rate led to a protocol amendment (Supplementary Methods 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40892/abstract). The final protocol specified a sample size of 5,400, which would have had 80% power to detect a 32% relative risk reduction in the primary end point in the atorvastatin versus placebo arms based on 434 primary events (Supplementary Methods 1 and Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40892/abstract). However, the ongoing much lower-than-anticipated CVE rate led to premature termination of the trial.

All randomized patients were included in the analysis up to December 20, 2012 or the end of trial visit, whichever was earlier, irrespective of whether the study drug was continued (i.e., intent-to-treat analyses). Cox regression models were developed for time to occurrence of a first CVE using treatment allocation as the independent variable. The models were stratified by center and adjusted for baseline imbalances, compliance,

and nonstudy statin use (the latter two as time-dependent variables using a previously described method [25]). All adjustments were prespecified in the protocol and the statistical analysis plan (Supplementary Methods 4, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40892/abstract). Treatment differences were expressed as hazard ratios (HRs) with 95% confidence intervals (95% CIs). *P* values less than 0.05 (2-sided) were considered significant. Kaplan-Meier product-limit estimates of the survival curves were calculated. Lipid levels and levels of blood tests monitoring statin safety were compared between groups using Mann-Whitney tests. All analyses were performed with SPSS Statistics for Windows, version 22.0 (IBM).

RESULTS

Trial progress. Between August 7, 2007 and November 21, 2011, 3,002 patients with RA from 102 centers were randomized (1,504 to receive atorvastatin and 1,498 to receive placebo). Their mean age was 61 years (228 [7.6%] of 3,002 patients were <50 years of age), and 74% were women. They were followed up for a median of 2.51 years (interquartile range [IQR] 1.90, 3.49 years), providing 7,827 person-years of follow-up. At the time of trial closure (December 31, 2011), the observed event rate in the 2 arms combined was 0.70% per annum compared with the expected 1.6% per annum. Trial progress is shown in Figure 1 and Supplementary Table 1, available

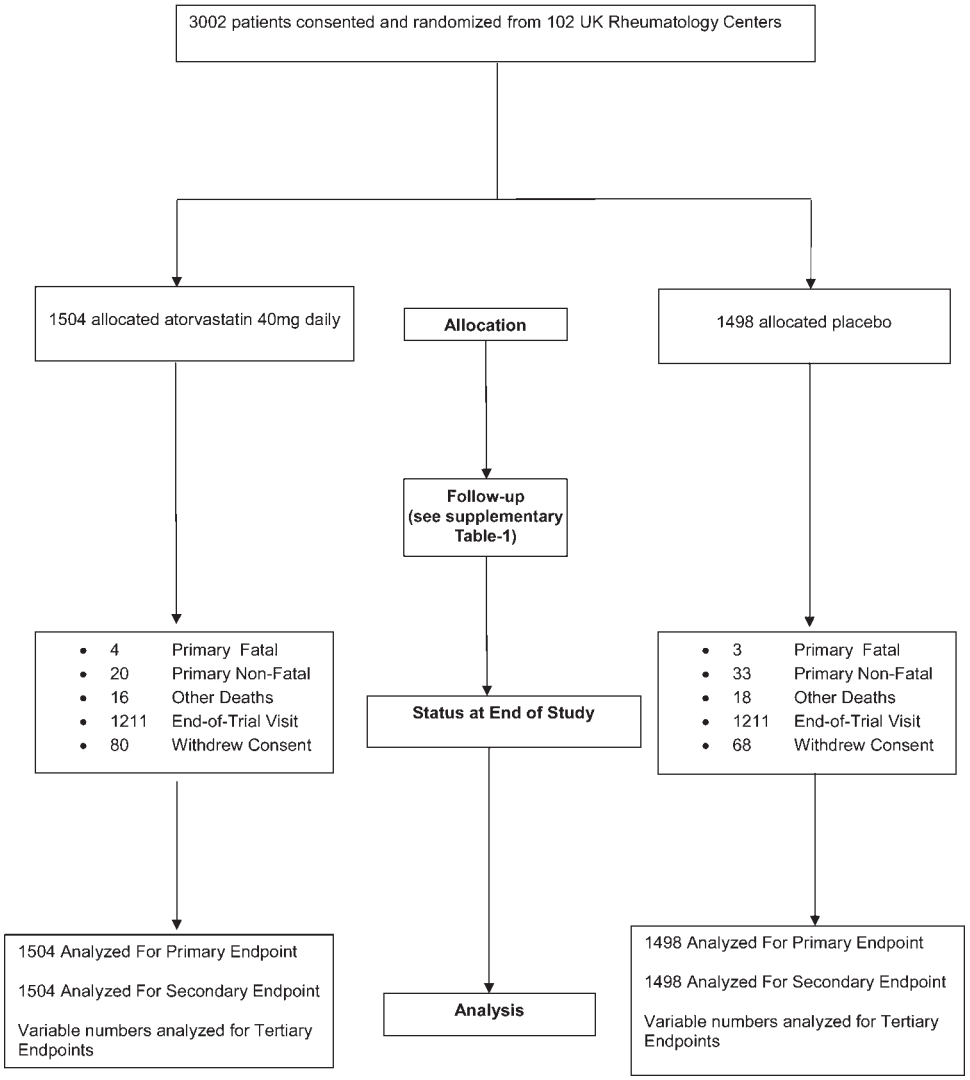

Figure 1. Trial profile. A total of 3,002 patients from 102 UK rheumatology centers were randomized in the Trial of Atorvastatin for the Primary Prevention of Cardiovascular Events in Patients with Rheumatoid Arthritis (TRACE RA). Of those, 1,504 were randomized to receive atorvastatin 40 mg daily and 1,498 were randomized to receive matching placebo. A detailed breakdown of follow-up during the course of the trial is shown in Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40892/abstract. All randomized patients were included in the intent-to-treat analysis for the primary and secondary end points. Variable numbers of patients, based on data availability, were used for analyses of other outcomes.

Table 1. Baseline characteristics of the patients randomized to receive atorvastatin or placebo*

	Atorvastatin 40 mg (n = 1,504)	Placebo (n = 1,498)
Demographic/anthropometric characteristics		
Sex, female	1,107/1,504 (74)	1,120/1,498 (75)
Age, mean ± SD years (n)	61.1 ± 8.3 (1,500)	60.9 (8.5) (1491)
Race, white	1,394/1,421 (98)	1,407/1,430 (98)
BMI, median (IQR) (n)	26.4 (23.7, 30.1) (1,466)	26.8 (24.0, 30.1) (1,432)
RA characteristics		
Time since symptom onset, median (IQR) years (n)	13 (6, 21) (1,471)	13 (6, 21) (1,460)
Time since diagnosis, median (IQR) years (n)	11 (4, 18) (1,499)	11 (5, 20) (1,489)
RF and/or ACPA positive	737/1,177 (63)	709/1,153 (62)
DAS28, median (IQR) (n)	3.7 (2.6, 4.7) (1,471)	3.5 (2.5, 4.6) (1,471)
HAQ DI score, median (IQR) (n)	1.25 (0.50, 1.88) (1,473)	1.25 (0.38, 1.88) (1,464)
EQ-5D, median (IQR) (n)	0.62 (0.52, 0.80) (1,422)	0.689 (0.52, 0.80) (1,408)
Treatment		
Biologic DMARDs	229/1,466 (16)	232/1,458 (16)
Conventional synthetic DMARDs	1,264/1,466 (86)	1,241/1,458 (85)
Steroids	253/1,466 (17)	241/1,458 (17)
NSAIDs/coxibs	629/1,466 (43)	554/1,458 (38)
Cardiovascular characteristics		
Smoking status		
Current smoker	260/1,422 (18)	209/1,431 (15)
Ex-smoker	606/1,422 (43)	637/1,431 (45)
Never smoked	556/1,422 (39)	585/1,431 (41)
Hypertension	322/1,456 (22)	335/1,437 (23)
First degree relative with premature CVD	285/1,321 (22)	263/1,304 (20)
Total cholesterol, median (IQR) mmoles/liter (n)	5.4 (4.8, 6.1) (845)	5.3 (4.8, 6.0) (832)
Triglycerides, median (IQR) mmoles/liter (n)	1.26 (0.90, 1.80) (673)	1.30 (0.90, 1.80) (652)
HDL cholesterol, median (IQR) mmoles/liter (n)	1.56 (1.2, 1.90) (719)	1.52 (1.25, 1.85) (700)
LDL cholesterol, median (IQR) mmoles/liter (n)	3.2 (2.7, 3.8) (544)	3.2 (2.7, 3.8) (530)
CRP, median (IQR) mg/liter (n)	5 (3, 11) (780)	5 (3, 12) (776)
Estimated GFR, median (IQR) ml/minute/1.73 m^2 (n)	79 (59, 110) (1,124)	79 (58, 111) (1,109)
Treatment		
Aspirin	3/116 (3)	3/126 (2)
ACE inhibitors	10/113 (9)	10/127 (8)
Other cardiac drugs	10/113 (9)	10/123 (8)

* The variable number of patients for each characteristic is due to missing data from incomplete case report forms. The low number of baseline lipid measurements is because, due to budgetary constraints, the protocol did not require measurement of lipid levels at baseline. In the UK, it is the responsibility of primary care physicians to assess their patients for cardiovascular risk and to prescribe statins for primary prevention where indicated according to national guidelines. The Trial of Atorvastatin for the Primary Prevention of Cardiovascular Events in Patients with Rheumatoid Arthritis (TRACE RA) aimed to recruit patients who did not have cardiovascular disease (CVD) at baseline and who were not already taking a statin for primary prevention. If lipid levels had been measured routinely in the 12 months prior to recruitment (in primary or secondary care), the results were recorded in the trial case report form. Except where indicated otherwise, values are the number of patients/number for whom data were available (%). BMI = body mass index; IQR = interquartile range; RA = rheumatoid arthritis; RF = rheumatoid factor; ACPA = anti–citrullinated protein antibody; DAS28 = Disease Activity Score in 28 joints; HAQ DI = Health Assessment Questionnaire disability index; EQ-5D = EuroQol 5-domain; DMARDs = disease-modifying antirheumatic drugs; NSAIDs = nonsteroidal antiinflammatory drugs; HDL = high-density lipoprotein; LDL = low-density lipoprotein; CRP = C-reactive protein; GFR = glomerular filtration rate; ACE = angiotensin-converting enzyme.

on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40892/abstract.

Baseline characteristics of the patients. At baseline, the randomized groups were well balanced for all demographic, anthropometric, and RA characteristics, antirheumatic and other therapies, and CVD risk factors, except for current smoking and nonsteroidal antiinflammatory drug (NSAID) or cyclooxygenase 2 inhibitor (coxib) treatment, which were higher in the atorvastatin group than in the placebo group (18.4% versus 14.5% for current smoking and 42.9% versus 38.0% for NSAID or coxib treatment). A total of 40.3% of the patients had low disease activity according to the DAS28 or were in remission (DAS28 ≤3.2), 86% were receiving stable doses of conventional synthetic DMARDs, 16% were receiving biologic DMARDs, and 17% were receiving steroid therapy (Table 1 and Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40892/abstract).

Compliance and nonstudy statin use. In the atorvastatin group, reported compliance fell from 89% at the 3-month visit to 39% by 60 months of follow-up, while nonstudy statin use increased from 0.5% to 5.6%. In the placebo group, compliance fell from 89% to 25% and nonstudy statin use increased from 0.7% to 7.8% (Supplementary Table 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40892/abstract). Time-weighted average compliance was 66% in the atorvastatin arm and 65% in the placebo arm. Time-weighted nonstudy statin use was 1.6% in the atorvastatin arm and 3.3% in the placebo arm.

Primary end point. Twenty-four patients allocated to receive atorvastatin (1.6%) had a confirmed CVE, compared to 36 (2.4%) of the patients allocated to receive placebo (HR 0.66 [95% CI 0.39, 1.11]; $P = 0.115$). After adjustment for baseline differences, compliance, and nonstudy statin use, the HR was 0.60 (95% CI 0.32, 1.15) ($P = 0.127$). Based on the number of events, the numbers of patients, and the mean follow-up time in each arm, the number needed to treat to prevent 1 CVE during the trial was 121. Kaplan-Meier analysis of time to primary end point in the 2 groups is shown in Figure 2, and the cumulative incidence of first CVE in the 2 groups is shown in Figure 3. The estimated reduction in CVE risk per 1 mmole/liter reduction in LDL cholesterol level was 42% (95% CI −14%, 70%) (Supplementary Figure 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40892/abstract). This was calculated by extrapolating the HR of 0.66 for a 0.77 mmoles/liter reduction to an HR of 0.66 to the power of (1/0.77) for a 1 mmole/liter reduction, i.e., an HR of 0.58 or a CVE risk reduction of 42%.

Secondary end points. *Individual components of the primary end point*. Coronary events (nonfatal MI, coronary death, or coronary revascularization) occurred in 13 (0.9%) of the patients in the atorvastatin group versus 23 (1.5%) of the patients in the placebo group. Presumed ischemic stroke or TIA occurred in 6 (0.4%) of the patients in the atorvastatin group versus 12 (0.8%) of the patients in the placebo group, and any non-coronary arterial revascularization occurred in 3 (0.2%) of the patients in the atorvastatin group versus 1 (0.1%) of the patients in the placebo group. No other cardiovascular death occurred in either group. A peripheral atherosclerotic event occurred in 1 (0.1%) of the patients in the atorvastatin group and none of the patients in the placebo

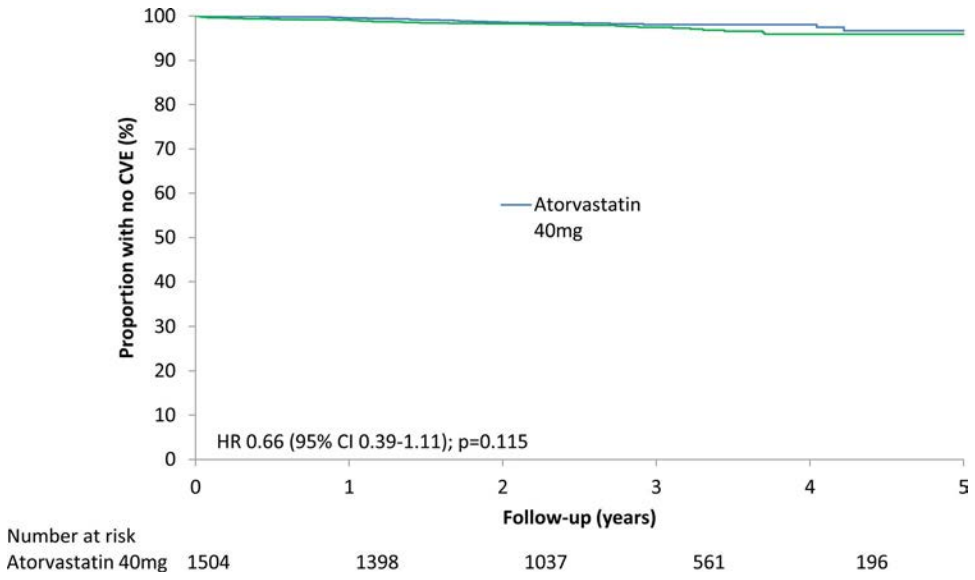

Figure 2. Kaplan-Meier plots of time to first cardiovascular event (CVE) for patients in the atorvastatin and placebo groups. HR = hazard ratio; 95% CI = 95% confidence interval.

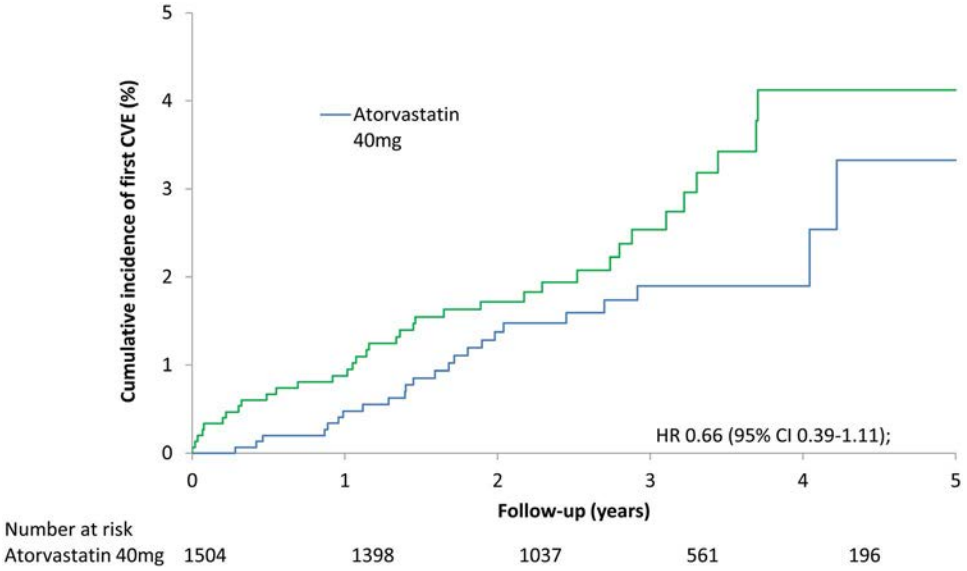

Figure 3. Cumulative incidence of first cardiovascular event (CVE) for patients in the atorvastatin and placebo groups. HR = hazard ratio; 95% CI = 95% confidence interval.

group, and suspected coronary heart disease death occurred in 2 (0.1%) of the patients in the atorvastatin group versus 1 (0.1%) of the patients in the placebo group (Supplementary Table 4, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40892/abstract).

Total and cause-specific mortality. Total and cause-specific mortality (coronary, other vascular, and nonvascular deaths separately) did not differ between the 2 arms (25 deaths in the atorvastatin arm [1.7%] and 27 deaths in the placebo arm [1.8%]) (Supplementary Table 4).

Safety outcomes. There were no suspected unexpected serious adverse reactions. There were 298 reported adverse events in the atorvastatin arm (19.8%) and 292 in the placebo arm (19.5%) ($P = 0.854$) (Table 2).

Two hundred fourteen (14.2%) of the patients in the atorvastatin group versus 223 (14.9%) of the patients in the placebo group had ≥1 hospitalization, with an identical median stay of 3 days (IQR 1, 6 days). There were no differences in the number of hospitalizations per patient ($P = 0.710$ by Kendall's tau-b) or in the proportion of patients with ≥1 hospitalization ($P = 0.641$ by Fisher's exact test) (Supplementary Table 5, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40892/abstract).

Life-threatening but nonfatal serious adverse events (SAEs) occurred in 22 patients in the atorvastatin group versus 24 patients in the placebo group, while SAEs resulting in death occurred in 19 and 18 patients, respectively. None were considered to be related to trial medication. The randomization code was broken at the site in 3 cases in the atorvastatin arm (due to liver cysts with elevated ALT level, acute hepatitis, and abnormal findings on liver function tests) and 2 cases in the placebo arm (due to high grade lymphoma and chest infection), none of which was attributed to trial medication.

There were 64 reports of "RA flare" (significant worsening of RA symptoms), 29 in the atorvastatin group and 35 in the placebo group. There were 249 reports of "new or significant muscle pain," 132 in the atorvastatin group versus 117 in the placebo group ($P = 0.354$). Of these, 13 (9 patients in the atorvastatin group versus 4 patients in the placebo group) had concurrent ALT or AST elevation of >2× the ULN, which was neither sustained nor considered to be related to the trial medication. Three of these patients (2 in the atorvastatin group and 1 in the placebo group) were withdrawn from the trial by the local principal investigator. None of the patients had a CK elevation of >10× the ULN. Two patients (1 in each group) had unsustained CK elevations of 3–10× the ULN; neither was considered to be due to trial medication. No asymptomatic cases of CK elevation were detected either on monitoring per protocol or during routine DMARD monitoring. There were no cases of ALT or AST elevation of >5× the ULN on per protocol testing, but there were 6 cases outside protocol testing (all unsustained and considered unrelated to trial medication). Overall, there were 159 cases of ALT or AST elevation of 2–5× the ULN (90 in the atorvastatin group and 69 in the placebo group; $P = 0.103$), none of which was sustained or attributed to trial medication.

Biochemical and arthritis outcomes at the end of the trial. At the end of the trial, mean LDL cholesterol levels were 0.77 mmoles/liter lower among those allocated to receive atorvastatin compared to those allocated to receive placebo. In the atorvastatin group, 54% of the patients were classified as compliant at the end-of-trial visit. There were no significant differences between groups

Table 2. Adverse events according to ICD-10 chapter by treatment arm*

	Atorvastatin 40 mg (n = 1,504)	Placebo (n = 1,498)
Infectious and parasitic disease	16 (1.1)	15 (1.0)
Neoplasms	28 (1.9)	30 (2.0)
Blood and blood-forming organs and immune system disease	5 (0.3)	2 (0.1)
Endocrine, nutritional, and metabolic disease	1 (0.1)	1 (0.1)
Mental and behavioral disorder	2 (0.1)	1 (0.1)
Nervous system	4 (0.3)	10 (0.7)
Eye and adnexa	8 (0.5)	5 (0.3)
Ear and mastoid disease	2 (0.1)	0 (0.0)
Circulatory disease	40 (2.7)	45 (3.0)
Respiratory disease	33 (2.2)	38 (2.5)
Digestive system disease	37 (2.5)	28 (1.9)
Skin and subcutaneous system disease	12 (0.8)	8 (0.5)
Musculoskeletal and connective tissue disease	20 (1.3)	22 (1.5)
Genitourinary system disease	13 (0.9)	11 (0.7)
Symptoms, signs, and abnormal clinical and laboratory findings not classified elsewhere	8 (0.5)	10 (0.7)
Injury, poisoning	18 (1.2)	16 (1.1)
External causes of morbidity and mortality	23 (1.5)	19 (1.3)
None	111 (7.4)	97 (6.5)
Missing	14 (0.9)	14 (0.9)
Any adverse event	298 (19.8)	292 (19.5)

* Values are the number (%) of patients. ICD-10 = International Statistical Classification of Diseases and Related Health Problems, Tenth Revision.

in RA disease activity (DAS28), severity (HAQ DI), or quality of life (EQ-5D). However, CRP levels were significantly lower in the atorvastatin group (median 2.59 mg/liter [IQR 0.94, 6.08]) than in the placebo group (median 3.60 mg/liter [IQR 1.47, 7.49]) ($P < 0.0001$). Although levels of CK and ALT (but not AST) were statistically significantly higher (by ~12–15%) in the atorvastatin group (Table 3), these differences are not considered clinically significant. The number of cases of myopathy and elevations of liver enzyme levels above the normal range were similar in the 2 groups. In the end-of-trial analysis of the atorvastatin group, lipid, ALT, and CRP levels were significantly associated with compliance (data not shown).

DISCUSSION

TRACE RA was designed to assess whether patients with RA who were not already receiving statin therapy would benefit from atorvastatin 40 mg daily for the primary prevention of CVEs. In this study, the largest ever academically-led clinical trial in RA, >3,000 RA patients were recruited and followed up for a median of 2.5 years. The unexpectedly low event rate and resulting limited statistical power to detect an effect during the planned 5 years of follow up led to premature termination of the trial. The best estimate of the "true" reduction in CVEs in the atorvastatin versus placebo arm is 34%. Using a 95% confidence level we cannot rule out any effect size between a 61% reduction and an 11% increase. Thus, our results were not statistically significant. The observed 34% reduction is consistent with the Cholesterol Treatment Trialists' Collaboration meta-analysis of the effect of statins in other populations (12). Furthermore, in this potentially vulnerable population, atorvastatin was safe, with no excess reports of muscle pain or other significant symptoms among those allocated atorvastatin compared to those receiving placebo.

There were several reasons such a trial was needed. CVD remains a major cause of death (5) and is significantly increased in people with RA compared to the general population (26), a fact recognized by the addition of RA as an independent risk factor in CVD risk algorithms such as QRISK2 (27) and QRISK3 (28). The relative contribution of classic CVD risk factors and novel mechanisms related to systemic inflammation to the excess CVD mortality of RA is still debated (4,29,30), and there have been no clinical end point trials assessing the effect of statins, or any other primary prevention strategy, in this population. Some small studies have shown that statins reduce surrogate measures of atherosclerotic events, for example, arterial stiffness (31) or carotid plaque (32), while a few cohort studies have suggested that statin use is associated with survival gains (33) and statin discontinuation with poorer survival (34) in RA. Finally, post hoc analyses of two trials of more intensive versus standard statin doses have suggested that the effect of statins, in terms of both LDL cholesterol reduction and CVE prevention, is similar in subjects with "inflammatory joint disease," including RA, and those without joint inflammation; however these findings were based on a very small number of patients and events (18).

Randomization in TRACE RA was stratified only by study site in the expectation that, given the large numbers, baseline variables would distribute evenly between the treatment arms. However, baseline current smoking and NSAID/coxib usage, both well-established risk factors for CVEs (35,36), were higher in the atorvastatin group. Although every effort was made to maximize adherence to the trial medication during the trial, adherence rates in TRACE RA were relatively low. Adherence to trial medication in statin trials varies widely (37). This appears to depend on many factors, including the population studied, whether it is for primary or secondary prevention, trial design (e.g. inclusion of a "run-in" period), trial duration, and method of assessing adherence, among others. Adherence to statin treatment in real-world use is generally accepted to be <50% (37,38). In this context, the adher-

Table 3. Lipid levels and other outcomes at trial end by treatment arm*

	Atorvastatin 40 mg (n = 1,504)	Placebo (n = 1,498)	Difference (atorvastatin minus placebo)	*P*†
Lipid variable, mean ± SEM (n)				
Total cholesterol, mmoles/liter	4.13 ± 0.04 (987)	4.86 ± 0.04 (973)	−0.72 ± 0.05	<0.0001
Triglycerides, mmoles/liter	1.10 ± 0.02 (987)	1.26 ± 0.03 (973)	−0.16 ± 0.03	<0.0001
HDL cholesterol, mmoles/liter	1.41 ± 0.01 (987)	1.30 ± 0.01 (972)	0.11 ± 0.02	<0.0001
LDL cholesterol, mmoles/liter	2.21 ± 0.03 (985)	2.98 ± 0.03 (965)	−0.77 ± 0.04	<0.0001
Other variables, median (IQR) (n)				
CK, units/liter	94 (69, 135) (986)	84 (60, 118) (971)	–	<0.0001
CRP, mg/liter	2.59 (0.94, 6.08) (987)	3.60 (1.47, 7.49) (972)	–	<0.0001
ALT, units/liter	24.0 (17.4, 33.0) (987)	20.8 (15.5, 27.7) (973)	–	<0.0001
AST, units/liter	36.2 (28.5, 46.7) (987)	35.6 (27.5, 46.6) (973)	–	0.185
Clinical outcomes, median (IQR) (n)				
EQ-5D score	0.66 (0.52, 0.80) (1,062)	0.70 (0.52, 0.80) (1,079)	–	0.301
HAQ DI score	1.25 (0.38, 1.88) (1,105)	1.25 (0.38, 1.97) (1,124)	–	0.644
DAS28 score	3.3 (2.3, 4.4) (997)	3.3 (2.4, 4.4) (1,023)	–	0.515
DAS28 category, no./no. available (%)				0.368
High (>5.1)	133/997 (13.3)	129/1,023 (12.6)	–	
Moderate (>3.2, ≤5.1)	391/997 (39.2)	428/1,023 (41.8)	–	
Low (>2.6, ≤3.2)	153/997 (15.3)	171/1,023 (16.7)	–	
Remission (≤2.6)	320/997 (32.1)	295/1,023 (28.8)	–	

* All patients attending the end-of-trial visit (1,211 per arm) were invited to provide blood samples for measurement of lipid levels and other variables. Of these patients, ~83% in each study arm agreed. HDL = high-density lipoprotein; LDL = low-density lipoprotein; IQR = interquartile range; CK = creatine kinase; CRP = C-reactive protein; ALT = alanine transaminase; AST = aspartate transaminase; EQ-5D = EuroQol 5-domain; HAQ DI = Health Assessment Questionnaire disability index.
† By *t*-test for lipid levels, by Kendall's tau-b for Disease Activity Score in 28 joints (DAS28) category, and by Mann-Whitney tests for all other comparisons.

ence observed in TRACE RA, although disappointing, is probably not particularly poor. Prespecified adjusted analyses for baseline differences, compliance, and nonstudy statin use resulted in an HR of 0.60 (95% CI 0.32, 1.15) ($P = 0.127$).

From a clinical perspective, the safety outcomes are as important as CVE reduction. RA patients typically have multiple comorbidities (39) and polypharmacy (40), often with potentially hepatotoxic drugs. Virtually all participants in TRACE RA were receiving potentially hepatotoxic therapies such as methotrexate, but all patients receiving methotrexate were also prescribed folic acid. The 40 mg daily dose of atorvastatin is also of interest, as there are few randomized data on safety for this dose. Reassuringly, the type and severity of adverse events, the rate of hospitalizations, elevations of liver or muscle enzyme levels, incidence of myalgia, and worsening of RA were all similar in the 2 arms. These results suggest that atorvastatin 40 mg (and lower doses) is safe to use in patients with RA who are already receiving DMARD therapy.

Clinically assessed RA disease activity, severity, and quality of life were not significantly different between the 2 groups at the end of the trial. However, consistent with data from other studies (16,17), levels of CRP were significantly lower, by ~1 mg/liter, in the atorvastatin group than in the placebo group. This difference is unlikely to be clinically significant in the context of RA disease activity.

Since TRACE RA was terminated early because the CVE rate was much lower than expected, it is not surprising that the HR for the primary end point was not significant. The observed number of primary outcomes provides <20% power to detect the relative risk reduction of 32% specified in the final protocol and provides adequate power (>80%) only to detect a relative risk reduction of >68%. The results for the primary outcome are therefore best represented as the estimated HR and its associated confidence interval. When the trial was designed (2002–2004), the assumption of a 1.6–1.8% annual event rate seemed, if anything, conservative. A meta-analysis of mortality studies in RA published prior to 2005 demonstrated a meta–standardized mortality ratio of 1.5 (1). Annual CVE rates ranged from 2.5–5% (26). Possible explanations for the lower-than-expected observed event rate in TRACE RA include: 1) event rates in randomized trials are always lower than in observational studies and the "healthy volunteer" effect may have been more pronounced than usual; 2) TRACE RA, by design, excluded patients with the highest baseline CVE risk since these patients were already being treated or had a recommendation for a statin; 3) TRACE RA participants were

younger than in other statin trials and were predominantly female (as expected from RA disease demographics); and 4) <20% of participants had high disease activity at baseline.

There is increasing evidence that good disease control reduces the progression of subclinical atherosclerosis in RA patients (41,42) and is associated with better cardiovascular outcomes. Therefore, an additional explanation for the low event rate observed in TRACE RA might have been a significant increase in the use of DMARDs, particularly biologic DMARDs, during the course of the trial. However, this was not the case. The use of prednisolone (in terms of frequency and average daily dosage), conventional synthetic DMARDs, and biologic DMARDs at baseline was balanced between the atorvastatin and placebo groups and remained so during the trial. There was not any significant increase in the use of biologic DMARDs during the trial in either group (Supplementary Section 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40892/abstract). Some recent studies suggest a modest decline in CVE rates, mirroring those observed in the general population (43,44), while others demonstrate a very substantial decline in CVE rates in RA (45). Although it is possible that CVEs may have been missed in both the atorvastatin and placebo arms, we believe this is unlikely due to the robust CVE capture system including regular patient review (80% of the patients attended the end-of-trial visit in both arms) and linkage with several national electronic data sources. Information from data linkage was available for all patients.

Overall, the findings of TRACE RA have important implications for clinical practice and research. The large randomized statin trials have shown that statin therapy reduces CVE risk by approximately one-third, regardless of the level of background risk. Nevertheless, most guidelines recommend therapy only for those whose estimated individual 10-year or lifetime risk falls above a certain threshold, for reasons of cost and risk/benefit ratio (46). TRACE RA suggests that contemporary RA patients are likely to derive the same level of benefit from statins as other populations. However, the low event rate shows that there is a sizeable population of RA patients (even among those older than 50 years or with >10 years of disease duration) who have a relatively low CVD risk. This finding does not support prescribing statins to all RA patients, one of the main questions addressed by this trial. Instead, the decision to prescribe should be based on assessment of the individual RA patient's risk using, at present, the relevant national or international recommendations and risk assessment tools (9), while disease-specific algorithms are developed and validated (47). In terms of future research, TRACE RA provides information about effect and sample sizes that may be helpful in the design of future trials investigating CVD prevention strategies in RA, whether these are based on cardiovascular interventions, intensive inflammatory disease control, or both.

In conclusion, TRACE RA suggests that atorvastatin 40 mg daily is safe for the primary prevention of CVEs in patients with RA and appears to confer a similar degree of risk reduction in these patients as in other populations. CVE rates are decreasing in this population. This finding requires further investigation and does not support a primary prevention strategy involving statin use in all RA patients.

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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. Kitas 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. Kitas, Nightingale, Armitage, Sattar, Belch, Symmons.

Acquisition of data. Kitas, Sattar, Belch, Symmons.

Analysis and interpretation of data. Kitas, Nightingale, Armitage, Sattar, Belch, Symmons.

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APPENDIX A: THE TRACE RA CONSORTIUM

For the TRACE RA Consortium, members of the Trial Management Committee were as follows: chief investigator, Dr. George D. Kitas (University of Manchester and Dudley Group NHS Foundation Trust); lead investigators, Dr. Jill J. F. Belch (University of Dundee) and Dr. Deborah P. M. Symmons (University of Manchester); trial managers, Dr. Hawys Williams (University of Manchester), Mrs. Shobna Vasishta (University of Dundee), and Mrs. Rebecca Storey (Dudley Group NHS Foundation Trust); trial statistician, Dr. Peter Nightingale (University Hospitals Birmingham); senior investigators, Dr. Ian Bruce (University of Manchester), Dr. Paul Durrington (University of Manchester), Dr. Iain McInnes (University of Glasgow), Dr. Naveed Sattar (University of Glasgow), Dr. Deva Situnayake (City Hospital, Birmingham), and Dr. Allan Struthers (University of Dundee).

Members of the Trial Steering Committee were as follows: chairman, Dr. Gordon Lowe (University of Glasgow); independent members, Dr. Jane Armitage (University of Oxford), Dr. Keith Fox (University of Edinburgh Centre for Cardiovascular Science), and Dr. Dorian Haskard (Eric Bywaters Centre for Vascular Inflammation, Imperial College London); Arthritis Research UK member, Ms Caroline Dore; lay member, Ms Ailsa Bosworth (National Rheumatoid Arthritis Society), investigators, Dr. George D. Kitas (University of Manchester and Dudley Group NHS Foundation Trust), Dr. Jill J. F. Belch (University of Dundee), Dr. Deborah P. M. Symmons (University of Manchester), and Dr. Hawys Williams (University of Manchester).

Members of the Data Monitoring Committee were as follows: chairman, Dr. Michael Frenneaux (University of Aberdeen); Dr. Christopher Edwards (University of Southampton), Dr. Jonathan Emberson (University of Oxford Clinical Service Unit), and Dr. Deborah Bax (University of Sheffield).

Members of the Endpoints Committee were as follows: chairman, Dr. Stuart Cobbe (University of Glasgow); Dr. David Stott (University of Glasgow), Dr. Roger Sturrock (University of Glasgow), and Dr. Peter Macfarlane (University of Glasgow).

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Risk Factors for Major Adverse Cardiovascular Events in Phase III and Long-Term Extension Studies of Tofacitinib in Patients With Rheumatoid Arthritis

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Objective. Tofacitinib is an oral JAK inhibitor for the treatment of rheumatoid arthritis (RA). This study was undertaken to evaluate the risk of major adverse cardiovascular events (MACE) in patients with RA receiving tofacitinib.

Methods. Data were pooled from patients with moderately to severely active RA receiving ≥1 tofacitinib dose in 6 phase III and 2 long-term extension studies over 7 years. MACE (myocardial infarction, stroke, cardiovascular death) were independently adjudicated. Cox regression models were used to evaluate associations between baseline variables and time to first MACE. Following 24 weeks of tofacitinib, changes in variables and time to future MACE were evaluated after adjusment for age, baseline values, and time-varying tofacitinib dose. Hazard ratios and 95% confidence intervals were calculated.

Results. Fifty-two MACE occurred in 4,076 patients over 12,873 patient-years of exposure (incidence rate 0.4 patients with events per 100 patient-years). In univariable analyses of baseline variables, traditional cardiovascular risk factors and glucocorticoid and statin use were associated with MACE risk; disease activity and inflammation measures were not. In subsequent multivariable analyses, baseline age, hypertension, and the total cholesterol to high-density lipoprotein (HDL) cholesterol ratio remained significantly associated with risk of MACE. After 24 weeks of treatment, an increase in HDL cholesterol and a decrease in the total to HDL cholesterol were associated with decreased MACE risk; changes in total cholesterol, low-density lipoprotein (LDL) cholesterol, and disease activity measures were not. Increased erythrocyte sedimentation rates trended with increased future MACE risk.

Conclusion. In this post hoc analysis, after 24 weeks of tofacitinib treatment, increased HDL cholesterol, but not increased LDL cholesterol or total cholesterol, appeared to be associated with lower future MACE risk. Further data are needed to test the cardiovascular safety of tofacitinib.

INTRODUCTION

Cardiovascular disease (CVD) is one of the most common comorbidities in patients with rheumatoid arthritis (RA), with a prevalence of 9.3% for any CV event (1). Compared with the general population, patients with RA have an increased risk of CVD (2) and higher rates of CVD-induced mortality (3). As a consequence, CVD is the leading cause of death in patients with RA, accounting for almost 31% of mortality (4).

Traditional CV risk factors, such as hypertension, smoking, and type 2 diabetes mellitus, contribute to the increased risk of CVD among patients with RA as they do in the general

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population (5,6); however, after adjustment for traditional risk factors, a proportion of the increased CV risk in patients with RA remains unexplained (7). A key driver of increased CV risk in RA appears to be the high systemic inflammatory burden. There is an apparent inverse relationship between inflammation and lipid levels in patients with RA, such that increased inflammation is associated with reduced lipid levels and also with changes in the composition of lipid profiles (8).

The relationship between lipid levels and CV risk in patients with RA is complex. Within the general population, an increased risk of CVD is associated with high serum total cholesterol, high serum low-density lipoprotein (LDL) cholesterol, and low serum high-density lipoprotein (HDL) cholesterol levels (9,10). Individuals with an elevated total cholesterol to HDL cholesterol ratio have an increased risk of developing CVD, and the total cholesterol to HDL cholesterol ratio has been shown to be a reliable predictor of CVD risk (11). In contrast, in patients with active RA, increased risk of CVD has been associated with relatively lower levels of serum total cholesterol and LDL cholesterol, as well as HDL cholesterol. These lower lipid levels may be driven by inflammation associated with RA (12,13). In RA, changes are also seen in the composition and function of HDL particles; previous studies have shown that active disease is associated with impaired antioxidative function of HDL, decreased HDL-mediated cholesterol efflux, and alterations in the levels and function of several HDL-associated proteins (14–16).

Overall CV risk in patients with RA is affected by disease activity and likely by the resultant systemic inflammation. In a previous study by Maradit-Kremers et al, high clinical disease activity, as measured by 3 erythrocyte sedimentation rates (ESRs) of ≥60 mm/hour, correlated with a 2-fold increased risk of death from CVD in an inception cohort of 603 patients (17). In the same cohort of 172 patients who developed congestive heart failure (CHF), the proportion of patients with an ESR of ≥40 mm/hour was highest during the 6-month period immediately preceding CHF onset (18). Other studies have demonstrated associations of markers of inflammation with subclinical atherosclerosis (19,20). Finally, in a post hoc analysis of data from patients with RA treated with the interleukin-6 (IL-6) receptor inhibitor tocilizumab, increases in disease activity measures, such as the Disease Activity Score in 28 joints (DAS28) score (21) and joint counts, were associated with risk of future major adverse CV events (MACE), while changes in lipid levels were not (22).

It is unclear whether there are differential effects of RA therapeutics on CV outcomes. In previous studies, treatment with disease-modifying antirheumatic drugs (DMARDs), such as methotrexate (MTX) and tumor necrosis factor inhibitors (TNFi), has been shown to reduce the probability of CV events (23,24). In the ENTRACTE study, which evaluated CV events in patients receiving tocilizumab versus etanercept, 83 MACE occurred over 4,900 patient-years in the tocilizumab arm, compared with 78 MACE over 4,891 patient-years in the etanercept arm (hazard ratio 1.05 [95% confidence interval 0.77–1.43]) (25). Taken together, these findings highlight the importance of investigating CV risk factors in patients with RA, as well as the effects of therapies for RA on the risk of MACE.

Tofacitinib is an oral JAK inhibitor for the treatment of RA. Formal comparison of the effect of tofacitinib on the risk of MACE is currently being investigated in a phase IIIb/IV prospective comparative study with TNFi (NCT02092467) (26). MACE is also being evaluated in patients receiving tofacitinib versus biologic DMARDs and conventional synthetic DMARDs in a real-world setting using data from the Corrona registry and the following European registries: British Society for Rheumatology Biologics Register (BSRBR), Anti-Rheumatic Therapy in Sweden (ARTIS), Rheumatoide Arthritis: Beobachtung der Biologika-Therapie (RABBIT), and Base de Datos de Productos Biológicos de la Sociedad Española de Reumatología (BIOBADASER). However, during phase II studies, tofacitinib treatment was associated with increased LDL cholesterol and HDL cholesterol levels in patients with RA (27–31). Consequently, phase III and long-term extension (LTE) studies included adjudication of potential CV events and deaths. In a pooled analysis of phase III data, tofacitinib treatment was associated ~10–20% of the increases in total cholesterol, HDL cholesterol, and LDL cholesterol levels from baseline to week 4, which were maintained to week 24. Changes in lipid levels stabilized after 12 weeks of tofacitinib treatment, and this was associated with a low incidence of CV events (32). The objective of the present post hoc analysis was to determine whether changes in lipids levels following administration of tofacitinib were associated with an increased risk of MACE in patients with RA enrolled in phase III and LTE studies.

PATIENTS AND METHODS

Design of phase III and LTE studies. Patients with RA participated in 1 of 6 randomized, double-blind phase III studies and/or 2 open-label LTE studies (for study names and details, see Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40911/abstract). Inclusion and exclusion criteria have been previously described (33–41). Briefly, patients were age ≥18 years, with active RA that fulfilled the American College of Rheumatology 1987 criteria (42), and had active disease at screening and baseline.

All studies were conducted in compliance with the Declaration of Helsinki and the Good Clinical Practice Guidelines established by the International Conference for Harmonisation. The study protocols were approved by the institutional review board or independent ethics committee at each center. All patients provided written informed consent.

Two of the phase III studies evaluated tofacitinib 5 and 10 mg twice daily as monotherapy: ORAL Solo (NCT00814307), a 6-month study of tofacitinib versus placebo in patients with an inadequate response to DMARDs (34) and ORAL Start

(NCT01039688), a 24-month study of tofacitinib versus MTX in MTX-naive patients (36).

The remaining 4 phase III studies evaluated tofacitinib 5 and 10 mg twice daily in combination with conventional synthetic DMARDs versus placebo, as follows: ORAL Scan (NCT00847613), a 24-month study of tofacitinib in patients with an inadequate response to MTX who were receiving background MTX (37); ORAL Standard (NCT00853385), a 12-month study in patients with an inadequate response to MTX who were receiving background MTX (38); ORAL Sync (NCT00856544), a 12-month study of tofacitinib in combination with conventional synthetic DMARDs in patients with an inadequate response to DMARDs (35); and ORAL Step (NCT00960440), a 6-month study in patients with an inadequate response to TNFi who were receiving background MTX (33).

The 2 open-label LTE studies (ORAL Sequel; NCT00413699 [database not locked at the time of analysis] and NCT00661661) enrolled patients who had completed phase I, phase II, or phase III index studies of tofacitinib. Regardless of treatment assignment in the qualifying index study, patients began treatment in the LTE studies with tofacitinib 5 or 10 mg twice daily (with the exception of Chinese and Japanese patients who received 5 mg twice daily per protocol), and were subsequently allowed to switch doses (39–41). In all studies, adverse events were recorded verbatim by the investigator and coded according to the Medical Dictionary for Regulatory Activities (MedDRA), version 13.0.

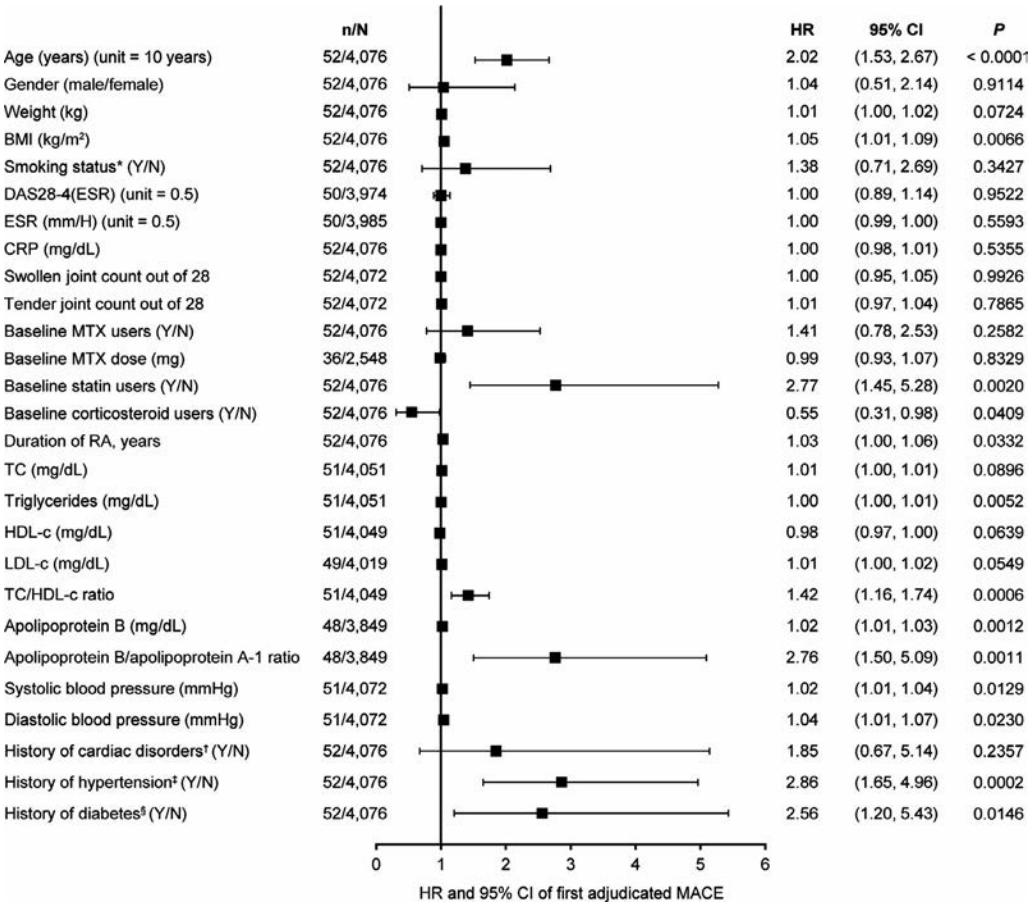

Figure 1. Univariable analyses of associations between baseline variables and the occurrence of major adverse cardiovascular events (MACE) during tofacitinib treatment. For all continuous variables, unit = 1 unless specified otherwise; in unit = x, "x" is the change in the continuous variable corresponding to which the change in hazards is observed. * = smoking status as recorded at baseline; † = including any of the Medical Dictionary for Regulatory Activities–coded terms: angina pectoris, arrhythmia, atrial flutter, atrial fibrillation, first-degree atrioventricular block, left bundle branch block, cardiac failure, cardiac failure congestive, cardiac valve disease, cardiomegaly, cardiomyopathy, coronary artery disease, hypertensive cardiomyopathy, left ventricular hypertrophy, mitral valve incompetence, mitral valve prolapse, valve prolapse, myocardial infarction, palpitations, sinus bradycardia, sinus tachycardia, tachycardia, tachycardia paroxysmal, tricuspid valve incompetence, and ventricular extrasystole; ‡ = based on prior medical history; § = based on both use of diabetes medication and medical history. n = number of patients with future MACE for each baseline variable; N = number of patients included in the analysis for each baseline variable; HR = hazard ratio; 95% CI = 95% confidence interval; BMI = body mass index; DAS28-4 (ESR) = 4-variable Disease Activity Score in 28 joints using the erythrocyte sedimentation rate; CRP = C-reactive protein; MTX = methotrexate; RA = rheumatoid arthritis; TC = total cholesterol; HDL-c = high-density lipoprotein cholesterol; LDL-c = low-density lipoprotein cholesterol.

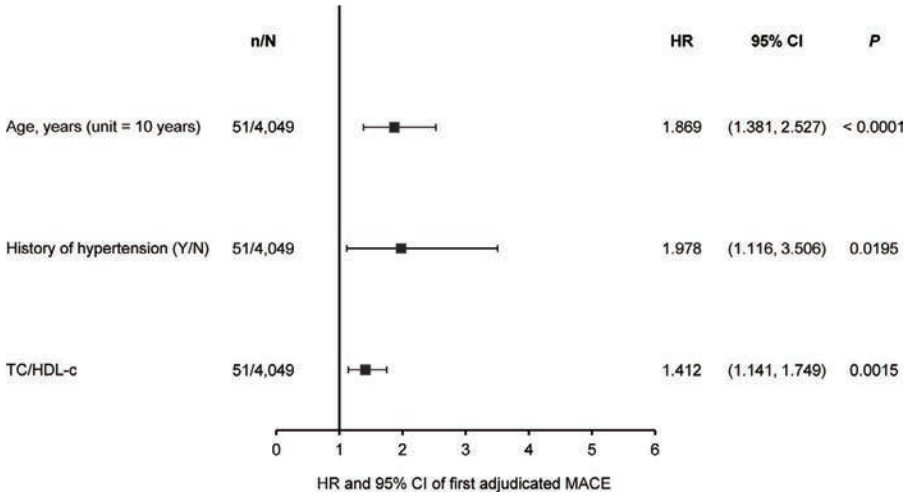

Figure 2. Multivariable analyses of associations between baseline variables and the occurrence of MACE during tofacitinib treatment. For all continuous variables, unit = 1 unless specified; in unit = x, "x" is the change in the continuous variable corresponding to which the change in hazards is observed. Only variables with significant associations with occurrence of MACE are shown; this analysis also included baseline body mass index and time-varying tofacitinib dosage (both not significant). See Figure 1 for definitions.

Post hoc analyses. The current post hoc analyses included all patients with RA who received at least 1 dose of tofacitinib 5 or 10 mg twice daily in the phase III and LTE studies and had exposure after week 24 (patients who had MACE before week 24, or who had withdrawn or completed the study before week 24 were excluded). As patients in the LTE studies were allowed to switch doses, patients were assigned into 5 or 10 mg twice daily treatment groups based on their average total daily dose (TDD; calculated by adding all doses received by each patient, and dividing by the number of days a dose was received). Patients were assigned to the 5 mg twice daily group if the TDD was <15 mg/day, and to the 10 mg twice daily group if it was ≥15 mg/day (43).

MACE, defined as any myocardial infarction (MI), cerebrovascular event (stroke), or CV death (defined as death caused by coronary, cerebrovascular, or cardiac events), were identified during the assessment of safety end points during the phase III and LTE studies. Patients were evaluated until withdrawal from the

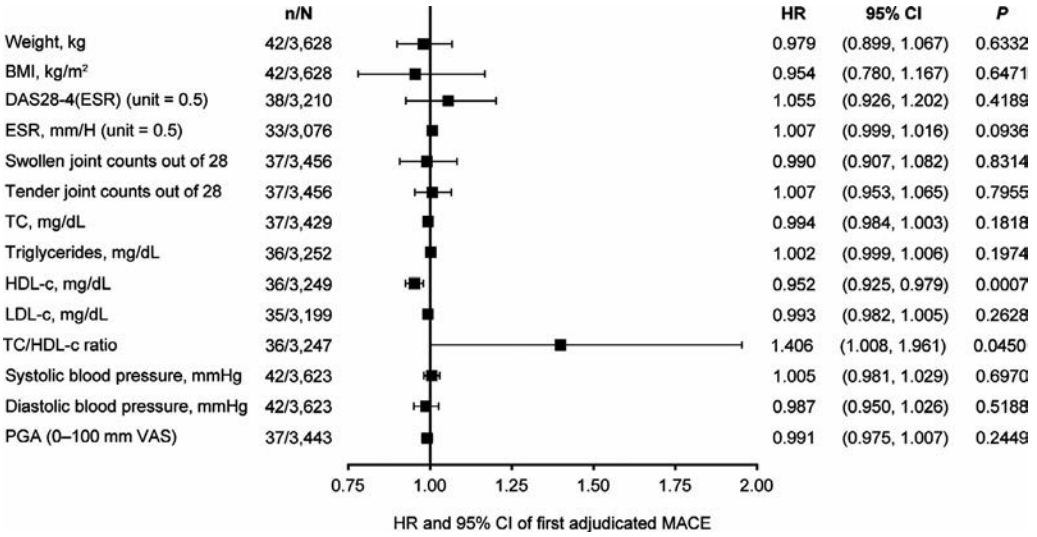

Figure 3. Multivariable analyses of associations between changes in variables after 24 weeks of tofacitinib treatment and the risk of future MACE. For each variable listed, a Cox regression model was fit, with change in the variable at week 24, the variable at baseline, age at baseline, and time-varying dosage as predictors. In this model, only patients with exposure after week 24 were considered (i.e., patients who had MACE before week 24 or who had withdrawn or completed the study by week 24 were excluded). Patients with missing data for the week-24 variable were excluded from the analysis of that variable (no imputation method). The HR corresponds to increased risk of MACE per 1-unit increase in the parameter. n = number of patients with future MACE for each predictor; N = number of patients included in the analysis for each predictor; PGA = patient global assessment; VAS = visual analog scale (see Figure 1 for other definitions).

study, completion of the study, or the initial occurrence of MACE, whichever occurred first. In the event of multiple occurrences of MACE, only the first was counted.

Only adjudicated events were included in the analysis. Adjudication of MACE started in October 2009 with the chartering of the CV Safety Event Adjudication Committee (CVSEAC). Events reported prior to October 2009 were not adjudicated and are therefore not included in this analysis. The CVSEAC was retired in November 2013 and a new committee, the CV Event Adjudication Committee, was established. Both committees comprised independent, external experts in the fields of CV and/or neurovascular disease.

Statistical analysis. Baseline demographic and disease characteristics were summarized descriptively for patients with and without MACE. Cox regression models were used to evaluate the associations between baseline (pre-tofacitinib) covariate values and time to first MACE, in univariable analyses (each covariate assessed singly) (Figure 1) and multivariable analyses (several covariates included together in 1 model) (Figures 2 and 3). The covariates included in the multivariable analysis were age, history of hypertension, total cholesterol to HDL cholesterol ratio, baseline body mass index (BMI), and time-varying tofacitinib dosage (Figure 2). A second multivariable analysis was carried out using the same variables, but included history of diabetes mellitus instead of baseline BMI. A final model was selected via backward elimination with stay criteria at 15%. Associations were expressed as hazard ratios and 95% confidence intervals.

Cox regression models were also used to separately evaluate the associations between changes in the predictors of MACE from baseline to week 24 and the time to future development of MACE (defined as the first occurrence of MACE after 24 weeks), after adjustment for age, baseline values of covariates, and time-varying tofacitinib dosage in a multivariable analysis (Figure 3). For each risk factor, the analysis included age, baseline value, and change from baseline to week 24; other risk factors were not included in the same model.

The time-varying tofacitinib dosage was a time-dependent covariate, and was determined by the time of first onset of MACE. If the first onset of MACE occurred during the index study, the randomized dosage in the index study was used. If the first onset of MACE occurred during the LTE study, then the average of the tofacitinib dosage (5 or 10 mg twice daily) was used.

For all analyses of covariates, unadjusted *P* values less than or equal to 0.05 were considered significant. No multiplicity adjustment was carried out, as this was a post hoc analysis for exploratory purposes.

RESULTS

Tofacitinib exposure and patient disposition. The analysis population included 4,076 patients, representing a total of 12,932 patient-years of tofacitinib exposure. In total, 52 patients had adjudicated MACE, resulting in a total of 12,873 patient-years of exposure for the event and an incidence rate of 0.4 patients with events per 100 patient-years of exposure (95% confidence interval 0.3–0.5), as of March 2015. There were 12 cases of CV death (cardiac death, n = 8; cerebrovascular death, n = 2; non-cardiac/other vascular death, n = 2 [acute cardiac failure, n = 1; cerebral hemorrhage, n = 1]), 19 cases of nonfatal MI, and 23

Table 1. Baseline demographic characteristics of patients with and patients without MACE*

Characteristic	No MACE (n = 4,024)	Adjudicated MACE (n = 52)
Age, mean ± SD years	52.7 ± 11.9	60.2 ± 10.4
Female, no. (%)	3,334 (82.9)	43 (82.7)
BMI, mean ± SD kg/m^2	27.0 ± 6.4	29.2 ± 8.2
History of CHD, no. (%)	21 (0.5)	0 (0.0)
History of cardiac disorders, no. (%)†	199 (4.9)	4 (7.7)
History of diabetes mellitus, no. (%)‡	307 (7.6)	8 (15.4)
Abnormal BP, no. (%)§	334 (8.3)	4 (7.7)
History of hypertension, no. (%)	1,358 (33.7)	30 (57.7)
Smoking status, no. (%)		
Never	676 (16.8)	14 (26.9)
Current	678 (16.8)	11 (21.2)
Ex-smoker¶	2,667 (66.3)	27 (51.9)
Concomitant medications		
Glucocorticoids, no. (%)	1,909 (47.4)	18 (34.6)
Statins, no. (%)	420 (10.4)	12 (23.1)
NSAIDs, no. (%)	2,817 (70.0)	34 (65.4)
MTX, no. (%)	2,443 (60.7)	36 (69.2)
MTX dose, mean ± SD mg	15.0 ± 4.7	14.9 ± 4.0

* MACE = major adverse cardiovascular event; BMI = body mass index; CHD = coronary heart disease; NSAIDs = nonsteroidal anti-inflammatory drugs; MTX = methotrexate.
† Including any of the Medical Dictionary for Regulatory Activities–coded terms: angina pectoris, arrhythmia, atrial flutter, atrial fibrillation, first-degree atrioventricular block, left bundle branch block, cardiac failure, cardiac failure congestive, cardiac valve disease, cardiomegaly, cardiomyopathy, coronary artery disease, hypertensive cardiomyopathy, left ventricular hypertrophy, mitral valve incompetence, mitral valve prolapse, valve prolapse, myocardial infarction, palpitations, sinus bradycardia, sinus tachycardia, tachycardia, tachycardia paroxysmal, tricuspid valve incompetence, and ventricular extrasystole.
‡ Based on both use of diabetes medication and medical history.
§ Defined as systolic blood pressure (BP) of >150 mm Hg or diastolic BP of >90 mm Hg.
¶ Defined as those who had smoked previously but were not smokers at baseline.

Table 2. Baseline disease characteristics in patients with and patients without MACE*

Characteristic	No MACE (n = 4,024)	Adjudicated MACE (n = 52)
Duration of RA, years	7.7 ± 7.9	10.1 ± 8.8
4-variable DAS28-ESR	6.3 ± 1.1	6.3 ± 1.3
Tender joint count	14.1 ± 7.3	14.3 ± 7.5
Swollen joint count	10.4 ± 5.6	10.3 ± 5.9
Total cholesterol, mg/dl	198.3 ± 42.1	208.2 ± 48.9
HDL cholesterol, mg/dl	59.4 ± 16.9	55.3 ± 16.0
LDL cholesterol, mg/dl	113.9 ± 34.2	123.3 ± 43.2
Total cholesterol to HDL cholesterol ratio	3.5 ± 1.1	4.0 ± 1.5
Triglycerides, mg/dl	125.3 ± 72.6	152.1 ± 86.9
Apolipoprotein A-1, mg/dl	153.6 ± 31.2	149.4 ± 27.8
Apolipoprotein B, mg/dl	94.4 ± 24.7	105.8 ± 29.4
CRP, mg/dl	17.1 ± 22.7	15.7 ± 16.9
ESR, mm/hour	50.4 ± 26.9	47.9 ± 23.8

* Values are the mean ± SD. MACE = major adverse cardiovascular event; RA = rheumatoid arthritis; DAS28-ESR = Disease Activity Score in 28 joints using the erythrocyte sedimentation rate; HDL = high-density lipoprotein; LDL = low-density lipoprotein; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate.

cases of nonfatal stroke. Two patients had multiple categories of MACE (1 patient had a nonfatal stroke and a nonfatal MI, and 1 patient had a nonfatal stroke, followed by cerebrovascular death).

The baseline demographic characteristics of patients with and without adjudicated MACE are shown in Table 1. Overall, compared with patients without MACE, patients with MACE were older (mean age 52.7 years versus 60.2 years), had a higher mean BMI (27.0 versus 29.2 kg/m^2), were more likely to have a history of diabetes mellitus (7.6% versus 15.4%) or hypertension (33.7% versus 57.7%), and were more likely to be receiving concomitant statins (10.4% versus 23.1%).

The baseline disease characteristics of patients with and without adjudicated MACE are shown in Table 2. Compared with patients without MACE, patients with MACE had a longer mean disease duration (7.7 years versus 10.1 years), slightly higher mean total cholesterol levels (198.3 versus 208.2 mg/dl), LDL cholesterol levels (113.9 versus 123.3 mg/dl), total cholesterol to HDL cholesterol ratio (3.5 versus 4.0), and triglyceride levels (125.3 versus 152.1 mg/dl), and slightly lower HDL cholesterol levels (59.4 versus 55.3 mg/dl) at baseline.

Baseline predictors of MACE. In univariable analyses, risk of MACE was significantly associated with older age, higher BMI, statin use, or longer duration of RA. Other baseline predictors that were associated with a significantly increased risk of MACE were elevated levels of triglycerides, higher total cholesterol to HDL cholesterol ratio, elevated apolipoprotein B (Apo B) levels, higher Apo B to Apo A-1 ratio, abnormal blood pressure, history of hypertension, and history of diabetes mellitus (Figure 1).

In contrast, glucocorticoid use was associated with a significantly lower risk of MACE (mean ± SD glucocorticoid dosage at baseline 3.5 ± 4.4 and 3.3 ± 6.4 mg/day for patients receiving tofacitinib 5 mg and 10 mg twice daily, respectively, and 3.9 ± 4.1 and 3.4 ± 3.9 mg/day for patients receiving placebo who advanced to tofacitinib 5 mg and 10 mg twice daily, respectively). Baseline disease activity and inflammation measures, including the 4-variable DAS28 using the ESR (21), ESR and C-reactive protein level, and swollen and tender joint counts, were not significantly associated with MACE risk (Figure 1).

In separate multivariable analyses, patient age, history of hypertension, and the total cholesterol to HDL cholesterol ratio continued to be significantly associated with an increased risk of MACE (Figure 2). The findings of the multivariable analyses were consistent across all selection methods used (backward, forward, and stepwise selection). Time-varying tofacitinib dosage and baseline BMI were not found to be associated with risk of MACE in this analysis. Furthermore, in a second multivariable analysis that included history of diabetes mellitus in place of baseline BMI, history of diabetes mellitus was also not associated with MACE risk.

Changes in predictors of MACE between baseline and week 24. Previously, tofacitinib treatment was found to be associated with increases in total cholesterol, HDL cholesterol, and LDL cholesterol levels (32), and baseline and week 24 values for the other covariates included in the analysis are shown in Supplementary Table 2 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40911/abstract). The effects of 24 weeks of treatment with tofacitinib (and tofacitinib-associated increases in lipid levels) on the risk of future MACE were assessed by multivariable analyses (Figure 3). After adjustment for age, time-varying tofacitinib dosage, and the baseline value for each variable, increases in HDL cholesterol levels and decreases in the total cholesterol to HDL cholesterol ratio after 24 weeks of tofacitinib were associated with a reduced risk of MACE. In contrast, increases in total cholesterol levels and LDL cholesterol levels were not associated with increased risk of future MACE. A trend was observed between increases in ESR after 24 weeks of tofacitinib treatment and an increase in the future risk of MACE, although this did not reach statistical significance. Changes in disease activity measures, such as the 4-variable DAS28 using the ESR, and swollen and tender joint counts, were not associated with increased risk of future MACE.

DISCUSSION

In this post hoc analysis of data from phase III and LTE studies of tofacitinib, we assessed the associations between

baseline variables and time to first MACE as well as the changes in variables after 24 weeks of tofacitinib treatment and time to future MACE.

In univariable analyses of baseline variables, an increased risk of MACE was associated with the presence of traditional risk factors at baseline (such as older age, higher BMI, abnormal blood pressure, and history of either hypertension or diabetes mellitus), elevated baseline triglyceride and Apo B levels, and higher baseline Apo B to Apo A-1 ratio and total cholesterol to HDL cholesterol ratio. In subsequent multivariable analysis of baseline measures, age, history of hypertension, and the total cholesterol to HDL cholesterol ratio continued to be associated with an increased risk of MACE.

The analyses of baseline variables in this study are consistent with large population studies of CV outcomes, reinforcing the finding that traditional CV risk factors are important to CV risk in patients with RA (17). They also reinforce the findings from previous studies that have demonstrated the association between MACE risk and both traditional risk factors and Apo B to Apo A-1 and total cholesterol to HDL cholesterol ratios in patients with RA (22,44). In addition, the European League Against Rheumatism guidelines on management of CV risk in patients with RA suggest that the total cholesterol to HDL cholesterol ratio is a particularly important indicator of CV risk (45).

Risk of MACE was not associated with measures of disease activity or inflammation at baseline in this analysis. A previous study by Rao et al, which investigated risk factors for MACE in patients with RA during treatment with tocilizumab, also showed no association between risk of MACE and baseline measures of inflammation, but did demonstrate an association between the risk of MACE and baseline disease activity measures (22).

The present analyses also suggest an association between baseline statin use and risk of future MACE. However, this observation was potentially due to confounding by indication, as patients with RA who had the highest CV risk as judged by their physicians were more likely to be taking a statin at baseline.

In a previous pooled analysis, which assessed lipid concentrations in the same patient population as the current analysis, increased total, HDL, and LDL cholesterol levels were observed in patients receiving tofacitinib (32). We evaluated the effects of 24 weeks of tofacitinib treatment on risk of MACE in multivariable analyses and found that increases in HDL cholesterol levels and decreases in the total cholesterol to HDL cholesterol ratio were associated with reduced risk of future MACE in multivariable analyses, while increases in total cholesterol and LDL cholesterol levels were not. Rao et al also found no association of CV risk with total cholesterol and LDL cholesterol levels following 24 weeks of tocilizumab treatment. However, in contrast with our findings, increases in HDL cholesterol were not associated with risk of MACE following tocilizumab treatment in their study (22).

Higher HDL cholesterol levels have previously been associated with a decreased risk of MI in patients with active RA (46,47). However, cholesteryl ester transfer protein inhibitors, which also increase HDL cholesterol levels, have failed to confer consistent reductions in CV events in clinical trials in the general population (48). Therefore, the association between modulation of HDL cholesterol level and risk of MACE remains unresolved. Consequently, additional consideration should be given to mechanisms independent of increases in HDL cholesterol levels, such as improved HDL particle function, which has been linked to CV outcomes in the general population (49). Previous studies have suggested that tofacitinib may improve the function of HDL particles via increases in the activity of the HDL-associated enzyme paraoxonase 1 (50). In addition, during the MEASURE study, "normalization" of HDL particle composition was observed in a detailed analysis of lipoprotein subfractions following tocilizumab treatment (51), and a small-scale study of rituximab in patients with RA showed a reduction in proatherogenic HDL particle composition (52). Furthermore, cholesterol ester fractional catabolism, which is higher in patients with active RA than in the general population, was reduced following tofacitinib treatment, and this reduction was also associated with improvements in HDL functional markers and correlated significantly with increased HDL cholesterol levels (53). Therefore, the effects of tofacitinib treatment on CV risk are likely to be multifactorial and may include changes in lipoproteins that are independent of cholesterol levels, such as changes in HDL composition and/or cholesterol ester fractional catabolism (53).

The present study also showed a trend toward an association of elevated ESR following tofacitinib treatment with an increased risk of future MACE, and this is consistent with findings in a population-based study of CV death, which suggested that patients with RA who have sustained elevation of ESR may have a higher risk of CV death (17). One possible explanation for this higher risk is that increased ESR may be a surrogate of failure to respond. In contrast to these findings, Rao et al (22) did not demonstrate changes in ESR to be associated with future risk of MACE following tocilizumab treatment; additionally, increases in DAS28 scores and swollen and tender joint counts were associated with higher CV risk in their study but not in ours. The disparities between our results and the findings of Rao et al may be due to differences in the pharmacokinetics/pharmacodynamics of tocilizumab and tofacitinib as well as the overall small number of CV events in both trial programs. In addition, while IL-6–induced JAK/STAT signal transduction as a driver of CV events is well established in atherosclerosis (54), inflammatory processes are myriad and complex, with both multiple upstream activators and diverse downstream targets in different cell types. Therefore, tocilizumab inhibition of IL-6 and tofacitinib inhibition of JAK/STAT may have different effects in the downstream inflammation process and may, therefore, have contrasting effects on lipid levels.

This study had a number of limitations. The data were obtained from a post hoc analysis of data pooled from 6 phase III and 2 LTE studies that were not designed to evaluate future

MACE risk. Consequently, the number of patients and exposure times in this study were low, as were the number of adjudicated MACE. The adjudication process changed in November 2013 from consensus-based to blinded independent review, and there were also changes in the individuals conducting adjudication as well as changes from committee-created definitions of events to Food and Drug Administration–approved definitions (55). These procedural changes may have introduced variables into the adjudication outputs. Also, several covariates, including disease activity and measures of inflammation, as well as time-varying confounders such as hypertension, statin use, and glucocorticoids, were only assessed at baseline in the multivariable analyses and this may not be sufficient to predict CV events with this sample size. Furthermore, we recognize that while the backward elimination method is the simplest to implement and most intuitive to explain, it may not always identify the best subset of variables or covariates for retention in the model. In this analysis, consistent results were also observed with forward and stepwise elimination methods. In addition, this study did not evaluate HDL and LDL particle size, or HDL particle function. As a consequence of these limitations, direct interpretation of the results may be confounded.

In conclusion, in pooled analyses of tofacitinib-treated patients, traditional CV risk factors at baseline appeared to be associated with an increased risk of future MACE, while no apparent association was observed between future risk of MACE and baseline disease activity or measures of inflammation. Following adjustment for age, baseline values, and time-varying tofacitinib dosage, increases in HDL cholesterol and decreases in the total cholesterol to HDL cholesterol ratio were associated with reduced future MACE risk after 24 weeks of tofacitinib treatment, while increases in LDL cholesterol and total cholesterol were not associated with future MACE risk. Increases in ESR after 24 weeks of tofacitinib therapy may be associated with increased future MACE risk and, conversely, a decrease in inflammation as measured by ESR with tofacitinib may convey some CV protection. More data are needed to confirm these findings, which could be beneficial in future profiling of tofacitinib-treated patients with RA who may be at greatest risk of MACE. The CV event safety of tofacitinib versus adalimumab or etanercept in patients with RA is currently being investigated in a phase IIIb/IV randomized open-label study (A3921133; NCT02092467) (26).

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 submitted for publication. Dr. Soma 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. DeMasi, Soma, Biswas.

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Long-Term Physical Activity and Subsequent Risk for Rheumatoid Arthritis Among Women: A Prospective Cohort Study

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Objective. To evaluate the effects of long-term physical activity on subsequent risk of rheumatoid arthritis (RA) in a prospective cohort study.

Methods. This study investigated physical activity and RA risk among women from the Nurses' Health Study II (1989–2015). Physical activity exposures and covariates were prospectively obtained using biennial questionnaires. Two rheumatologists independently reviewed the medical records of women who self-reported a new diagnosis of RA on biennial questionnaires and who screened positive for RA based on a supplemental survey. All incident RA cases met the 1987 American College of Rheumatology (ACR) or 2010 ACR/European League Against Rheumatism (EULAR) classification criteria for RA. The primary analysis investigated the long-term cumulative average number of hours spent in recreational physical activity 2–8 years prior to the RA diagnosis, a time span chosen to reduce the potential for reverse causation bias, since early RA affects physical activity prior to diagnosis. Estimated Cox regression hazard ratios (HRs) with 95% confidence intervals (95% CIs) were used to assess the risk of RA serologic phenotypes (all, seropositive, or seronegative) in relation to physical activity categories. The analyses were adjusted for body mass index (BMI) at age 18 years and time-varying potential confounders, and the mediating effect of updated BMI on the interaction between physical activity and RA risk was quantified.

Results. Among the 113,366 women analyzed, 506 incident RA cases (67.0% with seropositive RA) were identified during 2,428,573 person-years of follow-up. After adjustment for confounders, including smoking, dietary quality, and BMI at age 18 years, increasing cumulative average total hours of recreational physical activity was associated with a reduced risk of RA, as follows: HR 1.00 for <1 hour/week (reference), HR 1.00 (95% CI 0.78–1.29) for 1 to <2 hours/week, HR 0.92 (95% CI 0.72–1.17) for 2 to <4 hours/week, HR 0.84 (95% CI 0.63–1.12) for 4 to <7 hours/week, and HR 0.67 (95% CI 0.47–0.98) for ≥7 hours/week (P for trend = 0.02). The proportion of the effect between physical activity and RA mediated by updated BMI was 14.0% (P = 0.002) for all RA and 20.0% (P = 0.001) for seropositive RA.

Conclusion. Higher levels of physical activity were associated with reduced RA risk. These results add to the literature implicating metabolic factors in the pathogenesis of RA.

INTRODUCTION

While the etiology of rheumatoid arthritis (RA) is not fully elucidated, there has been substantial progress in identifying potentially modifiable risk factors. Metabolic risk factors, such as elevated body mass index (BMI) and low-quality or proinflammatory dietary intake, are associated with increased RA risk, particularly a risk of seropositive RA (1–3). These factors may have important downstream biologic consequences since they can increase the severity of systemic inflammation, resulting in autoimmunity which may, in turn, increase the susceptibility to RA (4,5).

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Physical activity is an important health behavior for reducing the risk of numerous serious chronic conditions and also improves general well-being and physical function (6). Physical activity may have beneficial biologic effects on the immune system by reducing inflammatory cytokines and regulating cellular immune function (7,8). Increasing physical activity has been recommended to patients with RA, and some studies have suggested that physical activity may also reduce the risk of subsequent RA development (9–12).

Since RA causes limitations in physical activity through joint inflammation, studies evaluating physical activity and RA risk require careful design to ensure that any associations detected are not actually attributable to symptoms of early RA that could affect the level of physical activity; this effect is referred to as "reverse causation bias" (13). In addition, since long-term physical activity likely affects BMI, longitudinal studies incorporating repeated measures of physical activity, BMI, and dietary intake are necessary to analyze these complex relationships with RA.

Therefore, we investigated long-term physical activity and RA risk using a large, prospective cohort study with long-term follow-up and repeated measures. We limited the potential for reverse causation bias by including a large time separation between the physical activity exposure measures and the window for RA case assessment. We hypothesized that increased levels of long-term physical activity would be associated with reduced RA risk, particularly the risk of seropositive RA. We further hypothesized that some of this association would be mediated by changes in BMI during follow-up.

PATIENTS AND METHODS

Study population. The Nurses' Health Study II (NHSII) is an ongoing prospective cohort study in the US that was established in 1989 and enrolled 116,430 registered nurses ages 25–42 years. Participants completed baseline and biennial questionnaires regarding lifestyle, health behaviors, medications, and diseases (14).

For this analysis, we excluded participants who reported a diagnosis of RA or other connective tissue diseases at baseline, those who had missing baseline physical activity information, or those who only answered the baseline questionnaire. After exclusions, 113,366 women were analyzed. The end of follow-up for the analysis of this study was June 1, 2015. All participants provided informed consent, and this study was approved by the Partners HealthCare institutional review board.

Assessment of physical activity. Detailed collection of data on physical activity was first included on the baseline questionnaire and was repeated approximately every 4 years (in 1991, 1997, 2001, 2005, and 2009). Women were asked to report their average weekly time spent during the preceding year in each of the following recreational activities: walking, jogging, running, bicycling, lap swimming, tennis/racquet sports, and aerobics/calisthenics. Response options for the time per week dedicated to each recreational activity included the following categories: 0 minutes, 1–4 minutes, 5–19 minutes, 20–59 minutes, 1 hour, 1–1.5 hours, 2–3 hours, 4–6 hours, 7–10 hours, and 11+ hours per week. Women also self-reported their usual walking pace as

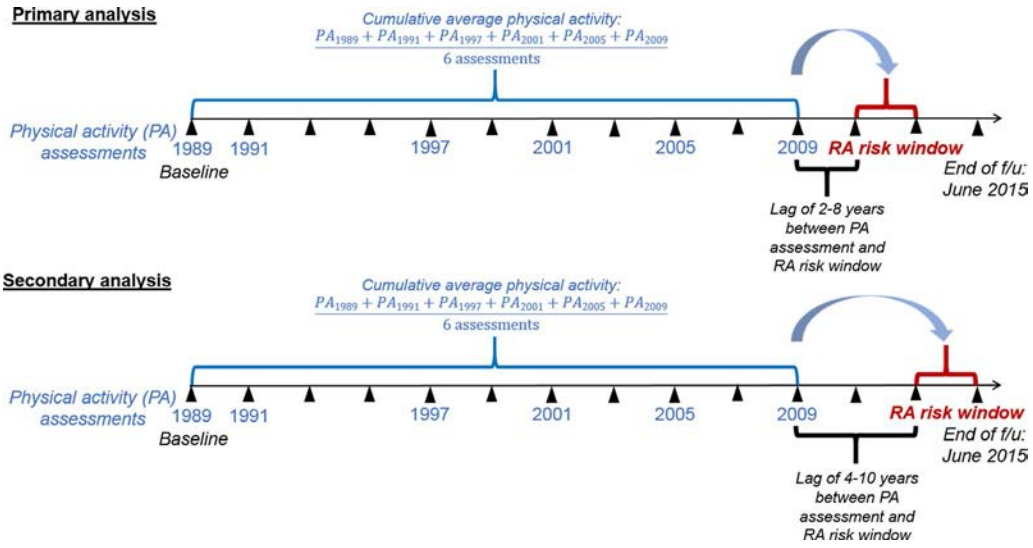

Figure 1. Study schematic illustrating the prospective cohort design. The primary exposure was cumulative average physical activity (PA) and the outcome was rheumatoid arthritis (RA) onset at least 2 years after the last physical activity assessment. The primary analysis was lagged so that there was always at least 2 years (and as many as 8 years) between the last PA exposure assessment and the outcome date of RA diagnosis that occurred in the RA risk window. The secondary analysis was lagged by another follow-up (f/u) cycle so that there was at least 4 years (and as many as 10 years) between the last PA exposure assessment and the outcome dates of RA diagnosis.

follows: unable to walk, easy/casual (<2.0 miles per hour [mph]), normal/average (2–2.9 mph), brisk (3–3.9 mph), or very brisk/striding (≥4 mph). Sedentary activity was also assessed by reporting the time spent per week in sitting activities (e.g., sitting at home watching television), with response categories as follows: 0, 1, 2–5, 6–10, 11–20, 21–40, 41–60, 61–90, and >90 hours per week. In a validation study within the NHSII (n = 151), total physical activity measured by this questionnaire was assessed for correlations with physical activity as determined by participants' 7-day recall (r = 0.79) and 7-day diary (r = 0.62) (15).

We hypothesized that long-term physical activity reduced the risk of developing RA. To evaluate the long-term effect of physical activity during the follow-up, we defined the primary exposure as the cumulative average total hours per week spent in recreational activity since the baseline time point of the study. The midpoint of each response category was assigned and summed across different recreational activities to estimate the continuous weekly total time spent in recreational activity, which was then cumulatively averaged for each period. Figure 1 illustrates the prospective cohort design and the calculation of cumulative average hours per week spent in recreational activity. The example illustrates how 6 repeated physical activity assessments (in 1989, 1991, 1997, 2001, 2005, and 2009) were averaged cumulatively in the 2009 follow-up cycle to predict whether RA occurred in the window between 2011 and 2013. Based on previous physical activity recommendations from the US Department of Health and Human Services (16) and as reported in previous studies (17,18), we categorized the cumulative average time spent in recreational activity as follows: <1, 1 to <2, 2 to <4, 4 to <7, and ≥7 hours per week.

We performed secondary analyses for the component of the recreational activity variable as well as the intensity of recreational activity. Walking was the main contributor to recreational activity, being analyzed in quartiles of cumulative average total time per week. To classify the intensity of recreational activity, each type of activity was assigned a typical metabolic equivalent (MET) score, as determined using published methods (19). The reported time spent in each activity was multiplied by the corresponding MET score to calculate the MET-hours/week, which was categorized into quartiles after calculating the cumulative average hours per week spent in recreational activity. Finally, since greater time spent in sedentary activities at home may take away from the opportunity to spend time in recreational activities, we also analyzed the cumulative average hours per week spent in sedentary activity at home, categorized into quartiles.

Identification of incident RA. RA cases were identified by a 2-stage procedure. Participants who self-reported a new diagnosis of RA were mailed a screening questionnaire (20). For those who screened positive for RA, we obtained medical records that were then reviewed independently by 2 rheumatologists to identify RA cases meeting the 1987 American College of Rheumatology (ACR) or 2010 ACR/European League Against Rheumatism classification criteria for RA (21,22). In addition, date of diagnosis and results of clinical laboratory tests for rheumatoid factor (RF) and anti–cyclic citrullinated peptide (anti-CCP) antibodies were collected. Subjects therefore had a confirmed incident RA case with documented serologic phenotype from their medical records. For women diagnosed as having RA prior to the clinical use of the anti-CCP test in the early 2000s, serologic phenotype was determined solely on the basis of the presence or absence of RF.

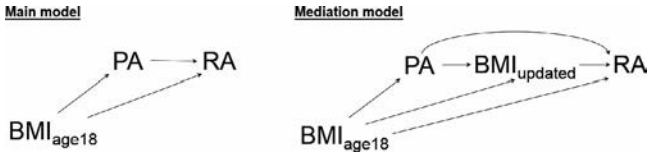

Figure 2. Directed acyclic graphs depicting the proposed causal relationship between body mass index (BMI), long-term physical activity (PA), and risk of rheumatoid arthritis (RA). In the main model (left), BMI at age 18 years was included as a confounder, since it occurred prior to the PA assessments and may be associated with long-term PA and RA risk. The effect size estimate in the main model is represented by the **horizontal arrow** from PA to RA, adjusted for other confounders in the model. In the mediation model (right), we additionally included BMI updated during the study follow-up, and considered this as a mediator, since long-term PA may be causally related to changes in BMI, which may lead to RA. The mediation analyses quantified the indirect effect of long-term PA on RA risk mediated through the updated BMI pathway. The effect size estimate in the mediation model (**curved arrow**) represents the direct effect of long-term PA on RA risk that does not include BMI at age 18 years, other confounders, or updated BMI.

Covariates. We considered factors related to both physical activity levels and RA risk as possible confounders. While many factors, such as pain, osteoarthritis, and comorbidities, are known to affect physical activity, these are not clearly established as RA risk factors, and therefore we did not consider them for multivariable models. Elevated BMI is associated with both decreased physical activity and increased RA risk (12,23,24). Figure 2 shows the proposed causal relationship between BMI, long-term physical activity, and RA risk. We analyzed BMI using 2 separate variables: BMI at age 18 years, and updated BMI during follow-up. We categorized BMI as <25.0, 25.0 to <30.0, or ≥30.0 kg/m^2. We considered BMI at age 18 years as a potential confounder, since this was recorded prior to the baseline physical activity assessment. We considered the biennially updated BMI variable as a mediator, such that long-term physical activity may affect BMI and this, in turn, may be associated with development of RA. BMI at age 18 years was included in both the main multivariable model and the mediation model. The mediation model included updated BMI to estimate the direct effect of long-term physical activity on

RA risk, as well as the indirect effect that was mediated through the updated BMI pathway.

Dietary intake was assessed using a semiquantitative food frequency questionnaire administered to women approximately every 4 years. We used the cumulative average Alternate Healthy Eating Index (AHEI) score, categorized into quartiles, as a measure of healthy dietary intake, since higher AHEI scores are associated with reduced RA risk (2,25). Cigarette smoking is a strong environmental RA risk factor (26), and we categorized time-updated smoking by pack-years (never smokers, >0 to 10 pack-years of smoking, and >10 pack-years of smoking). In addition, time-updated age, race, household income (categorized by quartile of US Census tract-based median household income at zip code level), residence of US region (West, Midwest, Mid-Atlantic, New England, or Southeast), and parity were also considered.

Statistical analysis. To reduce the potential for reverse causation bias, we lagged the analysis by 1 questionnaire cycle in

Table 1. Age-standardized characteristics of participants in the Nurses' Health Study II in 1989, by cumulative average total hours per week spent in recreational physical activity at baseline (n = 113,366)*

	Total recreational physical activity				
	<1 hour/week	1 to <2 hours/ week	2 to <4 hours/ week	4 to <7 hours/ week	≥7 hours/week
Participants, no. (%)	31,410 (27.7)	21,733 (19.2)	25,881 (22.8)	15,855 (14.0)	18,487 (16.3)
Age, mean ± SD years†	36.5 ± 4.6	36.2 ± 4.6	36.0 ± 4.6	35.9 ± 4.7	35.2 ± 4.8
White race, %	91.6	93.0	93.6	93.7	91.1
Median household income by quartile, %					
Q1 (lowest)	26.8	24.8	22.8	21.8	23.3
Q2	26.2	25.9	24.4	23.3	23.9
Q3	24.9	25.1	25.8	25.3	24.9
Q4 (highest)	22.1	24.2	27.0	29.6	27.9
US geographic region, %					
West	21.4	22.0	23.3	25.5	25.5
Midwest	37.0	37.0	35.9	34.1	32.6
Mid-Atlantic	29.4	28.9	29.0	28.5	30.7
New England	5.2	5.3	5.5	5.7	5.1
Southeast	6.9	6.8	6.4	6.0	6.0
Parous, %	75.2	72.4	69.7	65.5	61.0
Smoking by pack-years, %					
Never smokers	63.8	65.5	66.2	65.4	64.6
>0 to 10 pack-years	17.9	18.3	19.4	20.1	20.5
>10 pack-years	18.3	16.2	14.5	14.6	14.9
Alternate Healthy Eating Index by quartile, %‡					
Q1 (least healthy)	29.1	22.4	17.6	14.0	13.3
Q2	22.7	22.2	20.3	18.2	16.5
Q3	18.0	20.7	22.2	22.9	20.0
Q4 (most healthy)	12.2	17.4	22.6	27.6	29.6
BMI category at age 18 years, %					
<25.0 kg/m^2	88.4	89.5	90.1	90.5	90.1
25.0 to <30.0 kg/m^2	8.7	8.1	7.6	7.2	7.2
≥30.0 kg/m^2	2.9	2.4	2.3	2.3	2.7
Updated BMI category, %					
<25.0 kg/m^2	64.1	67.8	72.0	75.6	76.6
25.0 to <30.0 kg/m^2	20.2	19.7	18.5	16.4	15.7
≥30.0 kg/m^2	15.7	12.5	9.6	8.0	7.7

* Missing values are not shown. BMI = body mass index.
† Not age-standardized.
‡ Alternate Healthy Eating Index was first measured in 1991.

the primary analysis so that the physical activity exposures were measured at least 2 (and up to 8) years prior to RA diagnosis, and by an additional questionnaire cycle in a secondary analysis so that there were at least 4 (and up to 10) years between the physical activity assessment and window for RA risk in these analyses (Figure 1). When physical activity was not included on biennial questionnaires, physical activity data were carried forward from the prior cumulative average values (e.g., 1991 physical activity data were used in the 1993 and 1995 follow-up cycles). We reported baseline age-adjusted descriptive statistics for covariates across categorical total number of hours spent in recreational activity per week (the primary exposure variable).

We used Cox proportional hazards models to test for the association between the time-varying cumulative average total hours per week of recreational activity (categorized as <1 [reference], 1 to <2, 2 to <4, 4 to <7, and ≥7 hours per week) and RA, overall and by serologic phenotypes. The person-years of follow-up for each woman was ascertained as the number of person-years accrued from the date of return of the baseline questionnaire to the date of censoring (whichever came first) for the following reasons: RA diagnosis, report of connective tissue disease not confirmed as RA, loss to follow-up, date of death, or end of study. The age-adjusted models were adjusted for age and questionnaire period (equivalent to calendar year in this closed cohort with enrollment of all subjects in 1989, and follow-up occurring every other year). Based on previous studies (12,23,27,28), our main multivariable models were additionally adjusted for the following confounders: pack-years of smoking, median household income, US geographic region, parity, cumulative average AHEI score, and BMI at age 18 years. *P* for trend was calculated by assigning the median value within each category and using this as a continuous variable in the model.

By additionally including updated BMI in the main multivariable model, we evaluated the potential mediating effect of updated BMI on the association between cumulative average number of hours spent in recreational activity and risk of RA. The proportion mediated by updated BMI, including 95% CIs and *P* values, was calculated using the classic difference method with a publicly available macro (29,30). The mediation proportion is the percentage of the association between the cumulative average total hours per week spent in recreational activity and RA risk influenced by the change conferred by updated BMI (indirect effect).

In a secondary analysis, since higher walking pace has increased energy expenditure than lower walking pace in the same amount of time, we stratified by walking pace (easy/average or brisk/very brisk), and tested for the association between cumulative average total walking hours per week and risk of RA. We also analyzed the associations between the cumulative average MET-hours/week (a measure of physical activity intensity, categorized by quartile) and cumulative average sedentary hours per week at home (categorized by quartile) and risk of RA, overall and by serologic phenotypes, using the same multivariable models as in the primary analysis.

Since there is a complex bidirectional relationship between physical activity and other health factors, such as comorbidities, that could be important in the estimation of RA risk, we performed a sensitivity analysis in which only the baseline physical activity assessment was considered. This analysis treated the physical activity exposure variable as time-fixed such that subsequent measured and unmeasured mediators (such as changes in physical activity, BMI, and health status) are less likely to affect the relationship with RA risk in the analysis. For this analysis, we adjusted for covariates, including BMI, only as of the baseline time point. In an additional sensitivity analysis, we included cancer, cardiovascular disease, and diabetes in the multivariable models of our main analysis, to evaluate whether adjusting for potential confounding by comorbidities impacted the findings.

The proportional hazards assumption was tested by including interaction terms between physical activity and follow-up time, using likelihood ratio tests to compare nested models with and those without the interaction terms; the assumption was met in all analyses. Two-sided *P* values less than 0.05 were considered significant in all analyses. All of the statistical analyses were performed using SAS software version 9.4 (SAS Institute).

RESULTS

Characteristics of the study participants. Table 1 displays the age-adjusted baseline characteristics of the NHSII study participants categorized by cumulative average total hours per week of recreational activity. Most of the women were white and had a normal BMI at age 18 years. Women in the cumulative average recreational activity category of <1 hour/week (mean ± SD age at baseline 36.5 ± 4.6 years) were older than women in the ≥7 hours/week category (mean ± SD age at baseline 35.2 ± 4.8). Women in the ≥7 hours/week recreational activity category tended to have a lower BMI at age 18 years, a lower BMI at baseline, and a healthier diet than less active women.

Among the 113,366 women analyzed in the NHSII, we identified 506 incident RA cases during 2,428,573 person-years of follow-up (mean ± SD follow-up 21.4 ± 5.1 years per participant). Among the 506 women with incident RA, 339 (67.0%) had seropositive RA, and 167 (33.0%) had seronegative RA. Of the 506 women with RA, 495 (97.8%) satisfied the 1987 ACR classification criteria.

Recreational physical activity time and RA risk. Compared to women with cumulative average recreational activity of <1 hour/week, increasing total recreational activity hours per week was associated with a reduced risk of RA overall (Table 2). The HRs across categories of recreational activity were as follows: HR 1.00 for <1 hour/week (refer-

Table 2. Risk of RA and serologic phenotypes of RA by cumulative average total hours per week spent in recreational physical activity, lagged by 1 questionnaire cycle*

	Total recreational physical activity						
	<1 hour/week	1 to <2 hours/week	2 to <4 hours/week	4 to <7 hours/week	≥7 hours/week	*P* for trend	Mediation by updated BMI, % (95% CI) [*P*]†
All RA							
No. of cases/person-years	130/550,684	121/512,446	139/653,002	78/422,311	38/290,130		
Age-adjusted model	1.00 (reference)	0.96 (0.75–1.23)	0.86 (0.67–1.09)	0.76 (0.57–1.01)	0.60 (0.42–0.87)	0.002	
Multivariable model 1 (main)‡	1.00 (reference)	1.00 (0.78–1.29)	0.92 (0.72–1.17)	0.84 (0.63–1.12)	0.67 (0.47–0.98)	0.02	
Multivariable model 2 (main + updated BMI)§	1.00 (reference)	1.01 (0.78–1.29)	0.94 (0.73–1.20)	0.86 (0.65–1.15)	0.71 (0.49–1.02)	0.04	14.0 (4.5–36.0) [0.002]
Seropositive RA							
No. of cases/person-years	87/549,445	75/511,396	99/651,600	53/421,493	25/289,614		
Age-adjusted model	1.00 (reference)	0.88 (0.65–1.20)	0.90 (0.67–1.20)	0.76 (0.54–1.07)	0.59 (0.38–0.92)	0.01	
Multivariable model 1 (main)‡	1.00 (reference)	0.92 (0.67–1.25)	0.96 (0.72–1.29)	0.84 (0.59–1.19)	0.66 (0.42–1.04)	0.06	
Multivariable model 2 (main + updated BMI)§	1.00 (reference)	0.93 (0.68–1.27)	0.99 (0.74–1.33)	0.87 (0.61–1.24)	0.70 (0.44–1.11)	0.12	20.0 (5.0–54.2) [0.001]
Seronegative RA							
No. of cases/person-years	43/549,337	46/511,405	40/651,474	25/421,495	13/289,623		
Age-adjusted model	1.00 (reference)	1.13 (0.74–1.71)	0.77 (0.50–1.18)	0.76 (0.46–1.25)	0.63 (0.34–1.17)	0.05	
Multivariable model 1 (main)‡	1.00 (reference)	1.18 (0.77–1.79)	0.83 (0.53–1.29)	0.85 (0.51–1.41)	0.70 (0.37–1.33)	0.13	
Multivariable model 2 (main + updated BMI)§	1.00 (reference)	1.18 (0.77–1.79)	0.83 (0.53–1.29)	0.85 (0.51–1.42)	0.71 (0.38–1.35)	0.15	4.1 (0.1–60.1) [0.3]

* Lagging by 1 questionnaire cycle was defined as at least 2 years between physical activity assessment and rheumatoid arthritis (RA) diagnosis. Except where indicated otherwise, values are the hazard ratio (95% confidence interval [95% CI]).
† Mediation by updated body mass index (BMI) was calculated using the difference method comparing model 2 and model 1.
‡ Multivariable model 1 was adjusted for age, questionnaire period (calendar year), US geographic region (West, Midwest, Mid-Atlantic, New England, Southeast), median household income (quartile), smoking pack-years (never, >0 to 10, >10), parity (vs. nulliparous), cumulative average Alternate Healthy Eating Index (quartile), and BMI category at age 18 years (<25.0, 25.0 to <30.0, ≥30.0 kg/m^2).
§ Multivariable model 2 was adjusted for variables in model 1 as well as updated BMI category (<25.0, 25.0 to <30.0, ≥30.0 kg/m^2).

Table 3. Risk of RA and serologic phenotypes of RA by cumulative average total hours per week of recreational physical activity, lagged by 2 questionnaire cycles*

	Total recreational physical activity						
	<1 hour/week	1 to <2 hours/week	2 to <4 hours/week	4 to <7 hours/week	≥7 hours/week	*P* for trend	Mediation by updated BMI, % (95% CI) [*P*]†
All RA							
No. of cases/person-years	121/504,652	118/463,986	133/586,418	74/381,916	39/268,543		
Age-adjusted model	1.00 (reference)	1.03 (0.80–1.33)	0.91 (0.71–1.17)	0.80 (0.60–1.06)	0.66 (0.46–0.94)	0.005	
Multivariable model 1 (main)‡	1.00 (reference)	1.08 (0.83–1.39)	0.99 (0.77–1.27)	0.88 (0.66–1.19)	0.74 (0.51–1.06)	0.04	
Multivariable model 2 (main + updated BMI)§	1.00 (reference)	1.09 (0.84–1.41)	1.01 (0.79–1.30)	0.92 (0.68–1.24)	0.78 (0.54–1.13)	0.10	19.7 (6.0–48.3) [0.0001]
Seropositive RA							
No. of cases/person-years	81/503,578	76/463,104	91/585,218	49/381,226	26/268,089		
Age-adjusted model	1.00 (reference)	0.98 (0.72–1.34)	0.92 (0.68–1.24)	0.77 (0.54–1.10)	0.65 (0.42–1.02)	0.02	
Multivariable model 1 (main)‡	1.00 (reference)	1.03 (0.75–1.41)	1.00 (0.73–1.35)	0.86 (0.60–1.24)	0.74 (0.47–1.17)	0.11	
Multivariable model 2 (main + updated BMI)§	1.00 (reference)	1.04 (0.76–1.43)	1.03 (0.76–1.40)	0.91 (0.63–1.31)	0.80 (0.51–1.26)	0.23	26.6 (5.5–69.3) [0.0001]
Seronegative RA							
No. of cases/person-years	40/503,507	42/463,113	42/585,118	25/381,222	13/268,098		
Age-adjusted model	1.00 (reference)	1.14 (0.74–1.75)	0.90 (0.58–1.39)	0.84 (0.51–1.39)	0.66 (0.35–1.24)	0.09	
Multivariable model 1 (main)‡	1.00 (reference)	1.18 (0.76–1.83)	0.96 (0.62–1.50)	0.93 (0.56–1.54)	0.73 (0.38–1.37)	0.19	
Multivariable model 2 (main + updated BMI)§	1.00 (reference)	1.19 (0.77–1.84)	0.98 (0.63–1.52)	0.95 (0.57–1.58)	0.75 (0.39–1.42)	0.24	8.8 (0.9–50.9) [0.1]

* Lagging by 2 questionnaire cycles was defined as at least 4 years between physical activity assessment and rheumatoid arthritis (RA) diagnosis. Except where indicated otherwise, values are the hazard ratio (95% confidence interval [95% CI]).
† Mediation by updated body mass index (BMI) was calculated using the difference method comparing model 2 and model 1.
‡ Multivariable model 1 was adjusted for age, questionnaire period (calendar year), US geographic region (West, Midwest, Mid-Atlantic, New England, Southeast), median household income (quartile), smoking pack-years (never, >0 to 10, >10), parity (vs. nulliparous), cumulative average Alternate Healthy Eating Index (quartile), and BMI category at age 18 years (<25.0, 25.0 to <30.0, ≥30.0 kg/m^2).
§ Multivariable model 2 was adjusted for variables in model 1 as well as updated BMI category (<25.0, 25.0 to <30.0, ≥30.0 kg/m^2).

ence), HR 1.00 (95% CI 0.78–1.29) for 1 to <2 hours/week, HR 0.92 (95% CI 0.72–1.17) for 2 to <4 hours/week, HR 0.84 (95% CI 0.63–1.12) for 4 to <7 hours/week, and HR 0.67 (95% CI 0.47–0.98) for ≥7 hours/week, adjusted for potential confounders including age, questionnaire period, smoking, median household income, US geographic region, parity status, dietary quality, and BMI at age 18 years. There was a significant trend toward a reduced overall RA risk (*P* for trend = 0.02) with increasing cumulative average total recreational activity hours per week. There was a similar but nonsignificant trend toward a reduced risk of seropositive RA. The HRs for seropositive RA across recreational physical activity categories were as follows: HR 1.00 for <1 hour/week (reference), HR 0.92 for 1 to <2 hours/week, HR 0.96 for 2 to <4 hours/week, HR 0.84 for 4 to <7 hours/week, and HR 0.66 for ≥7 hours/week (*P* for trend = 0.06). The point estimates for seronegative RA were also similar, but the results were not statistically significant (*P* for trend = 0.13).

When the mediating effect of updated BMI was additionally accounted for in these analyses, the indirect effect (proportion of effect) between recreational activity and RA risk mediated through the updated BMI was as follows: 14.0% (95% CI 4.5–36.0%; *P* = 0.002) for all RA and 20.0% (95% CI 5.0–54.2%; *P* = 0.001) for seropositive RA. No statistically significant mediation between physical activity, updated BMI, and risk of seronegative RA was detected (*P* = 0.3).

Table 3 shows the results of the secondary analysis in which we lagged the assessment of physical activity and RA risk window by an additional questionnaire cycle (at least 4–10 years between physical activity assessment and the RA risk window). This analysis had fewer RA cases, since women who were diagnosed as having RA during the first 4 years of follow-up were not included in this analysis. These results were similar to those of the primary analysis; there was a statistically significant trend toward reduced RA risk with increasing cumulative average total recreational activity hours/week (*P* for trend = 0.04). The proportions of effect between recreational activity and RA mediated by updated BMI were as follows: 19.7% (*P* = 0.0001) for all RA and 26.6% (*P* = 0.0001) for seropositive RA.

Time spent walking and RA risk. To further investigate the association between recreational activity and RA, we individually analyzed walking activity, since this was the main contributor to the composite variable. Table 4 shows the risk of RA according to the cumulative average total walking hours/week stratified by walking pace. We did not observe a statistically significant association between walking time and all RA risk in the subgroup with easy/average walking pace. Among women with brisk/very brisk walking pace, there was a statistically significant effect, in which longer cumulative average walking hours/week was associated with lower RA risk (for quartile 4 [most walking] versus quartile 1 [least walking], HR 0.76, 95% 0.53–1.09; *P* for trend = 0.05).

Physical activity intensity, sedentary activity at home, and RA risk. Supplementary Table 1 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40899/abstract) presents the association between

Table 4. Risk of rheumatoid arthritis overall by quartile of cumulative average walking hours per week, according to walking pace and lagged by 1 questionnaire cycle*

	Quartile of cumulative average walking hours per week				*P* for trend
	Q1 (least walking)	Q2	Q3	Q4 (most walking)	
Easy/normal walking pace					
Median walking hours/week	0.03	0.67	1.00	2.50	
No. of cases/person-years	82/422,466	83/356,666	87/389,650	97/443,930	
Age-adjusted model	1.00 (reference)	1.14 (0.84–1.56)	1.13 (0.83–1.53)	1.08 (0.81–1.46)	0.76
Multivariable model 1 (main)†	1.00 (reference)	1.16 (0.85–1.58)	1.16 (0.86–1.57)	1.13 (0.84–1.52)	0.60
Multivariable model 2 (main + updated BMI)‡	1.00 (reference)	1.16 (0.85–1.58)	1.16 (0.85–1.57)	1.13 (0.84–1.53)	0.57
Brisk/very brisk walking pace					
Median walking hours/week	0.20	1.00	2.50	5.00	
No. of cases/person-years	85/356,503	113/424,218	91/404,176	46/279,626	
Age-adjusted model	1.00 (reference)	1.10 (0.83–1.46)	0.93 (0.69–1.25)	0.72 (0.50–1.03)	0.03
Multivariable model 1 (main)†	1.00 (reference)	1.13 (0.85–1.50)	0.98 (0.73–1.32)	0.76 (0.53–1.09)	0.05
Multivariable model 2 (main + updated BMI)‡	1.00 (reference)	1.13 (0.85–1.50)	0.98 (0.73–1.33)	0.76 (0.53–1.09)	0.06

* Lagging by 1 questionnaire cycle was defined as at least 2 years between physical activity assessment and rheumatoid arthritis diagnosis. Except where indicated otherwise, values are the hazard ratio (95% confidence interval).
† Multivariable model 1 was adjusted for age, questionnaire period (calendar year), US geographic region (West, Midwest, Mid-Atlantic, New England, Southeast), median household income (quartile), smoking pack-years (never, >0 to 10, >10), parity (vs. nulliparous), cumulative average Alternate Healthy Eating Index (quartile), and body mass index (BMI) category at age 18 years (<25.0, 25.0 to <30.0, ≥30.0 kg/m^2).
‡ Multivariable model 2 was adjusted for the variables in model 1 as well as updated BMI category (<25.0, 25.0 to <30.0, ≥30.0 kg/m^2).

RA risk and cumulative average MET-hours/week (as a measure of intensity of physical activity). Compared to women in the lowest quartile of physical activity intensity, women in the highest quartile had a reduced risk of RA overall (HR 0.72, 95% CI 0.55–0.93), when the analysis was adjusted only for age and for questionnaire period (*P* for trend = 0.02). However, this was no longer statistically significant (HR 0.79, 95% CI 0.60–1.03) when the analysis was adjusted for other potential confounders (*P* for trend = 0.09).

Supplementary Table 2 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40899/abstract) shows the association between RA risk and cumulative average sedentary hours/week at home. We did not observe any statistically significant association of sedentary activity at home with RA risk.

Supplementary Table 3 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40899/abstract) shows the results of the sensitivity analysis in which we analyzed only baseline physical activity levels for subsequent RA risk. These results were similar to those in the primary analysis, with increasing categories of physical activity associated with a reduced risk of RA overall, in the age-adjusted model (*P* for trend = 0.004), in the multivariable model using BMI at age 18 years (*P* for trend = 0.03), and in the multivariable model additionally adjusted for baseline BMI (*P* for trend = 0.04).

Supplementary Table 4 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40899/abstract) shows the results of the sensitivity analysis in which we additionally adjusted for comorbidities (cancer, cardiovascular disease, and diabetes) in the multivariable models. These results were similar to those of the primary analysis, with increasing categories of physical activity associated with a reduced risk of RA overall, as determined in the multivariable model using BMI at age 18 years (*P* for trend = 0.02) and in the multivariable model additionally adjusted for updated BMI (*P* for trend = 0.048).

DISCUSSION

In this large, prospective cohort study, we found that increasing physical activity was associated with reduced RA risk. For women with ≥7 total recreational activity hours/week, RA risk was reduced by 33% compared to women with <1 hour/week, independent of other RA risk factors. In our study design, we excluded physical activity measures in the years prior to RA diagnosis so that these results would be less likely explained by early disease manifestations affecting physical activity before clinical diagnosis. We found that BMI was a significant mediator in the association between physical activity and RA risk, but physical activity also contributed to RA risk through pathways that did not include BMI. Overall, these results suggest that long-term physical activity may be an important contributor to RA risk independent of dietary and BMI history.

Several previous studies investigated physical activity and RA risk. The Swedish Mammography Cohort study (12) analyzed 30,112 women using a single questionnaire on physical activity at baseline, and identified 201 incident RA cases. Women in the highest category (≥20 minutes/day of walking/bicycling and ≥1 hour/week of exercise) had a reduced risk of developing RA (multivariable relative risk 0.65, 95% CI 0.43–0.96) compared to women in the lowest category (<20 minutes/day of walking/bicycling and <1 hour/week of exercise). However, those results may have been explained partially by reverse causation bias, particularly in cases diagnosed early during follow-up. Furthermore, those previous studies used only a single measure of physical activity, and there were no data on RA serologic phenotype.

Krok-Schoen et al (31) recently reported on leisure physical activity (measured as MET-hours/week) and RA risk among 80,551 postmenopausal women (with 3,348 incident RA cases by self-report) in the Women's Health Initiative. Those authors found a reduced risk of RA in women who reported a physical activity intensity level of ≥17.5 MET-hours/week (HR 0.83, 95% CI 0.74–0.93) compared to those who reported being inactive, similar to our present results. However, physical activity was assessed only at baseline, RA cases were by self-report (and therefore may have been misclassified), the results were adjusted only for age, and therefore may have been confounded by other factors such as smoking, and results could also have been explained by reverse causation. Finally, the Iowa Women's Health Study (32) analyzed 31,336 women, and identified 158 incident RA cases. They found no association between a baseline 3-level physical activity variable and RA risk (relative risk 0.90, 95% CI 0.61–1.32 for high versus low physical activity).

The results of our study add to the literature, as this study is the first to use repeated measures of physical activity for RA risk, investigate physical activity and risk of RA by serologic phenotype, incorporate a study design to protect against reverse causation bias, and quantify the potential mediating effect of BMI on physical activity and RA risk. The point estimates for physical activity were similar for both RA serologic phenotypes. However, the statistical significance of the association differed, perhaps because of the reduced power of the seronegative RA subgroup. We found that BMI was a strong mediator in the relationship between physical activity and RA. These results suggest that modifying either physical activity or BMI may be potential strategies to mitigate the risk of RA, but randomized trials would be needed to establish a true causal relationship.

Updated BMI was a strong mediator between physical activity and seropositive RA, but not seronegative RA. These results also suggest that physical activity may have different biologic effects on each RA subphenotype. An alternative strategy for the mediation analysis would be to perform analyses using marginal structural models, but previous literature suggests that a marginal structural

model would produce findings similar to those obtained with our approach (33,34). Our findings add to the literature demonstrating differences based on BMI and other measures of adiposity for RA risk by serologic phenotype (23,35–37). Although we had repeated measures of physical activity available to use, this introduces complexity with regard to whether factors associated with reductions in physical activity may have mediated the relationship. We carefully analyzed the effect of time-updated BMI on the association, but other factors, such as chronic pain and comorbidities, may have complex relationships with the exposure and outcome in our study. To mitigate this, we performed a sensitivity analysis in which we considered only physical activity and confounders as assessed at baseline, such that subsequent mediating health factors were not included in the model. Since we found a similar association in this sensitivity analysis, we suspect that measured and unmeasured subsequent clinical mediating factors did not have a large impact on our findings.

We also investigated the association between walking hours and RA risk, stratified by walking pace. We observed a statistically significant effect among women with brisk/very brisk walking pace, in which longer cumulative average walking hours/week appeared to reduce the risk of RA, which was similar to the results in the Swedish Mammography cohort (12). We also investigated intensity of physical activity and RA risk, and our findings suggested that RA risk was reduced among women with the highest intensity of recreational activity. However, we observed no significant association between sedentary activities at home and RA risk. Overall, these findings suggest a beneficial effect of increasing time spent in recreational activity on RA risk. These results may have important public health consequences for patients with RA, since a recent report suggested that up to 80% of the US population may be insufficiently active (6).

Physical activity is generally recommended for patients with RA to maintain conditioning, improve quality of life, and decrease systemic inflammation (9,38–40). Physical activity may also have health benefits in terms of reducing the risk of developing RA by decreasing inflammation and regulating the immune system (7). Stimulated myocytes may secrete antiinflammatory myokines that decrease the levels of tumor necrosis factor, among other proinflammatory cytokines (7,41). Physical activity is also thought to have positive effects on immune system regulation (4,8). Increasing physical activity has been associated with a reduced risk of inflammatory bowel disease, which could potentially be related to similar mechanisms (42).

Our study has some limitations to consider. We designed the study to minimize the potential for reverse causation bias being an explanation for our results, but it is still possible that the association we observed may be explained in part by symptoms of early RA. This is unlikely, however, since the secondary analysis extending the lag period to 4–10 years showed similar results. The study sample consisted of mostly white women who were healthy, educated, and working at baseline, and therefore the results may not be generalizable to other, more diverse populations or to men.

While we applied repeated measures of physical activity during many years of follow-up, these measures were ascertained by self-report and only gathered approximately every 4 years. While the questionnaire instrument has been validated, we were unable to analyze physical activity changes occurring in the interim (15). We identified cases meeting accepted RA criteria, with rigorous methods of case ascertainment, all verified by medical record review, an approach that enabled analyses based on RA serologic phenotype. However, we were unable to analyze subjects based solely on anti-CCP status, since we relied on clinical testing and some of the cases were diagnosed prior to the clinical use of this test. It is possible that some of the women may have been clinically inappropriately diagnosed as having RA, in particular seronegative RA, being particularly more likely to occur in women with concurrent fibromyalgia, osteoarthritis, or obesity (with elevated levels of serum inflammation markers) who also may have had lower levels of physical activity due to these conditions. However, all of the incident RA cases were self-reported, and these patients screened positive for RA on a supplemental questionnaire and met accepted research classification criteria for RA using objective criteria, including objective documentation of synovitis, on the independent reviews of their medical records by 2 separate board-certified rheumatologists. This analysis censored women as of the date of self-reported RA or other related connective tissue disease not classified as an RA case, such that women were free of reported RA or other connective tissue disease in the analyzed person-years of follow-up. Nearly all of the RA cases met the 1987 ACR criteria, which are thought to be more specific for RA than the 2010 ACR/EULAR criteria. Therefore, we believe that misclassification of the RA outcome is unlikely to explain our results.

Inherent to all observational studies, the results may have been explained by residual confounding. However, we had detailed data available on important confounders, including smoking, dietary intake, and BMI. We considered the updated BMI variable as a mediator, but this may alternatively be considered as a confounder. Although data on BMI at age 18 years were available throughout the follow-up, we did not have data on BMI between age 18 years and baseline, which may have impacted our findings.

Even though comorbidities have a clear impact on physical activity, it is less clear that they are related to a subsequent risk of RA. The results were similar when we additionally adjusted the analyses for cancer, cardiovascular disease, and diabetes, and therefore these comorbidities did not explain our findings. Other potentially important factors, such as pain and other comorbidities, that may have been related to both physical activity and RA development were unmeasured. A randomized controlled trial evaluating physical activity interventions for RA risk or surrogate outcomes such as RA-related autoantibody presence/levels would be necessary to determine a true biologic effect of physical activity on RA risk. However, such a study would need a

large sample size and lengthy follow-up with a strategy for blinding assignment of the intervention, and therefore it would be difficult to implement. We encourage further studies aimed at understanding the potential biologic effects of physical activity and other metabolic interventions on RA risk or RA-related autoimmunity.

Our study also has strengths. We analyzed a large, prospective cohort with a lengthy follow-up, and identified validated incident RA cases during 2.5 million person-years over up to 26 years of follow-up. We had detailed data on a variety of physical activity measures updated approximately every 4 years, as well as detailed information on important potential confounders, allowing for time-varying analysis and long-term measures of physical activity. We identified cases by medical record review to ensure that all cases of RA fulfilled accepted criteria and were truly incident, while allowing for analyses based on RA serologic phenotype. Finally, we censored women at the time of their self-reported diagnosis of RA who were not subsequently confirmed to be an RA case, to help ensure that the sample was free of RA during all analyzed person-years of follow-up.

In conclusion, we found that increasing time spent in recreational activity was associated with a reduced risk of RA, independent of other RA risk factors including smoking, BMI history, and dietary intake. Long-term physical activity had similar associations with both seropositive RA and seronegative RA. We found that some of the effect of physical activity on seropositive RA was mediated by changes in BMI, suggesting that both physical activity and weight loss interventions could delay or even prevent the onset of seropositive RA. These findings add to the accumulating evidence that metabolic factors are important in the pathogenesis of RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual contact, and all authors approved the final version to be published. Dr. Sparks 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.

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Rheumatoid Arthritis Disease Activity Predicting Incident Clinically Apparent Rheumatoid Arthritis–Associated Interstitial Lung Disease: A Prospective Cohort Study

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Objective. To evaluate rheumatoid arthritis (RA) disease activity and risk of RA-associated interstitial lung disease (RA-ILD).

Methods. We investigated disease activity and risk of RA-ILD using the Brigham RA Sequential Study (BRASS, 2003–2016). All patients were diagnosed as having RA according to accepted criteria. Disease Activity Scores in 28 joints (DAS28) and covariate data were measured prospectively at annual study visits. Diagnosis of RA-ILD was determined by review of images from clinically indicated chest computed tomography scans. We analyzed patients without RA-ILD at baseline. We used Cox regression to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for RA-ILD, using annually updated DAS28 data, with adjustment for known RA-ILD risk factors (age, sex, smoking status, RA duration, and serologic status). We performed alternative analyses that did not censor at the time of missing DAS28 data and included adjustment for use of methotrexate, use of glucocorticoids, presence of bone erosions, and presence of rheumatoid nodules.

Results. Among 1,419 participants, the mean ± SD age was 55.8 ± 14.2 years, and 68.6% were seropositive for either cyclic citrullinated peptide or rheumatoid factor. We identified 85 incident cases of RA-ILD during a mean ± SD follow-up duration of 8.9 ± 4.2 years per patient. The moderate/high disease activity group had a multivariable HR of 2.22 (95% CI 1.28–3.82) for RA-ILD compared to the remission/low disease activity group. Risk of RA-ILD increased across disease activity categories: multivariable HR 1.00 (reference) for remission, 1.41 (95% CI 0.61–3.28) for low disease activity, 2.08 (95% CI 1.06–4.05) for moderate disease activity, and 3.48 (95% CI 1.64–7.38) for high disease activity (P for trend = 0.001). For each unit increase in the DAS28, the risk of RA-ILD increased by 35% (95% CI 14–60%). Results were similar in analyses that included follow-up for missing DAS28 data and with adjustment for use of methotrexate, use of glucocorticoids, presence of bone erosions, or presence of rheumatoid nodules.

Conclusion. Active articular RA was associated with an increased risk of developing RA-ILD. These results suggest that decreasing systemic inflammation may alter the natural history of RA-ILD development.

The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of Harvard University, its affiliated academic health care centers, or the National Institutes of Health.

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INTRODUCTION

Rheumatoid arthritis–associated interstitial lung disease (RA-ILD) is one of the most serious extraarticular RA manifestations, with a median survival of only 3 years postdiagnosis (1–3). Among patients with RA, the prevalence range of RA-ILD is 3–10% and may have increased in recent years (4–6).

While the natural history of RA-ILD has yet to be fully elucidated, previous studies have identified some risk factors, including older age, male sex, cigarette smoking, later onset RA, longer RA duration, elevated rheumatoid factor (RF) level, and elevated cyclic citrullinated peptide (CCP) level (1,7–13). However, none of these factors except smoking are potentially modifiable, so identifying other RA-ILD risk factors that may allow for intervention is of crucial importance.

Articular disease activity in RA drives treatment, including the use of disease-modifying antirheumatic drugs (DMARDs), which typically treat to a target of remission or low disease activity (14,15). Active joint involvement in RA reflects ongoing levels of systemic inflammation, which may include inflammation in the lungs, potentially impacting the pathogenesis of RA-ILD (11). A previous cross-sectional study suggested that patients with RA-ILD had higher levels of articular disease activity compared to patients with RA and without ILD (8). However, no previous study has investigated dynamic measures of RA disease activity for subsequent risk of RA-ILD.

Therefore, we examined whether RA disease activity is associated with a subsequent risk of RA-ILD, using data from an RA cohort that included repeated measures of disease activity and chest computed tomography (CT) scans (obtained during a lengthy follow-up) to identify incident RA-ILD. We hypothesized that patients with moderate/high disease activity have an increased risk of developing RA-ILD compared to patients with disease in remission or with low disease activity, independent of known RA-ILD risk factors.

PATIENTS AND METHODS

Study population. We analyzed data from the Brigham RA Sequential Study (BRASS), a single-center research cohort of patients with RA at Brigham and Women's Hospital in Boston, MA that began enrolling patients in 2003 (16). All patients in the BRASS registry were diagnosed as having RA according to a treating physician and accepted criteria (17,18). Approximately 10% of patients in the BRASS study enrolled within the first year of diagnosis, so most had prevalent RA at baseline. Validated measures of disease activity incorporating joint counts evaluated by rheumatologists, RA characteristics, medications, lifestyle factors, and detailed patient-reported outcomes were prospectively assessed at baseline and at annual study visits. Blood was obtained for research purposes at each annual study visit to measure the following: CCP level, determined by 3rd generation validated enzyme-linked immunosorbent assay (Inova Diagnostics); RF level, determined by immunoturbidimetric technique on a Cobas Integra 700 analyzer (Roche Diagnostics); C-reactive protein (CRP) level, determined by commercial assay; and other research laboratory tests. Data from the electronic medical record were available to link clinically indicated test results and clinical notes to the research data. Most patients in the BRASS study received all medical care in the Partners HealthCare system. All aspects of this study were approved by the Partners HealthCare Institutional Review Board.

Study design. We conducted a prospective cohort study (secondary analysis of prospectively collected data) among patients in the BRASS study to investigate whether disease activity was associated with incident RA-ILD. We excluded patients without disease activity at baseline and those with prevalent RA-ILD. The study concluded on April 14, 2016, when chest CT scans were last examined for RA-ILD for research purposes (Figure 1).

Disease Activity Score in 28 joints exposure measures. We analyzed validated disease activity by reviewing the Disease Activity Scores in 28 joints (DAS28) (19) that were prospectively collected at annual study visits. We used the 3-variable DAS28 consisting of tender joint count, swollen joint count, and laboratory results for CRP level. We did not use the 4-variable DAS28 in order to minimize missing data at baseline and during follow-up, since we planned to investigate dynamic changes in the DAS28 over time. Our primary analysis relied on the measure of binary DAS28 classification: moderate/high disease activity

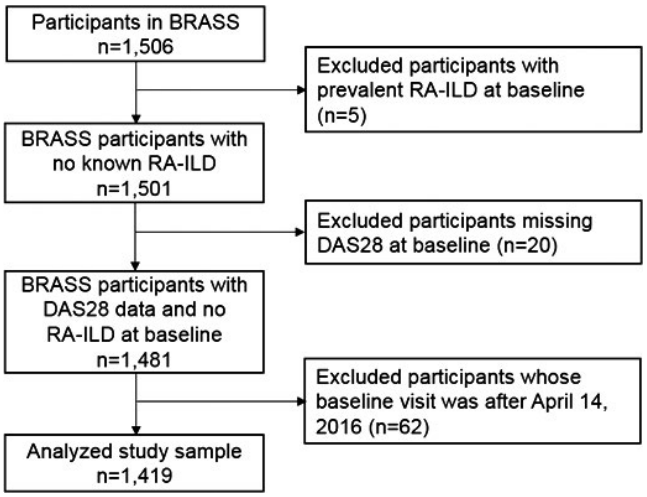

Figure 1. Flow diagram of the analyzed study population. BRASS = Brigham Rheumatoid Arthritis Sequential Study; RA-ILD = rheumatoid arthritis–associated interstitial lung disease; DAS28 = Disease Activity Score in 28 joints.

compared to the reference group of remission/low disease activity, using a cutoff score of 3.2 as recommended by the American College of Rheumatology (ACR) for clinical practice (20). We chose this as the primary exposure variable because many RA guidelines recommend treating to a target of low disease activity or remission (14,15).

We also performed secondary analyses for 2 additional disease activity measures: 1) a 4-level ordinal DAS28 variable consisting of categories using ranges recommended by the ACR (20): remission (<2.6, the reference group), low (2.6 to 3.2), moderate (>3.2 to 5.1), and high (>5.1); and 2) a continuous DAS28 variable.

Identification of incident RA-ILD. We identified all chest CT scans that were performed for clinical indications among patients in the BRASS study. We identified new chest CT scans occurring during follow-up by periodically querying the electronic medical record at fixed intervals according to our research protocol. Each CT chest scan was visually inspected for research purposes by 3 independent adjudicators: 2 attending chest radiologists and 1 attending pulmonologist. As previously suggested (21), we excluded scans with radiologic changes suggesting concurrent illness that limited the CT scan interpretation (e.g., lung cancer, active infection, or pleural effusions), and then each scan was classified according to the following descriptions: 1) no interstitial lung abnormalities, 2) indeterminate, 3) early/mild interstitial lung abnormalities, or 4) radiologically severe interstitial lung abnormalities. Interstitial lung abnormalities were defined as any of the following changes affecting >5% of any lobar region: nondependent ground-glass or reticular abnormalities, diffuse centrilobular nodularity, nonemphysematous cysts, honeycombing, or traction bronchiectasis, as described in previous studies (22,23). Radiologically severe interstitial lung abnormalities were defined as bilateral fibrosis in multiple lobes associated with honeycombing and traction bronchiectasis in a subpleural distribution. Discrepancies between adjudicators were discussed until there was complete agreement. No clinical data, including radiologic or pathology reports, if available, were used to supplement the research abstraction of the chest CT imaging. In this study, 195 unique patients had chest CT images reviewed.

Each chest CT scan with interstitial lung abnormalities was categorized using subtypes of RA-ILD by a senior chest radiologist. These subtypes included acute interstitial pneumonia (AIP), diffuse alveolar damage (DAD), cellular or fibrotic nonspecific interstitial pneumonia (NSIP), and usual interstitial pneumonia (UIP), using standard definitions (24). More details about the characteristics used for RA-ILD subtyping can be found in Supplementary Methods (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40904/abstract).

In addition, pulmonary function test (PFT) results from around the time of diagnosis of RA-ILD were collected if available, including the forced expiratory volume in 1 second (FEV_1, as percent predicted), forced vital capacity (FVC, as percent predicted), FEV_1/FVC (as a ratio), total lung capacity (TLC, as percent predicted), and diffusing capacity for carbon monoxide (DL_{CO}, as percent predicted).

In this study, we defined the date of diagnosis of incident RA-ILD as the date of the first chest CT scan that showed either early/mild or radiologically severe lung interstitial lung abnormalities. We did not analyze patients who were known to have RA-ILD at baseline.

To determine whether the RA-ILD cases identified using these methods had clinical significance, we performed targeted medical record review. First, we reviewed the clinical radiographic report of the initial chest CT scan showing interstitial lung abnormalities. We noted whether RA-ILD or related changes were detected by the clinical radiologist. Second, we determined whether additional clinical evaluation or management of RA-ILD occurred after the chest CT scan (e.g., repeat chest imaging, PFT). Third, we reviewed the medical records to note whether RA-related medications were changed. We were unable to utilize the research data for medications since these were not collected to coincide with clinical events.

Covariates. We prospectively measured the following established risk factors for RA-ILD (10,11) as key covariates, at the BRASS baseline and at yearly study visits: age in years (as a continuous variable), sex (male or female), smoking status (never, past, or current) and pack-years (as a continuous variable), RA duration in years (as a continuous variable), and RA serologic status (seropositive or seronegative). Seropositivity was defined as either CCP- or RF-positive, and seronegativity was both CCP- and RF-negative.

We collected other demographics and lifestyle and clinical factors including race (white or nonwhite), education level (college degree or more versus some college or less), body mass index (kg/m^2, categorized as underweight or normal/overweight/obese, as a continuous variable), physical activity (metabolic equivalent hours per week, as a continuous variable) (25), and a multimorbidity index validated for patients with RA (0, 1, or >1, as a continuous variable) (26).

Throughout the study, we also collected data on other RA characteristics, including the use of methotrexate, nonbiologic disease-modifying antirheumatic drugs (DMARDs), biologic DMARDs, or glucocorticoids; functional status (measured by the Multidimensional Health Assessment Questionnaire [MDHAQ], as a continuous variable) (27); bone erosions or radiographic changes (absent or present); and soft tissue rheumatoid nodules (absent or present).

Statistical analysis. We reported descriptive statistics for the entire analyzed population and also stratified data using the primary exposure variable of moderate/high disease activity versus remission/low disease activity, as measured at the baseline study visit. Proportions of each RA-ILD subtype and PFT results, if available, were used to describe cases of incident RA-ILD.

The primary analysis examined annually collected DAS28 data to investigate changes during follow-up and subsequent risk of RA-ILD. DAS28 data were updated at each yearly study visit and were used to predict the risk of RA-ILD over the next year (until the subsequent study visit). In this analysis, patients with missing DAS28 data were censored without analyzing subsequent person-time, even if RA-ILD was subsequently identified, in order to ensure that changes in the DAS28 near the time of diagnosis were captured. We used Cox regression to estimate hazard ratios (HRs) and 95% confidence intervals (95% CIs) for RA-ILD, with DAS28 as the exposure variable of interest. Person-time accrued from the time point of the baseline BRASS study visit. Censoring, if applicable, occurred at the date of outcome, missing DAS28, loss to follow-up, death, or at the end of study (April 14, 2016), whichever came first. The primary exposure variable was the binary DAS28 measure, based on comparison of the moderate/high disease activity category to the reference category of remission/low disease activity. We initially performed unadjusted Cox proportional hazards regression models. We then included known risk factors for RA-ILD in a multivariable model: age, sex, smoking status, RA duration, and RA serologic status. We performed similar analyses using the 4-level ordinal and continuous DAS28 variables. In the 4-level ordinal DAS28 model, we obtained *P* for trend values by first calculating the mean DAS28 value within each category and then using this as a continuous variable in a separate model.

We performed several alternative analyses to assess the robustness of our findings. First, we defined a cumulative average updated DAS28 score as a long-term measure of all previous DAS28 measures in the BRASS study. For example, for a patient at their third annual study visit, DAS28 values were summed at the baseline, month 12, and month 24 visits and divided by 3 to categorize the long-term disease activity at the current visit (month 36). There were no disease activity measures available for this analysis prior to the BRASS baseline. Second, we performed an analysis in which the DAS28 assessment and the window for assessment of RA-ILD was established as a time span of ≥1 year. Because the onset of RA-ILD may have preceded the date that the clinical CT chest scan was performed, it is possible that active lung inflammation affected joint inflammation and not vice versa. To mitigate this possibility, we performed analyses using a lag to ensure that there was ≥1 year between the date of DAS28 scoring and the date of the chest CT scan identifying RA-ILD. Third, we analyzed all follow-up data instead of censoring at the date of missing DAS28 data, so that all incident RA-ILD cases were captured regardless of whether this occurred in proximity to the DAS28 scoring. We achieved this by using the time-updated DAS28 analysis, as in the primary analysis, and we carried forward the most recent DAS28 observation for this analysis. We also performed these analyses by carrying forward the most recent cumulative average updated observation. Fourth, we analyzed DAS28 at the BRASS baseline only, in order to predict RA-ILD throughout all follow-up, similar to a previous study that did not take into account dynamic changes in DAS28 over time (2).

We also performed alternative analyses with adjustment for additional factors not included in the multivariable model of the primary analysis, in case they affected the relationship between DAS28 and RA-ILD. We did not include DMARD use, glucocorticoid use, or RA severity factors in the primary analysis, because they have complex causal relationships with RA disease activity, they are not clearly related to RA-ILD, and a limited number of RA-ILD outcomes prevented us from including all covariates. In the alternative analyses, we included adjustment for methotrexate use, glucocorticoid use, presence of bone erosions or radiographic changes, and presence of rheumatoid nodules, in separate models that also included the covariates in the primary multivariable model.

To address whether systemic inflammation may have explained the results, we performed an alternative analysis analyzing CRP level as an exposure variable. For this analysis, we considered CRP as the following: 1) a continuous variable, 2) a log-transformed continuous variable, 3) a 3-level variable with clinical cutoffs related to cardiovascular disease risk (3.1–10 mg/liter) or systemic inflammation (>10 mg/liter; reference group 0–3 mg/liter), and 4) divided into quartiles based on the baseline values (reference group: quartile 1).

We verified the proportional hazard assumption in each analysis by including an interaction term between time after index date and the DAS28 variable in each model and verified no statistically significant interaction. We used a *P* value of 0.05 by 2-sided test to define statistical significance. All analyses were performed using SAS version 9.4.

RESULTS

Patient characteristics. A flow diagram of the analyzed study population (n = 1,419) is shown in Figure 1. The mean ± SD age was 55.8 ± 14.2 years, 82.3% of the patients were female, 92.1% were white, and 68.6% were seropositive for either CCP

or RF (Table 1). At the baseline visit, 627 patients (44.2%) were in remission or had low disease activity, and 792 (55.8%) had moderate/high disease activity. Patients in the moderate/high disease activity group were older (age 57.9 ± 13.3 years versus 53.0 ± 14.8 years), were heavier smokers (mean ± SD pack-years 11.0 ± 19.1 versus 8.0 ± 16.8), had longer RA duration (median 11.0 years versus 6.0 years), and were more likely to be seropositive (71.7% versus 64.6%), compared to those in the remission/low disease activity group.

Features of RA-ILD and mortality. We identified a total of 85 cases of incident RA-ILD after the baseline visit. In the primary analysis that censored patients with missing DAS28 data, there were a total of 61 cases of incident RA-ILD during 7,967 person-years of follow-up. The mean ± SD duration of follow-up per patient was 5.6 ± 3.6 years, and the median was 4.5 years (interquartile range [IQR] 2.8–7.9). In the alternative analyses that included person-time even if a patient was missing DAS28 data, there were a total of 12,650

Table 1. Baseline characteristics of the patients overall and according to RA disease activity*

	Total (n = 1,419)	Remission/low disease activity (n = 627)	Moderate/high disease activity (n = 792)
Sociodemographics			
Age, mean ± SD years	55.8 ± 14.2	53.0 ± 14.8	57.9 ± 13.3
Female	1,168 (82.3)	509 (81.2)	659 (83.2)
White	1,297 (92.1)	575 (92.2)	722 (92.0)
College degree	801 (56.8)	422 (67.5)	379 (48.3)
Lifestyle and clinical factors			
BMI, mean ± SD kg/m^2	26.8 ± 5.7	26.1 ± 5.3	27.4 ± 5.9
BMI category			
Underweight/normal	576 (43.4)	288 (49.7)	288 (38.5)
Overweight	423 (31.9)	166 (28.6)	257 (34.4)
Obese	329 (24.7)	126 (21.7)	203 (27.1)
Smoking pack-years, mean ± SD	9.7 ± 18.2	8.0 ± 16.8	11.0 ± 19.1
Smoking status			
Never	679 (47.9)	325 (51.8)	354 (44.7)
Past	503 (35.5)	209 (33.3)	294 (37.1)
Current	98 (6.9)	28 (4.5)	70 (8.8)
MET hours/week, mean ± SD	5.6 ± 5.6	6.7 ± 6.2	4.7 ± 4.9
Multimorbidity index count, mean ± SD	0.7 ± 1.3	0.5 ± 1.1	0.8 ± 1.4
Multimorbidity index count			
0	965 (68.0)	458 (73.1)	507 (64.0)
1	210 (14.8)	85 (13.6)	125 (15.8)
>1	244 (17.2)	84 (13.4)	160 (20.2)
RA characteristics			
RA duration, median (IQR) years	9.0 (3.0–20.0)	6.0 (3.0–15.0)	11.0 (3.0–24.0)
Seropositive for CCP or RF	973 (68.6)	405 (64.6)	568 (71.7)
MDHAQ score, mean ± SD	0.6 ± 0.5	0.4 ± 0.4	0.8 ± 0.5
Methotrexate use	1,061 (74.8)	471 (75.1)	590 (74.5)
Nonbiologic DMARD use	1,336 (94.2)	604 (96.3)	732 (92.4)
Biologic DMARD use	663 (46.7)	313 (49.9)	350 (44.2)
Glucocorticoid use	1,129 (79.6)	487 (77.7)	642 (81.1)
Bone erosion/radiographic changes present	712 (59.4)	239 (43.8)	473 (72.4)
Rheumatoid nodule(s) present	386 (27.6)	102 (16.5)	284 (36.3)

* Except where indicated otherwise, values are the number (%) of patients. Missing data are not shown. RA = rheumatoid arthritis; BMI = body mass index; MET = metabolic equivalent; IQR = interquartile range; CCP = cyclic citrullinated peptide; RF = rheumatoid factor; MDHAQ = Multidimensional Health Assessment Questionnaire; DMARD = disease-modifying antirheumatic drug.

Table 2. Pulmonary features of patients with incident RA-ILD (n = 85)*

RA-ILD subtype, no (%)	
Cellular NSIP	42 (49.4)
Fibrotic NSIP	27 (31.8)
UIP/AIP/DAD	16 (18.8)
Pulmonary function test results†	
FEV, mean ± SD % predicted	73.5 ± 22.1
FVC, mean ± SD % predicted	77.6 ± 17.6
FEV_1/FVC	0.74 ± 0.11
TLC, mean ± SD % predicted	85.4 ± 17.5
DLco, mean ± SD % predicted	62.3 ± 20.8

* NSIP = nonspecific interstitial pneumonia; UIP = usual interstitial pneumonia; AIP = acute interstitial pneumonia; DAD = diffuse alveolar damage; FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; TLC = total lung capacity; DLco = diffusing capacity for carbon monoxide.
† Pulmonary function test results were available for 64 patients (75.3%) with rheumatoid arthritis–associated interstitial lung disease (RA-ILD).

person-years of follow-up (mean ± SD 8.9 ± 4.2 years and median 11.4 years [IQR 4.0–12.5] per patient).

Table 2 describes the total 85 incident RA-ILD cases. Most patients had either cellular NSIP (49.4%; n = 42) or fibrotic NSIP (31.8%; n = 27) based on the review of chest CT scans. A minority of patients (18.8%; n = 16) were diagnosed as having UIP, AIP, or DAD. Among the RA-ILD cases with PFT results available (75.3%; n = 64), the mean ± SD of FVC, TLC, and DL_{CO} percent predicted was 77.6% ± 17.6, 85.4% ± 17.5, and 62.3% ± 20.8, respectively.

Of the 78 patients with RA-ILD for whom clinical radiographic reports were available, 68 (87.2%) had RA-ILD, or features consistent with the diagnosis, noted on the report. After chest CT scans were completed, 64 (75.3%) of the 85 patients with RA-ILD had additional evaluations or follow-up for the condition. All patients who had scans in which severe interstitial lung abnormalities were detected also had RA-ILD or features consistent with the diagnosis noted on the clinical radiographic report, and they had additional evaluations or follow-up for RA-ILD. During medical record review, we observed that 32 patients (37.6%) with RA-ILD had documented changes to RA-related medications after the chest CT scan. We identified a total of 126 deaths (8.9%) among the analyzed study population. Among patients with incident RA-ILD, 32 (37.6%) died.

DAS28 and risk of incident RA-ILD. Table 3 shows the results of the analyses of annual DAS28 scores and risk of RA-ILD, censored after a patient had missing DAS28 data. During follow-up, there was more person-time categorized as remission/low disease activity (5,459 person-years; 68.5%) than as moderate/high disease activity (2,508 person-years; 31.5%). In the primary analysis using annual DAS28 score categories as a binary variable, the moderate/high disease activity group had an HR of 3.11 (95% CI 1.86–5.20) for RA-ILD compared to the remission/low disease activity group, in the unadjusted analysis. After adjustment for age, sex, smoking pack-years, RA duration, and serologic status, the results were attenuated, but there was still a statistically significant association of moderate/high disease activity with increased risk of RA-ILD (HR 2.22 [95% CI 1.28–3.82]).

In the secondary analysis that used the 4-level ordinal DAS28 variable, both high disease activity (multivariable HR 3.48 [95% CI 1.64–7.38]) and moderate disease activity (HR 2.08 [95% CI 1.06–4.05]) were associated with an increased risk of RA-ILD, compared to the remission category. There was a statistically significant trend toward increased risk of RA-ILD across the 4 disease activity categories (*P* for trend = 0.001). When annual DAS28 data were analyzed as a continuous vari-

Table 3. HRs for incident RA-ILD according to annual DAS28 measures in sample population (n = 1,419)*

	No. of cases/person-years	Unadjusted HR (95% CI)	Multivariable adjusted HR (95% CI)†
DAS28 as binary variable			
Remission/low (≤3.2)	26/5,459	1.00 (referent)	1.00 (referent)
Moderate/high (>3.2)	32/2,508	3.11 (1.86–5.20)	2.22 (1.28–3.82)
DAS28 as 4-level ordinal variable‡			
Remission (<2.6)	18/4,232	1.00 (referent)	1.00 (referent)
Low (2.6 to 3.2)	8/1,227	1.57 (0.68–3.62)	1.41 (0.61–3.28)
Moderate (>3.2 to 5.1)	20/1,828	2.73 (1.44–5.17)	2.08 (1.06–4.05)
High (>5.1)	15/680	5.88 (2.92–11.82)	3.48 (1.64–7.38)
DAS28 as continuous variable			
Per unit increase	61/7,967	1.53 (1.31–1.79)	1.35 (1.14–1.60)

* HRs = hazard ratios; RA-ILD = rheumatoid arthritis–associated interstitial lung disease; DAS28 = Disease Activity Score in 28 joints; 95% CI = 95% confidence interval.
† The multivariable model included adjustment for age, sex, smoking pack-years, RA duration, and serologic status.
‡ *P* for trend in the unadjusted model was <0.001 in the unadjusted model and 0.001 in the adjusted model.

able, findings were similar. For every unit increase in the DAS28, there was a 35% increase in the risk of RA-ILD (HR 1.35 [95% CI 1.14–1.60]).

Alternative analyses. Figure 2 shows results of the primary analysis as well as alternative analyses, using different follow-up strategies for the binary DAS28 measure and with adjustment for additional covariates. These results were similar to those of the primary analysis. With use of the cumulative average of DAS28 data, moderate/high disease activity remained significantly associated with RA-ILD compared to remission/low disease activity (HR 1.83 [95% CI 1.03–3.22]). Moderate/high disease activity was also associated with an increased risk of RA-ILD in the lagged analysis that ensured ≥1 year between DAS28 assessment and onset of the disease (HR 2.63 [95% CI 1.51–4.60]). In the analyses that included all follow-up data instead of censoring after the date of missing DAS28 data, moderate/high disease activity remained significantly associated with an increased risk of RA-ILD. When only the DAS28 data obtained at the baseline study visit were taken into account, moderate/high disease activity was associated with a >2-fold increased risk of RA-ILD (HR 2.55 [95% CI 1.45–4.49]), compared to remission/low disease activity.

To assess whether residual confounding by other factors may have explained our results, we performed additional analyses that included other adjustment variables beyond those included in the multivariable model in our primary analysis. Moderate/high disease activity remained significantly associated with RA-ILD in these analyses, and results were similar to those of the primary analysis. The 2-fold increased risk of RA-ILD with moderate/high disease activity compared to remission/low disease activity remained statistically significant after adjustment for methotrexate use, glucocorticoid use, presence of bone erosions or radiographic changes, and presence of rheumatoid nodules. In these multivariable models, methotrexate use (HR 0.67 [95% CI 0.37–1.22]), presence of bone erosions (HR 0.58 [95% CI 0.28–1.24]), and presence of rheumatoid nodules (HR 1.44 [95% CI 0.80–2.60]) were not associated with a risk of RA-ILD, but glucocorticoid use was associated with an increased risk (HR 2.49 [95% CI 1.06–5.81]), independent of disease activity and the other risk factors.

In the adjusted analyses that took into account only CRP results in relation to RA-ILD risk, there was no association of risk with continuous CRP level (multivariable HR 1.003 per mg/liter [95% CI 0.997–1.011]) or log-transformed continuous CRP level (HR 1.19 per log-mg/liter [95% CI 0.97–1.46]). For the 3-level categorical variable, neither a CRP level of >10 mg/liter (HR 1.80 [95% CI 0.86–3.78]) nor a CRP level of 3.1–10 mg/liter (HR 1.15 [95% CI 0.55–2.39]) was associated with risk of RA-ILD compared to a CRP level of 0–3 mg/liter (*P* for trend = 0.12) in

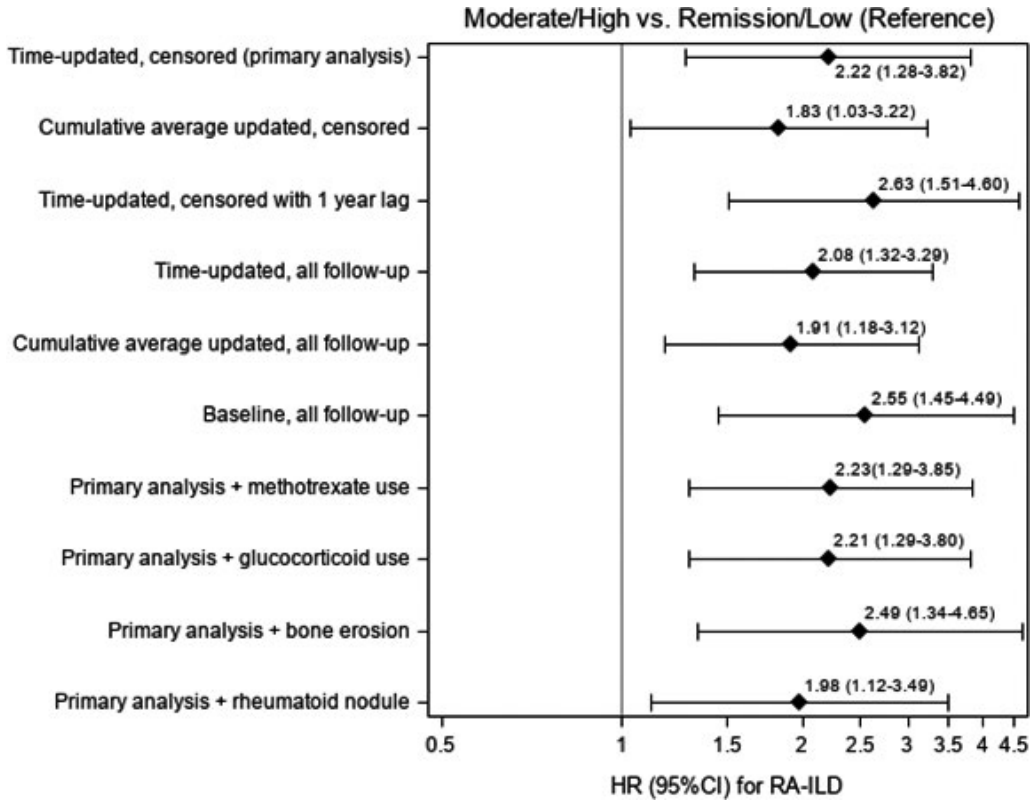

Figure 2. Multivariable hazard ratios (HRs) for incident rheumatoid arthritis–associated interstitial lung disease (RA-ILD) for moderate/high disease activity compared to remission/low disease activity, in the primary and alternative analyses (n = 1,419). Results include adjustment for the covariates listed in Table 3, unless otherwise stated. 95% CI = 95% confidence interval.

adjusted models. There was also no association of CRP quartile with RA-ILD (quartile 4 versus quartile 1 multivariable HR 2.09 [95% CI 0.67–6.50]).

DISCUSSION

In this large secondary analysis of prospectively collected data, we identified active RA as being associated with an increased risk of subsequent development of RA-ILD. Patients with moderate/high RA disease activity, as defined by the DAS28, had a 2-fold increased risk of developing RA-ILD compared to those in the remission/low disease activity group. This association was independent of known risk factors for RA-ILD, including sex, smoking, RA duration, and RA serologic status. These results suggest that systemic inflammation contributes to the pathogenesis of RA-ILD and that reducing disease activity could influence the natural history of RA-ILD development. These results may have important implications for rheumatologists and RA patients, as they could provide additional motivation to achieve and maintain remission or low disease activity in order to possibly reduce the future risk of developing RA-ILD, a devastating extraarticular RA manifestation.

Some earlier studies have investigated articular RA involvement and RA-ILD risk (8,9). In a previous single-center US study, patients with prevalent RA-ILD were compared to patients with RA and without ILD (8). In that cross-sectional analysis, DAS28 as a continuous variable was significantly associated with increased odds of having prevalent RA-ILD (odds ratio [OR] 1.49 [95% CI 1.23–1.80]). However, a smaller cross-sectional study in Mexico did not demonstrate an association between continuous DAS28 and prevalent RA-ILD (OR 0.29 [95% CI 0.03–1.48]), but there were few RA-ILD cases (9). Both of these studies investigated other factors in addition to disease activity, so they did not focus on analyzing DAS28 measures prior to RA-ILD diagnosis. In a previous prospective cohort study conducted in the UK that analyzed data from 1,460 patients, 52 incident cases of RA-ILD (3.6%) were identified during a median of 10 years of follow-up (2). In that study, continuous DAS28 at baseline had no association with subsequent risk of developing RA-ILD (HR 1.11 [95% CI 0.94–1.31]) (2). Similarly, 46 (7.9%) of 582 patients developed RA-ILD during a median of 16.4 years of follow-up in Olmsted County, MN (1). DAS28 was not investigated as a factor for RA-ILD risk in this study. However, swelling of a large joint(s) (any occurrence during follow-up) had no statistically significant association with RA-ILD (HR 1.90 [95% CI 0.83–4.36]) (1). The aim of these cohort studies was to investigate the epidemiology and mortality outcomes of RA-ILD, so they were not specifically focused on the relationship of DAS28 and RA-ILD.

We found that active RA, defined by moderate/high disease activity according to the DAS28, was associated with a 2-fold increased risk of RA-ILD compared to remission/low disease activity, even with adjustment for known RA-ILD risk factors such as age, cigarette smoking status, and RA serologic status. We found a strong dose effect across the 4 levels of DAS28 and when analyzing DAS28 as a continuous variable. The current paradigm to treat to a target of remission or low disease activity implies that treating articular inflammation with DMARDs may also alter the natural history of the development of RA-ILD (15). This also suggests that articular and pulmonary inflammation may be intrinsically related (28,29). Similar to results of previous studies (1,2,4,13,30–33), we observed high mortality rates in patients who developed RA-ILD. More than one-third of patients with RA-ILD died by the end of the follow-up period. This finding underscores the importance of further elucidating the natural history of RA-ILD prior to clinical manifestation, in order to develop interventions that may delay or prevent RA-ILD onset or alter its course.

Many DMARDs have been reported to possibly contribute to the onset of RA-ILD, but published reports have typically been limited to descriptions of individual cases or series, so causality is difficult to assess (34). In particular, methotrexate has been reported to cause, in rare cases, pneumonitis or fibrosis, which may be similar to RA-ILD (35). Conversely, we found no association of methotrexate use with risk of RA-ILD, and a previous secondary analysis of placebo-controlled studies showed no association of methotrexate use with pulmonary outcomes (36). An analysis of a double-blind randomized placebo-controlled trial of methotrexate is ongoing (37). Further studies are needed to establish whether disease activity affects the onset and course of RA-ILD and to understand the role that particular DMARDs may play in its pathogenesis.

To our knowledge, the present study is the first to comprehensively investigate dynamic changes in disease activity and risk of RA-ILD. We performed these analyses using a large prospective RA cohort with a lengthy follow-up period that included repeated measures of DAS28 and other potential confounders of RA-ILD risk. Our study design accounted for the dynamic nature of the DAS28 instead of relying only on a single measure at baseline as previously done. We also had clinical data available, with lengthy follow-up, in order to capture RA-ILD outcomes. We used expert adjudication that systematically reviewed clinically indicated chest CT scans to identify the presence and date of onset of incident RA-ILD and to determine the subtype of the condition (21). We performed targeted medical record review and determined that the majority of RA-ILD cases in our analysis had findings noted on the clinical radiographic report that were consistent with the diagnosis, and many had additional evaluations and changes to their RA-related medications. This suggests that most of the cases in this analysis had clinically significant RA-ILD. Finally, we performed many analyses to ensure that our results remained robust when using other DAS28 measure definitions and alternative censoring/follow-up analysis strategies.

Our study has some limitations to consider. We only performed this study at a single tertiary care center, where patients were relatively well-educated, were mostly white, and agreed to participate in a research study. Replication of these results in other geographic settings and using populations with different socio-

demographics is needed. The BRASS registry is not restricted to incident RA, so some patients had longstanding disease at baseline, which may have affected the rate of progression to RA-ILD (31,38,39). DAS28 data from prior to enrollment were not available for analysis. Additionally, DAS28 data were only available annually, so we were unable to analyze intermittent changes or flares that may have affected the exposure variable classification. While we had rich data on many confounding factors, including smoking, serologic status, duration of RA, and the presence of rheumatoid nodules or bone erosions, there may be residual confounding of RA disease severity that could have been adjusted for if information on quantity or size of nodules, or Sharp/van der Heijde scores (40) of hand radiographs, were available for all patients. Treat-to-target guidelines for RA typically advocate for assessments of disease activity that are more frequent than once per year, particularly for patients with early or active RA (15). The cohort we analyzed was part of an observational study and was not created to encourage treatment to target. The frequency of clinical visits and treatment decisions for these patients were at the discretion of the treating rheumatologist, and we were unable to extract disease activity measures from clinical notes for this research study. Therefore, the observational research assessments of disease activity in our study may not completely mirror clinical care, since disease activity was only measured annually.

We may not have detected periods of high or low disease activity occurring between the annual research visits. However, this non-differential misclassification of the exposure variable would bias the effect size toward the null, so that it would not explain the positive association that we report. It is possible that other strategies for analyzing disease activity, such as area under the curve (AUC) as a time-integrated approach for disease activity, may have affected the relationship between disease activity and RA-ILD risk that we identified. AUC strategies are typically used in studies investigating patients with early, active RA, including frequent measures of disease activity to quantify duration of time spent in active disease (41). This would not apply to the BRASS study, which included some patients with prevalent RA, some patients who enrolled during disease remission, less frequent disease activity measures, and varying durations of follow-up. We find it reassuring that all strategies used to analyze disease activity (4-level variable, continuous variable, binary variable, annually updated, cumulative average update, baseline only, censoring at last follow-up, and including all follow-up) yielded a similar association, showing that patients with active disease had an increased RA-ILD risk.

Our study focused on the association of disease activity and risk of RA-ILD, rather than on particular DMARDs. There are currently conflicting reports on whether specific DMARDs may reduce or increase the risk of RA-ILD. Our results showing that disease activity may impact RA-ILD risk provide rationale for pharmacoepidemiologic comparative safety studies or randomized controlled trials for the risk and progression of RA-ILD.

While we used a stringent system to identify patients with incident RA-ILD, we relied on clinically indicated chest CT scans, so some cases of subclinical RA-ILD may not have been detected if there were no clinical signs or symptoms that would prompt the performance of imaging studies. Incorporating serial chest CT scans for research purposes during follow-up would likely have led to the identification of more patients with subclinical RA-ILD, but it is unclear whether those imaging findings have clinical significance. Since mortality rates were high among those with incident RA-ILD, we can conclude that many of the patients in our study with clinically apparent incident RA-ILD likely had clinically significant disease. In future studies, chest CT scans should be performed serially for research purposes to establish the prevalence, clinical significance, and natural history of subclinical RA-ILD.

We designed this study using the hypothesis that articular inflammation may influence development of RA-ILD. However, there is likely a period of subclinical RA-ILD that may influence articular disease and could explain our results. Since RA-ILD had not been clinically detected in certain patients, treatment choices were directed by rheumatologists for articular RA manifestations, so the results of the present study may have clinical implications regardless of the underlying mechanisms. Further studies are necessary to better understand the association of active articular disease and risk of RA-ILD. Our results showed no statistical association of CRP levels with RA-ILD, which suggests that the articular RA involvement may be responsible for the association of DAS28 scores with RA-ILD risk.

We used the date of chest CT scan as the date of onset of RA-ILD, but this may not reflect the true date of onset of RA-ILD. However, we found similar results when ensuring that there was ≥1 year between DAS28 assessment and evaluation for RA-ILD. Future studies are needed to understand the timing and trajectory of PFT and imaging abnormalities in relation to clinical RA-ILD onset (42). While the majority of patients in the BRASS study received all of their clinical care in the Partners HealthCare system, it is possible that some may have had chest CT scans elsewhere that we were unable to review. This misclassification of the outcome typically biases toward the null, so we think it is unlikely to explain our results. While we analyzed categories of disease activity based on ACR recommendations (20), it is possible that the DAS28 may have misclassified some patients compared to alternative measures or different category cutoffs (43). As in all observational studies, our results may have been confounded by other factors. We performed alternative analyses with adjustment for additional factors including medication use and RA severity factors, which produced similar results. There may be other unmeasured factors including biomarkers and a recently identified genetic risk factor in the *MUC5B* gene, the inclusion of which may have affected our results (44–47). We encourage continued collaborative studies to identify and validate additional risk factors for RA-ILD, as well as to develop and

test interventions that may reduce RA-ILD susceptibility and improve outcomes.

In conclusion, we identified active RA as a potential risk factor for the development of RA-ILD. We found that moderate/high disease activity was associated with a 2-fold increase in risk of RA-ILD compared to remission/low disease activity. This association was independent of known RA-ILD risk factors as well as DMARD use, glucocorticoid use, and RA disease severity factors. These results suggest that decreasing systemic inflammation by treating RA signs and symptoms may delay or even prevent the onset of RA-ILD.

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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. Sparks 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. Sparks, Karlson, Shadick, Doyle.
Acquisition of data. Sparks, Fletcher, Zaccardelli, Friedlander, Gill, Hatabu, Nishino, Murphy, Iannaccone, Mahmoud, Frits, Weinblatt, Shadick, Doyle.
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Malondialdehyde–Acetaldehyde Adducts and Antibody Responses in Rheumatoid Arthritis–Associated Interstitial Lung Disease

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Objective. To compare serum anti–malondialdehyde–acetaldehyde (anti-MAA) antibody levels and MAA expression in lung tissue from patients with rheumatoid arthritis–associated interstitial lung disease (RA-ILD) to those found in controls.

Methods. Anti-MAA antibody (IgA, IgM, IgG) concentrations were measured in patients with RA-ILD and compared to those of RA patients with chronic obstructive pulmonary disease (COPD) and RA patients without lung disease. Associations between anti-MAA antibody with RA-ILD were assessed using multivariable logistic regression. Lung tissue from patients with RA-ILD, other ILD, or emphysema, and from controls (n = 3 per group) were stained for MAA, citrulline, macrophages (CD68), T cells (CD3), B cells (CD19/CD27), and extracellular matrix proteins (type II collagen, fibronectin, vimentin). Tissue expression and colocalization with MAA were quantified and compared.

Results. Among 1,823 RA patients, 90 had prevalent RA-ILD. Serum IgA and IgM anti-MAA antibody concentrations were higher in RA-ILD than in RA with COPD or RA alone ($P = 0.005$). After adjustment for covariates, the highest quartiles of IgA anti-MAA antibody concentration (odds ratio 2.09 [95% confidence interval 1.11–3.90]) and IgM (odds ratio 2.23 [95% confidence interval 1.19–4.15]) were significantly associated with the presence of RA-ILD. MAA expression in RA-ILD lung tissue was greater than in tissue from all other groups ($P < 0.001$), and it colocalized with citrulline (r = 0.79), CD19+ B cells (r = 0.78), and extracellular matrix proteins (type II collagen [r = 0.72] and vimentin [r = 0.77]) to the greatest degree in RA-ILD.

Conclusion. Serum IgA and IgM anti-MAA antibody is associated with ILD among RA patients. MAA is highly expressed in RA-ILD lung tissue, where it colocalizes with other RA autoantigens, autoreactive B cells, and extracellular matrix proteins, highlighting its potential role in the pathogenesis of RA-ILD.

INTRODUCTION

Interstitial lung disease (ILD) is a major determinant of poor long-term outcomes in rheumatoid arthritis (RA) patients, who already experience high rates of premature mortality. Median survival rates following a diagnosis of RA-associated ILD (RA-ILD) have been reported to be as short as 3 years (1), and trends in mortality related to RA-ILD do not appear to be declining (2). The estimated prevalence of clinically apparent ILD is 5–15% in RA patients, with up to 30% showing subclinical disease on high-resolution computed tomography (HRCT) (1–4). Wide-ranging epidemiologic estimates contribute to the difficulty in establishing a diagnosis of RA-ILD, which relies on multidisciplinary evaluation that often includes pulmonary function testing, HRCT of the chest,

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and/or lung biopsy (5,6). With a poorly understood pathogenesis and development of clinical symptoms well after radiologic or physiologic abnormalities have been established (4,7), delays in diagnosis of RA-ILD are common. These delays in detection may be particularly harmful if substantial irreversible decline occurs before effective management or other preventative strategies are initiated.

Recognizing the occurrence of diagnostic uncertainties and associated delays, there have been efforts to identify biomarkers capable of accurately identifying patients with or at risk of developing RA-ILD. Candidate biomarkers have included Krebs von den Lungen 6, matrix metalloproteinase 7 (MMP-7), interferon-γ–inducible protein 10 (IP-10), pulmonary and activation-regulated chemokine (PARC), surfactant protein D (Sp-D), antibody to citrullinated–heat-shock protein 90 (anti–cit-Hsp90), and a *MUC5B* promoter variant (8–12). While these have shown promise and provided important insight into putative pathways driving disease, their utility in clinical practice has yet to be demonstrated. Of the biomarkers reported to date, some appear to lack specificity for RA-ILD, while others have been subject to limited testing in RA patients with other lung diseases (such as chronic obstructive pulmonary disease [COPD]) or have not been applied more broadly to large RA patient populations. Thus, there is a need for ongoing identification and characterization of biomarkers for RA-ILD (13).

The pathophysiology of RA-ILD encompasses multiple complex, interrelated processes, including inflammation, autoimmunity, fibrosis, and oxidative stress (6,14). Malondialdehyde–acetaldehyde (MAA) adducts are highly immunogenic products of oxidative stress with the potential to facilitate tolerance loss in the absence of adjuvant (15). Antibody responses to MAA in RA patients have been described by our group and are associated with both anti–citrullinated protein antibody (ACPA) responses and disease activity (16). Additionally, MAA colocalizes with citrulline and immune cells in RA synovium, and both MAA and anti-MAA antibody expression are enriched in RA synovial tissue (16,17). Beyond its potential contributions to articular disease, MAA has been demonstrated to stimulate inflammation and fibrosis in airway epithelial cells in animal models and in vitro (18,19).

Recognizing the proinflammatory and profibrotic properties of MAA and considering our observations of increased anti-MAA antibody responses in RA, we hypothesized that MAA expression and anti-MAA antibody concentrations would be increased in RA-ILD. We tested this hypothesis by comparing circulating anti-MAA antibody concentrations in patients with RA-ILD to those in other RA patients, including patients with other chronic lung conditions. Additionally, we examined MAA expression in lung tissue from patients with RA-ILD, other ILD (non–RA-ILD), or emphysema, and in normal lung tissue, assessing colocalization with other RA autoantigens, as well as immune cells that have been consistently implicated in RA pathogenesis.

PATIENTS AND METHODS

Study population and samples. Serum analyses were conducted among participants in the Veterans Affairs Rheumatoid Arthritis (VARA) registry (20). The VARA registry is a multicenter prospective observational study of US veterans with RA who meet the 1987 American College of Rheumatology (ACR) criteria (21), and it includes patients from 13 sites. Participants provided informed consent prior to enrollment, all sites obtained local institutional review board approval, and the present study was approved by the VARA Scientific Ethics and Advisory Committee. At enrollment, data on participant demographics, smoking status, education, disease onset, medications, and comorbidities were recorded. At enrollment and follow-up visits, ACR core measures including the Multidimensional Health Assessment Questionnaire (22), 28-joint tender and swollen joint counts, and patient and provider global assessments were collected, acute-phase reactants were measured, and composite disease activity measures were scored (e.g., Disease Activity Score in 28 joints [DAS28]) (23).

Lung tissue was obtained from the National Heart, Lung, and Blood Institute Lung Tissue Research Consortium (https://ltrcpublic.com). Samples (n = 3 per group) were obtained according to a standard protocol from individuals with RA-ILD, ILD (non–RA-related: nonspecific interstitial pneumonia [NSIP] [n = 2] and idiopathic pulmonary fibrosis [n = 1]), and emphysema (pathologic diagnosis), and from controls who underwent transplant procedures, lung volume reduction surgery, or biopsies. The latter control samples were typically collected during evaluation of suspected malignancy and had normal surrounding tissue.

Characterization of lung disease in the VARA. International Classification of Diseases (ICD) Ninth and Tenth Revision codes (ICD-9: 515, 516.3, 516.8, 516.9, 714.8; ICD-10: M05.1, J84.1, J84.9, J99.0) (2,24–26) were used for initial ILD case finding within the VARA registry. Inpatient and outpatient visit diagnoses in the Corporate Data Warehouse were queried within the VA informatics and computing infrastructure (27). Medical record review was performed within the Compensation and Pension Record Interchange for all participants with ≥2 outpatient or ≥1 inpatient diagnostic codes for ILD. Diagnoses by provider specialty (pulmonologist, rheumatologist, or other physician), imaging findings (CT and chest radiography), lung pathology, pulmonary function test (PFT) results, and corresponding dates were abstracted. Participants were classified as having RA-ILD if they had a pulmonologist diagnosis and imaging findings of ILD, or if they had a non-pulmonologist physician diagnosis plus 2 of the following: CT or chest radiography findings interpreted by the reading radiologist as ILD, pathology from a lung biopsy consistent with ILD, or PFTs as restrictive disease interpreted by the reading pulmonologist. COPD diagnoses (clinical diagnoses of chronic bronchitis and emphysema) were extracted from medical records

and recorded in the VARA registry by treating rheumatologists at the time of VARA enrollment.

Patients were categorized into 1 of 3 mutually exclusive groups: 1) RA-ILD (with or without comorbid COPD), 2) COPD in the absence of ILD, or 3) neither RA-ILD nor COPD. Recognizing that pathophysiologic processes, radiologic and physiologic abnormalities, and clinical symptoms precede a formal diagnosis of ILD (resulting in diagnostic delays) (4,7), a 2-year period following VARA enrollment (time of serum collection) was used for classifying prevalent ILD. We excluded patients with indeterminate ILD (physician diagnosis, CT evidence, or biopsy findings, but failure to fulfill the aforementioned algorithm) (see Supplementary Figure 1, on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40900/abstract).

Measurement of serum and tissue analytes. Anti-MAA antibodies (IgA, IgM, and IgG isotypes) were measured by enzyme-linked immunosorbent assay (ELISA) in VARA participants using banked serum from enrollment, and reported in relative units (RU) as previously described (16). We categorized anti-MAA antibody values into quartiles to assess trends over the range of values as well as to dichotomize the anti-MAA antibody isotypes into high versus low concentrations, with the upper 3 quartiles considered high (approximating the frequency of other RA-related autoantibodies, including both anti–cyclic citrullinated peptide antibody [anti-CCP] and rheumatoid factor [RF]). Anti-CCP antibodies were measured using a second generation ELISA, while RF was measured by nephelometry (28).

Lung tissue was stained for MAA using an in-house MAA-specific rabbit polyclonal antibody that was labeled with a Zenon 405 reporter (Molecular Probes) and for citrullinated proteins using a citrulline-specific mouse IgM monoclonal antibody, clone F95 (Millipore). A C3-conjugated AffiniPure F(ab')$_2$ fragment goat anti-mouse IgM, μ chain–specific (Jackson ImmunoResearch) was used as the detection antibody for clone F95. Immune cell types (macrophages, T cells, and B cells) were stained using Alexa Fluor 594–conjugated polyclonal anti-CD68 and anti-CD27 antibodies, and Alexa Fluor 647–conjugated polyclonal anti-CD3 and anti-CD19 antibodies (Bioss). Tissue was incubated with isotype controls using Alexa Fluor 594– or Alexa Fluor 647–conjugated rabbit IgG. Based on prior analyses of paired lung and synovial tissue (29), we also stained for extracellular matrix proteins: Alexa Fluor 488–conjugated polyclonal type II collagen antibodies, Alexa Fluor 647–conjugated polyclonal vimentin antibodies, and Alexa Fluor 555–conjugated polyclonal fibronectin antibodies. Tissue was imaged using a confocal laser scanning microscope, and staining was quantified using pixel densities, as in prior studies (16,17).

Statistical analysis. Baseline characteristics were compared between those with RA-ILD, RA with COPD, and RA alone, using chi-square test or analysis of variance (ANOVA). Anti-MAA antibodies were compared between groups using the Kruskal-Wallis test with Dunn's post hoc test including Bonferroni correction. Two multivariable logistic regression models were used to assess the association of anti-MAA antibody with RA-ILD status, with covariates specified a priori; the RA with COPD and RA alone groups were combined and served as the comparator group, because there were not significant differences in anti-MAA antibody concentration between these groups in unadjusted comparisons. The first (model A) included adjustment for known patient characteristics associated with RA-ILD: age, sex, ethnicity, and smoking status. The second (model B) included covariates from model A in addition to RA-specific factors reported to be associated with ILD: anti-CCP antibody positivity and disease activity (DAS28) (1,30–32). Anti-MAA antibody isotypes were tested in separate models because of collinearity. Missing data were handled by complete-case analysis with complete data available for >98% of participants.

Tissue staining of MAA, citrulline, immune cells, and extracellular matrix proteins was compared between RA-ILD, other ILD, emphysema, and normal tissue controls via ANOVA with Tukey's post hoc test to account for multiple comparisons. Colocalization of MAA with immune cells and extracellular matrix proteins was determined using the Fiji plug-in, Coloc 2 in ImageJ, as previously reported (17). To confirm the validity of this approach, we also measured colocalization between MAA and citrulline using Zen blue software (Zeiss) in normal and RA-ILD tissue. Pearson's correlations were compared across groups using ANOVA. Results were consistent between the 2 approaches (Coloc 2: normal r = 0.12, RA-ILD r = 0.79, $P < 0.001$; Zen blue: normal r = 0.19, RA-ILD r = 0.72, $P < 0.001$). Thus, the remainder of colocalization analyses were completed using Coloc 2 in ImageJ. *P* values less than 0.05 were considered significant. Analyses were completed using Stata version 15.0. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated.

RESULTS

Study cohort derivation and characteristics. Of the 2,695 patients in the VARA registry, 1,885 had anti-MAA antibody measurements from a prior study (measured in the entire cohort at that time) (16). Diagnostic code screening and subsequent chart review confirmed 90 prevalent ILD cases; an additional 63 participants were excluded because of indeterminate ILD status (see Supplementary Figure 1, http://onlinelibrary.wiley.com/doi/10.1002/art.40900/abstract). Baseline characteristics of the eligible participants in the VARA registry (n = 1,823), stratified by lung disease status, are shown in Table 1. Those with RA-ILD were older, more often male, had at least a high school education, were seropositive, and had received biologic disease-modifying antirheumatic drugs or prednisone. Methotrexate use was taken less frequently by those with RA-ILD. RA patients with

Table 1. Baseline characteristics of Veterans Affairs Rheumatoid Arthritis registry participants*

	Overall (n = 1,823)	RA-ILD (n = 90)	RA with COPD (n = 294)	RA alone (n = 1,439)	*P*
Age, mean ± SD years	63.5 ± 11.0	67.0 ± 9.9	65.8 ± 9.7	62.8 ± 11.3	<0.001
Male sex	90.1	95.6	92.5	89.2	0.05
Caucasian	76.7	76.7	83.7	76.2	0.02
High school education	86.4	91.7	78.9	87.5	<0.001
Smoking status					<0.001
Current	26.1	27.8	31.0	25.1	
Former	53.4	58.9	58.8	52.0	
Never	20.4	13.3	10.2	23.0	
BMI, mean ± SD kg/m^2	28.4 ± 5.7	27.8 ± 5.1	28.3 ± 6.1	28.4 ± 5.7	0.67
RDCI score, mean ± SD	1.9 ± 1.5	3.2 ± 1.6	3.9 ± 1.1	1.4 ± 1.2	<0.001
RA duration, mean ± SD years	11.1 ± 11.5	13.3 ± 13.1	11.1 ± 11.9	10.9 ± 11.3	0.17
SE-positive	68.8	65.6	73.0	68.2	0.22
Anti-CCP–positive	77.3	86.7	80.3	76.0	0.03
RF-positive	79.8	92.2	80.6	78.9	0.009
MDHAQ score, mean ± SD	0.9 ± 0.6	0.9 ± 0.5	1.1 ± 0.6	0.9 ± 0.6	0.004
DAS28, mean ± SD	4.0 ± 1.6	4.1 ± 1.4	4.4 ± 1.5	3.9 ± 1.6	0.003
Methotrexate usage	51.9	21.0	47.6	54.7	<0.001
Biologic usage	22.9	30.0	16.3	23.8	0.005
Prednisone usage	43.5	63.0	43.1	42.4	0.01

* Except where otherwise indicated, values are percentage of patients. *P* values for group differences were determined by analysis of variance or chi-square test. RA-ILD = rheumatoid arthritis–associated interstitial lung disease; COPD = chronic obstructive pulmonary disease; BMI = body mass index; RDCI = rheumatic disease comorbidity index; SE = shared epitope; anti-CCP = anti–cyclic citrullinated peptide; RF = rheumatoid factor; MDHAQ = Multidimensional Health Assessment Questionnaire; DAS28 = Disease Activity Score in 28 joints.

COPD were less likely to be Caucasian, less likely to have a high school education, and were more likely to be current smokers.

Characteristics of RA-ILD cases are shown in Supplementary Table 1 (http://onlinelibrary.wiley.com/doi/10.1002/art.40900/abstract). The vast majority of cases were confirmed based on a pulmonologist diagnosis (97.8%) and CT evidence (94.4%). PFTs showing restrictive disease were present in 60.0% of cases with biopsy confirmation in 13.3%. ILD was present for a mean of 2.3 years prior to enrollment and attributed to RA in 93.3% of cases. ILD pattern was reported for only 38.9% of cases, with usual interstitial pneumonia (UIP) being the most common pattern.

Serum anti-MAA antibody and RA-ILD. Median serum concentrations of IgA and IgM anti-MAA antibody were higher

Table 2. Anti-MAA antibody concentrations among RA patients*

	RA-ILD (n = 90)	RA with COPD (n = 294)	RA alone (n = 1,439)	*P*
IgA anti-MAA antibody	891 (501–1,624)†	869 (399–1,665)†	689 (323–1,440)	0.005
IgM anti-MAA antibody	3,582 (1,302–11,141)†‡	2,332 (888–5,649)	2,094 (843–5,610)	0.005
IgG anti-MAA antibody	2,226 (1,353–3,781)	1,996 (1,039–3,701)	1,868 (943–3,415)	0.09

* Values are the median (interquartile range) in relative units. *P* values were determined by Kruskal-Wallis test using unadjusted comparisons. Anti-MAA = anti–malondialdehyde–acetaldehyde (see Table 1 for other definitions).
† $P < 0.05$ versus RA alone (using Dunn's test including Bonferroni correction).
‡ $P < 0.05$ versus RA with COPD (using Dunn's test including Bonferroni correction).

among those with RA-ILD than with RA alone (all *P* values <0.05) (Table 2). Additionally, median serum concentrations of IgM anti-MAA antibody were significantly higher in patients with RA-ILD (3,582 RU) than in patients with RA with COPD (2,332 RU) (*P* = 0.01). IgG anti-MAA antibody was not significantly different between RA-ILD, RA with COPD, and RA alone (*P* = 0.09).

After multivariable adjustment for patient characteristics and RA-related factors, higher quartiles of IgA and IgM anti-MAA antibody remained significantly associated with RA-ILD (Table 3). Notably, inclusion of anti-CCP antibody positivity and DAS28 in multivariable models had minimal impact on the associations between anti-MAA antibody and RA-ILD. High values of IgA anti-MAA antibody, defined as the upper 3 quartiles, were associated with >2-fold higher odds of RA-ILD (OR 2.09 [95% CI 1.11–3.90] in fully adjusted model) in the absence of a dose-dependent relationship across quartiles (*P* for trend = 0.07). As with IgA isotypes, higher values of IgM anti-MAA antibody were also significantly associated with RA-ILD (OR 2.23 [95% CI 1.19–4.15]) but demonstrated a dose-dependent relationship between anti-MAA antibody quartiles and prevalent ILD (*P* for trend = 0.004). The 2 highest quartiles of IgG anti-MAA antibody showed a trend toward an association with RA-ILD, though this did not reach statistical significance (quartile 3, *P* = 0.15 and quartile 4, *P* = 0.17). We assessed all 3 isotypes together by categorizing individuals according to the number of positive anti-MAA antibody isotypes. Individuals with 3 positive isotypes had 2.5-fold higher odds of having RA-ILD than those with 0–1 positive isotypes (OR 2.56 [95% CI 1.29–5.09]).

Characteristics of lung tissue donors. The mean ± SD age of participants from whom tissue samples were obtained was 56.4 ± 11.7 years, and 75.0% were female. A smoking history

Table 3. Multivariable associations of anti-MAA antibodies with RA-ILD*

	Model A (n = 1,820)			Model B (n = 1,792)		
	OR (95% CI)	*P*	*P* for trend	OR (95% CI)	*P*	*P* for trend
IgA anti-MAA						
By quartile			0.04			0.07
Quartile 1	Referent	–		Referent	–	
Quartile 2	2.27 (1.12–4.59)	0.02	–	2.09 (1.03–4.27)	0.04	–
Quartile 3	2.20 (1.09–4.43)	0.03	–	2.07 (1.02–4.18)	0.04	–
Quartile 4	2.26 (1.12–4.56)	0.02	–	2.10 (1.04–4.25)	0.04	–
Antibody-positive	2.24 (1.20– 4.18)	0.01	–	2.09 (1.11–3.90)	0.02	–
IgM anti-MAA						
By quartile			0.001			0.004
Quartile 1	Referent	–		Referent	–	
Quartile 2	1.87 (0.91–3.86)	0.09	–	1.84 (0.89–3.81)	0.10	–
Quartile 3	2.26 (1.11–4.60)	0.03	–	2.08 (1.02–4.27)	0.05	–
Quartile 4	2.93 (1.49–5.78)	0.002	–	2.73 (1.38–5.41)	0.004	–
Antibody-positive	2.35 (1.26–4.38)	0.007	–	2.23 (1.19–4.15)	0.01	–
IgG anti-MAA						
By quartile			0.09			0.14
Quartile 1	Referent	–		Referent	–	
Quartile 2	1.34 (0.69–2.61)	0.39	–	1.33 (0.68–2.59)	0.41	–
Quartile 3	1.73 (0.91–3.27)	0.09	–	1.61 (0.84–3.06)	0.15	–
Quartile 4	1.67 (0.88–3.18)	0.12	–	1.58 (0.83–3.02)	0.17	–
Antibody-positive	1.58 (0.91–2.75)	0.11	–	1.50 (0.86–2.63)	0.15	–

* Anti–malondialdehyde–acetaldehyde (anti-MAA) antibodies were tested for associations with RA-ILD in separate logistic regression models. Model A was adjusted for age, sex, ethnicity, and smoking status. Model B was adjusted for all of the variables in model A plus anti-CCP positivity and the DAS28. Patients who were positive for anti-MAA antibodies were assessed in total and by quartiles of increasing antibody concentrations. OR = odds ratio; 95% CI = 95% confidence interval (see Table 1 for other definitions).

was present in 66.7% of patients overall (100% of other ILD and emphysema, 33.3% of normal and RA-ILD). The mean ± SD pack-years of smoking history was 17.5 ± 14.3. Anti-CCP antibodies and IgM-RF were positive in two-thirds of patients with RA-ILD. Anti-CCP antibodies, but not IgM-RF, were also detected in one-third of other patients with ILD.

MAA and citrulline expression in lung tissue. MAA expression was highest in RA-ILD lung tissue ($P < 0.001$ versus all other groups) (Figures 1A and B). Citrulline levels were also higher in RA-ILD lung tissue (Figures 1C and D), relative to normal and other ILD lung tissue ($P < 0.001$), but not significantly different than emphysematous lung tissue ($P = 0.91$). Expression of both MAA and citrulline was highly colocalized in RA-ILD lung tissue (r = 0.79) (Figures 1E and F), significantly higher than in lung tissue from other patient groups ($P < 0.001$ versus normal [r = 0.12] and other ILD [r = 0.38]; $P = 0.002$ versus emphysema [r = 0.47]).

Colocalization of MAA and citrulline with immune cells in lung tissue. Staining for CD68+ macrophages and CD3+ T cells was higher in all diseased tissue relative to normal lung tissue (all P values <0.01) (Figure 2A). Macrophage staining was higher in other ILD than in RA-ILD and emphysema ($P < 0.05$). In contrast, CD19+ and CD27+ (memory) B cells were more abundant in RA-ILD lung tissue than in tissue from all other groups ($P \leq 0.02$). There was minimal-to-moderate colocalization between MAA and macrophages or T cells (r = 0.12–0.54), with no significant differences between lung tissue types (all P values >0.10) (Figure 2B). In contrast, we observed strong colocalization of MAA with CD19+ B cells, with the highest correlation identified in RA-ILD (r = 0.78, $P \leq 0.02$ versus all other lung tissue). Colocalization of MAA with CD27+ B cells was more modest (r = 0.02–0.30), with other ILD yielding the highest correlation (r = 0.30, $P \leq 0.004$ versus RA-ILD and normal; $P = 0.06$ versus emphysema).

Citrulline colocalized with CD68+ macrophages to a greater degree in RA-ILD ($P = 0.04$) and emphysema ($P < 0.001$) than in normal lung tissue (Figure 2C). There was minimal colocalization of citrulline with T cells (r = 0.07–0.18). There was moderate colocalization of citrulline with CD19+ B cells in both RA-ILD (r = 0.53) and other ILD (r = 0.44) that exceeded the degree of colocalization observed for emphysema and normal tissue ($P < 0.01$). Colocalization of citrulline with CD27+ (memory) B cells was highly prevalent in diseased lung tissue (all P values <0.001 versus normal) but not different between specific types of diseased lung tissue (all P values >0.29).

Colocalization of MAA with extracellular matrix proteins. Staining for type II collagen was higher in

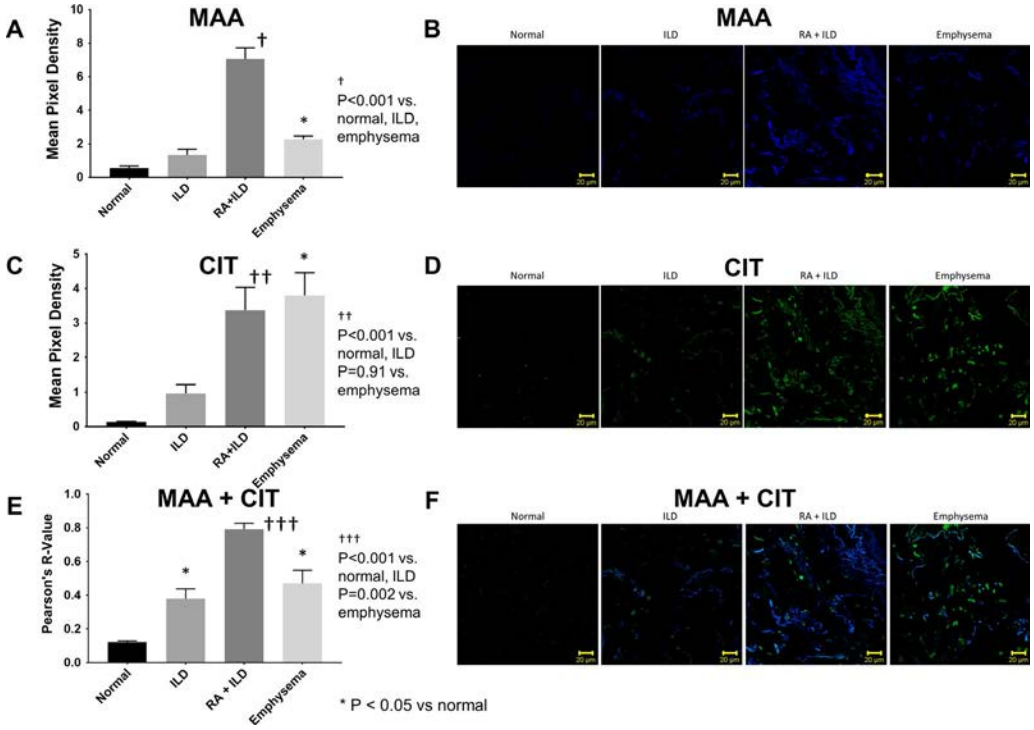

Figure 1. Lung tissue expression of malondialdehyde–acetaldehyde (MAA) (**A** and **B**), citrulline (CIT) (**C** and **D**), and their colocalization (**E** and **F**) in patients with rheumatoid arthritis–associated interstitial lung disease (RA-ILD), compared to patients with ILD alone, patients with emphysema, and normal controls. Lung tissue was examined for the expression of MAA (**A**) and citrulline (**C**), and colocalization of their staining was quantified using Pearson's correlation coefficient (**E**). Representative images of immunohistochemical staining of the lung tissue are shown for MAA (**B**) and citrulline (**D**), and their colocalization is shown in overlapping images (**F**). Bars show the mean ± SEM of 3 patients per group.

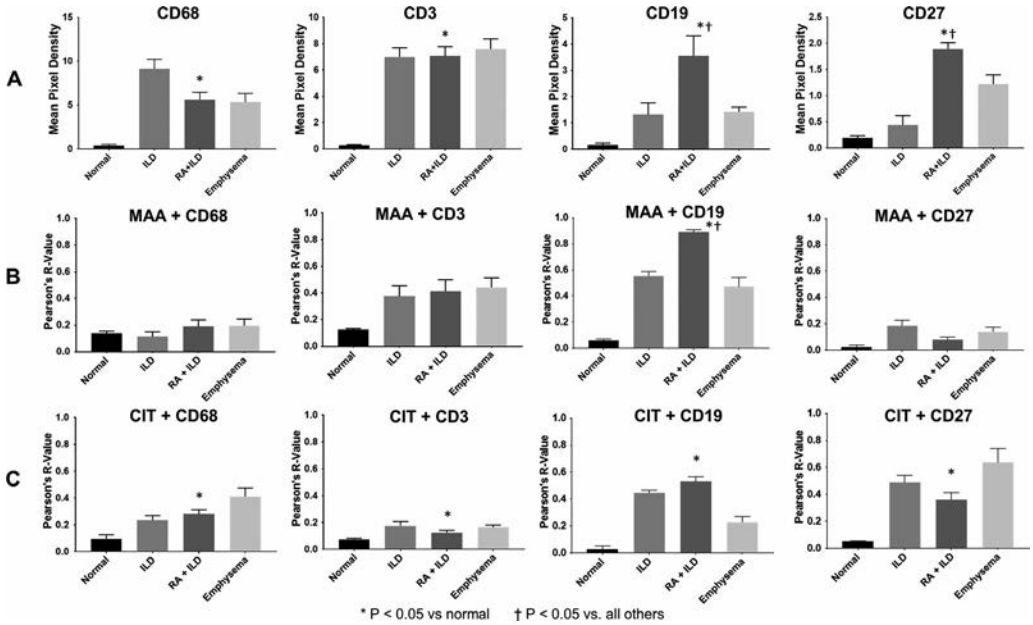

Figure 2. Colocalization of MAA with citrulline and immune cells in lung tissue from patients with RA-ILD, compared to patients with ILD alone, patients with emphysema, and normal controls. **A**, Lung tissue from each group was stained for expression of macrophages (CD68), T cells (CD3), and B cells (CD19 and CD27). **B** and **C**, Colocalization of MAA (**B**) and citrulline (**C**) with macrophage, T cell, and B cell expression in lung tissue from each group was assessed for correlations using Pearson's correlation coefficient. Bars show the mean ± SEM of 3 patients per group. See Figure 1 for definitions.

RA-ILD and other ILD than in normal lung tissue ($P \leq 0.002$) (Figure 3A). However, colocalization of MAA with type II collagen was greater in RA-ILD (r = 0.72) compared to other lung tissue (r = 0.12–0.49, all P values ≤0.02) (Figure 3B). Fibronectin staining was higher in both RA-ILD and emphysema relative to normal lung tissue ($P \leq 0.03$), with only weak colocalization of MAA and fibronectin in RA-ILD (r = 0.21). Vimentin staining was higher in all diseased lung tissue compared to normal lung tissue (all P values ≤0.03), although colocalization of MAA and vimentin was higher in RA-ILD than in other ILD ($P < 0.001$), without significant differences compared to other lung tissue (all P values ≥0.09).

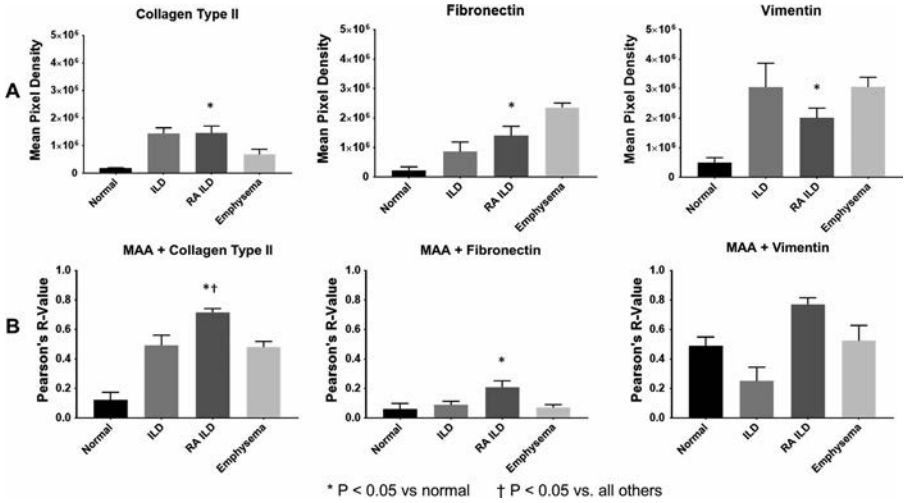

Figure 3. Colocalization of MAA with extracellular matrix proteins in lung tissue from patients with RA-ILD, compared to patients with ILD alone, patients with emphysema, and normal controls. **A**, Lung tissue from each group was stained for expression of the extracellular matrix proteins type II collagen, fibronectin, and vimentin. **B**, Colocalization of MAA with extracellular matrix protein expression in the lung tissue from each group was assessed for correlations using Pearson's correlation coefficient. Bars show the mean ± SEM of 3 patients per group. See Figure 1 for definitions.

DISCUSSION

ILD complicates the disease course for 5–15% of RA patients (1–4), resulting in potentially devastating complications of functional decline and premature mortality. Enhancing the identification of RA-ILD is an important area of translational research in RA, with serum biomarkers emerging as candidates to fulfill this need. The present study is, to our knowledge, the first to investigate serum anti-MAA antibody as a potential biomarker of RA-ILD and to characterize the expression of MAA in lung tissue from these patients. We found that IgA and IgM anti-MAA antibody concentrations were higher in patients with RA-ILD than in other RA patients, including those with other forms of chronic lung disease (IgM only). In parallel studies, we found MAA adduct expression to be higher in RA-ILD lung tissue than in other chronic lung diseases including other ILD. Importantly, MAA adducts demonstrated marked colocalization with citrulline, CD19+ B cells, and type II collagen, most frequently observed in RA-ILD lung tissue. This study is among the first to characterize a biomarker for RA-ILD that has leveraged a comparator population incorporating RA patients with other chronic lung diseases that may be overrepresented in RA (33). Taken together, our findings suggest that MAA-modified proteins and resulting immune responses may serve as useful biomarkers for RA-ILD and that MAA-modified proteins may contribute to the pathogenesis of RA-ILD.

Serum biomarkers have been increasingly investigated for their potential role in identifying RA-ILD. Protein candidates have included biomarkers widely used in RA (anti-CCP antibody and RF) (30,32,34), novel autoantibodies (anti–cit-Hsp90) (11), cytokines/chemokines (MMP-7, IP-10, PARC) (9,10), and Sp-D (10). Oxidative stress represents a potentially relevant biologic pathway that has not been investigated in prior studies of biomarkers in RA-ILD. Oxidative stress, a disruption of the balance of free radicals and antioxidants, is believed to be intimately involved in the development of diffuse lung diseases because of the continuous exposure to oxygen, high surface area, and robust blood supply in the lungs. MAA, which is generated from lipid peroxidation during oxidative stress, has the potential to link multiple pathways implicated in pathogenesis of RA-ILD: oxidative stress, autoimmunity, inflammation, and fibrosis. MAA induces tolerance loss (15), elicits robust adaptive immune responses (anti-MAA antibody), and up-regulates proinflammatory and profibrotic pathways (18,19). Notably, our study began to characterize lung tissue expression of MAA in different lung disease states and serum anti-MAA antibody responses in RA patients with and without lung diseases. Confirming our hypothesis, MAA expression in lung tissue and serum anti-MAA antibody concentrations were highest in patients with RA-ILD.

Although we found >2-fold higher odds of ILD among RA patients with serum IgA or IgM anti-MAA antibody concentrations in the top 3 quartiles, it is important to note that these antibodies are not specific to RA-ILD. Anti-MAA antibodies are present in RA patients in the absence of chronic lung disease, as well as in other disease states (35). However, specificity of a candidate biomarker of RA-ILD may be less important than initial case finding, given that HRCT and PFT results are ultimately needed to confirm the presence and subtype of ILD, which influences prognosis. Translating these novel findings of anti-MAA antibody in RA-ILD into clinical practice will require additional work. As several other serum biomarkers have shown promise in identifying RA-ILD, biomarker panels that include anti-MAA antibody and other analytes are likely to outperform models based on a single analyte. To date, the measurement of anti-MAA antibody has leveraged the use of adducted albumin as the plating antigen, a protein that has no known pathogenic role in RA. Identification of the precise antigenic targets of anti-MAA antibody is likely to allow for improved assay performance in identifying RA patients with ILD. Finally, our current results enable the assessment of the ability to identify established RA-ILD using anti-MAA antibody. Further study will be needed to examine the value of anti-MAA antibody for predicting future risk of RA-ILD. This could be of even greater value than identifying prevalent RA-ILD, as it may identify patients with earlier disease that might be more amenable to therapeutic and/or preventative interventions (36), though specific data on RA-ILD are lacking.

Corresponding to serum findings, staining for MAA adducted antigens was highest in lung tissue from patients with RA-ILD. Importantly, this occurred preferentially in RA-ILD lung tissue, with significantly higher staining than in tissue from patients with other ILD or emphysema. In contrast to MAA, citrulline was expressed in both RA-ILD and emphysema. Although the specificity of serum anti-CCP antibodies for RA approaches 96% (37), others have similarly found citrulline and ACPA responses to accompany chronic obstructive lung diseases in the absence of RA (38–40). Given the strong colocalization of MAA with citrullinated antigens in RA-ILD, we postulate that MAA could act as a "second hit" in RA pathogenesis by facilitating loss of tolerance to colocalized citrullinated antigens. Although further testing will be needed to address this hypothesis, the colocalization of CD19+ B cells with MAA and citrulline would support the concept that these posttranslational changes (both of which likely result from injurious stimuli) conspire in autoantibody generation. This is further supported by preliminary work in animal models suggesting that immunization with comodified albumin (with MAA and citrulline) leads to greater ACPA responses than occur with citrullinated albumin alone (41). Finally, vimentin is an extracellular matrix protein that has previously been shown to be a shared target of citrullination/ACPAs in the synovium and lung (29). While we did not find vimentin expression to be increased in RA-ILD compared to other lung conditions, we observed marked colocalization of MAA with vimentin in RA-ILD lung tissue, which was significantly more robust than that seen with other ILD.

Our group previously characterized anti-MAA antibodies in sera from RA patients and patients with other rheumatic and musculoskeletal diseases (16,35). Circulating anti-MAA antibody

concentrations are higher in RA patients than those with osteoarthritis, are associated with serum ACPAs, and are enriched within RA synovium (16,17). As we found in the RA-ILD lung tissue assessed in the present study, MAA and citrulline colocalized in RA synovium (17). Also consistent with our present findings on RA-ILD lung tissue, prior work by our group has shown that MAA and citrulline both colocalize with B cells in the synovium. However, there are differences in B cell subsets according to site. In the synovium, MAA and citrulline colocalized most strongly with CD27+ memory B cells (17). In the lung tissue from patients with RA-ILD, MAA colocalized most strongly with CD19+ B cells but not with CD27+ memory B cells. While further research is needed to elucidate the temporal evolution of immune responses to MAA, it is intriguing that immature B cells are associated most strongly with MAA adduct expression in the lung, given the emerging evidence that the lungs may be a site of immune tolerance breakdown contributing to the early development of RA (42).

There are limitations to the present study. Male predominance, military veteran status, and lower prevalence of biologic use within our patient group may affect generalizability. ILD data were collected retrospectively, and not all data were available within the medical records. This may underestimate the cross-sectional prevalence of ILD (4.7%) in the cohort. However, misclassification of ILD cases as non-ILD would bias our results toward the null. Distinguishing between clinical and subclinical ILD cannot be definitive based on retrospective classification. Because we confirmed diagnoses based on physician reports in the medical records, rather than relying on diagnostic codes or diagnostic testing alone, we believe the majority of ILD cases were clinically evident. Given the low frequency with which ILD pattern (UIP versus NSIP versus other) was specified, we were not able to compare anti-MAA concentrations by RA-ILD pattern. Likewise, anti-MAA antibody measurements were not available for all registry participants, which may also have reduced study power. Again, this should not have introduced bias, as antibody measurements were performed on the entire cohort at the time of the prior study without any relation to ILD status.

Reflecting the prevalence of seropositivity for RF and anti-CCP antibody, we dichotomized anti-MAA antibody according to levels in the upper 3 quartiles on in the lowest quartile. Only increasing IgM anti-MAA antibody quartiles were more strongly associated with the presence of ILD. Further work is needed to determine clinically important cutoffs for these antibodies. Sample sizes were limited for lung tissue studies, with lung tissue obtained from 3 individuals with each lung condition, prohibiting multivariable analyses. One of the patients with non–RA-ILD had detectable ACPAs but was not classified as having RA. Given the cross-sectional nature of the study, it is unknown if that patient later developed RA. This potential misclassification of RA-ILD as non–RA-ILD would only bias our results toward the null. Lung tissue samples were not matched, so there may be unmeasured confounding.

There are important strengths to this study, as well. We performed a detailed review of the medical records to validate ILD diagnoses in RA patients from a well-characterized registry that includes robust data, including many relevant covariates (20). We not only evaluated serologic anti-MAA antibody concentrations but also investigated tissue expression of MAA and its colocalization with citrulline, immune cells, and extracellular matrix proteins that have been consistently implicated in disease pathogenesis. Finally, we characterized MAA and anti-MAA immune responses in RA-ILD by using comparator subjects who were free of lung disease in addition to comparators with other chronic lung diseases.

In conclusion, we found higher levels of serum IgA and IgM anti-MAA antibody to be associated with RA-ILD in a large cohort of US veterans with RA. Lung tissue expression of MAA is similarly higher in RA-ILD lung tissue, where it colocalizes with citrulline, CD19+ B cells, and extracellular matrix proteins. These findings suggest that MAA immune responses could play an important role in the pathogenesis of RA-ILD and that anti-MAA antibodies may be promising serum biomarkers in the identification of this extraarticular disease manifestation.

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. England 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. England, Duryee, Ascherman, Demoruelle, Deane, Thiele, Mikuls.

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Analysis and interpretation of data. England, Duryee, Poole, Ascherman, Caplan, Demoruelle, Deane, Klassen, Thiele, Mikuls.

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A Prospective Study of the Development of Inflammatory Arthritis in the Family Members of Indigenous North American People With Rheumatoid Arthritis

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Objective. To determine the incidence of inflammatory arthritis and autoantibody prevalence in Indigenous North American people.

Methods. Unaffected relatives of Indigenous North Americans with rheumatoid arthritis (RA) from central Canada and Alaska were systematically monitored from 2005 to 2017. Rheumatoid factor (RF) and anti–citrullinated protein antibodies (ACPAs) were tested at every visit, and a subset was tested for ACPA fine specificity using a custom multiplex assay. Multistate models based on all available study visits were developed to determine the likelihood of transitioning between autoantibody states, or to inflammatory arthritis.

Results. Eighteen of 374 relatives (4.8%) developed inflammatory arthritis during follow-up (after a mean ± SD of 4.7 ± 2.4 years), yielding a transition rate of 9.2 cases/1,000 person-years. Thirty percent of those who developed inflammatory arthritis were seronegative at baseline, but all were seropositive at inflammatory arthritis onset. Although 30% of ACPA/RF double-seropositive individuals developed inflammatory arthritis (after 3.2 ± 2.2 years), the majority of these individuals did not develop inflammatory arthritis. Multistate modeling indicated a 71% and 68% likelihood of ACPA and RF seropositive states, respectively, reverting to a seronegative state after 5 years, and a 39% likelihood of an ACPA/RF double-seropositive state becoming seronegative. Fine specificity testing demonstrated an expansion of the ACPA repertoire prior to the development of inflammatory arthritis.

Conclusion. Despite a high incidence of inflammatory arthritis in this cohort of at-risk relatives of Indigenous North Americans with RA, a large proportion of autoantibody-positive individuals do not develop inflammatory arthritis and revert back to an autoantibody-negative state.

INTRODUCTION

Seropositive rheumatoid arthritis (RA) is a systemic autoimmune disease which causes inflammation and progressive destruction of synovial joints after a varying period of preclinical autoantibody seropositivity (1–4). Indigenous North Americans have some of the highest rates of RA worldwide, with varying estimated rates of incidence and prevalence reported among different Indigenous North American populations (5). A recent analysis from a large administrative database in Manitoba, Canada showed the prevalence of RA in an Indigenous North American population to be 0.9%, compared to 0.6% in a non-Indigenous North American population (6). In a southeast Alaska Indigenous North American population, the prevalence of RA is estimated to be 2.4%, with incidence rates of 46/100,000 males per year and 122/100,000 females per year (7). Indigenous North Americans have a greater frequency of poor prognostic factors, such as extraarticular manifestations, seropositivity, and large joint involvement, compared to white populations (8). At the onset of RA, Indigenous North Americans exhibit higher disease activity compared to white populations, and less improvement in disease activity and patient-reported outcomes (8–10).

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The increased burden of RA in Indigenous North Americans is multifactorial. We have shown that Indigenous North Americans have increased genetic risk related to the carriage of shared epitope alleles, in particular HLA–DRB1*1402, and single-nucleotide polymorphisms that have been shown to be associated with RA in other populations (11,12). It should be noted that HLA–DRB1*1402 is highly prevalent and unique to Indigenous populations (13,14). RA tends to be familial in Indigenous North Americans and is associated with the clustering of RA autoantibodies and serum cytokine profiles in the relatives of the Indigenous North Americans with RA (15). Other factors associated with increased risk of RA, such as high prevalence of rheumatoid factor (RF) (16), increased smoking rates (17), increased rates of obesity (18), and high prevalence of periodontal disease (19,20), further compound the risk for RA in this population.

Prospectively collected data on RA in the Pima population in the southwestern US from 1983 to 1990 showed an incidence rate of 3.8 cases/1,000 person-years and a prevalence of 1% in men and 3.4% in women; however, longitudinal data on autoantibodies in this population are not available (21). European-based studies suggest that the risk of RA in first-degree relatives of individuals with RA is 3-fold, with increasing risk associated with anti–citrullinated protein antibodies (ACPAs) and RF, or a diagnosis made prior to 40 years of age (22). In the present study, we followed up relatives of Indigenous North Americans with RA to generate a real-world estimate of the incidence of seropositive inflammatory arthritis, autoantibody prevalence, and autoantibody fluctuation over time. To our knowledge, this is the first prospective cohort study examining the development of RA and autoantibodies in a high-risk Indigenous North American group comprising the relatives of individuals with RA.

SUBJECTS AND METHODS

Study subjects. From April 15, 2005 through December 31, 2017, the relatives of Indigenous North Americans with RA (the latter being designated RA probands who met the 1987 revised American College of Rheumatology [ACR] classification criteria [23] or the 2010 ACR/European League Against Rheumatism [EULAR] classification criteria [24]), were recruited from urban and rural areas in Manitoba, Canada and Alaska for this longitudinal prospective study (25). The study was approved by the Research Ethics Board of the University of Manitoba, the Alaska Area Institutional Review Board, the Band Councils of the individual study communities in Manitoba, and the Tribal Health Organizations of the study communities in Alaska. RA probands were eligible for participation if they were older than age 18 years and self-identified as being an Indigenous North American. Relatives of these RA probands who met the same eligibility criteria were recruited to participate in this present study (Supplementary Figure 1, available on *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40880/abstract). In summary, the majority (75%) were first-degree relatives (FDR), and a total of 621 relatives of these Indigenous North Americans with RA were enrolled, of whom 374 were followed up longitudinally. There was no significant difference in sex, age, smoking history, body mass index (BMI), prevalence of diabetes, or frequency of joint symptoms between the cohort who were longitudinally followed up and those who had only 1 study visit (data not shown).

Study design and inflammatory arthritis case definition. Our primary objective was to determine the incidence of inflammatory arthritis in this at-risk population of relatives of Indigenous North Americans with RA. The secondary goal of this study was to explore the stability of RA autoantibody states over time. At baseline, relatives given a standardized 68-joint examination for swollen and tender joints by a rheumatologist to confirm the clinical absence of synovitis, and were stratified based on the presence of ACPAs and RF (Supplementary Figure 1, http://onlinelibrary.wiley.com/doi/10.1002/art.40880/abstract). Those who were ACPA and/or RF seropositive returned annually for a study visit, while relatives who were seronegative for these antibodies returned in 3 years. This asymmetric follow-up protocol was a logistic decision that was made in order to focus on individuals who were most likely to develop inflammatory arthritis. Follow-up visits consisted of examination by a rheumatologist (DR, HEG, or EDF) for evidence of synovitis, autoantibody testing, and symptom reporting. All participants were provided information regarding the typical symptoms of RA and instructed to report these symptoms immediately to the study coordinator so that they could be examined by a rheumatologist at the time of symptom onset. Participants could contribute follow-up time to the study if they had 2 or more visits and a minimum of 6 months of follow-up duration.

Since the onset of RA can feature a range of articular involvement patterns ranging from monoarthritis to polyarthritis, the development of inflammatory arthritis was defined as 1 or more swollen joints deemed to represent active synovitis by 1 of 3 study rheumatologists (DR, HEG, or EDF). Inflammatory arthritis, rather than RA, was selected as an end point because it would have been unethical to delay treatment of patients with autoantibody-positive inflammatory arthritis, since treatment could alter the disease course. Joint swelling most likely caused by other arthropathies (e.g., crystalline arthritis or osteoarthritis) were excluded from the definition of inflammatory arthritis, although in most cases, the analysis of synovial fluid was not undertaken. Joints that were tender but not swollen were also not considered to represent synovitis. Subjects who met this definition of inflammatory arthritis were deemed to be "progressors." Once a study subject developed inflammatory arthritis, they no longer contributed to the follow-up period calculations.

Symptom reporting. Details of the musculoskeletal questionnaire used in this study have been previously published (25). The questionnaire focuses on 6 symptoms suggestive of inflammatory arthritis and is answered in a yes/no format.

Laboratory parameters and HLA testing. Screening for ACPAs and IgM RF was performed at a clinical and/or research laboratory at a single tertiary care hospital (Health Sciences Centre). Because of the longitudinal study design, testing for ACPAs was performed using one of the following assays: anti-CCP IgG, anti-CCP2, anti-CCP3, or anti-CCP3.1. ACPA seropositivity status was categorized as negative (below manufacturer's standardized assay cutoff), weak positive (≤3 times the upper limit normal [ULN]), or strong positive (>3 times the ULN) according to the ACR classification criteria for RA (24). Because the anti-CCP3 test has been shown to be more sensitive than the anti-CCP2 test (26), all subjects contributing to the longitudinal follow-up period had baseline and last study visit samples tested using either anti-CCP3 or anti-CCP3.1 assays. Samples obtained during other study visits (between the baseline and last visit) were tested using any of the assays listed above and contributed to the multistate modeling described below.

Baseline IgM RF was tested by nephelometry at a clinical laboratory, using 20 IU/ml as the cutoff value for positive versus negative. In the early stages of the study, IgM RF testing used enzyme-linked immunosorbent assay (ELISA) with appropriate positive and negative controls. IgM RF seropositivity was defined as weak positive (≤3 times the ULN) or strong positive (>3 times the ULN). C-reactive protein was measured by the clinical laboratory in mg/liter.

HLA–DRB1 typing was performed by polymerase chain reaction using sequence-specific oligonucleotide primers and sequence-based typing as previously described (27). The following DRB1 alleles were included as SE-encoding alleles: DRB1*0101, 0102, 0401, 0404, 0405, 0408, 0410, 1001, and 1402.

ACPA fine specificity testing. Comprehensive analysis of ACPA fine specificity was undertaken on available stored plasma samples in relatives enrolled from 2005 to 2012. Autoantibodies toward a broad spectrum of citrullinated and noncitrullinated peptides from known RA autoantigens were coupled to a bead-based assay, and ACPAs were detected quantitatively by anti-human phycoerythrin-conjugated IgG antibodies after being passed through a laser detector, as previously described (3). To generate an ACPA score, each ACPA value was divided by the mean value of the cohort, then all ACPA values were added together, calculated as [Σ(ACPA value/mean ACPA value)] as previously described (28). An ELISA assay was used to test for serum anti–citrullinated histone 4 antibodies (anti–Cit-H4), as we have previously reported (29).

Statistical analysis and multistate modeling. Descriptive statistics (mean values with SD, median values with interquartile range [IQR]) were used to summarize the cohort characteristics. Comparisons between the progressor and nonprogressor groups were made using the chi-square test (for categorical variables and, as appropriate, independent *t*-tests), the Mann-Whitney test, or the Kruskal-Wallis test for continuous variables. To calculate rates of the development of inflammatory arthritis, the number of cases of inflammatory arthritis was used as the numerator and the denominator was the total number of person-years of follow-up. Correlations between Cit-H4 antibody, anti-CCP3.1, and ACPA scores were calculated using Pearson's correlation coefficient.

To characterize fluctuating autoantibody states over time and in recognition of the asymmetric nature of follow-up, we created multistate models to analyze the likelihood of converting from one autoantibody state to another (e.g., ACPA and RF positive to ACPA negative and RF positive). A multistate model is a model for time-to-event data in which all subjects start at an initial state and visit intermediate states (ACPA/RF +/–), possibly more than once (30), or enter an absorbing state of inflammatory arthritis at which time the end point is met and observation/follow-up ceases. Multistate models can be thought of as a generalization of both survival analysis and discrete-time Markov models, with the rates of transition between states being described as hazards or instantaneous risks (31). The higher the hazard, the more likely a subject makes the transition between 2 given states. Multistate models also allow the estimation of the probability of being in a future state after a specified amount of time. To model such data, we used the multistate model package in R (32). We included ACPA and RF seropositivity status from all available study visits (n = 1,181; median 3 visits/individual [IQR 3–5]) in the cohort of 374 relatives who were longitudinally followed up.

The first multistate model focused on transitions between ACPA states (Supplementary Figure 2, http://onlinelibrary.wiley.com/doi/10.1002/art.40880/abstract), and the second multi-

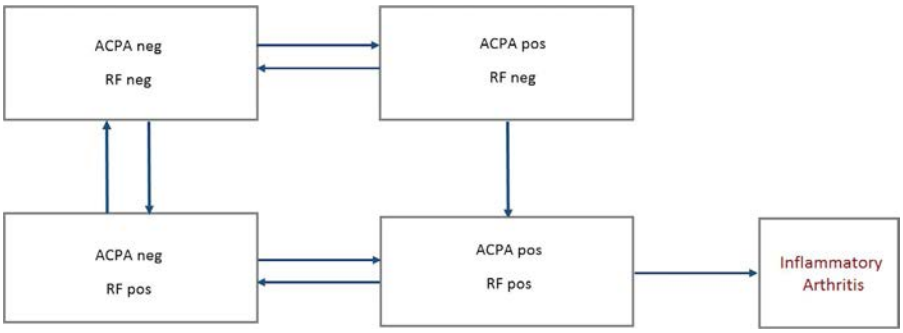

Figure 1. Transition paradigm between autoantibody states in multistate models. ACPA = anti–citrullinated protein antibody; RF = rheumatoid arthritis. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40880/abstract.

state model included ACPA and RF status, and the absorbing state of inflammatory arthritis (Figure 1). The transition from RF and ACPA positive to RF negative and ACPA positive was included in the model a priori but later removed when the data did not support its existence.

RESULTS

Participant characteristics. Baseline characteristics of the study population are shown in Table 1. In total, 314 (84%) of 374 participants were from Manitoba, and 60 (16%) of 374 were from Alaska. Forty-one participants (11.1%) were anti-CCP3 positive (4.9% strongly positive), and 16.4% were RF positive. Twelve participants (3.2%) were positive for both ACPAs and RF at baseline.

Table 1. Baseline characteristics of the subjects with longitudinal follow-up*

	Progressor (n = 18)	Nonprogressor (n = 356)	*P*
Female sex	14 (77.8)	234 (65.7)	0.29
Age, mean ± SD years	30.0 ± 11.5	37.1 ± 12.8	0.02
Duration of follow-up, mean ± SD years	4.7 ± 2.4	5.2 ± 2.7	0.57
History of smoking	16 (88.9)	272 (76.5)	0.24
Pack years smoking, median (IQR)	3.5 (1.2–12.0)	5 (0.8–12.6)	0.41
BMI, kg/m^2, mean ± SD	28.9 ± 7.9	32.4 ± 7.1	0.06
Type 2 diabetes	0 (0)	58 (19.5)	0.13
Any HLA SE	13/14 (92.9)	191/234 (81.6)	0.29
HLA–DRB1*1402 positive	9/14 (64.3)	93/234 (39.7)	0.07
HLA SE double positive	4/14 (28.6)	54/234 (23.1)	0.64
CRP, median (IQR) mg/liter	2.4 (1.1–5.7)	3.4 (1.7–7.0)	0.44
Autoantibody status			
ACPA negative	8 (44.4)	321/352 (91.2)	0.01
ACPA positive	10 (55.5)	31/352 (8.8)	0.01
ACPA strong positive†	7 (38.9)	11/352 (3.1)	0.01
RF negative	11 (61.1)	295/348 (84.7)	0.02
RF positive	7 (38.9)	53/348 (15.2)	0.01
RF strong positive†	4 (22.2)	11/348 (3.2)	0.01
RF and ACPA negative	6 (30.0)	271/348 (77.9)	0.01
RF and ACPA positive	5 (27.8)	7/348 (2.0)	0.01
Symptoms			
Hand pain	10/16 (62.5)	143/279 (51.3)	0.381
Other joint pain	10/15 (66.7)	155/280 (55.4)	0.390
Hand swelling	6/16 (37.5)	99/282 (35.1)	0.845
Other joint swelling	7/16 (43.8)	79/279 (28.3)	0.405
Hand stiffness	8/17 (47.1)	109/281 (38.3)	0.498
Other joint stiffness	8/16 (50)	114/280 (40.7)	0.407

* Except where indicated otherwise, values are the number/number assessed (%). IQR = interquartile range; BMI = body mass index; SE = shared epitope; CRP = C-reactive protein; ACPA = anti–citrullinated protein antibody; RF = rheumatoid factor.
† Strong positivity was classified if the value was ≥3 times the upper limit of normal based on the manufacturer's cutoff.

Comparisons of anti-CCP assays. Concordance between CCP2 and CCP3.1 findings was tested in 426 samples, which had been tested by both assays. Overall, there was only modest agreement between the 2 tests (κ = 0.521, $P = 0.01$). We found 94% of strong positive CCP2 results were also strong positive by CCP3.1 assay, whereas only 54% of CCP3.1 strong positive tests were strong positive for CCP2. Overall, there were more weak positives associated with anti-CCP3.1 testing but 3 individuals were positive only for anti-CCP2 and not for CCP3.1.

Follow-up and development of inflammatory arthritis. At the conclusion of the study, the majority of participants (83.7%) were autoantibody negative. Autoantibody prevalence did not differ in those who were followed up for 5 or more years versus those who were followed up for fewer than 5 years (r = 0.065, $P = 0.25$).

A total of 18 (4.8%) of 374 relatives developed inflammatory arthritis after being followed up an average of 4.7 ± 2.4 years (mean ± SD). Of these, 15 (83%) of 18 progressors met the 2010 ACR/EULAR criteria for RA at the onset of inflammatory arthritis. The total cumulative duration of follow-up for the entire cohort was 1,940 person-years, giving a transition rate of 9.2 cases of inflammatory arthritis per 1,000 person-years (i.e., 0.9% annually).

Details of baseline characteristics of the cohort are in Table 1. At the time of enrollment, the arthritis symptom profile of the progressors and nonprogressors was similar. Progressors were younger (mean age 30.0 years versus 37.1 years; $P = 0.02$) and tended to have a lower BMI (28.9 kg/m^2 versus 32.4 kg/m^2; $P = 0.06$). Interestingly, none of the progressors had type 2 diabetes, whereas almost 20% of the nonprogressors had diabetes, a prevalence that is similar to what has been previously documented in the Indigenous North American population (33).

Although the frequency of HLA–DRB1*1402 carriage was higher in progressors, there was no difference in the SE allele carriage (Table 1). HLA–DRB1*0901, a non-SE allele, is associated with seropositive RA in Indigenous North American adults and children (27,34), and when we included HLA–DRB1*0901 as a risk allele in addition to SE alleles, this increased the strength of the association between inflammatory arthritis and having 2 RA risk alleles (53% [8 of 15] versus 29% [69 of 234]; $P = 0.05$).

As shown in Table 2, 28% (5 of 18) progressors were ACPA/RF double positive at baseline and developed inflammatory arthritis after a mean ± SD 3.2 ± 2.2 years. The 6 progressors who were seronegative at baseline all developed ACPAs prior to, or at, the onset of inflammatory arthritis, occurring a mean ± SD 2.3 ± 1.7 years prior to the diagnosis of inflammatory arthritis. The calculated rate of developing inflammatory arthritis was 4.1

Table 2. Baseline characteristics and development of inflammatory arthritis, by autoantibody group*

	ACPA−/RF− (n = 277)	ACPA−/RF+ (n = 48)	ACPA+/RF− (n = 29)	ACPA+/RF+ (n = 12)
Age, years	36.7 ± 12.8	34.6 ± 12.7	39.2 ± 13.8	38.7 ± 13.2
Female sex, no. (%)	185 (67.3)	30 (62.5)	19 (63.3)	9 (75.0)
BMI, kg/m^2	32.2 ± 6.7	33.1 ± 8.7	30.8 ± 8.0	35.1 ± 8.0
HLA SE positive, no./no. assessed (%)	155/192 (80.1)	27/32 (84.4)	11/12 (91.7)	6/7 (86.0)
HLA SE, 2 alleles, no./no. assessed (%)	46/192 (24.0)	6/32 (18.8)	4/12 (33.3)	1/7 (14.3)
Time at risk, total person-years	1,472.1	276.2	137.3	51.5
Cases of inflammatory arthritis (% in autoantibody group)	6 (2.1)	2 (4.2)	5 (17.2)	5 (41.7)
Cases of inflammatory arthritis per 1,000 person-years	4.1	7.2	36.4	97.1
Time to inflammatory arthritis, years	5.6 ± 1.1	5.5 ± 5.0	4.7 ± 2.7	3.2 ± 2.2
Time to ACPA positivity, years	2.3 ± 1.7	2.2 (n=1)	-	-
No. of cases meeting ACR RA criteria	5/6	1/2	5/5	4/5

* Autoantibody groups were determined by enrollment autoantibody status. Seropositivity for anti–citrullinated protein antibody (ACPA) and rheumatoid factor (RF) is based on the manufacturer's cutoff level. Except where indicated otherwise, values are the mean ± SD. BMI = body mass index; SE = shared epitope; ACR = American College of Rheumatology; RA = rheumatoid arthritis.

cases/1,000 person-years for the autoantibody-negative group and increased to 97.1 cases/1,000 person-years in the case of ACPA/RF double-positive individuals. At the time that progressors were classified as having developed inflammatory arthritis, 15 of 18 met the classification criteria for RA. The remaining 3 progressors went on to subsequently meet the RA classification criteria after the end point of the study. None of the incident cases of inflammatory arthritis were seronegative inflammatory arthritis.

Multistate model of transition between autoantibody states. To better understand the likelihood of transitions between different levels of ACPA positivity, a multistate model was developed (see Supplementary Table 1, http://onlinelibrary.wiley.com/doi/10.1002/art.40880/abstract). The model suggests that reversions to seronegativity after 5 years are common, including when the ACPA status was strongly positive, although the strongly positive ACPAs were much less likely to

Table 3. Multistate models predicting the likelihood of being in an autoantibody state or development of inflammatory arthritis after specific follow-up periods*

Years of followup, baseline state	Autoantibody negative	ACPA+/RF−	ACPA−/RF+	ACPA+/RF+	Inflammatory arthritis
1-year followup					
Baseline state					
Autoantibody negative	0.82	0.08	0.09	0.001	0.001
ACPA+/RF−	0.74	0.15	0.06	0.04	0.01
ACPA−/RF+	0.44	0.03	0.48	0.04	0.01
ACPA+/RF+	0.08	0.01	0.20	0.53	0.18
2-year followup					
Baseline state					
Autoantibody negative	0.77	0.08	0.12	0.02	0.01
ACPA+/RF−	0.75	0.08	0.12	0.04	0.01
ACPA−/RF+	0.59	0.06	0.28	0.05	0.02
ACPA+/RF+	0.20	0.04	0.21	0.28	0.27
5-year followup					
Baseline state					
Autoantibody negative	0.72	0.08	0.15	0.03	0.02
ACPA+/RF−	0.71	0.08	0.13	0.04	0.04
ACPA−/RF+	0.68	0.07	0.16	0.04	0.05
ACPA+/RF+	0.39	0.05	0.12	0.06	0.38

* Values are the likelihood of being in the given state, with the sum of likelihoods totaling 100%. ACPA = anti–citrullinated protein antibody; RF = rheumatoid arthritis.

transition to a negative state after only 1 or 2 years. The estimated time spent in each state was 2.46 years (95% confidence interval [95% CI] 1.02–5.96) for ACPA negative, 0.2 years (95% CI 0.09–0.47) for ACPA weak positive, and 1.65 years (95% CI 1.02–2.67) for ACPA strong positive. Although weakly positive ACPA is a very transitory state, the majority of strongly positive ACPAs had also reverted to a seronegative state after prolonged follow-up.

In the second multistate model which includes ACPA, RF, and inflammatory arthritis (Figure 1), transition probabilities were estimated for 1, 2, and 5 years of follow-up, as shown in Table 3. Double negative was the most common state (n = 832 visits, 70%), while double ACPA/RF positive was the least common (n = 50, 4.2%). Subjects who were both ACPA negative and RF negative were 72% (95% CI 67–75%) likely to remain in this state after 5 years. Single positive states of either ACPA or RF had similar likelihoods of transitioning to inflammatory arthritis after 5 years (4.2% and 4.8%, respectively). The ACPA/RF double-positive state, which comprised seropositivity at any level above the assay cutoff, carried an estimated risk of transitioning to inflammatory arthritis of 18% (95% CI 12–27%) after 1 year, 27% (95% CI 18–41%) after 2 years, and 38% (95% CI 24–54%) after 5 years. A more striking finding is that 7 (58%) of 12 of the ACPA/RF double-positive subjects at baseline had not developed inflammatory arthritis after being followed up for a mean ± SD of 5.1 ± 2.2 years, and indeed this state carried an estimated 36% (95% CI 27–51%) likelihood in the multistate model of becoming autoantibody negative after 5 years.

In analyzing all of the study visits from individuals who were ACPA/RF double positive at any time point during the follow-up period, but who did not develop inflammatory arthritis, it was evident that strong positivity (>3 times the assay cutoff) for both ACPAs and RF rarely reverted to a seronegative state but remained in this state for an extended time period. Sex, age, and HLA status were introduced into the modeling as covariates (see Supplementary Tables S2, S3, and S4; http://onlinelibrary.wiley.com/doi/10.1002/art.40880/abstract), but the 95% CIs were too broad to be interpreted.

ACPA fine specificity in progressors and nonprogressors. Longitudinal analysis of the ACPA repertoire revealed an incremental increase in both the total number of ACPAs and intensity of reactivity in the progressors (Figure 2). Similarly, the ACPA+/RF+ double-positive nonprogressors also displayed intense reactivity to many citrullinated epitopes. In contrast, individuals with intermittent ACPA positivity by stan-

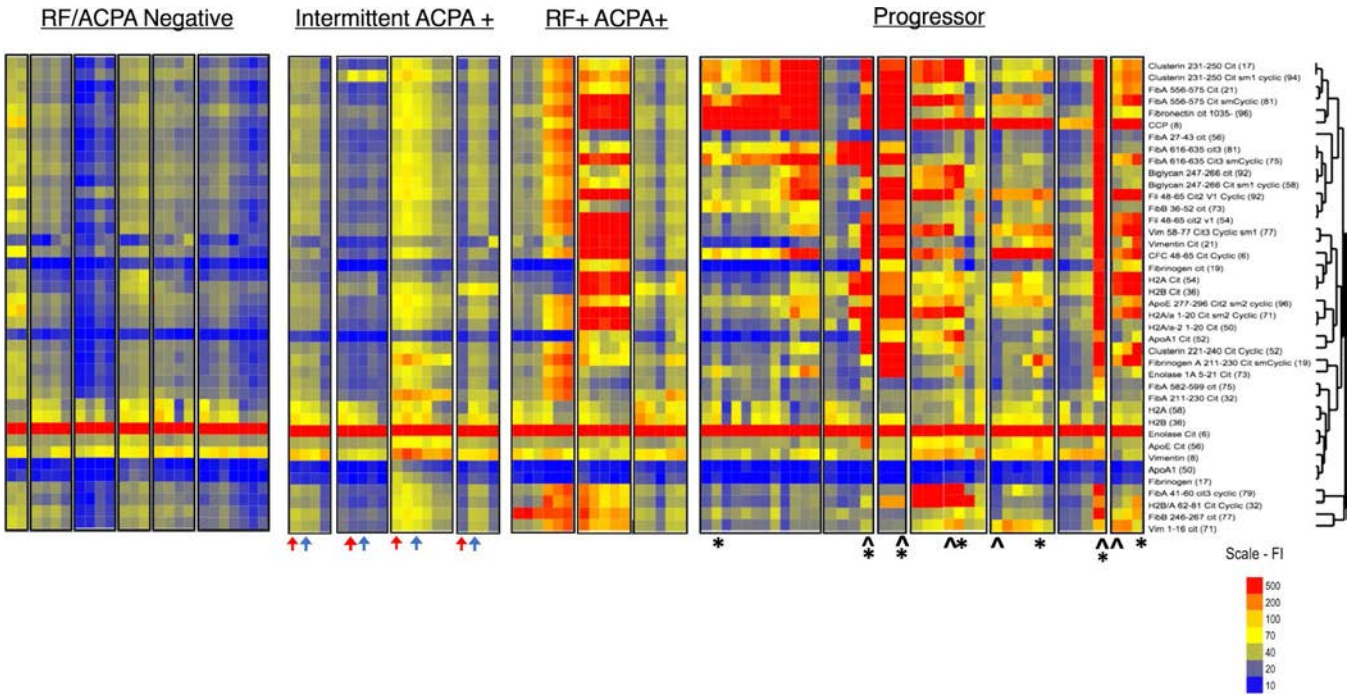

Figure 2. Heatmaps of anti–citrullinated protein antibody (ACPA) fine specificities in subjects at multiple follow-up visits, grouped by autoantibody status and development of inflammatory arthritis (progressor). Each distinct block of columns (outlined in black) represents an individual, with smaller undefined columns within the block representing individual visits when ACPA fine specificities were tested. Scale below is based on fluorescence intensity. The time of development of anti–cyclic citrullinated peptide 2 (anti–CCP2) seropositivity (↑) and the time of loss of ACPA seropositivity (↑) are shown. ^ = acquisition of IgM rheumatoid factor (RF); * = time of onset of inflammatory arthritis. FibA = fibrinogen A; FIL = filaggrin; CFC = Cit filaggrin cyclic; ApoE = apolipoprotein E; Vim 1 = vimentin.

dard commercial ELISA testing had reduced scope of ACPA reactivity over time. The enolase epitope was strongly positive in all samples because it was a defective peptide in the assay, making that particular epitope uninterpretable. In the available samples tested, the calculated ACPA score correlated with anti-CCP titers (r = 0.55, *P* = 0.01; n = 214). ACPA levels directed toward an immunodominant citrullinated epitope derived from the histone 4 protein (29) showed a high degree of correlation between the levels of anti–Cit-H4 antibodies and the ACPA score in sequential samples from the progressors (r = 0.66, *P* = 0.0001; n = 42).

DISCUSSION

This prospective observational study of the relatives of Indigenous North Americans with RA in Manitoba and Alaska has demonstrated a relatively high frequency of development of seropositive RA, with an overall rate of 9.2 cases per 1,000 person-years of follow-up. The incidence rate of inflammatory arthritis in at-risk relatives of Indigenous North Americans with RA was not previously described.

Large prospective observational studies of RA onset, such as those emanating from the Nurses' Health Study, have shown age-adjusted incidence rates to be 26.7 cases/100,000 person-years (35) in a primarily white female North American population. Data from Olmsted County, Minnesota indicated a slightly higher RA incidence rate of 40.9 cases/100,000 person-years (36). A study of combined data from Swedish patient registries demonstrated an ~4 times increased risk of developing RA in FDRs of patients with autoantibody-positive RA (22), and a rate of 4.9 cases/1,000 person-years was observed at follow-up in a cohort of Mexican Mestizo RA FDRs (37). Thus, even considering methodologic differences between these studies, an incidence rate of 9.2 cases per 1,000 person-years in our study population of first- and second-degree relatives of Indigenous North Americans with RA is substantially higher than that reported in other populations. These findings suggest that the previously reported genetic and environmental risk factors in this Indigenous North American population culminate in a high incidence of seropositive RA.

In the current study, symptoms suggestive of RA (such as arthralgia) were not used to select study participants, and we found that articular symptoms were not more frequent in progressors compared to nonprogressors. We previously found that self-reported symptoms of pain, stiffness, and swelling are more frequent in relatives of Indigenous North Americans with RA compared to either Indigenous North Americans or non-Indigenous North Americans with no family history of autoimmune diseases (25).

A prospective cohort study from The Netherlands that recruited individuals based on ACPA or RF seropositivity and presence of arthralgia demonstrated an inflammatory arthritis incidence rate of 20% after a median of 11 months of follow-up (38). In a separate study of a Dutch cohort, subjects were designated as having "clinically suspect arthralgia" if they had inflammation of the small joints, and in that cohort, the incidence rate of inflammatory arthritis was ~20% over a median follow-up of 1.4 years (39). Importantly, in this latter study, the median time to development of inflammatory arthritis was 7 weeks after enrollment, and >80% of those individuals had subclinical inflammation on magnetic resonance imaging (MRI) at enrollment. Preclinical RA is difficult to define and these studies identified synovitis as possibly occurring during the inflammatory state labeled as clinically suspect arthralgia. Because the date at which inflammatory arthritis was diagnosed, rather than the exact date of onset of symptoms pertaining to inflammatory arthritis, was used, it is possible that the date of inflammatory arthritis diagnosis does not reflect very early development of synovitis. Systematic application of imaging modalities such as MRI and ultrasound may be of value in classifying preclinical stages of RA.

Commercial anti-CCP assays have been progressively developed to optimize the sensitivity and specificity for the diagnosis of RA. Data regarding their performance in preclinical cohorts are more limited. We found the baseline prevalence of ACPA seropositivity, based on CCP3 testing, in unaffected relatives of Indigenous North Americans with RA to be 11.1%, which is similar to the 9.5% prevalence found in an FDR cohort in a study of the etiology of RA (26).

Double seropositivity for ACPAs and RF was associated with the highest rates of inflammatory arthritis development and the shortest latency period, consistent with findings in other cohorts. Some practitioners treat ACPA/RF double-seropositive individuals with hydroxychloroquine, and several ongoing clinical trials are targeting this patient population using other strategies (40,41). Surprisingly, the multistate modeling indicated that the likelihood of ACPA/RF double seropositivity reverting to seronegativity after 5 years was >30%. The model also indicated a high likelihood of all seropositive states ultimately reverting to a seronegative state after an extended follow-up period. These findings suggest that our current understanding of RA risk based simply on the presence of ACPAs and/or RF seropositivity remains insufficient to provide clinically actionable guidelines.

ACPA fine specificity testing in the progressors confirmed previous findings that there is a protracted period of epitope spreading that appears to accelerate prior to inflammatory arthritis onset (3,4,42). In progressors who were RF negative at baseline, RF seropositivity tended to develop in parallel with, or after, the ACPA epitope spreading, and shortly before the development of inflammatory arthritis. Levels of ACPA targeting the specific peptide Cit-H4 were closely correlated with increasing ACPA scores, indicating a relationship between these 2 events. We have previously shown that this peptide is an immunodominant autoantigen in RA synovial fluid, where ongoing NETosis may fuel the autoimmune response (29). These findings suggest that the autoim-

mune response to Cit-H4 likely develops outside the joint as part of a broader ACPA response, and then becomes amplified in the inflammatory synovial microenvironment.

Although the role of HLA–DRB1 risk alleles in the progression to inflammatory arthritis in this population is of considerable interest, it was difficult to show a clear association. This is likely due to a high background frequency of the risk alleles. The inclusion of HLA–DRB1*0901 as a risk allele increased the strength of association with carriage of 2 risk alleles. HLA–DRB1*0901 has been associated with RA despite the fact that Asian populations are known to lack the Q(R)RRAA sequence in position 71–74 (43). We could not demonstrate any impact of HLA–DRB1 alleles in the autoantibody transitions analyzed in the multistate model, possibly because our model lacked the power to analyze their impact.

Limitations of the study include uncertainty as to whether the findings can be extended to the general population of Indigenous North Americans and non-Indigenous North Americans. Familial risk of RA is increased ~2–4 fold in most populations, particularly in Indigenous North Americans (15,22). Thus, based on shared genetic and environmental risk factors, relatives of Indigenous North American RA patients may have an increased propensity for developing ACPAs and/or RF, and also for developing inflammatory arthritis once they have autoantibodies. Given that a high proportion of individuals with autoantibodies reverted to a seronegative state, this phenomenon likely applies to some degree to other populations. Data from other prospectively followed up preclinical cohorts will be valuable in this respect.

Cohort studies in geographically dispersed Indigenous North American populations are logistically challenging and carry inherent limitations. Follow-up frequency for autoantibody-positive and negative individuals was asynchronous based on practical considerations and may have introduced a bias that led to a distortion in the incidence rate. Furthermore, a considerable number of individuals dropped out of the study, which may have introduced a further selection bias into the cohort. A comparison between the demographic and serologic characteristics of individuals who were included in the current analysis and those who were lost to follow-up after the baseline visit did not suggest any significant differences (data not shown).

Because this study spanned more than 15 years, commercially available anti-CCP testing evolved over this period. The anti-CCP results were generated by a single clinical laboratory at the time of each study visit, but the commercial assays used changed. We tried to reduce this variability by ultimately testing all participants at the baseline and final visits using the currently available commercial CCP3.1 kits. We also assigned categorical values to negative, weak positive, and strong positive anti-CCP results based on the cutoff of the specific assay used on each visit. Nevertheless, the variability of anti-CCP testing in intervening visits and moderate concordance among assays may have impacted on the multistate modeling in particular.

We acknowledge that an accurate and generalizable definition of inflammatory arthritis onset is difficult to achieve. The study relied on the experience of 1 of 3 study rheumatologists to make a diagnosis of inflammatory arthritis. This was not confirmed by a second rheumatologist, and neither imaging nor synovial fluid analysis was undertaken in most cases, which is a limitation of the study. Progressors could have been misdiagnosed at the time of inflammatory arthritis diagnosis, but the fact that they were all seropositive at that point and all ultimately met RA criteria makes this unlikely. It should be added that seronegative RA is highly infrequent in this, and other, Indigenous North American populations.

In conclusion, we showed a high incidence of seropositive inflammatory arthritis in a longitudinal cohort of high-risk relatives of Indigenous North Americans with RA who were prospectively followed up. Prediction models based on repeated measurements of ACPAs and RF showed that the development of inflammatory arthritis and reversion to a seronegative state were equally likely outcomes in ACPA+/RF+ subjects. Although we performed this study in a unique population of subjects who are known to have a high burden of disease, our findings should be considered in the future to appropriately power intervention studies designed to potentially prevent the onset of clinically detectable RA.

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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. El-Gabalawy 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. Hitchon, Robinson D, Sokolove, Ferucci, El-Gabalawy.

Acquisition of data. Smolik, Meng, Sokolove, Ferucci.

Analysis and interpretation of data. Tanner, Dufault, Anaparti, Robinson W, Sokolove, Lahey, Ferucci, El-Gabalawy.

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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

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Objective. Smoking is associated with an increased risk of rheumatoid arthritis (RA) in subsets of patients defined according to the presence or absence of anti–citrullinated protein antibodies (ACPAs) and rheumatoid factors (RFs). Moreover, an interaction between smoking and the HLA–DRB1 shared epitope (SE) has been demonstrated to be a risk factor for seropositive RA. The aim of this study was to investigate the interplay between smoking and the HLA–DRB1 SE with regard to risk of RA in different patient subsets based on ACPA and RF status.

Methods. Incident cases of RA (3,645 cases, 5,883 matched controls) were divided into 4 subgroups based on the presence or absence of RF and anti–cyclic citrullinated peptide 2 (anti-CCP2) antibodies. The influence of smoking on the risk of disease was determined in each RA subgroup, using logistic regression models with calculation of odds ratios and 95% confidence intervals (95% CIs). The potential interaction between smoking and HLA–DRB1 SE genes was evaluated by calculating the attributable proportion due to interaction (AP).

Results. In the RF+/anti-CCP2+ subset of RA patients, both smoking and the presence of the HLA–DRB1 SE conferred independent disease risks, and there was a strong interaction between the 2 risk factors (AP 0.4, 95% CI 0.3, 0.5). In the RF–/anti-CCP2+ patient subset, the HLA–DRB1 SE conferred an increased risk of RA, whereas the independent influence of smoking was limited. However, there was a significant interaction between the HLA–DRB1 SE and smoking (AP 0.2, 95% CI 0.02, 0.5). In the RF+/anti-CCP2– patient subset, there was an increased risk of disease among smokers, which was only marginally affected by the presence of the HLA–DRB1 SE, and no interaction between the 2 factors was observed (AP 0.002, 95% CI –0.3, 0.3). In the RF–/anti-CCP2– patient subset, neither smoking nor the presence of the HLA–DRB1 SE conferred an increased risk of RA.

Conclusion. These findings demonstrate different effects of smoking and HLA–DRB1 in the 4 serologically defined RA subsets.

INTRODUCTION

Rheumatoid arthritis (RA) is an immune-mediated inflammatory disease resulting from the complex interaction between genetic constitution and environmental triggers. The most important genetic risk factor for RA defined to date is the shared epitope (SE) of HLA–DRB1 (1–3), and smoking has been identified as the most important environmental factor in the development of RA (4–6).

The effects of these 2 risk factors, the HLA–DRB1 SE and smoking, and the interaction between them have been shown to be confined to the subset of RA patients whose disease is defined by the presence of anti–citrullinated protein antibodies (ACPAs) and/or rheumatoid factors (RFs), and a hypothesis regarding the etiology of this subset has been formulated based on the interaction between the HLA–DRB1 SE and smoking, as well as between the HLA–DRB1 SE and other airway exposures (7,8). However, the potential roles of RF and ACPAs in the pathogenesis of different subsets of RA have not yet been fully elucidated. We used an updated version of the Swedish population-based case–control study Epidemiological Investigation of Rheumatoid Arthritis (EIRA)

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to investigate the interplay between smoking and the HLA–DRB1 SE with regard to risk of RA in different serologically defined patient subsets grouped according to ACPA and RF status.

PATIENTS AND METHODS

Study design and study subjects. The present study investigated data from the ongoing EIRA project, which is a population-based case–control study comprising subjects ages 18–70 years in the middle and southern parts of Sweden. All hospital-based and most privately run rheumatology units in the study area participated in recruiting incident RA cases to the study. All patients identified as an incident case fulfilled the American College of Rheumatology 1987 classification criteria for RA (9). During the study period (November 1996 to September 2014), completed questionnaires were obtained from 3,724 RA cases and 5,935 matched healthy controls. Subjects who could not provide detailed information on smoking habits were excluded, as were patients whose ACPA or RF status was not available. A flow chart depicting the distribution of subjects is presented in Supplementary Figure 1 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40852/abstract). For each case recruited between November 1996 and October 2005, 1 control subject was randomly selected from the national population register, matched by age in 5-year age strata, by sex, and by residential area (EIRA I). For each case recruited between October 2005 and September 2014, 2 control subjects were selected using the same matching criteria (EIRA II). The response proportion was 92% for the cases and 75% for the controls. All aspects of the study were approved by the ethics committee of the Karolinska Institutet.

Anti–cyclic citrullinated peptide 2 (anti-CCP2) and RF analyses. Cases were categorized into either anti-CCP2 positive or anti-CCP2 negative based on the results of an Immunoscan-RA Mark2 enzyme-linked immunosorbent assay (anti-CCP2 test). An antibody level exceeding 25 AU/ml was regarded as a positive result. RF positivity or RF negativity was determined locally by the unit entering the case into the study.

Data collection and definition of smoking status. Information regarding lifestyle factors and different environmental exposures was collected using a standardized questionnaire. Detailed information on smoking was obtained by asking each subject about current and previous smoking habits, including duration of smoking, average number of cigarettes smoked per day, and type of cigarettes. For each case, the time of the initial appearance of RA symptoms was used as an estimate of the date of disease onset, and the year in which this occurred was defined as the index year. The corresponding controls were given the same index year. Information regarding smoking was considered prior to or during the index year in the cases and during the same period of time in the corresponding controls. Subjects who had smoked during the index year were defined as current smokers, those who had stopped smoking prior to the index year were defined as past smokers, and those who had never smoked before or during the index year were defined as never smokers.

Genotyping. Blood samples were available from participants who answered the questionnaire between November 1996 and May 2012. HLA–DRB1 genotypes were obtained using a previously described method (10). Data on genotypes were available for 3,355 cases (63%) and 2,840 controls (48%). The HLA–DRB1*01, HLA–DRB1*04, and HLA–DRB1*10 alleles were classified as the SE alleles.

Statistical analysis. Using logistic regression analyses with calculation of odds ratios (ORs) and 95% confidence intervals (95% CIs), the risk of occurrence of each RA serologic subset in patients with different smoking habits was compared with that in never smokers. The occurrence of RA among those who had started and stopped smoking in different life periods was compared with that among never smokers. A trend test for a dose-response relationship regarding cumulative dose of smoking and risk of each subset of RA was performed using a continuous

Table 1. Rate of anti-CCP2 positivity among RA cases categorized by RF status, number of SE alleles, and smoking status*

	No. of RA cases	Anti-CCP2 positive, no. (%)
RF-negative		
0 alleles		
Never smoker	179	18 (10.1)
Ever smoker	254	23 (9.1)
1 allele		
Never smoker	209	59 (28.2)
Ever smoker	317	104 (32.8)
2 alleles		
Never smoker	73	38 (52.1)
Ever smoker	111	60 (54.1)
RF-positive		
0 alleles		
Never smoker	128	86 (67.2)
Ever smoker	296	205 (69.3)
1 allele		
Never smoker	313	265 (84.7)
Ever smoker	808	701 (86.8)
2 alleles		
Never smoker	192	177 (92.2)
Ever smoker	496	473 (95.4)

* Anti-CCP2 = anti–cyclic citrullinated peptide 2; RA = rheumatoid arthritis; RF = rheumatoid factor; SE = shared epitope.

variable for cumulative dose of smoking (expressed in pack-years) in a logistic regression model.

In addition, we investigated the interaction between smoking and SE genes with regard to each RA subset. The potential interaction was analyzed using departure from additivity of effects as the criterion for interaction and was evaluated by calculating the attributable proportion due to interaction (with 95% CIs) (11,12).

All analyses were adjusted for age, sex, residential area, ancestry, and study. Assessment of ancestry was based on whether or not the subject was born in Sweden, and whether or not either of the subject's parents had immigrated to Sweden. A subject who was born in Sweden and whose parents had not immigrated was classified as Swedish. Adjustments were also made for educational level (university degree or no university degree), exposure to passive smoking (yes or no), alcohol consumption (number of standardized drinks per week at study inclusion), and body mass index at inclusion in the study (≤25 kg/m^2 or >25 kg/m^2). However, these factors had only a minor influence on the results and were therefore not retained in the final analyses. All analyses were conducted using SAS software version 9.4 (SAS Institute).

RESULTS

The majority of the patients with incident RA were both RF positive and ACPA positive (57%), whereas 25% were negative for both classes of antibodies. Nine percent of patients were ACPA positive only, and 9% were RF positive only. The characteristics of the cases and controls are presented in Supplementary Table 1 (on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40852/abstract). There were no significant

Table 2. Odds of developing rheumatoid arthritis, stratified by serologic subset, according to different categories of smokers compared with never smokers, in total and by cumulative dose of smoking*

	Anti-CCP2−, RF−		Anti-CCP2+, RF−		Anti-CCP2−, RF+		Anti-CCP2+, RF+	
	No. cases/no. controls†	OR (95% CI)	No. cases/no. controls†	OR (95% CI)	No. cases/no. controls†	OR (95% CI)	No. cases/no. controls†	OR (95% CI)
Total								
Never smoker	367/2,655	1.0 (reference)	133/2,655	1.0 (reference)	111/2,655	1.0 (reference)	594/2,655	1.0 (reference)
Past smoker	330/1,909	1.1 (0.9–1.4)	109/1,909	1.2 (0.98–1.6)	116/1,909	1.3 (1.02–1.8)	774/1,909	1.8 (1.6–2.1)
Current smoker	218/1,319	1.1 (0.9–1.3)	89/1,319	1.3 (1.004–1.7)	109/1,319	1.8 (1.3–2.3)	695/1,319	2.4 (2.1–2.7)
<10 pack-years								
Never smoker	367/2,655	1.0 (reference)	133/2,655	1.0 (reference)	111/2,655	1.0 (reference)	594/2,655	1.0 (reference)
Past smoker	179/1,062	1.1 (0.9–1.4)	58/1,062	1.1 (0.8–1.5)	62/1,062	1.2 (0.9–1.7)	320/1,062	1.2 (1.001–1.5)
Current smoker	68/476	1.1 (0.8–1.5)	28/476	1.0 (0.7–1.3)	28/476	1.2 (0.8–1.8)	148/476	1.3 (1.1–1.5)
10–20 pack-years								
Never smoker	367/2,655	1.0 (reference)	133/2,655	1.0 (reference)	111/2,655	1.0 (reference)	594/2,655	1.0 (reference)
Past smoker	79/495	1.0 (0.7–1.2)	30/495	1.3 (0.9–2.0)	25/495	1.2 (0.8–1.9)	205/495	1.9 (1.6–2.3)
Current smoker	52/325	1.1 (0.8–1.5)	26/325	1.6 (1.04–2.5)	37/325	2.4 (1.6–3.6)	201/325	2.7 (2.2–3.3)
>20 pack-years								
Never smoker	367/2,655	1.0 (reference)	133/2,655	1.0 (reference)	111/2,655	1.0 (reference)	594/2,655	1.0 (reference)
Past smoker	72/352	1.2 (0.9–1.6)	21/352	1.5 (0.9–2.4)	29/352	1.9 (1.3–2.7)	249/352	3.1 (2.6–3.7)
Current smoker	98/518	1.2 (0.9–1.5)	35/518	1.6 (1.05–2.4)	44/518	2.0 (1.2–3.1)	346/518	3.6 (2.9–4.4)

* All analyses were adjusted for age, sex, residential area, and ancestry. Anti-CCP2 = anti–cyclic citrullinated peptide 2; RF = rheumatoid factor; OR = odds ratio; 95% CI = 95% confidence interval.
† Values are the number of exposed cases and controls.

differences between the RA subsets with regard to age, sex, or ancestry.

Among the RA patients, ACPA positivity independently correlated with both the HLA–DRB1 SE status and the RF status (Table 1). Smoking habits did not significantly influence these correlations, but there was a significant dose-dependent relationship between smoking and RF positivity irrespective of anti-CCP2 and HLA–DRB1 SE status ($P < 0.0001$).

Compared with never smokers, the overall OR for developing RF–/anti-CCP2– RA among ever smokers was 1.1 (95% CI 0.96–1.3). The corresponding ORs for the other subsets of patients who were ever smokers were as follows: for RF–/anti-CCP2+ RA, OR 1.2 (95% CI 0.98–1.6); for RF+/anti-CCP2– RA, OR 1.6 (95% CI 1.2–1.9); for RF+/anti-CCP2+ RA, OR 2.0 (95% CI 1.8–2.2) (Table 2). The association between smoking and risk of RA increased numerically with increasing exposure to smoking (i.e., increasing pack-years of smoking) in all 3 antibody-dependent subsets, but was largest in the subsets positive for RF (P for trend < 0.0001 in the RF-positive subsets).

The influence of smoking on the risk of developing RF+/anti-CCP2+ RA increased significantly with the number of SE alleles (P for trend < 0.0001) (Table 3).

The risk of RA conferred by HLA–DRB1 SE seropositivity was mainly observed in anti-CCP2+ RA patients, irrespective of RF status (Table 4). The interaction between smoking and HLA–DRB1 SE genes, measured as the attributable proportion due to interaction, was highest in the subset positive for both RF and anti-CCP2, but a notable interaction was observed also in the RF–/anti-CCP2+ RA subset for the group consisting of individuals who had smoked more than 10 pack-years (Table 4). This interaction was also stronger among HLA–DRB1 SE homozygotes than among HLA–DRB1 SE heterozygotes (Table 5). No significant interaction was observed between smoking and HLA–DRB1 SE genes with regard to the risk of anti-CCP2– RA, regardless of RF status (Table 4).

A summary of the risk of RA conferred by smoking and the presence of the HLA–DR SE in the 4 different serologically defined subsets of RA patients is provided in Figure 1.

Table 3. Odds of developing rheumatoid arthritis, stratified by serologic subset, according to HLA SE status and different categories of smokers compared with never smokers*

	Anti-CCP2–, RF–		Anti-CCP2+, RF–		Anti-CCP2–, RF+		Anti-CCP2+, RF+	
	No. cases/no. controls†	OR (95% CI)	No. cases/no. controls†	OR (95% CI)	No. cases/no. controls†	OR (95% CI)	No. cases/no. controls†	OR (95% CI)
HLA SE negative								
Never smoker	161/592	1.0 (reference)	18/592	1.0 (reference)	42/592	1.0 (reference)	86/592	1.0 (reference)
Past smoker	142/501	1.0 (0.7–1.3)	12/501	1.0 (0.4–1.8)	43/501	1.1 (0.7–1.7)	117/501	1.6 (1.2–2.2)
Current smoker	89/263	1.2 (0.9–1.6)	11/263	1.3 (0.6–2.8)	48/263	2.4 (1.5–3.7)	88/263	2.3 (1.6–3.2)
HLA SE heterozygote								
Never smoker	151/521	1.0 (reference)	59/521	1.0 (reference)	49/521	1.0 (reference)	265/521	1.0 (reference)
Past smoker	125/464	0.9 (0.7–1.1)	58/464	1.2 (0.8–1.8)	58/464	1.6 (0.9–2.0)	369/464	1.6 (1.3–2.0)
Current smoker	95/272	1.1 (0.8–1.5)	46/272	1.5 (0.97–2.3)	49/272	1.7 (1.1–2.7)	336/272	2.4 (1.9–2.9)
HLA SE homozygote								
Never smoker	35/137	1.0 (reference)	38/137	1.0 (reference)	15/137	1.0 (reference)	177/137	1.0 (reference)
Past smoker	35/92	1.3 (0.9–2.6)	29/92	1.3 (0.7–2.2)	11/92	1.1 (0.5–2.5)	238/92	2.2 (1.6–3.1)
Current smoker	16/60	1.0 (0.6–2.0)	31/60	1.9 (1.04–3.4)	12/60	1.8 (0.8–3.9)	235/60	3.2 (2.2–4.6)

* All analyses were adjusted for age, sex, residential area, and ancestry. SE = shared epitope; anti-CCP2 = anti–cyclic citrullinated peptide 2; RF = rheumatoid factor; OR = odds ratio; 95% CI = 95% confidence interval.
† Values are the number of exposed cases and controls.

Table 4. Interaction between the HLA SE and smoking in relation to odds of developing rheumatoid arthritis*

	Anti-CCP2−, RF−		Anti-CCP2+, RF−		Anti-CCP2−, RF+		Anti-CCP2+, RF+	
	No. cases/no. controls†	OR (95% CI)	No. cases/no. controls†	OR (95% CI)	No. cases/no. controls†	OR (95% CI)	No. cases/no. controls†	OR (95% CI)
Total								
HLA SE negative								
Never smoker	161/592	1.0 (reference)	18/592	1.0 (reference)	42/592	1.0 (reference)	86/592	1.0 (reference)
Ever smoker	231/764	1.1 (0.9, 1.4)	23/764	1.1 (0.6, 2.0)	91/764	1.8 (1.2, 2.6)	205/764	1.9 (1.5, 2.5)
HLA SE positive								
Never smoker	184/636	1.0 (0.8, 1.3)	97/636	5.0 (3.0, 8.4)	63/636	1.4 (0.9, 2.1)	441/636	4.8 (3.7, 6.1)
Ever smoker	261/848	1.1 (0.9, 1.4)	160/848	6.7 (4.0, 11.1)	126/848	2.2 (1.5, 3.1)	1166/848	10.0 (7.8, 12.8)
AP‡		0.03 (−0.3, 0.3)		0.2 (0.02, 0.5)		0.002 (−0.3, 0.3)		0.4 (0.3, 0.5)
<10 pack-years of smoking								
HLA SE negative								
0 pack-years	161/592	1.0 (reference)	18/592	1.0 (reference)	42/592	1.0 (reference)	86/592	1.0 (reference)
<10 pack-years	101/340	1.1 (0.8, 1.4)	10/340	1.0 (0.5, 2.0)	35/340	1.4 (0.8, 2.2)	62/340	1.2 (0.8, 1.7)
HLA SE positive								
0 pack-years	184/636	1.0 (0.8, 1.3)	97/636	5.0 (3.0, 8.4)	63/636	1.4 (0.9, 2.1)	441/636	4.8 (3.7, 6.2)
<10 pack-years	119/402	1.1 (0.8, 1.4)	68/402	5.1 (3.0, 8.8)	52/402	1.7 (1.1, 2.6)	374/402	6.3 (4.8, 8.3)
AP‡		−0.06 (−0.5, 0.3)		0.04 (−0.3, 0.4)		−0.02 (−0.5, 0.5)		0.2 (0.05, 0.4)
10–20 pack-years of smoking								
HLA SE negative								
0 pack-years	161/592	1.0 (reference)	18/592	1.0 (reference)	42/592	1.0 (reference)	86/592	1.0 (reference)
10–20 pack-years	59/198	1.1 (0.8, 1.5)	6/198	1.2 (0.5, 3.0)	25/198	2.0 (1.2, 3.5)	59/198	2.3 (1.6, 3.4)
HLA SE positive								
0 pack-years	184/636	1.0 (0.8, 1.3)	97/636	5.0 (3.0, 8.4)	63/636	1.4 (0.9, 2.1)	441/636	4.8 (3.7, 6.3)
10–20 pack-years	61/220	1.0 (0.7, 1.4)	47/220	8.5 (4.8, 15.3)	33/220	2.4 (1.4, 3.9)	317/220	11.4 (8.5, 15.3)
AP‡		−0.09 (−0.6, 0.4)		0.4 (0.1, 0.7)		−0.06 (−0.6, 0.5)		0.5 (0.3, 0.6)
>20 pack-years of smoking								
HLA SE negative								
0 pack-years	161/592	1.0 (reference)	18/592	1.0 (reference)	42/592	1.0 (reference)	86/592	1.0 (reference)
>20 pack-years	71/226	1.2 (0.8, 1.6)	7/226	1.5 (0.6, 3.6)	31/226	2.5 (1.5, 4.1)	84/226	3.3 (2.3, 4.6)
HLA SE positive								
0 pack-years	184/636	1.1 (0.8, 1.3)	97/636	5.0 (3.0, 8.4)	63/636	1.4 (0.9, 2.1)	441/636	4.8 (3.7, 6.2)
>20 pack-years	81/226	1.3 (0.98, 1.8)	45/226	9.2 (5.1, 16.6)	41/226	3.1 (2.0, 5.0)	475/226	18.2 (14, 24)
AP‡		0.09 (−0.3, 0.5)		0.4 (0.1, 0.7)		0.09 (−0.4, 0.5)		0.6 (0.5, 0.7)

* All analyses were adjusted for age, sex, residential area, and ancestry. Anti-CCP2 = anti–cyclic citrullinated peptide 2; RF = rheumatoid factor; OR = odds ratio; 95% CI = 95% confidence interval.
† Values are the number of exposed cases and controls.
‡ Attributable proportion (AP) due to interaction between the HLA shared epitope (SE) and smoking.

Table 5. Odds of developing rheumatoid arthritis, stratified by serologic subset, in subjects categorized by the number of HLA SE alleles and smoking status*

	Anti-CCP2+, RF−			Anti-CCP2+, RF+		
	No. cases/no. controls†	OR (95% CI)	AP‡	No. cases/no. controls†	OR (95% CI)	AP‡
HLA SE						
0 alleles						
Never smoker	18/592	1.0 (reference)		86/592	1.0 (reference)	
Ever smoker	23/764	1.1 (0.6–2.0)		205/764	1.8 (1.4–2.4)	
1 allele						
Never smoker	59/501	4.0 (2.3–6.8)		264/501	3.6 (2.7–4.7)	
Ever smoker	100/697	5.3 (3.2–8.8)	0.2 (0.02–0.5)	693/697	6.9 (5.3–8.8)	0.4 (0.2–0.5)
2 alleles						
Never smoker	38/135	7.8 (5.4–17.8)		177/135	8.8 (6.4–12.1)	
Ever smoker	60/151	14.0 (8.0–24.3)	0.3 (0.04–0.7)	473/151	21.5 (16.0–28.8)	0.6 (0.4–0.7)

* Anti-CCP2 = anti–cyclic citrullinated peptide 2; RF = rheumatoid factor; OR = odds ratio; 95% CI = 95% confidence interval.
† Values are the number of exposed cases and controls.
‡ Attributable proportion (AP) due to interaction between the HLA shared epitope (SE) and ever smoking.

DISCUSSION

The main finding of the present study is that the impact of smoking and HLA–DRB1 genes and their interaction with regard to risk of RA varied between the 4 serologically defined subsets of RA. It has previously been difficult to distinguish the association between the HLA–DRB1 SE and ACPAs from the unique association between smoking and RF, due to the fact that sample sizes have been limited. By using well-defined serologic subsets of RA, we have demonstrated that smoking is indeed a prominent risk factor for RF+/anti-CCP2– RA, whereas the effect of smoking is more limited, but still existing, in RF–/anti-CCP2+ RA. Our results are consistent with those of several previous studies in which it was suggested that smoking may generate RF and ACPAs as well as other autoantibodies in RA (13). The situation with regard to the association with the HLA–DRB1 SE was even more obvious. A clear association with the class II genes was observed for both anti-CCP2–positive RA subsets irrespective of RF status, whereas no association was observed for the RF+/anti-CCP2– RA subset.

Another recent study showed that RF levels are associated with ACPA positivity irrespective of smoking history, and noted that there seemed to be a difference in the importance of the number of SE alleles in determining ACPA positivity between RF-negative and RF-positive RA patients (10). The results of the present study confirmed a correlation between RF and anti-CCP2 positivity in RA patients that was independent of smoking habits. However, the correlation was present regardless of HLA–DRB1 SE status ($P < 0.0001$).

A question that can now be addressed more distinctly than before is how smoking may be involved in the induction of RF and ACPAs (herein measured as anti-CCP2 antibodies) in individuals with different genetic setups. Our study in the RF+/anti-CCP2– RA subset clearly showed that smoking may induce RF independent of both the HLA–DRB1 SE and presence/induction of ACPAs. This is consistent with findings in previous studies in healthy individuals, which showed that smoking can induce RF production (14). This lack of relationship with the HLA–DRB1 SE status is also compatible with the notion of T cell–independent triggering mechanisms, as demonstrated recently by the findings of a low number of T cell–mediated somatic mutations in single RF-producing B cells from RA patients (15). The situation was substantially different in the RF–/anti-CCP2+ RA group, in whom there was a major and gene dose–dependent effect of HLA–DRB1 and a more limited, but still visible, effect of smoking, particularly in heavy smokers. This finding is compatible with prior reports of high numbers of T cell–dependent somatic mutations in genes coding for anticitrulline-reactive antibodies (15–17).

The situation in the major subset of RA patients who were positive for both RF and anti-CCP2 was also different, with major effects of both smoking and the HLA–DRB1 SE and with a pronounced interaction between the 2 risk factors. Thus, the challenge is to understand the mechanisms that explain why the gene–environment interaction between the HLA–DRB1 SE and smoking is most pronounced in conjunction with the presence of both RF and anti-CCP2 antibodies. As previously described, the data suggest that class II major histocompatibility complex (MHC)–dependent immunity may be triggered at sites in which smoke primarily interacts with the immune system, i.e., in the lungs and related mucosal tissues (18,19). A more precise molecular definition with regard to which structures in the HLA–DRB1 molecule are involved in this interaction has also been provided recently (20).

An obvious hypothesis for a triggering scenario would be that RF generated by T cell–independent effects of smoking (14) would enhance class II MHC–dependent T cell activation against

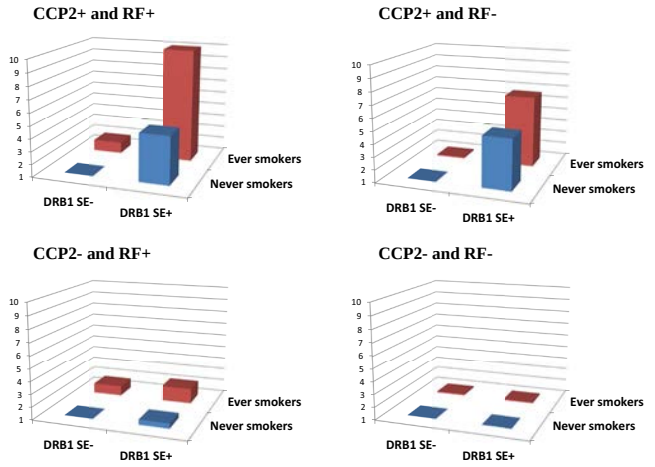

Figure 1. Odds ratios for the different serologic subsets of rheumatoid arthritis stratified by positivity or negativity for rheumatoid factor (RF) and anti–cyclic citrullinated peptide 2 (anti-CCP2) antibodies, according to different combinations of HLA–DRB1 shared epitope (SE) and smoking status. Data are shown in Table 4.

citrullinated proteins at mucosal sites where smoke encounters the immune system. Such enhancing effects on antigen presentation from RF and other mechanisms that generate immune complexes are well known (21). This scenario may be further strengthened from the generation of RF due to reactivity of T cells against antigens present in local immune complexes (22). Taken together, these data suggest that the dramatic interaction between the HLA–DRB1 SE and smoking in conferring a risk of RF-positive RA (8) and ACPA-positive RA (6,23) requires the simultaneous presence of both of these antibodies.

Interestingly, the presence of both RF and ACPAs also appears to provide the highest risk for subsequent development of RA in antibody-positive, but still nonarthritic, individuals (24,25). The synergizing effects between ACPAs, RF, and immune complexes have also been described in models of effector phases of joint inflammation in RA (26–28). All of these prior studies addressed in vitro–formed immune complexes, but the first report of an evaluation of ACPA-containing immune complexes obtained in vivo was recently published (29).

Our study was designed as a case–control study with incident RA cases, and information regarding smoking habits and exposure to passive smoking was collected retrospectively. Recall bias was minimized by using incident cases of RA. We took great effort to obtain information on lifestyle factors and environmental exposures from the RA patients in a way that was identical to that used for the controls. Furthermore, the questionnaire contained a wide range of questions regarding many potential environmental risk factors, and no section in the questionnaire was given prime focus.

A potential selection bias may arise when recruiting cases and controls. The proportion of respondents with regard to participation in the EIRA study was 92% for cases and 75% for controls. Since the structure of the Swedish public health care system provides equal access to medical services for all Swedish citizens, it is most likely that almost all cases of RA are referred to public rheumatology units, and it is not likely that the few unidentified cases would cause a substantial bias in our calculations. Selection bias among controls is likely to be modest, since the prevalence of smoking among controls, seen as an indicator of lifestyle, was consistent with that observed in the general population at equivalent ages (30).

In summary, our findings describe how smoking and the HLA–DRB1 SE may play different roles in the pathogenesis of different serologically defined subsets of RA, and that RF and ACPAs appear to act together in both the triggering and the effector phases in the major RF+/ACPA+ subset of RA.

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. Hedström 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. Rönnelid, Klareskog, Alfredsson.
Acquisition of data. Klareskog, Alfredsson.
Analysis and interpretation of data. Hedström.

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Selective Sexual Dimorphisms in Musculoskeletal and Cardiopulmonary Pathologic Manifestations and Mortality Incidence in the Tumor Necrosis Factor–Transgenic Mouse Model of Rheumatoid Arthritis

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Objective. To examine and quantify the sexual dimorphism in pathologic features manifested in the musculoskeletal and cardiopulmonary systems and incidence of mortality in the tumor necrosis factor–transgenic (TNF-Tg; Tg3647 strain) mouse model of inflammatory erosive arthritis.

Methods. Kaplan-Meier survival estimates were determined in male and female Tg3647 mice and sex-matched wild-type (WT) littermate mice. Longitudinal and cross-sectional pathologic outcomes in the musculoskeletal and cardiopulmonary systems were assessed via ultrasound, micro–computed tomography, grip strength measurements, histologic and serologic analyses, flow cytometry, and skeletal muscle physiologic measures.

Results. Compared to male Tg3647 mice (n = 30), female Tg3647 mice (n = 34) had significantly shorter lifespans ($P < 0.001$) and exhibited the following pathologic features (n = 4–6 per group; $P < 0.05$ versus male Tg3647 littermates): gross deficits in body mass and muscle weight, early-onset inflammatory arthritis with severity of end-stage arthritis that was as severe as that seen in male transgenic mice, and early onset and increased severity of inflammatory interstitial lung disease (ILD). Histologically, the ILD observed in Tg3647 mice was characterized by inflammatory cell accumulation and pulmonary arteriole thickening, which was concomitant with the presence of right ventricular hypertrophy, a feature that was also more severe in the female compared to male Tg3647 mice ($P < 0.05$). No sexual dimorphisms in TNF-induced deficient grip strength, axial skeletal growth, or bone loss were found. Globally, the extent of the pathologic changes observed in female Tg3647 mice was greater than that observed in male Tg3647 mice when each group was compared to their sex-matched WT littermates.

Conclusion. These findings indicate that TNF selectively drives the early onset of arthritis and progression of pathologic changes in the cardiopulmonary system in female Tg3647 mice. These results in the Tg3647 mouse identify it as a suitable model to better understand the mechanisms underlying sexual dimorphism and cardiopulmonary disease in the setting of inflammatory arthritis and other connective tissue diseases.

INTRODUCTION

Autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus, Graves' disease, Sjögren's syndrome, and scleroderma, exhibit remarkable sexual dimorphism, with female:male incidence ratios of at least 2:1, and some of these diseases are 10 times more frequent in females (1). RA is one of the most prevalent of these autoimmune diseases, affecting 0.6–0.8% of the adult population in the US, second only to thyroid disease (2,3). Conceptually, 3 main factors may account for the predominance of these disorders in females: steroid hormones, chromosomal differences based on the number of X

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chromosomes, and environmental or societal differences (1). The analysis of environmental or societal differences in preclinical models presents major obstacles, and a significant body of work has focused on the interaction between autoimmunity and sex hormones and chromosome-linked traits (1,4–6). Of particular relevance, however, is the absence of current models to study the role of sexual dimorphism in the development of inflammatory arthritis and concomitant extraarticular features (7).

The tumor necrosis factor–transgenic (TNF-Tg) mouse serves as a model of multiple diverse inflammatory phenotypes (8–10). Specifically, the Tg197 and Tg3647 mouse strains possess the full-length human TNF gene with a modified 3′–untranslated region (3′-UTR) exchanging for the β-globin 3′-UTR, and they develop spontaneous inflammatory erosive arthritis at ages 1 month and 3 months, respectively (8,10). This difference in the age at disease onset is thought to be due to the number of transgenes inserted, as the Tg197 strain carries ~5 copies and the Tg3647 strain carries 1 copy (8,10). However, differences in the timing of disease onset and disease severity between male and female mice have not been fully explored. Additionally, other murine models of spontaneous arthritis, such as the SKG, K/BxN, and other TNF-induced murine arthritis models (i.e., models driven by different TNF transgenes), have not been described as displaying sexual dimorphism (7,11).

Herein, we expand upon anecdotal evidence within our laboratory showing that female TNF-Tg (Tg3647 line) mice have shorter lifespans and develop arthritis earlier than their male counterparts. These studies were motivated by recent epidemiologic evidence describing increased mortality, earlier arthritis onset, and increased arthritis severity in female patients with RA (12–14). We investigated the incidence of mortality in Tg3647 mice, along with supporting necropsy analysis of female mice that were euthanized at age 6 months. These analyses demonstrated that the dramatically shortened lifespan of the female mice compared to their male littermates may be related to the development of cardiopulmonary disease in the females. We also performed longitudinal and cross-sectional analyses of the cardiopulmonary and musculoskeletal systems to investigate sexual dimorphism in both the progression and pathophysiologic manifestations of rheumatic disease related to the increased incidence of mortality. We found that female Tg3647 mice manifested symptoms of arthritis at earlier time points than males, and also developed severe cardiopulmonary disease, which explains the high incidence of mortality and shorter lifespans of the female mice.

MATERIALS AND METHODS

The present study was conducted with prior approval from the University of Rochester Medical Center University Committee for Animal Resources. The Tg3647 strain of TNF-transgenic mice was originally obtained from Dr. George Kollias (8,10) and has been maintained across multiple generations. All studies were performed with littermate wild-type (WT) control mice. An initial cohort composed of male WT mice (n = 28), female WT mice (n = 27), male Tg3647 mice (n = 30), and female Tg3647 mice (n = 34) was observed for 400 days under standard microisolator conditions, with autoclaved food (Rodent Diet 5010; LabDiet) and water available ad libitum. Date of birth and date of death were recorded for Kaplan-Meier survival estimates. Subsequently, 4 female Tg3647 mice that were euthanized at age 6 months were submitted for necropsy analysis of the internal organs.

For the present analyses, a prospective study was designed and performed to assess longitudinal outcomes in the mice from ages 2 months to 5.5 months, as well as to assess outcomes at cross-sectional time points of 3, 4, and 5.5 months (1 month = 28 days). In total, 3 cohorts of 4 groups of mice, composed of male WT, female WT, male Tg3647, and female Tg3647 mice (n = 6 mice per group), were created, and the mice were euthanized at either 3, 4, or 5.5 months of age (for a flow chart of the study design, see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract). Confirmatory studies with additional mice were performed to assess complementary outcomes as needed. Details on the methods used for histology, ultrasound, micro–computed tomography (micro-CT), grip strength measurements, muscle force generation, tibialis anterior dissection, spine analysis, flow cytometry analysis, measurement of cytokine levels, and statistical analyses are all presented in the Supplementary Materials and Methods (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract).

RESULTS

Earlier mortality and concomitant pathologic manifestations within the cardiopulmonary and musculoskeletal systems in female Tg3647 mice. Kaplan-Meier estimates of survival in female versus male Tg3647 and WT mice revealed a significant decrease in the lifespan of female Tg3647 mice compared to their male Tg3647 counterparts (median 166 days versus 229 days; $P < 0.001$) (Figure 1A). In order to understand the etiology of the accelerated mortality in transgenic female mice, an internal organ necropsy study was performed on 4 female Tg3647 mice that had been euthanized at age 6 months. This investigation revealed significant cardiopulmonary pathologic manifestations in all 4 mice, including marked numbers of plasma cells, lymphocytes, and macrophages surrounding pulmonary vessels and airways, as well as thickened right ventricles (RVs) (for more details, see Supplementary Materials and Methods [http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract]).

These unexpected findings triggered a comprehensive assessment of the temporal progression of pathologic features in the cardiopulmonary and musculoskeletal systems in the male and female Tg3647 mice and their WT littermates. The purpose

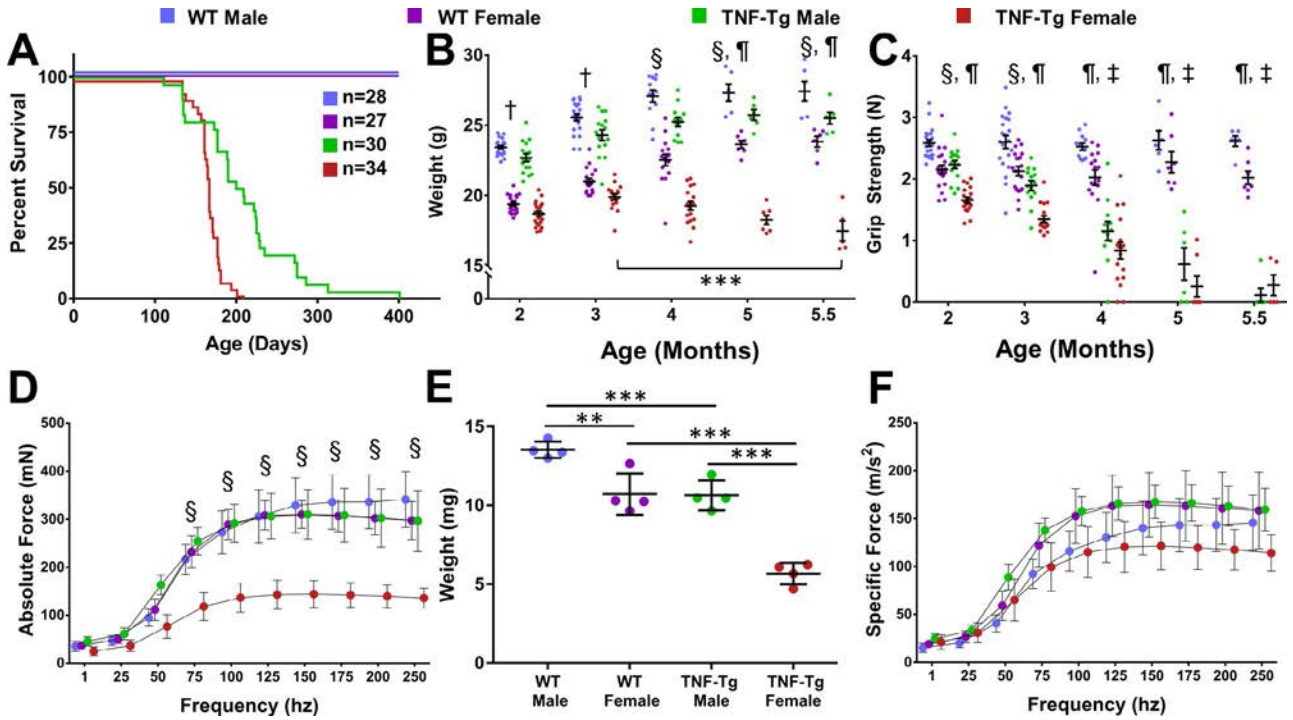

Figure 1. Increased morbidity and accelerated mortality in female Tg3647 mice (a tumor necrosis factor–transgenic [TNF-Tg] mouse strain). Female and male Tg3647 mice and sex-matched wild-type (WT) littermate mice were compared for survival expectancy (**A**), total body weight (**B**), and grip strength (**C**) from ages 2 months to 5.5 months. Absolute extensor digitorum longus (EDL) muscle force (**D**) and specific muscle force normalized to cross-sectional area of the muscle (**F**) were compared between groups, and the potential contribution of muscle mass to EDL muscle force was evaluated (**E**). Bars show the mean ± SD. † = $P < 0.05$ for female Tg3647 versus male Tg3647; § = $P < 0.05$ for female Tg3647 versus all other groups; ¶ = $P < 0.05$ for male Tg3647 versus male WT; ‡ = $P < 0.05$ for female Tg3647 versus female WT; ** = $P < 0.01$; *** = $P < 0.001$. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract.

of these studies was to formally define the female–male temporal pathologic divergence in this model of RA. Of note, no remarkable pathologic features were identified in the liver, kidney, large intestine, small intestine, brain, and salivary glands in any of the mice (details in Supplementary Materials and Methods [http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract]), but pathologic analysis did reveal musculoskeletal and splenic abnormalities in the Tg3647 mice, which are symptoms that have been well-established in TNF-Tg mice (8–10,15).

Specific musculoskeletal sexual dimorphism in Tg3647 mice. Total body weights of both the male and female Tg3647 mice were significantly decreased ($P < 0.05$) compared to their sex-matched WT littermates at ages 4, 5, and 5.5 months (Figure 1B). Importantly, the body weight of female Tg3647 mice decreased from age 3 months to age 5.5 months ($P < 0.001$), while there was no difference in the body weight of male Tg3647 mice or male WT mice during this period (Figure 1B). No differences in bone growth were observed until age 4 months, when differences in the L5 height between Tg3647 mice and WT mice became apparent (see Supplementary Figures 2A–E, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract). Evidence of generalized osteopenia in Tg3647 mice, as compared to WT mice, was similar to that previously reported (16). No differences in the L5 trabecular bone volume/total volume and cortical bone volume were found between male and female Tg3647 mice (see Supplementary Figures 2F–O at http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract).

Grip strength was significantly decreased ($P < 0.05$) at all time points in both male and female Tg3647 mice when compared to their sex-matched WT littermate controls (Figure 1C). Moreover, at ages 2 and 3 months, female Tg3647 mice had significantly reduced grip strength ($P < 0.05$) compared to age-matched male Tg3647 mice. However, post hoc analysis revealed no interaction of sex and genotype at these time points (Figure 1C), suggesting that female mice have less grip strength independent of genotype.

In terms of skeletal muscle, absolute extensor digitorum longus (EDL) muscle force showed severe deficits in the female Tg3647 mice at age 5.5 months ($P < 0.05$ versus all other groups). However, this was primarily attributable to the lack of EDL muscle mass in Tg3647 mice compared to WT mice ($P < 0.001$) (Figures 1D and E). When the values were normalized to account for cross-sectional area of the muscle, there were no differences in specific EDL muscle force between the groups (Figure 1F).

Arthritic changes in the knee occurred earlier in female Tg3647 mice compared to male Tg3647 mice. The female mice

demonstrated significant increases ($P < 0.001$) in total synovial area, numbers of cells within the synovium, total histology score, and inflammatory infiltrates at age 3 months as compared to the male mice at age 3 months (Figures 2A–F and Supplementary Figures 3A–J, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract). In contrast, the severity of ankle arthritis was similar between the sexes at age 3 months (see results in Supplementary Figures 4A–D, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract). Histologic features of the knees and ankles of WT mice remained normal over the study period (data not shown).

Analysis of the popliteal lymph node (PLN) volume, a biomarker of arthritic changes in the knee (15,17,18), also showed changes at earlier time points in female Tg3647 mice compared to male Tg3647 mice. While both male and female Tg3647 mice experienced a progressive increase in their PLN volumes at the same rate, vascularity of the PLNs was greater in females than in males at age 3 months, as determined by normalized power Doppler volume analysis. Compared to that in male mice at age 5.5 months, the total PLN volume in female mice began to decrease at an earlier age (Figures 2G and H). The earlier changes in PLN dynamics in female mice were clearly demonstrated when the classification scheme was applied to categorize the LNs into expanding and collapsed phases (see Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract). Significantly more expanding PLNs ($P < 0.05$) were identified in female Tg3647 mice compared to male Tg3647 mice at ages 3 and 4 months (early arthritis). Furthermore, the PLNs of 8 female mice collapsed by age 5.5 months (advanced arthritis), whereas the PLNs of only 3 male mice collapsed by age 5.5 months ($P < 0.05$).

Moreover, the trends in PLN volume individually showed that PLNs in female mice reached peak volume and began to collapse much earlier than PLNs in the majority of male mice (Figures 2I and J). Overall, these data indicate that female Tg3647 mice developed knee arthritis at an earlier age and also had earlier pathologic complications in the draining lymph nodes of the joints and significant musculoskeletal morbidity compared to their male counterparts.

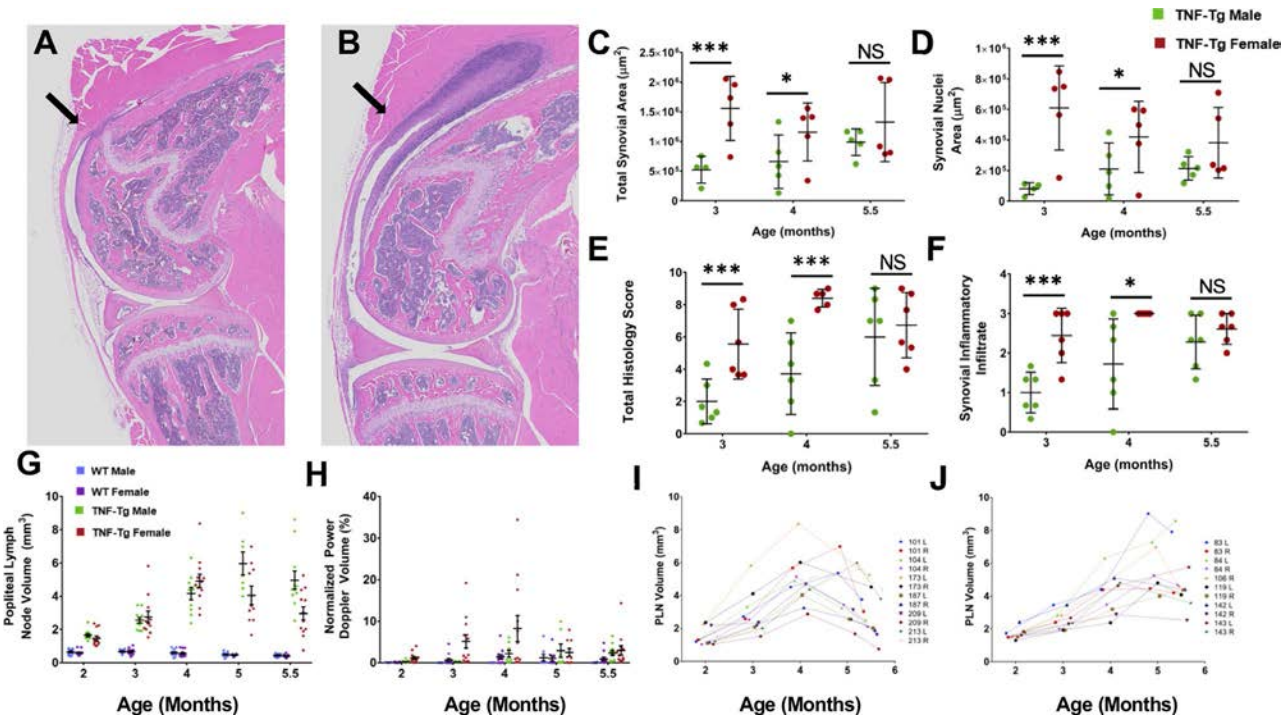

Figure 2. Earlier onset of knee arthritis and dysfunction of the joint draining lymph nodes in female Tg3647 mice. **A** and **B**, Arthritic synovial changes (indicated by the **arrow**) were assessed in the knees of male Tg3647 mice (**A**) and female Tg3647 mice (**B**) at age 3 months. **C–F**, Histomorphometric analysis of the mouse knees was performed at each cross-sectional time point (ages 3, 4, and 5.5 months) for total synovial affected area (**C**), affected synovial cellular area (**D**), total histology score from blinded scoring of the synovium for arthritic changes (**E**), and score of pannus invasion and tartrate-resistant acid phosphatase–positive area of the synovial inflammatory infiltrate (**F**). **G–J**, Popliteal lymph node (PLN) size (**G**) and PLN blood flow (determined using normalized power Doppler volume) (**H**), as biomarkers of knee arthritis progression, were assessed over time in each group, and individual PLN volume was assessed over time in female Tg3647 mice (**I**) and male Tg3647 mice (**J**). Bars show the mean ± SD (group sizes shown in Figure 1). * = $P < 0.05$; *** = $P < 0.001$. NS = not significant (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.40903/abstract.

Accelerated and more severe cardiopulmonary disease in female Tg3647 mice. Although prior reports have documented the presence of interstitial lung disease (ILD) (19,20) in Tg3647 mice, sexual dimorphism in pulmonary pathologic features has not been previously studied. We therefore performed a comprehensive assessment of the lungs and hearts of male and female Tg3647 and WT mice in longitudinal and cross-sectional cohorts. Representative images of the lung sections demonstrated dramatic differences at age 4 months between age-matched male and female mice, including increased perivascular and peribronchiolar inflammatory infiltrates as well as thickened alveolar septums and interstitial infiltrates in female

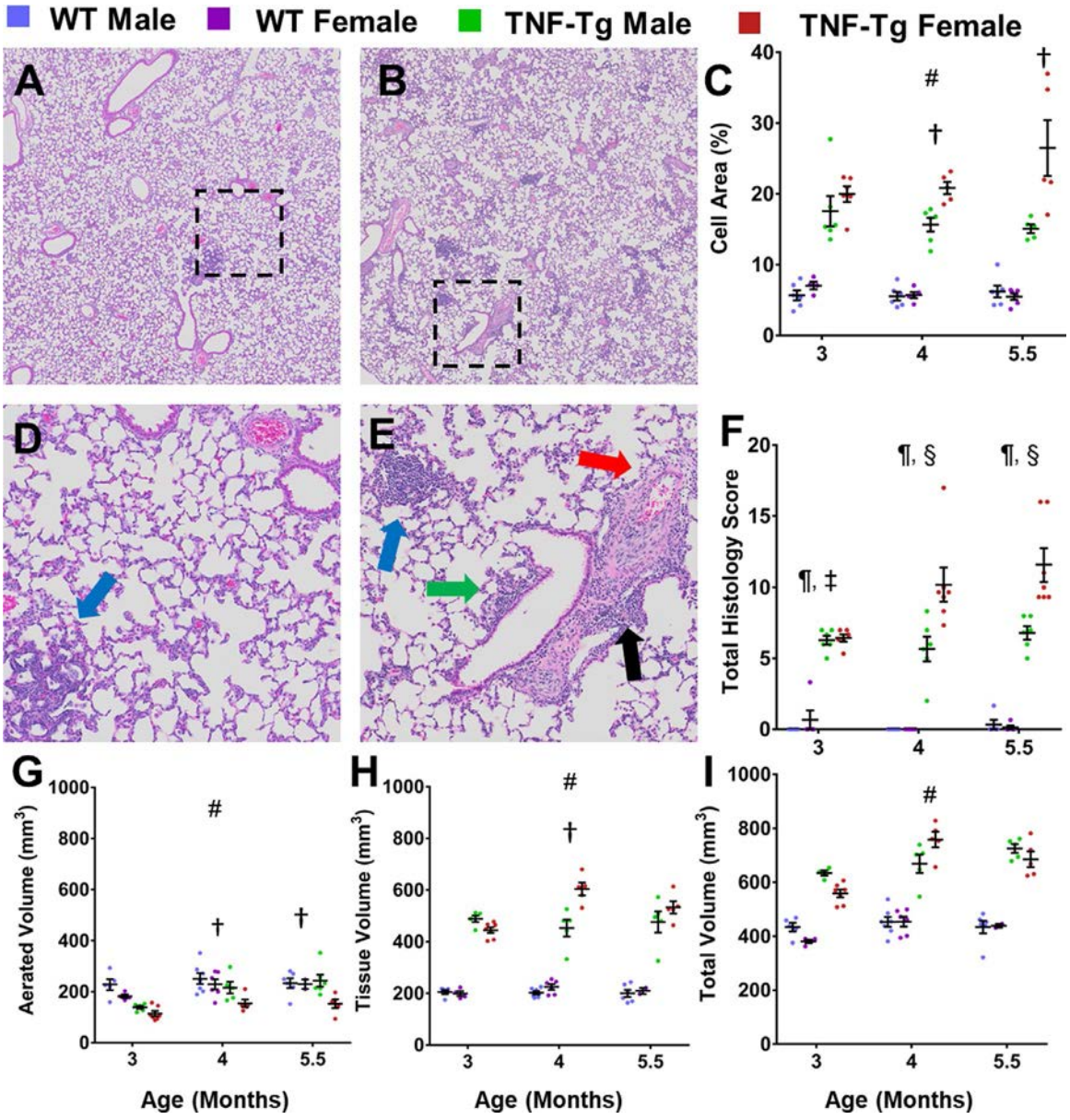

Figure 3. Increased interstitial and vascular pulmonary pathologic manifestations in female Tg3647 mice. **A**, **B, D**, and **E**, Representative micrographs of hematoxylin and eosin–stained lung tissue are shown. The lungs were assessed for pulmonary pathologic features in a 4-month-old male Tg3647 mouse (**A** and **D**) and 4-month-old female Tg3647 mouse (**B** and **E**). Panels **D** and **E** are high-magnification images of the boxed area in **A** and **B**, respectively. Original magnification ×16 in **A** and **B**; ×40 in **D** and **E**. **Arrows** in **D** and **E** show peribronchiolar (green), perivascular (black), and interstitial (blue) infiltrates, as well as dramatic arteriole thickening (red). **C**, Histomorphometric analysis was used to assess the affected nuclei area in the lungs of mice at ages 3, 4, and 5.5 months. **F**, The total histology score was assessed in a blinded manner for pathologic features in the lungs of all mice at ages 3, 4, and 5.5 months. **G–I**, Micro–computed tomography was used to assess changes in air volume (**G**), tissue volume (**H**), and total volume (**I**) in the lungs of all mice at ages 3, 4, and 5.5 months. Bars show the mean ± SD (group sizes shown in Figure 1). # = $P < 0.05$ for genotype main effect (Tg3647 versus WT at all time points); † = $P < 0.05$ for female Tg3647 versus male Tg3647; ¶ = $P < 0.05$ for male Tg3647 versus male WT; § = $P < 0.05$ for female Tg3647 versus all other groups; ‡ = $P < 0.05$ for female Tg3647 versus female WT. See Figure 1 for definitions.

mice (Figures 3A, B, D, and E and Supplementary Figures 5A–D, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract).

When histomorphometry was performed to calculate the percentage of cell area containing nuclei within the lung tissue sections, we observed a striking increase in percent cell area ($P < 0.05$) in the lung tissue of female Tg3647 mice at ages 4 and 5.5 months compared to male Tg3647 mice at these ages (Figure 3C). Blinded scoring of each lung tissue section for specific pulmonary pathologic features indicated a similar trend, in which female Tg3647 mice had significantly increased total histology scores ($P < 0.05$) compared to male Tg3647 mice at ages 4 and 5.5 months (Figure 3F). When histomorphometry subscores were investigated, the scores for the extent of peribronchiolar infiltrates (male versus female Tg3647 mice at age 4 months, mean ± SD 0.7 ± 0.3 versus 1.4 ± 0.5; at age 5.5 months, 1.0 ± 0.1 versus 2.1 ± 0.6), perivascular infiltrates (male versus female Tg3647 mice at age 4 months, mean ± SD 1.2 ± 0.4 versus 2.0 ± 0.6; at age 5.5 months, 1.2 ± 0.4 versus 2.3 ± 0.5), and arteriole thickness (male versus female Tg3647 mice at age 4 months, mean ± SD 0.9 ± 0.3 versus 2.1 ± 0.6; at age 5.5 months, 1.1 ± 0.5 versus 2.3 ± 0.4) were found to contribute the most to the

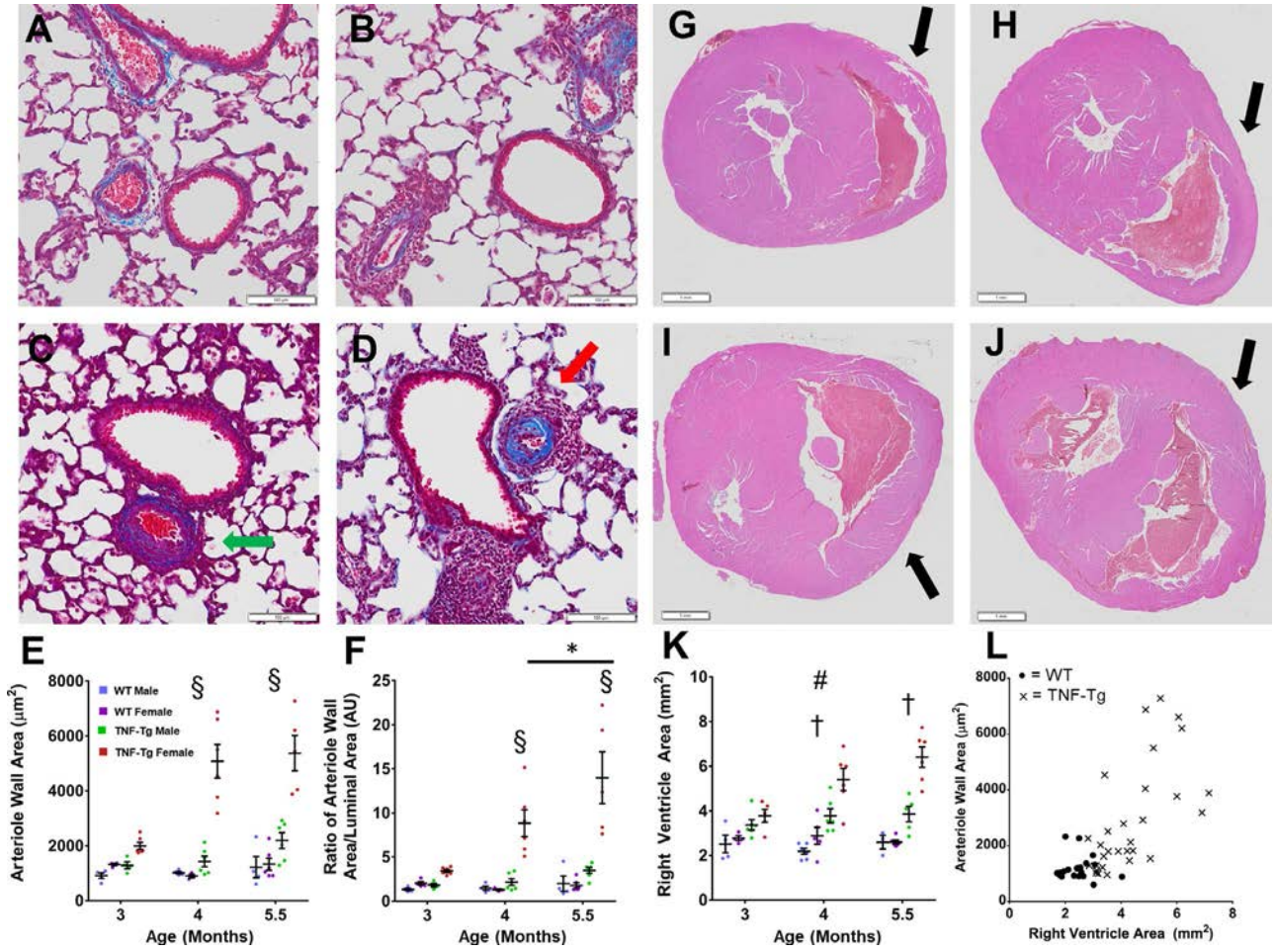

Figure 4. Significant vascular pulmonary pathologic manifestations concomitant with right ventricular enlargement in female Tg3647 mice. **A–D**, Representative Masson's trichrome–stained lung sections are shown. The lungs were assessed for arteriole thickening in a 4-month-old male Tg3647 mouse (**A**), 5.5-month-old male Tg3647 mouse (**B**), 4-month-old female Tg3647 mouse (**C**), and 5.5-month-old female Tg3647 mouse (**D**). In **C**, the **green arrow** shows pronounced arteriole thickening in a female mouse at age 4 months. In **D**, the **red arrow** shows closing off of the arterioles of a female mouse at age 5.5 months. **E**, Arteriole thickening was quantified as arteriole wall area and compared between the groups. **F**, The ratio of arteriole wall area to luminal area was compared between the groups. **G–J**, Cardiac histopathologic features were assessed in the hearts of a representative 4-month-old male Tg3647 mouse (**G**), 5.5-month-old male Tg3647 mouse (**H**), 4-month-old female Tg3647 mouse (**I**), and 5.5-month-old female Tg3647 mouse (**J**). **Arrows** indicate significant enlargement of the right ventricle in the female mice at ages 4 and 5.5 months compared to their male counterparts. **K** and **L**, The right ventricular wall area was assessed for correlation with age (**K**) and the arteriole wall area was assessed for correlation with the right ventricular wall area (**L**) using Spearman's rho correlation coefficients (Spearman's rho = 0.7, $P < 0.0001$). Bars show the mean ± SD (group sizes shown in Figure 1). § = $P < 0.05$ for female Tg3647 versus all other groups; † = $P < 0.05$ for female Tg3647 versus male Tg3647; # = $P < 0.05$ for genotype main effect (Tg3647 versus WT at all time points); * = $P < 0.05$. 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.40903/abstract.

histomorphometric differences in the lung tissue between male and female Tg3647 mice at these ages (see descriptions of the scoring systems and score scales in Supplementary Materials and Methods, and results in Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract).

In vivo micro-CT measurements of the lungs for aerated volume, tissue volume, and total volume confirmed the massive cellular burden in the lungs of female Tg3647 mice, since female Tg3647 mice exhibited a significant decrease in air volume at ages 4 and 5.5 months and a significant increase in lung tissue volume at age 4 months (each $P < 0.05$) as compared to male Tg3647 mice (Figures 3G–I) . Interestingly, interstitial fibrosis was not significantly different between the groups when scored pathologically (data not shown) nor when analyzed directly with histomorphometry (see Supplementary Figures 5E–J [http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract]). However, the increased arteriole thickness score and intense Masson's trichrome staining around the small arteries in female transgenic mice warranted further investigation.

Representative images of the arterioles of male Tg3647 mice (Figures 4A and B) and female Tg3647 mice (Figures 4C and D)

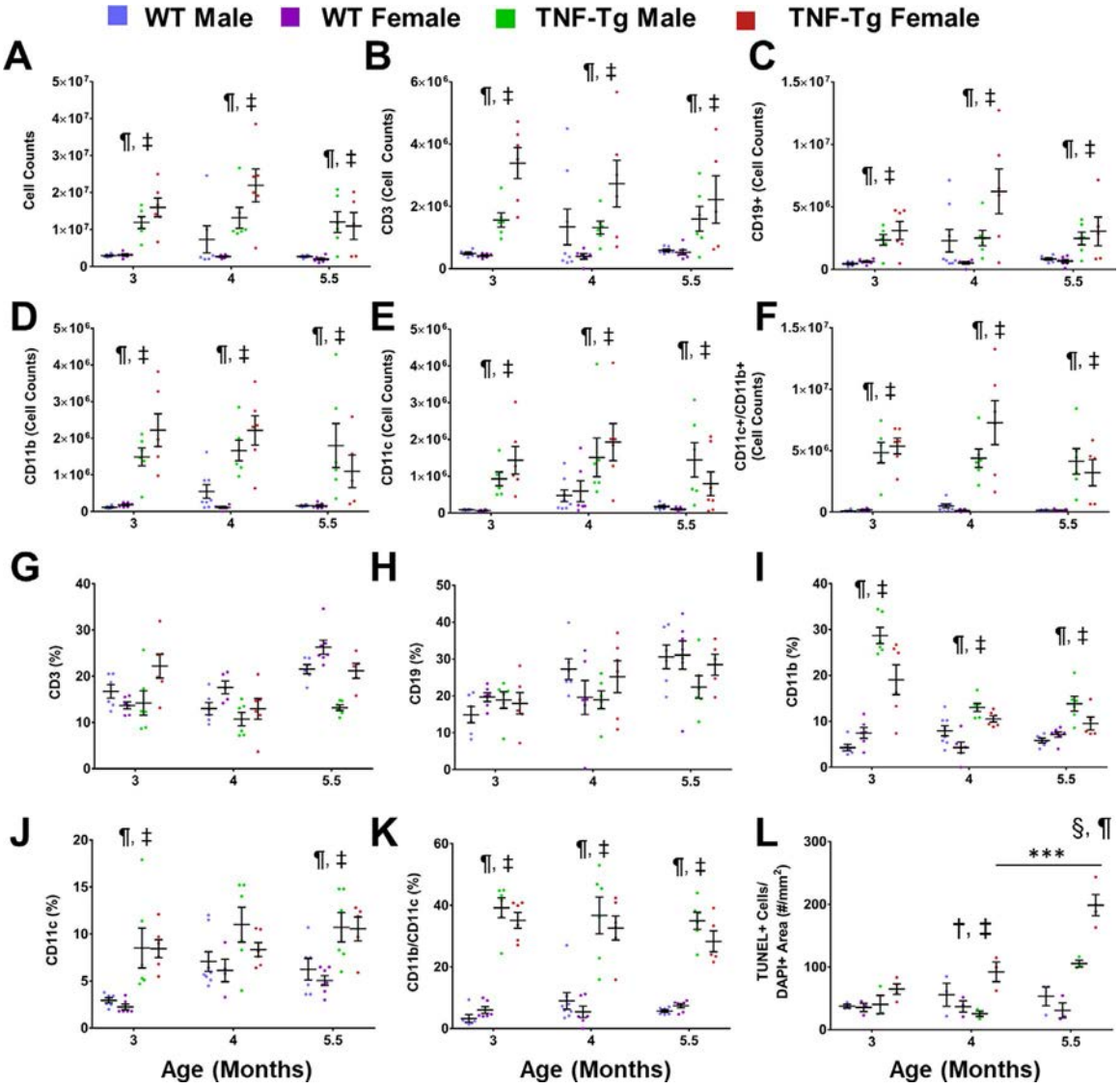

Figure 5. Increased numbers of CD11b+/CD11c+ double-positive cells in Tg3647 mice compared to their WT littermates. **A**, Total live cell counts were determined by flow cytometry in the lungs of mice at all time points. **B–F**, Specific cell counts in the lungs were done for CD3+ (**B**), CD19+ (**C**), CD11b+ (**D**), CD11c+ (**E**), and CD11c+/CD11b+ double-positive cells (**F**). **G–K**, The percentages of CD3+ (**G**), CD19+ (**H**), CD11b+ (**I**), CD11c+ (**J**), and CD11c+/CD11b+ double-positive cells (**K**) were determined in the lungs of mice. **L**, TUNEL staining was performed to determine the extent of cell death. Bars show the mean ± SD (group sizes shown in Figure 1). ¶ = $P < 0.05$ for male Tg3647 versus male WT; ‡ = $P < 0.05$ for female Tg3647 versus female WT; § = $P < 0.05$ for female Tg3647 versus all other groups; *** = $P < 0.001$. 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.40903/abstract.

stained with Masson's trichrome showed an intense blue staining as well as a thickened medial layer around the vessels (green arrow in Figure 4C) in the mice at ages 4 and 5.5 months. The arteriole wall displayed dramatic thickening in the 4-month-old and 5.5-month-old female Tg3647 mice compared to all other groups (each $P < 0.05$) (Figure 4E). No differences in vessel wall thickening among the male WT, female WT, or male Tg3647 mice was found at any age. Furthermore, the ratio of wall area to luminal area was increased in female Tg3647 mice from age 4 months to age 5.5 months ($P < 0.05$ in female Tg3647 mice at age 4 months versus at age 5.5 months), suggesting that progressive narrowing of the arterioles was occurring (Figures 4D and F).

Based on such severe pulmonary pathologic manifestations in the mouse arterioles, we next evaluated RV enlargement and found it was significantly increased in female transgenic mice compared to male transgenic mice at ages 4 months and 5.5 months and compared to WT littermates (each $P < 0.05$) (Figures 4G–K and Supplementary Figures 6A and B, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract). Furthermore, a significant correlation between lung arteriole wall area and RV area was noted in the transgenic mice (Spearman's rho = 0.70, $P < 0.0001$) (Figure 4L).

Assessment of the liver, kidneys, and spleens from 5.5-month-old male and female Tg3647 and WT mice (3 mice per sex and genotype) was performed. In agreement with the findings from the initial necropsy study of 4 female Tg3647 mice, the necropsy study of the internal organs of WT mice revealed no pathologic manifestations, whereas Tg3647 mice had severe cardiopulmonary disease with mild liver and kidney congestion, indicative of heart failure. In addition, splenomegaly was present in the transgenic mice, a finding that was consistent with prior observations in Tg3647 mice (8,9,15,21).

Increased numbers of CD11b+/CD11c+ double-positive cells in Tg3647 mouse lungs. In order to evaluate the cellular and molecular characteristics related to the histologic changes in the cardiopulmonary system, we performed flow cytometry and cytokine analyses in our cohorts of mice. Total cell numbers in the enzymatically digested lungs of transgenic mice were increased at all time points, with total cell numbers peaking at age 4 months in female Tg3647 mice (Figure 5A). The numbers of all immune cell populations studied were also increased at all time points in transgenic mice compared to their WT counterparts (Figures 5B–F). Interestingly, when the percentages of specific cell populations were calculated, the percentages of CD3+ and CD19+ cells in the lungs were not elevated in the transgenic mice (Figures 5G and H), and yet there was a dramatic increase in the percentages of CD11b+/CD11c+ double-positive cells in both male and female Tg3647 mice compared to their WT counterparts at all time points (Figures 5I–K). Notably, CD11b+ cell percentages were significantly increased at all ages in both male and female Tg3647 mice compared to WT controls (Figure 5I). CD11c+ cell percentages were increased in the transgenic mice at age 3 months, but the percentages were not different between transgenic mice and WT animals at age 4 months, and increased again in the transgenic mice at age 5.5 months (Figure 5J).

Because there was a lack of consistency between the immune cell counts and total synovial cell area measured by histomorphometry in the mice at age 5.5 months (Figure 5A versus Figure 2C), lung slides were stained with a TUNEL assay to assess cell apoptosis. The results showed that there was a significant increase in apoptosis ($P < 0.05$) in the lung tissue of female Tg3647 mice at ages 4 months and 5.5 months compared to all other groups, and also a significant increase in apoptosis ($P < 0.001$) in the female transgenic mice from age 4 months to age 5.5 months, suggesting that the lower cell counts determined by flow cytometry when compared to the total synovial cell area determined by histomorphometry could be attributed to an occurrence of substantial cell loss ex vivo.

Because the Tg3647 mouse line contains a copy of the human TNF gene as well as its own mouse TNF gene, we measured the serum levels of both human and mouse TNF in the mice (Figures 6A–J). Cytokine analysis revealed a significant increase in the levels of human TNF ($P < 0.05$) in the serum of all Tg3647 mice, at all time points, compared to WT mice (Figure 6A). The levels of interleukin-9 (IL-9) and IL-10 were each significantly increased ($P < 0.05$) only in the serum of female Tg3647 mice (Figures 6D and E), while IL-17 serum levels were significantly increased ($P < 0.05$) in both female WT and female Tg3647 mice compared to their male littermates (Figure 6F). The serum levels of interferon-γ–inducible protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), leukemia inhibitory factor (LIF), and keratinocyte chemoattractant (KC) were all significantly increased ($P < 0.05$) in Tg3647 mice of both sexes (Figures 6G–J).

DISCUSSION

RA is a systemic autoimmune disease and there are many established animal models that can be used to study the joint and extraarticular features of RA-like diseases (11), but to our knowledge, this is the first study to longitudinally characterize sexual dimorphism in a spontaneous murine model of chronic inflammatory erosive arthritis with concomitant cardiopulmonary pathologic manifestations. In female mice, the progressive cardiopulmonary disease becomes lethal in the first several months after birth, which would explain the mechanism of early mortality in female mice in this well-established model of RA. We also describe significant sexual dimorphism in muscle dysfunction, timing of onset of arthritis, timing of collapse of the joint draining lymph nodes, development and severity of ILD, and pulmonary arteriole thickening.

Extraarticular manifestations of RA, in particular lung disease, are a common occurrence. Recent estimates report that up to 10% of the RA population experience pulmonary involve-

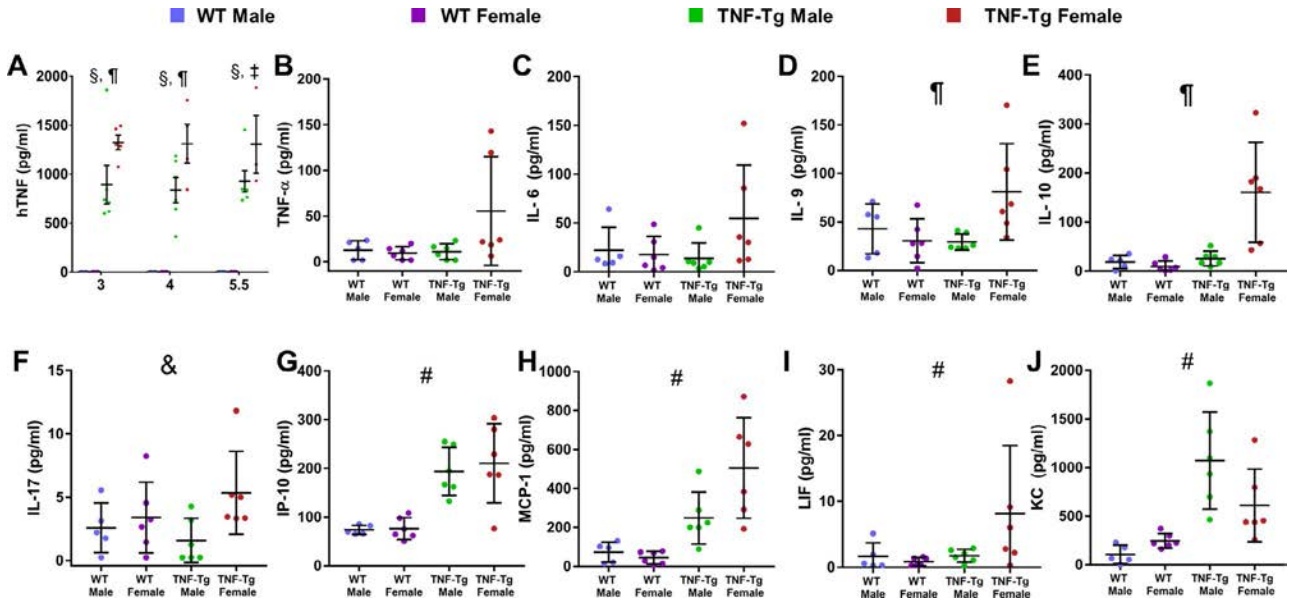

Figure 6. Increased serum levels of cytokines and chemokines in Tg3647 mice compared to their WT littermates. Serum from each group of mice at age 5.5 months was assayed and compared for the concentrations of human TNF (hTNF) (**A**), mouse TNF (**B**), interleukin-6 (IL-6) (**C**), IL-9 (**D**), IL-10 (**E**), IL-17 (**F**), interferon-γ–inducible protein 10 (IP-10) (**G**), monocyte chemoattractant protein 1 (MCP-1) (**H**), leukemia inhibitory factor (LIF) (**I**), and keratinocyte chemoattractant (KC) (**J**). Bars show the mean ± SD (group sizes shown in Figure 1). § = $P < 0.05$ for female Tg3647 versus all other groups; ¶ = $P < 0.05$ for male Tg3647 versus male WT; ‡ = $P < 0.05$ for female Tg3647 versus female WT; & = $P < 0.05$ for sex main effect (male versus female); # = $P < 0.05$ for genotype main effect (Tg3647 versus WT). 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.40903/abstract.

ment (22–24). Furthermore, patients with RA who are diagnosed as having ILD have poor survival expectancy, with some studies reporting a median survival time as low as 2.6 years after diagnosis, and growing evidence suggests an increased mortality incidence in female patients with RA (23,25). In a recent review from our group focusing on RA-associated ILD, we discussed the lack of consensus regarding the relationship between sex and morbidity and mortality in patients with RA-associated ILD (26). Whereas men with RA have historically been presumed to have an increased incidence of all-cause mortality compared to women with RA, more recent data suggest that women may be at higher risk of death from RA, in particular those with RA-associated ILD. In a large retrospective study of more than 2 million decedent records from the National Center for Health Statistics in the US, Olson et al found that in decedents with RA-associated ILD, mortality rates on average were increased in women compared to men in all age groups (23). Although the authors did not speculate on the potential etiologic mechanisms of this increase in women, we posit that the sexual dimorphism observed in the Tg3647 mouse may inform this discussion. Furthermore, a recent study showed that sexual dimorphism is present in subtypes of RA-associated ILD (i.e., nonspecific interstitial pneumonia [NSIP] versus usual interstitial pneumonia [UIP], as discussed further below), which may explain some of the incongruous findings in clinical studies of sex differences and mortality in RA-associated ILD (27).

The pathogenesis of RA-associated ILD is poorly understood, and diagnosis of lung disease is primarily based on findings from imaging tests. The predominant types of RA-associated ILD are a fibrotic type, UIP, and a cellular infiltrate type, NSIP (27). Frequently, however, both imaging and histology studies of lung biopsy tissue have shown that some patients may have characteristics of both subtypes (24), suggesting that there is a spectrum of disease rather than distinctly different types of RA-associated ILD. In our mouse model, we observed an interstitial cellular infiltrate similar to that seen in human NSIP. While the fibrotic UIP subtype is often thought to have a worse prognosis, recent evidence suggests that both types have a similar frequency of mortality (28,29).

A notable phenomenon of the phenotype in the Tg3647 mouse model is the pulmonary arteriole thickening that likely leads to RV hypertrophy (see Figure 4), as well as the increased mortality with dramatic cardiopulmonary pathologic manifestations. Clinically, RA-associated pulmonary hypertension is rare, but one of the recognized predominant causes is ILD (30), as was seen in our mouse model. Other causes include vasculitis and thromboembolic disease, neither of which was identified in our mouse model. Interestingly, development of pulmonary hypertension is much more common in other connective tissue diseases, such as systemic sclerosis, mixed connective tissue disease, and lupus (31,32). Furthermore, the role of TNF in the development of pulmonary vasculopathy has been intensely studied and has been described in animal models of arthritis induction with TNF overexpression (33,34). However, to date, there is no preclinical model to recapitulate the cellular ILD-related effects on vasculopathy and mortality outcomes. Therefore, the use of this sexually dimorphic

Tg3647 mouse model in future research studies is warranted in order to better understand the pathophysiologic processes that may be clinically relevant to connective tissue diseases.

Importantly, other investigators have seen similar sexual dimorphism in acute arthritis animal models of RA. Keith and colleagues investigated the role of testosterone in the development and progression of arthritis and lung disease in the SKG mouse model of zymosan-induced arthritis (4). They found increased incidence and severity of arthritis in female SKG mice compared to male mice injected with zymosan, and orchiectomized male SKG mice displayed a similar incidence and severity of arthritis as that in the females. Furthermore, they identified an inflammatory, but not fibrotic, lung disease in both the female SKG mice and orchiectomized male mice, but not in the male SKG mice that were only injected with zymosan. Interestingly, arthritic disease worsened in ovariectomized SKG mice, and arthritis was ameliorated in sham control animals treated with estrogen (35). Studies utilizing the collagen-induced arthritis animal model demonstrated increased incidence of arthritis in male mice after immunization, and castration of male mice exacerbated disease (36,37). When either testosterone or estrogen was exogenously delivered to immunized animals of either sex, both hormones suppressed disease (38–40). These data indicate that sex hormones can play both a protective and an accelerating role in autoimmune disease, depending on the source of inflammation and the type of response initiated by the immune system. Thus, studies modulating the sex hormones in Tg3647 mice are necessary to better understand their interactions within the immune system, the sexual dimorphism, and the multisystem pathologic features.

Contrasting effects of sex hormones on autoimmunity are also seen in RA patients. Women are significantly more likely to have RA than men, and there is a modest reduction in disease activity during pregnancy, with women who have moderate or severe disease seeing the largest effect (41). There is also a significant risk of flare postpartum. Yet, women with RA who take oral contraceptives do not show a clear reduction in the risk of RA (relative risk 0.88, 95% confidence interval 0.751–1.03) (42). Androgen replacement therapy may be effective at improving disease outcomes, especially in men and women with low testosterone levels (43,44). Overall, several factors, including serum versus tissue hormone concentrations, timing of onset, and clinical features may dictate the effect of sex hormones on arthritis.

Recently, Ntari et al described a strictly cardiovascular disease in the Tg197 line of TNF-Tg mice (45), which is a murine model of more aggressive disease (10) than that seen in the Tg3647 TNF-Tg mouse line described herein. In the Tg197 mouse line, Ntari and colleagues found left-sided aortic and mitral valve thickening and significant fibrosis, with no evidence of lung inflammation or right-sided heart disease. This effect was driven by TNF receptor signaling, which was dependent on valvular interstitial cells. In our model, however, we found no evidence of left-sided heart disease in the Tg3647 mice (see Figure 4 and Supplementary Figure 7, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract). We did observe significant collagen deposition in the interstitium of the myocardium of the RV, suggesting that TNF signaling may be conserved in both processes (see Supplementary Figures 6C–F [http://onlinelibrary.wiley.com/doi/10.1002/art.40903/abstract]). These differences between the 2 prominent TNF-Tg mouse lines are likely due to the number of transgenes inserted, the age of onset of disease manifestations, and the relative amounts of circulating human TNF. As mentioned earlier, the Tg197 mouse has an earlier onset of arthritis and cardiovascular disease than the Tg3647 mouse, and no sexual dimorphism has been reported (8,9). In addition, pulmonary vasculopathy is primarily restricted to female mice in the Tg3647 line, suggesting that TNF may be interacting significantly with female hormones, whereas the Tg197 mouse develops disease before sexual maturity. This combination of factors could likely account for the differences in pathologic manifestations between the 2 primary TNF-Tg mouse models.

Interestingly, when we performed flow cytometry on the lungs of Tg3647 mice, CD11b+/CD11c+ double-positive cells were significantly increased, both in total numbers and by percentage, in the male and female mice at all time points (Figure 5). This cell population is likely to originate from dendritic cells (46), which has been suggested to play a key role in the immunopathologic development of lung disease (47), and is likely the major component of the interstitial inflammatory infiltrate into the lung. Furthermore, ectopic lymphoid follicles were seen in histologic analyses (see Supplementary Figure 6), and total B cell numbers were increased, as demonstrated by flow cytometry. The presence of ectopic lymphoid tissue in our transgenic mice is similar to the inducible bronchus-associated lymphoid tissue (iBALT) seen in patients with pulmonary complications of RA and Sjögren's syndrome (48). Consistent with the idea that iBALT supports local production of autoantibodies, antibodies against vimentin and H2B detected in the serum of Tg3647 mice may be produced in the lung (49).

A limitation of our flow cytometry data was that the total cell counts in female Tg3647 mouse lung tissue did not correlate with the histologic cell counts (Figure 5A versus Figure 2C). One explanation is that the cells in these lungs were either primed to undergo programmed cell death or were currently undergoing apoptosis. To confirm this hypothesis, we stained lung tissue from all cohorts with a TUNEL assay and found a dramatic increase in TUNEL+ cells (~2-fold increase) in the female Tg3647 mice from age 4 months to age 5.5 months. This critical and normal process observed in both inflammation and wound healing was likely limiting the number of live cells isolated and processed for flow cytometry at the later stages of disease (50).

Analysis of serum cytokine levels in the Tg3647 mice revealed that the serum levels of IL-9 and IL-10 were increased in female Tg3647 mice, while IP-10, MCP-1, LIF, and KC serum levels were increased in Tg3647 mice regardless of sex (Figure 6). IL-9 is significantly increased in the serum of patients with intersti-

tial lung inflammation associated with connective tissue disorders and is known to enhance recruitment of B cells while reducing the incidence of interstitial fibrosis (51,52). Thus, it is feasible that IL-9 may be playing a role in the lack of interstitial fibrosis development in this TNF-Tg mouse model. IL-10 is a known antiinflammatory cytokine and is produced to dampen the immune response in inflammatory settings. Therefore, increased IL-10 levels are likely a compensatory mechanism to counter the severe inflammation noted at age 5.5 months in female Tg3647 mice. IP-10 (CXCL10), MCP-1, and KC (CXCL1) are all potent chemoattractants for leukocytes; consistent with this, we found a dramatic abundance of leukocytes in the interstitium of all Tg3647 mice. In summary, serum cytokine analysis complements the histopathology and flow cytometry results in describing lymphoid and myeloid cell migration and proliferation, with activated compensatory antiinflammatory mechanisms, in the Tg3647 mice.

In conclusion, herein we longitudinally phenotyped the specific sexual dimorphisms in pathologic features within the musculoskeletal and cardiopulmonary systems in Tg3647 TNF-Tg mice. We found that the early mortality in female mice could be attributed to heart failure secondary to severe pulmonary vasculopathy. This mouse model, most commonly studied for its inflammatory erosive arthritis, contains additional musculoskeletal and cardiopulmonary pathologic manifestations that can be studied to better understand the role of sex in TNF-mediated diseases, and specifically to assess the effects of sexual dimorphism on these affected systems. Elucidating these mechanisms may have broad impact on the extraarticular pathologic features that develop in RA, systemic sclerosis, and other connective tissue diseases.

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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. Rahimi 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. Bell, Wood, Ritchlin, Schwarz, Rahimi.

Acquisition of data. Bell, Wu, Rudmann, Forney, Chakkalakal, Paris, Klose, Xiao, Rangel-Moreno, Garcia-Hernandez, Rahimi.

Analysis and interpretation of data. Bell, Wu, Kaiser, Wood, Chakkalakal, Paris, Klose, Xiao, Rangel-Moreno, Garcia-Hernandez.

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Randomized, Double-Blind, Placebo-Controlled Trial of Intraarticular Trans-Capsaicin for Pain Associated With Osteoarthritis of the Knee

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Objective. To assess the efficacy and safety of high-purity synthetic trans-capsaicin (CNTX-4975) in patients with chronic moderate-to-severe osteoarthritis (OA)–associated knee pain.

Methods. In this phase II multicenter double-blind study, patients ages 45–80 years who had stable knee OA were randomized in a 2:1:2 ratio to receive a single intraarticular injection of placebo, CNTX-4975 0.5 mg, or CNTX-4975 1.0 mg. The primary efficacy end point was area under the curve (AUC) for change from baseline in daily Western Ontario and McMaster Universities Osteoarthritis Index pain with walking score (range 0–10, 0 = none and 10 = extreme) through week 12. Secondary efficacy end points included a similar AUC analysis of outcomes in patients treated with CNTX-4975 0.5 mg, and evaluations extending to 24 weeks.

Results. Efficacy was evaluated in 172 patients (placebo group, n = 69; CNTX-4975 0.5 mg group, n = 33; CNTX-4975 1.0 mg group, n = 70). At week 12, greater decreases in the AUC for the pain score were observed with CNTX-4975 in the 0.5 mg and 1.0 mg groups versus placebo (0.5 mg group least squares mean difference [LSMD] –0.79, $P = 0.0740$; 1.0 mg group LSMD –1.6, $P < 0.0001$). Significant improvements were maintained at week 24 in the 1.0 mg group (LSMD –1.4, $P = 0.0002$). Treatment-emergent adverse events were similar in the placebo and CNTX-4975 1.0 mg groups.

Conclusion. In this study, CNTX-4975 provided dose-dependent improvement in knee OA–associated pain. CNTX-4975 1.0 mg produced a significant decrease in OA knee pain through 24 weeks; CNTX-4975 0.5 mg significantly improved pain at 12 weeks, but the effect was not evident at 24 weeks.

INTRODUCTION

Knee osteoarthritis (OA) affects >10% of individuals ages 60 years and older, and current treatment options for pain control are considered inadequate (1,2). Management includes nonpharmacologic and pharmacologic options, many of which have at least short-term benefits (3,4). Intraarticular therapies, including injections of viscosupplements and glucocorticoids, may have limited efficacy (3–6). In a randomized clinical trial, intraarticular injection of glucocorticoids every 12 weeks for 2 years was associated with significantly greater loss of cartilage volume compared to placebo, with no significant difference in knee pain (6). Whether the loss of cartilage volume has clinical significance is unknown. Pharmacologic treatments have been recommended for properly selected patients, but they carry risks of adverse effects involving the gastrointestinal, cardiovascular, renal, and central nervous systems (3). The presence of comorbidities may make individuals with knee OA more susceptible to these adverse effects, thus limiting treatment options (3,7). A total knee joint replacement often provides longer-term benefits. However, this surgery entails serious risks, and many patients continue to have pain and disability following surgery (7,8). In addition, many patients are not candidates for major surgery (8). Therefore, there is an unmet need for effective therapies to mitigate risks and provide effective pain management.

Capsaicin, the pungent ingredient in chili peppers, is a potent agonist for the transient receptor potential cation channel

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subfamily V member 1 (TRPV1) (9). TRPV1 is a nonspecific cation channel that opens with exposure to heat, acid, and certain fatty acids (10). Within the peripheral nervous system, this channel is selectively expressed on the terminals of nociceptors (pain sensory fibers). After a brief period of activation, capsaicin induces a long-term desensitization of nociceptors related to calcium influx into the nociceptive nerve terminals (Aδ and C fibers). This desensitization is likely due to a reversible retraction of innervation (9,11). Based on studies of the skin, it is known that the nociceptors grow back during a period of weeks to months (12,13). In the meantime, there is a profound attenuation of pain sensibility but not of other sensory functions (11). A topical formulation of capsaicin has been approved by the US Food and Drug Administration for the treatment of postherpetic neuralgia (14,15).

In this study, the strategy was to take advantage of the selective long-term analgesic effects of capsaicin to address the moderate-to-severe pain associated with OA of the knee. An injectable form of highly purified trans-capsaicin, CNTX-4975, was developed using proprietary technology. A single intraarticular injection of CNTX-4975 was expected to provide rapid-onset long-term analgesia, with a duration of effect commensurate with the time required for the nociceptors to regenerate. Because trans-capsaicin at a concentration needed to affect the nociceptors is confined to the joint, the effects were expected to be restricted to within the joint. The elimination half-life of CNTX-4975 is <4 hours (data on file; Centrexion Therapeutics Corp.), which establishes a favorable ratio of pharmacokinetic and pharmacodynamic properties, namely, a brief systemic exposure with the prospect of long-term clinical benefit.

We report findings from the TRIUMPH study, a phase IIb randomized, double-blind, placebo-controlled, dose-ranging trial (ClinicalTrials.gov identifier: NCT02558439) designed to evaluate the efficacy and safety of a single intraarticular injection of CNTX-4975 for up to 24 weeks in patients with chronic, stable, moderate-to-severe OA knee pain in whom previous treatment was not successful.

PATIENTS AND METHODS

Patient characteristics. Patients were enrolled between August 2015 and April 2016 at 22 sites in the US. Eligible patients were adults ages 45–80 years who had a body mass index (BMI) of ≤45 kg/m^2, radiographic evidence of chronic OA (Kellgren/Lawrence [K/L] grade 2–4) (16) in the index knee, moderate-to-severe pain in the index knee that was stable for ≥2 months prior to screening, and a mean pain score of 5–9 (range 0–10, 0 = none and 10 = extreme) at screening and baseline (day 1) according to the question in the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) (17) that addresses pain with walking. The mean pain score in the contralateral knee had to be ≤3.

Additionally, patients must have had an inadequate response, an adverse event resulting in discontinuation of prior treatment, or an absolute or relative contraindication (based on product labeling) to what would otherwise be standard-of-care treatment(s). Prior standard-of-care may have included ≥1 of the following: systemic nonsteroidal antiinflammatory drugs (NSAIDs) (oral, rectal, or injection), opioid analgesics (oral or transdermal), intraarticular glucocorticoid, or intraarticular hyaluronic acid.

Exclusion criteria included pain in the index knee from a joint disease other than OA; pain in the nonindex knee rated at >3 according to the WOMAC pain with walking score; topical capsaicin, glucocorticoid injection, or intraarticular viscosupplementation in the index knee within 90 days of screening; joint replacement surgery at any time or open surgery on the index knee during the preceding 12 months; arthroscopic surgery on the index knee within 3 months of screening; non-OA chronic pain that required use of analgesic medications (e.g., pregabalin, duloxetine); current use of opioids for any condition other than OA of the index knee (maximum dose of 15 mg/day of hydrocodone [or equivalent]); secondary OA of the knee due to traumatic injury; significant current or past instability (e.g., cruciate ligament tear or rupture or previous repair) or misalignment (>10 degrees varus or valgus) of the index knee; documented history of neuropathic arthropathy or finding of bony fragmentation in the index knee with imaging; regular use of anticoagulant blood thinners (except low-dose aspirin or clopidogrel); or ulcer or open wound anywhere on the index knee.

Study design. Patients were randomly assigned in a 2:1:2 ratio to 1 of 3 treatment groups (placebo, CNTX-4975 0.5 mg, or CNTX-4975 1.0 mg) and stratified for balance across treatment groups by K/L grade (2–3 and 4 [≤10% had grade 4]) and BMI (<30 kg/m^2 and ≥30 kg/m^2). Randomization schedules were computer-generated using a permuted block algorithm that randomly allocated study drug to the randomization numbers. The numbers generated were assigned sequentially using a central interactive response system as patients entered the study. No one involved in study conduct had access to the randomization schedule before official unblinding of assignments. A central reader assessed all radiographs at baseline to determine the K/L grade. All patients, investigators, and study personnel involved in the conduct of the study (including data management personnel and the sponsor) were blinded with regard to treatment assignment, except for a randomization statistician and programmer from the contract research organization who had access to randomization code, a pharmacist who prepared study drug and provided a labeled syringe of masked study drug product for administration, and a pharmacy clinical research associate.

This study consisted of a screening period, a single treatment day (day 1), and a 24-week follow-up period. At the investigators' discretion, patients could be premedicated using an opioid, NSAID, or local anesthetic (e.g., ethyl chloride, topical or subcutaneous lidocaine), with a maximum of 2 premedications. After 15 minutes of joint cooling with a wrap placed around the knee,

patients received 15 ml of intraarticular 2% lidocaine, without epinephrine, for the purpose of (in order of importance): 1) achieving the targeted concentration of capsaicin, 2) improving distribution of capsaicin within the joint, and 3) decreasing the initial pain associated with injection. Cooling was reapplied for 30 minutes and then the study drug was provided in a vehicle consisting of polyethylene glycol 300, which was diluted to 30% (volume/volume) at the point of care with sterile water for injection. A single intraarticular injection (4 ml) of placebo (vehicle control), CNTX-4975 0.5 mg, or CNTX-4975 1.0 mg was administered. The CNTX-4975 and placebo injections were identical in appearance and viscosity. Injection into the joint was confirmed by ultrasound and/or joint fluid aspiration. Cooling was removed for injection and then reapplied immediately for 30 minutes–1 hour. Patients were advised not to take a hot bath or shower or to expose the injected knee to external heat within 24 hours after the injection.

Throughout the study, patients were permitted to take oral rescue medications (see Supplementary Table 1, on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40894/abstract) for OA pain in the index knee. Rescue medication was not permitted within 12 hours preceding any planned posttreatment study visit. Use of topical medication for OA knee pain during the trial was not permitted. Physical therapy was not permitted within 30 days prior to screening and throughout the study.

Patients used an interactive web-based response system to record index knee pain felt with walking during the previous 24 hours. Patients rated their pain daily from baseline to week 12 and weekly from week 12 to 24. In-clinic assessments were conducted at weeks 4, 8, 12, 16, and 24, and telephone assessments were conducted on day 3 and at weeks 14, 18, and 22.

This study was conducted in accordance with the principles of the Declaration of Helsinki, the International Conference on Harmonisation Guidelines for Good Clinical Practice, and applicable regulations of the country in which the study was conducted. The protocol was approved by the institutional review board (IRB) at each academic center, or a central IRB (Sterling IRB, Atlanta, GA) at nonacademic sites that were able to have a central review, prior to study initiation. Written informed consent was provided at the screening visit, before study-related procedures were initiated.

Efficacy and safety evaluations. The primary efficacy end point was area under the curve (AUC) for the change from baseline through week 12 in daily WOMAC pain with walking scores in patients treated with CNTX-4975 1.0 mg versus placebo. Secondary efficacy end points included a similar AUC analysis of scores in patients treated with CNTX-4975 0.5 mg and an evaluation of 24-week outcomes. Time points for the primary and secondary efficacy variables were changed from week 4 to week 12 in a protocol amendment to better address the study objectives using data collected for a longer period of time. Week 12 was selected because it is considered to be a criterion for considering whether a therapy addresses "chronic" pain (18). This amendment was made prior to database lock and unmasking of the data.

Prespecified exploratory efficacy analyses of both doses of CNTX-4975 versus placebo were performed to ascertain the mean changes from baseline in WOMAC scores addressing pain with walking (range 0–10), knee stiffness (range 0–20), and physical function (range 0–170) at each visit through week 24, and to assess the frequency of use of rescue medication for the index knee pain throughout the study period. Additional analyses included the Patient Global Impression of Change (PGIC) (7-point scale ranging from very much improved to very much worse) (19) at each postinjection visit, and an adapted Patient-Specific Functional Scale (PSFS) to assess functional activity of the index knee (range 0–10, 0 = able to perform activity and 10 = unable to perform the activity at the same level as before injury or problem) (20).

Safety assessments included monitoring for treatment-emergent adverse events (TEAEs), serious adverse events, and laboratory abnormalities. Procedural pain ratings (range 0–4, 0 = none and 4 = severe) were obtained at different intervals up to 2 hours postinjection of study drug. The number needed to treat (NNT), defined as the average number of patients treated to prevent 1 unfavorable outcome, and the number needed to harm (NNH), defined as the number of patients treated before 1 patient has an adverse event beyond what would occur with placebo, were assessed at 12 and 24 weeks.

Statistical analysis. For an effect size of 0.45, a sample size of 157 evaluable patients (63 each in the placebo and CNTX-4975 1.0 mg groups and 31 in the CNTX-4975 0.5 mg group) was needed to achieve 80% statistical power for a significant dose–placebo comparison using a 2-sided test at the 10% significance level (prespecified alpha level, $P \leq 0.10$). Assuming a 10% dropout rate, the initial planned enrollment was 173 patients, with 69 each in the placebo and CNTX-4975 1.0 mg groups and 35 in the CNTX-4975 0.5 mg group. For consistency with the method of sample size estimation and the study's power to detect a statistical difference in the primary end point, all analyses were performed using a prespecified alpha level of 0.10, with corresponding 90% confidence intervals (90% CIs).

Demographics, baseline characteristics, and safety end points were analyzed in the safety population, which included all patients who received any study medication. All efficacy end points were analyzed in the modified intent-to-treat population, which included all randomized patients who had ≥1 postbaseline efficacy assessment.

Primary and secondary efficacy end points were analyzed by analysis of covariance, with treatment as the main effect and with sex, pooled site, baseline K/L grade, baseline BMI, and baseline WOMAC knee pain with walking score as covariates. AUCs for pain rating values were converted to the 0–10 pain rating scale. The AUC was calculated using a time-weighted average standardized by length of time in the study for each patient through

week 12 or 24, depending on the end point. Standardization was performed by dividing a patient's total AUC by their time in the study, which allowed comparison of average daily pain for both completers and noncompleters to avoid attributing a low AUC value to patients who discontinued the study early. This method was also used to calculate a rescue-adjusted AUC for daily WOMAC pain with walking scores, removing scores from days when rescue medication was used. In the event of missing pain scores, the difference in time was considered in the calculation. If there were days missing in a study week, the calculated average for that study week included only nonmissing values; if no values were recorded for the study week, the average weekly WOMAC score for that study week was recorded as missing.

Exploratory efficacy end points of mean changes from baseline in WOMAC scores (for pain with walking, knee stiffness, and physical function) and PSFS scores were analyzed using a mixed model for repeated measures (MMRM). The MMRM included the same covariates as the primary analysis model. Study week and treatment by study week interaction were included as categorical variables. An unstructured within-patient covariance matrix was used. Least squares mean difference (LSMD) and 90% CIs were provided for each study week by treatment group. This analysis included all available data on patients who completed the study and those who discontinued early. In this analysis population, loss to follow-up was minimal, as few patients in each treatment group discontinued the study early and none discontinued because of an adverse event.

A responder analysis was performed for the PGIC, in which patients with significant clinical improvement (very much improved or much improved) in the index knee were compared to patients in all other categories. Proportions were compared between each CNTX-4975 treatment group and the placebo group using Pearson's chi-square test or Fisher's exact test. All analyses were performed using SAS version 9.3 or later. For safety assessments, no formal inferential statistical analyses were performed.

RESULTS

Patients. A total of 175 eligible patients were enrolled and included in the safety population (placebo, n = 70; CNTX-4975 0.5 mg, n = 34; CNTX-4975 1.0 mg, n = 71) (Figure 1). All patients had radiographic evidence of knee OA (K/L grade 2–4,

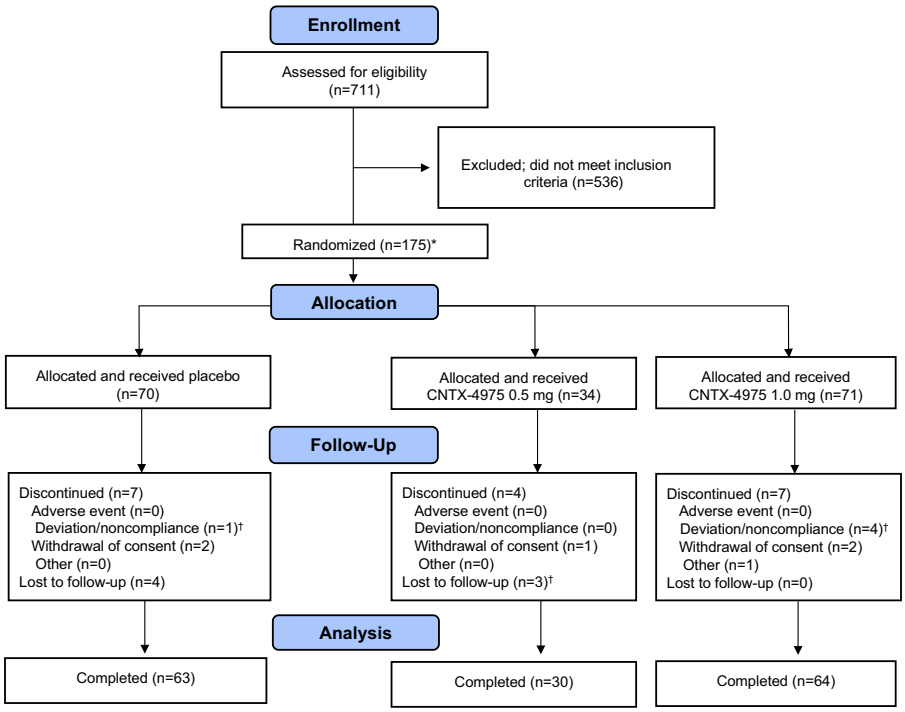

Figure 1. Disposition of the study patients. Reasons for exclusion at screening included Kellgren/Lawrence grade outside of range 2–4 (320 patients [60%]); inability to understand and follow study requirements, including diary entry via computer (64 [12%]); failure to meet the requirement for moderate-to-severe pain (29 [5%]); history of allergic reaction to the planned local anesthesia regimens, polyethylene glycol, or capsaicin (19 [3%]); baseline and screening scores outside of a 5–9 range on the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) pain with walking assessment (12 [2%]); >2-point difference in WOMAC pain with walking score between screening and baseline (11 [2%]); prior participation in an ALGRX4975 or CNTX-4975 study (10 [2%]); and positive urine drug screen or active/past substance use disorder within prior year (10 [2%]). Other inclusion/exclusion criteria each contributed ≤1% to exclusions at screening. * = Number of patients in the safety analysis. † Three patients were excluded from the efficacy analysis (modified intent-to-treat population, n = 172). One patient was excluded (prior to unblinding) due to deviation/noncompliance, as this patient was injected at 2 different study sites (CNTX-4975 1.0 mg, n = 1; placebo, n = 1). A third patient was lost to follow-up in the CNTX-4975 0.5 mg group.

as determined by a central reader [radiologist]). Three patients were excluded from the efficacy analysis prior to unblinding: 1 patient entered the study at 2 centers, received 2 injections, and was initially counted as 2 separate patients, and 1 patient received study medication but left the study site and could not be contacted. Thus, 172 patients were included in the modified intent-to-treat population (placebo, n = 69; CNTX-4975 0.5 mg, n = 33; CNTX-4975 1.0 mg, n = 70). A total of 157 patients (90%) completed the study (Figure 1). Demographics and baseline disease characteristics are summarized in Table 1.

Efficacy. In the placebo group, the CNTX-4975 0.5 mg group, and the CNTX-4975 1.0 mg group, the mean baseline scores for pain with walking were 7.4, 7.2, and 7.2, respectively. In the primary AUC efficacy analysis, the reduction in pain scores from baseline through week 12 was significantly greater in the CNTX-4975 1.0 mg group compared to placebo (LSMD –1.6 [90% CI –2.2, –1.0], $P < 0.0001$; mean ± SD change –4.1 ± 2.1 versus –2.6 ± 2.2) (Figure 2). Based on the primary end point and the pooled SD, the Cohen's *d* standardized effect was calculated as 0.68. A smaller but significant improvement versus placebo was observed with the 0.5 mg dose (LSMD –0.8 [90% CI –1.5, –0.06], $P = 0.07$; mean ± SD change –3.3 ± 2.1). The AUC for change from baseline through week 24 (same efficacy measure as week 12) showed significant improvements with the CNTX-4975 1.0 mg dose versus placebo (LSMD –1.4 [90% CI –1.9, –0.77], $P < 0.001$; mean ± SD change –3.9 ± 2.2 versus –2.7 ± 2.2), but not with the CNTX-4975 0.5 mg dose (LSMD –0.6 [90% CI –1.3, 0.15], $P = 0.19$; mean ± SD change –3.2 ± 1.9) (Figure 2).

In the analysis of the primary end point adjusted for use of rescue medications, the reduction in rescue-adjusted WOMAC pain with walking scores from baseline through week 12 was significantly greater with CNTX-4975 1.0 mg versus placebo (LSMD –0.9 [90% CI –1.5, –0.3], $P = 0.01$; mean ± SD change –2.75 ± 2.61 versus –1.95 ± 2.16), consistent with results for the primary end point. More rescue medication was taken in the placebo group and the CNTX-4975 0.5 mg group than in the CNTX-4975 1.0 mg group. For patients who took acetaminophen, the mean per patient total dose during the 12 weeks was 21,006 mg in the placebo group (n = 50) compared to 13,392 mg in the CNTX-4975 1.0 mg group (n = 47). The most commonly taken NSAID was ibuprofen. The mean per patient total ibuprofen dose was greater in the placebo group (9,403 mg; n = 18) than in the CNTX-4975 1.0 mg group (7,446 mg; n = 13).

In the MMRM analysis, significant improvements in the WOMAC pain with walking score with CNTX-4975 0.5 mg were demonstrated, compared to placebo, at week 12 (LSMD –0.9 [90% CI –1.7, –0.03], $P = 0.09$; mean ± SD change –3.8

Table 1. Demographics and baseline disease characteristics*

	Placebo (n = 70)	CNTX-4975 0.5 mg (n = 34)	CNTX-4975 1.0 mg (n = 71)	Total (n = 175)
Age, mean ± SD years	61 ± 9	60 ± 6	59 ± 8	60 ± 8
Female	64	59	63	63
BMI				
<30 kg/m^2	33	38	30	33
≥30 kg/m^2	67	62	70	67
Index knee				
Right	46	38	51	46
Left	54	62	49	54
K/L grade (index knee)†‡				
2	36	27	45	38
3	53	65	47	53
4	11	9	9	10
WOMAC pain with walking score§				
Moderate (>4–7)	34	38	47	40
Severe (>7–10)	63	59	54	58
Missing¶	3	3	0	2

* Except where indicated otherwise, values are the percent of patients. BMI = body mass index; K/L = Kellgren/Lawrence; WOMAC = Western Ontario and McMaster Universities Osteoarthritis Index.
† Range 0 (no radiographic features of osteoarthritis are present) to 4 (large osteophytes, marked joint space narrowing, severe sclerosis, and definite bony deformity).
‡ Chi-square test indicated no association ($P = 0.4007$) between treatment and baseline severity.
§ Range 0 (none) to 10 (extreme).
¶ Patients did not have 7 days of response data from baseline to randomization. Calculated baseline value required 7 of 14 days of diary data to calculate baseline pain with walking on a flat surface but did not require a diary entry at baseline.

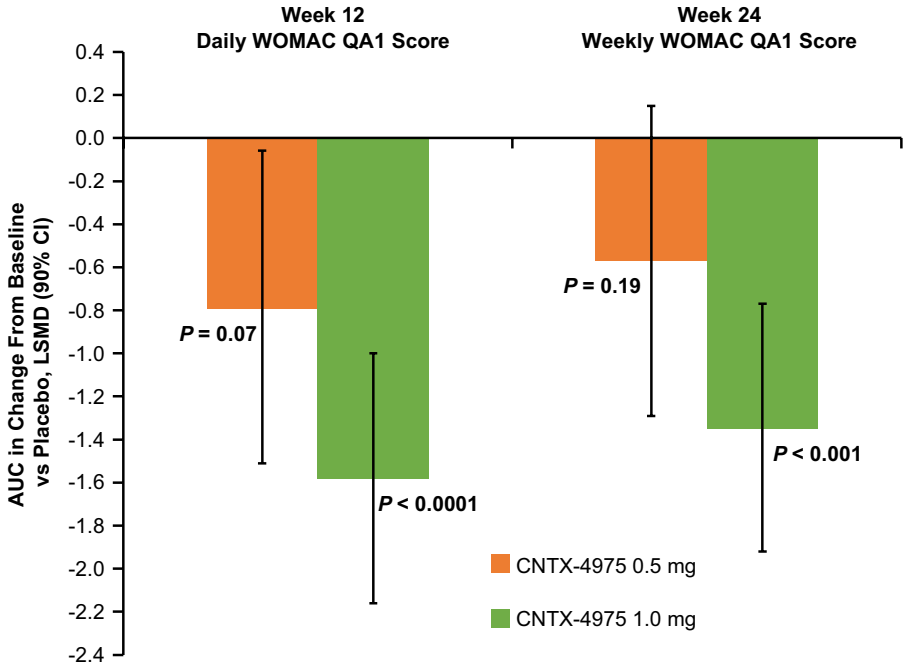

Figure 2. Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) pain with walking on a flat surface (QA1) scores. Standardized area under the curve (AUC), normalized to the 0–10 rating scale, for change from baseline with CNTX-4975 versus placebo in daily pain with walking scores through week 12 and in average weekly pain with walking scores through week 24 were evaluated. Analysis of covariance was performed in the modified intent-to-treat population. LSMD = least squares mean difference; 90% CI = 90% confidence interval.

± 2.5 versus –3.0 ± 2.5), but not at week 24 (LSMD –0.5 [90% CI –1.5, 0.5], $P = 0.41$; mean ± SD change –3.6 ± 2.0 versus –3.0 ± 2.8). At the CNTX-4975 1.0 mg dose, significant divergence from placebo was evident at week 12 (LSMD –1.5 [90% CI –2.2, –0.8], $P < 0.001$; mean ± SD change –4.4 ± 2.6 versus –3.0 ± 2.5) and at week 24 (LSMD –0.9 [90% CI –1.6, –0.1], $P = 0.07$; mean ± SD change –3.3 ± 2.6 versus –3.0 ± 2.8) (Table 2). A significant improvement was evident with the 1.0 mg dose as early as 1 week after treatment (Figure 3). The 1.0 mg dose was associated with significantly improved WOMAC knee stiffness scores (LSMD –2.5 [90% CI –3.8, –1.2], $P = 0.001$; mean ± SD change –6.7 ± 5.2 versus –4.8 ± 6.6) and knee function scores (LSMD –18.3 [90% CI –28.6, –7.9], $P = 0.004$; mean ± SD change –59.3 ± 39.8 versus –46.2 ± 46.0) versus placebo at week 12 (MMRM) (Table 2 and Supplementary Figures 1 and 2, http://onlinelibrary.wiley.com/doi/10.1002/art.40894/abstract). Numerical improvements in the CNTX-4975 1.0 mg group versus placebo were observed at week 24 for knee stiffness (LSMD –1.2 [90% CI –2.5, 0.1], $P = 0.14$; mean ± SD change –5.7 ± 5.5 versus –5.1 ± 6.2) and for knee function (LSMD –7.2 [90% CI –18.3, 3.8], $P = 0.28$; mean ± SD change –51.6 ± 44.8 versus –49.4 ± 49.2). The improvements in scores for these WOMAC questions at week 12 with CNTX-4975 0.5 mg versus placebo were not significant.

Based on the PGIC responder analysis, >50% of patients treated with either dose of CNTX-4975 reported significant improvement (much improved or very much improved) in the index knee at each follow-up visit. At no point did >50% of patients treated with placebo report comparable improvement. Improvements at weeks 4, 8, 12, and 16 in patients receiving the 1.0 mg dose were statistically significant at the prespecified alpha level of ≤0.1 versus placebo; improvements at 24 weeks did not reach statistical significance (Supplementary Table 2, http://onlinelibrary.wiley.com/doi/10.1002/art.40894/abstract). At weeks 12 and 16, patients achieved significant improvement with CNTX-4975 0.5 mg versus placebo ($P < 0.10$).

On the PSFS, functional activity of the index knee was significantly improved with CNTX-4975 1.0 mg versus placebo at each follow-up visit from week 4 through week 16 ($P < 0.10$ at each time point) (Supplementary Table 3, http://onlinelibrary.wiley.com/doi/10.1002/art.40894/abstract). Changes in PSFS score were not significantly different between CNTX-4975 0.5 mg and placebo treatment at any time point.

The NNT to determine ≥50% pain improvement was calculated using data from the CNTX-4975 1.0 mg and placebo groups in the modified intent-to-treat population. The NNT at weeks 12 and 24 was 3.6 and 10.3 patients, respectively.

Safety. Ten patients (all at 1 site) were premedicated with ibuprofen prior to injection of the study drug. No other premedications were used. TEAEs were reported by 30%, 47%, and 30% of patients in the placebo, CNTX-4975 0.5 mg, and CNTX-4975 1.0 mg groups, respectively, and were generally

Table 2. Mean change from baseline in weekly average WOMAC scores at weeks 12 and 24*

	Placebo (n = 69)		CNTX-4975 0.5 mg (n = 33)		CNTX-4975 1.0 mg (n = 70)	
End point†	Week 12	Week 24	Week 12	Week 24	Week 12	Week 24
WOMAC pain with walking on a flat surface score‡						
Baseline score, mean ± SD	7.4 ± 0.9		7.2 ± 1.1		7.2 ± 1.2	
Change from baseline, mean ± SD	−3.0 ± 2.5	−3.0 ± 2.8	−3.8 ± 2.5	−3.6 ± 2.0	−4.4 ± 2.6	−3.3 ± 2.6
LSM ± SE	−2.9 ± 0.4	−2.9 ± 0.4	−3.8 ± 0.5	−3.4 ± 0.5	−4.4 ± 0.4	−3.8 ± 0.4
LSMD vs. placebo (90% CI)			−0. 9 (−1.7, −0.0)§	−0.5 (−1.5, 0.5)	−1.5 (−2.2, −0.8)¶	−0.9 (−1.6, −0.1)§
WOMAC knee stiffness score#						
Baseline score, mean ± SD	13.1 ± 3.8		12.9 ± 3.3		12.3 ± 3.8	
Change from baseline, mean ± SD	−4.8 ± 6.6	−5.1 ± 6.2	−5.7 ± 5.0	−5.3 ± 4.5	−6.7 ± 5.2	−5.7 ± 5.5
LSM ± SE	−4.4 ± 0.7	−4.8 ± 0.7	−5.2 ± 0.9	−4.6 ± 0.9	−6.9 ± 0.7	−6.0 ± 0.7
LSMD vs. placebo (90% CI)			−0.8 (−2.4, 0.8)	0.3 (−1.4, 1.9)	−2.5 (−3.8, −1.2)¶	−1.2 (−2.5, 0.1)
WOMAC physical function score**						
Baseline score, mean ± SD	114.1 ± 24.7		108.4 ± 24.2		106.9 ± 27.9	
Change from baseline, mean ± SD	−46.2 ± 46.0	−49.4 ± 49.2	−49.3 ± 34.6	−45.7 ± 35.5	−59.3 ± 39.8	−51.6 ± 44.8
LSM ± SE	−46.3 ± 6.0	−50.4 ± 6.2	−51.3 ± 7.4	−46.3 ± 7.8	−64.5 ± 6.0	−57.6 ± 6.2
LSMD vs. placebo (90% CI)			−5.0 (−17.9, 7.9)	4.1 (−9.7, 17.9)	−18.3 (−28.6, −7.9)††	−7.2 (−18.3, 3.8)

* WOMAC = Western Ontario and McMaster Universities Osteoarthritis Index; LSMD = least squares mean difference; 90% CI = 90% confidence interval.
† Mixed model for repeated measures in modified intent-to-treat population. Negative numbers reflect a reduction in pain and stiffness and an improvement in function.
‡ Range 0 (none) to 10 (extreme).
§ $P < 0.10$ versus placebo.
¶ $P \leq 0.001$ versus placebo.
# Sum of the 2 stiffness responses (range 0–20).
** Sum of the 17 function responses (range 0–170).
†† $P \leq 0.01$ versus placebo.

mild (19%, 29%, and 20%) or moderate (11%, 18%, and 10%) in severity (Table 3). On day 1, TEAEs were reported by 2 patients (3%), 1 patient (3%), and 3 patients (4%), respectively. Only 1 patient in the CNTX-4975 0.5 mg group reported a serious TEAE (intractable shoulder pain from previous OA), which was not considered treatment-related. No deaths were reported.

The most frequent TEAEs, reported by ≥5% of patients in any treatment group, are summarized in Table 3. Most TEAEs were considered unrelated to study treatment. Four patients reported 7 TEAEs that were considered possibly or probably related to study medication; there was 1 report each of erythema, peripheral edema, and nausea (on treatment day 1) in the CNTX-4975 0.5 mg group, and dizziness, oral hypoesthesia, malaise, and hypotension (all on treatment day 1) in the CNTX-4975 1.0 mg group. One patient in the CNTX-4975 0.5 mg group developed an effusion that was tapped at 8 and 21 weeks into the study. The investigator did not believe this was study drug–related, and the patient had no other safety issues. Few laboratory abnormalities were observed, with similar profiles between placebo and CNTX-4975.

Pain was assessed at specific times both immediately before and after injection of intraarticular 2% lidocaine (without epinephrine), and study drug, using a 0–4 categorical scale (0 = no pain and 4 = severe pain). The average pain score before the intraarticular lidocaine injection, while patients were in a resting position, ranged from 1.6 to 1.7 for each of the 3 groups. Ten minutes after lidocaine injection, most patients (70%, 71%, and 66% in the placebo, CNTX-4975 0.5 mg, and CNTX-4975 1.0 mg groups, respectively) reported no procedural pain. The maximal recorded pain score typically occurred 30 minutes after injection of study drug. No-to-moderate pain was recorded in 93%, 85%, and 80% and moderately severe–to-severe pain in 7%, 15%, and 20%, respectively. The maximum average pain scores at rest (range 0–4) before injection were 1.6, 1.7, and 1.7, respectively, and at 30 minutes after injection of study treatment

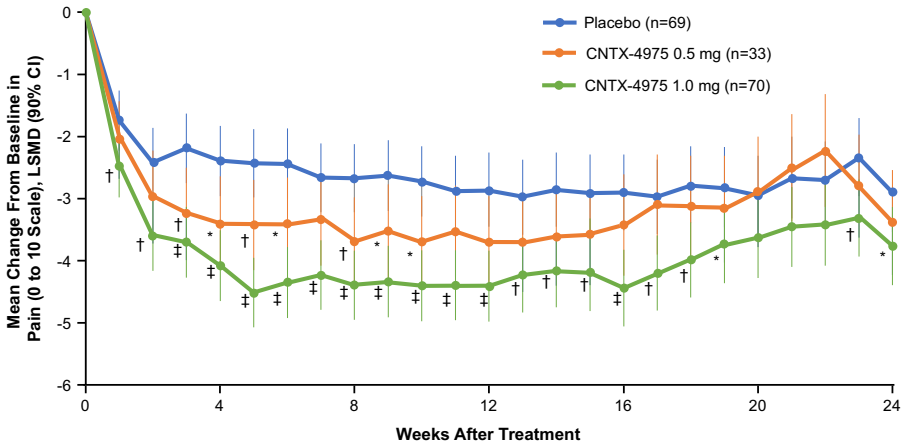

Figure 3. Change in average weekly WOMAC pain with walking scores. Change from baseline in average weekly scores through week 24 in patients treated with CNTX-4975 versus placebo is shown. A mixed model for repeated measures was used in the modified intent-to-treat population. Week 12 was the prespecified landmark end point; other *P* values were considered nominal and are presented for summary purposes only. Baseline scores (range 0–10): placebo 7.4, CNTX-4975 0.5 mg 7.2, CNTX-4975 1.0 mg 7.2. * = $P < 0.1$; † = $P < 0.05$; ‡ = $P < 0.001$, versus placebo. See Figure 2 for definitions.

they were 0.7, 1.2, and 1.6. Pain scores in each group declined to minimal levels in the subsequent 1.5 hours. Supplementary Figure 3 (http://onlinelibrary.wiley.com/doi/10.1002/art.40894/) includes a summary of procedural pain. An additional post hoc analysis indicated that procedural pain was not a significant covariate with regard to efficacy.

The NNH was evaluated in the CNTX-4975 1.0 mg and placebo groups. There were a total of 42 TEAEs, with 21 events occurring in each of these groups (Table 3). Based on these events, the NNH at 12 and 24 weeks was 58 and 237 patients, respectively.

DISCUSSION

The findings of this study demonstrated that a single intraarticular injection of CNTX-4975 1.0 mg was effective in providing significant and clinically meaningful reduction (≥50%) in pain that occurs while walking on a flat surface in patients with chronic moderate-to-severe OA knee pain (21,22), with the effect persisting for up to 24 weeks. Onset of improvement was rapid, with significant reduction in pain with walking, compared to placebo, as early as 1 week after treatment. The improvement in pain was associated with a reduction in knee stiffness and an improvement in function, as well as a positive PGIC score, through week 12, compared to placebo. The standardized effect size at 12 weeks for the CNTX-4975 1.0 mg dose using the primary end point was 0.68, which compares favorably to other approved therapies for OA-related knee pain (3). The CNTX-4975 0.5 mg dose was associated with a decrease in pain that was intermediate between that observed with placebo and with the CNTX-4975 1.0 mg dose.

CNTX-4975 1.0 mg was well tolerated, with a safety profile comparable to that of the placebo throughout the study.

Table 3. TEAEs through week 24*

Parameter/TEAE	Placebo (n = 70)	CNTX-4975 0.5 mg (n = 34)	CNTX-4975 1.0 mg (n = 71)
≥1 TEAE	21 (30)	16 (47)	21 (30)
≥1 serious TEAE	0	1 (3)†	0
Arthralgia	4 (6)	3 (9)	5 (7)
Upper respiratory tract infection	3 (4)	2 (6)	3 (4)
Increased hepatic enzyme	0	2 (6)	1 (1)
Joint effusion	0	3 (9)	0
Osteoarthritis	1 (1)	2 (6)	0

* Values are the number (%) of patients. Treatment-emergent adverse events (TEAEs) reported by ≥5% of patients in any treatment group within the safety population are shown. Procedural pain was not counted as a TEAE and therefore is not included.
† Patient reported intractable shoulder pain from previous osteoarthritis, which was not considered treatment-related.

Procedural pain was higher with CNTX-4975 and tapered to minimal levels by 2 hours after injection (Supplementary Figure 3, http://onlinelibrary.wiley.com/doi/10.1002/art.40894/). There was substantial overlap in postinjection pain with study drug in all 3 arms of the study. No patient withdrew due to an adverse event. Within each group, there was no relationship between procedural pain and outcome.

The AUC method was chosen for evaluation of the primary end point in this study. This method, while generally used in acute pain studies, also applies to chronic pain studies (23). The AUC analysis seemed most appropriate for the following reasons: 1) the profile of CNTX-4975 in previous studies included early onset of action with sustained pain relief through week 24; 2) the AUC method shows the entirety of benefit over time; and 3) the AUC method has potentially greater assay sensitivity because it more accurately shows the effects during the entirety of the study instead of at a single time point. The week-to-week mean numerical pain rating scale scores (Figure 3) were evaluated as a secondary end point using MMRM analysis. At week 12, the time of the designated primary end point, divergence from placebo was highly significant ($P < 0.001$) with the 1 mg dose. By week 18, the treatment effects of CNTX-4975 compared to placebo began to taper, although evidence showed divergence even at week 24 ($P = 0.067$).

The effects of trans-capsaicin are not dependent on ongoing exposure to the drug; the elimination half-life is <4 hours (24), whereas efficacy extends for months following a single injection. This reduces the safety risk of continued drug exposure effects in the long term. Due to the short exposure time and low systemic drug concentrations observed in clinical studies of injectable capsaicin (data on file; Centrexion Therapeutics Corp.), as well as the lipophilic nature of the drug (24), no effect outside of the knee joint is expected. The reduction in pain with the 1.0 mg dose was evident at 24 weeks, although there was a suggestion of diminution of effect after 16 weeks. Onset, maximum effect, and duration of action demonstrated dose dependency. Pain and loss of function are arguably the most important clinical features of OA (2), and an intervention that meaningfully improves pain and function is worth pursuing, given the limited choices currently available to patients.

This study has several limitations. Because it was a small randomized study in a specific population of patients with moderate-to-severe OA knee pain, the findings cannot be generalized to the knee OA population at large. In addition, as a small study, data regarding the safety profile are limited, although the findings are consistent with the safety profile of other capsaicin products.

In conclusion, the present results support the efficacy and safety of the intraarticular injection of trans-capsaicin to manage moderate-to-severe pain associated with knee OA. The findings indicate that further clinical development is warranted.

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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. Stevens 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. Stevens, Ervin, Guedes, Burges, Hanson, Campbell.

Acquisition of data. Stevens, Ervin, Guedes, Burges, Hanson, Campbell.

Analysis and interpretation of data. Stevens, Ervin, Nezzer, Nieves, Guedes, Burges, Hanson, Campbell.

ROLE OF THE STUDY SPONSOR

Centrexion Therapeutics Corp facilitated the study design and reviewed and approved the manuscript prior to submission. The authors independently collected the data, interpreted the results, and had the final decision to submit the manuscript for publication. Medical writing assistance was provided by Lauren Gallagher, PhD, and Illyce Nunez, PhD, of Peloton Advantage, LLC, an OPEN Health company, and supported by Centrexion Therapeutics Corp. Publication of this article was not contingent upon approval by Centrexion Therapeutics Corp.

ADDITIONAL DISCLOSURES

Author Ervin is an employee of the Center for Pharmaceutical Research. Authors Nezzer and Nieves were employees of Premier Research during the time the study was conducted.

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BRIEF REPORT

Variability in Antinuclear Antibody Testing to Assess Patient Eligibility for Clinical Trials of Novel Treatments for Systemic Lupus Erythematosus

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Objective. In the development of novel therapies for systemic lupus erythematosus, antinuclear antibody (ANA) positivity represents a criterion for trial eligibility. Since as many as 30% of patients enrolled in trials have been ANA negative, we evaluated the performance characteristics of immunofluorescence assays (IFAs) for ANA determinations for screening.

Methods. This study used 5 commercially available IFAs to assess the ANA status of 181 patients enrolled in a phase II clinical trial for an anti–interleukin-6 antibody. Enrollment included a detailed review of medical records to verify a historical ANA value. IFA results were related to various clinical and serologic features at enrollment.

Results. While the frequency of ANA negativity assessed by the central laboratory was 23.8% in a cohort of 181 patients, the evaluated IFA kits demonstrated frequencies of negativity from 0.6 to 27.6%. With 2 IFA kits showing a significant frequency of ANA negativity, positive and negative samples differed in levels of anti–double-stranded DNA, C3, and presence of other ANAs as well as the frequency of high interferon (IFN) expression.

Conclusion. These findings indicate that, when used for screening, IFAs can vary because of performance characteristics of kits and thus can affect determination of trial eligibility. With kits producing a significant frequency of ANA negativity, ANA status can be associated with other serologic measures as well as the presence of the IFN signature, potentially affecting responsiveness to a trial agent.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a serious autoimmune disease characterized by multisystem organ involvement associated with the production of antibodies to components of the cell nucleus (antinuclear antibodies [ANAs]) (1). ANA positivity is a criterion for disease classification, and serologic studies have provided evidence that ANA positivity is almost invariable in SLE. In addition to serving as biomarkers, ANAs can promote disease pathogenesis by forming immune complexes (ICs) that can deposit in the kidney, inducing nephritis (1). ICs containing antibodies directed to DNA (anti–double-stranded [anti-dsDNA]) or RNA binding proteins (anti-RBPs) can also drive the production of type 1 interferon (IFN) since ICs promote the uptake of nucleic acids into innate immune cells to stimulate internal nucleic acid sensors (2–4).

To improve therapy for SLE, there is intense interest in developing innovative agents such as belimumab, a monoclonal antibody to B cell–activating factor (BAFF) or B lymphocyte stimulator (BLyS) and the first biologic to receive regulatory approval for treating active nonrenal lupus. While belimumab did not reach the end points in phase II trials, a post hoc analysis indicated efficacy of this agent in patients who were positive for either an ANA or anti-dsDNA; in the phase II studies, about 30% of enrolled patients were serologically negative at screening (5,6). For the subsequent phase III trials, only patients who were ANA and/or anti-dsDNA positive at screening were enrolled. Since these trials were successful, other sponsors are adapting a similar screening strategy (7).

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Studies on belimumab have raised important questions about the use of serology to determine trial eligibility. The first question relates to the high frequency of ANA negativity among patients in the phase II studies. ANA testing usually involves indirect immunofluorescence assays (IFAs) using HEp-2 cells. Kits from different manufacturers, however, may differ in performance characteristics, and there can also be observer differences (8). Thus, in a trial setting, a negative ANA at screening may reflect assay performance rather than any fundamental change in ANA production. Another question concerns the reasons why serologic status can influence treatment responses and whether it predicts response only to agents directed at B cells.

A cross-sectional analysis of sera in patients with established SLE showed that assay format (IFA, enzyme-linked immunosorbent assay, or multiplex) can affect the frequency of ANA positivity (9); this study involved patients in routine care and did not address the relationship to disease activity. In view of the emerging use of ANA testing in the trial setting, we have therefore extended this investigation to specifically assess responses of patients with sufficient disease activity for clinical trial eligibility. For this purpose, we evaluated samples from a phase II study of a monoclonal antibody to interleukin-6 (IL-6) (10). At study enrollment, 23.8% of the patients had a negative ANA value at 1:80 dilution as assessed at the central laboratory, although a detailed review of medical records by experts confirmed historically positive ANA status and clinically active disease.

In the current study, we used 5 different ANA kits to test the sera of trial subjects and assess the relationship with clinical and laboratory features. We were especially interested in the IFN signature because IFN production may reflect the activity of ICs formed by ANAs. As results presented herein demonstrate, the frequency of ANA responses in patients with active lupus can vary significantly depending on the assay kit. Further, the results of this study indicate that IFA status can be associated with other immunologic findings, including the IFN signature, that could influence responsiveness to therapy.

PATIENTS AND METHODS

For this study, samples were collected at baseline from 181 patients enrolled in a phase II randomized clinical trial (ClinicalTrials.gov identifier NCT01405196) in order to evaluate the efficacy and safety of a monoclonal antibody to IL-6 in SLE where historical ANA positivity was allowed (10). All potentially eligible subjects underwent a careful review of medical history, reports of current lupus symptoms, laboratory findings (i.e., ANA, anti-dsDNA, and anti-RBPs [anti-Sm, anti-RNP, anti-SSA, and anti-SSB]), C3, and C4. Patients were required to have an SLE Disease Activity Index 2000 (SLEDAI-2K) (11) score of $\geq$6, with musculoskeletal and mucocutaneous manifestations being the most commonly reported symptoms (98.3% and 85.2%, respectively). Of patients in the present study, 43 (23.8%) had a <1:80 ANA titer at screening assessed at the central laboratory that used the Kallestad HEp-2 Cell Line Substrate (Bio-Rad Laboratories). For patients who were ANA negative, diagnosis and eligibility were verified by an independent panel of experts per protocol for study inclusion based on a documented historical ANA or anti-dsDNA value, clinical and other serologic findings such as neutropenia or hypocomplementemia, and appropriate level of disease activity.

Anti-RBPs and anti-dsDNA were measured using a bead-based immunoassay (AtheNA Multi-Lyte ANA Test System). The complement C3 and C4 assays were performed by immunonephelometry using the Siemens BNII Nephelometer. IFN classification was performed as described previously (12).

For the present study, sera obtained from patients were retested with the following ANA kits: ANA IFA: HEp-20-10 Test (EuroImmun), Nova Lite HEp-2 ANA (Inova Diagnostics), ANA/HEp-2 Cell Culture IFA Test System (Zeus Laboratories), Kallestad HEp-2 Cell Line Substrate (Bio-Rad Laboratories), and HEp-2000 Fluorescent ANA-Ro Test System (Immuno Concepts).

All assays were performed according to the recommended protocol by the manufacturers. Samples were run with positive and negative controls at 1:40 and 1:80 dilutions using an EVOS FL Cell Imaging System.

RESULTS

The original study population included 183 patients with a mean age of 40.4 years, mean disease duration of 8.1 years, and mean SLEDAI score of 9.5. From this cohort, there were ANA results from 182 subjects that were analyzed by the central laboratory, and samples were available from 181 patients for the present study. Samples were screened by a single experienced observer (DKT) using 5 commercially available ANA kits with HEp-2 cells. All assays were performed according to recommended manufacturer protocol using dilutions of 1:40 and 1:80 and included positive and negative controls. Results indicated that the frequency of ANA negativity varied from 0.6 to 27.6% (Table 1). Of samples tested, 70.7% were positive in all assays. No sample was consistently negative in either 4 or 5 assays, and only 1 sample was negative in 3 assays (data not shown).

Table 1. Comparison of 5 commercial antinuclear antibody (ANA) assays

Assay	No. (%) ANA-positive samples at 1:80	No. (%) ANA-negative samples at 1:80
EuroImmun	180 (99.4)	1 (0.6)
Nova Lite	175 (96.7)	6 (3.3)
Zeus	171 (94.5)	10 (5.5)
Kallestad	156 (86.2)	25 (13.8)
Immuno Concepts	131 (72.4)	50 (27.6)

Table 2. Comparison of Kallestad ANA assay results between the Duke laboratory and central laboratory*

Assay result	No. (%) of subjects (n = 180)
ANA+ at both Duke laboratory and central laboratory	126 (70.0)
ANA− at both Duke laboratory and central laboratory	14 (7.7)
ANA+ at Duke laboratory/ANA− at central laboratory	29 (16.1)
ANA− at Duke laboratory/ANA+ at central laboratory	11 (6.1)

* Antinuclear antibodies (ANAs) were tested at a dilution of 1:80. In these results, 1 sample was not included in the analysis of 181 subjects due to lack of availability.

For this phase II study, the original screening at the central laboratory utilized the Kallestad kit, which is 1 of the kits we studied. We therefore compared the results of the determinations performed at the central laboratory to those performed at the Duke laboratory; 1 sample was not included in the analysis due to lack of availability (Table 2). The results indicate substantial agreement in either reading since 70% of results were positive and 7.7% were negative when assessed at both laboratories (Table 2). The remaining samples showed discrepancies in positive and negative results.

To evaluate the association of ANA response with clinical or laboratory findings, the population was divided into groups by ANA positivity or negativity using the results of the Kallestad kit from either laboratory. A similar comparison was performed with the Immuno Concepts kit, which showed the highest frequency of negative determinations. As shown in Table 3, compared to those who were ANA negative, patients who were ANA positive had higher baseline disease activity, mean SLEDAI-2K scores, anti-dsDNA levels, lower C3 concentrations, and a higher frequency of anti-RBPs. Using results from the original ANA testing performed at the central laboratory, seropositive and seronegative patients showed no differences in the frequency of use of immunosuppressive agents (i.e., methotrexate, azathioprine, leflunomide, and/or mycophenolate mofetil) or glucocorticoid dosages of ≥7.5 mg/day. Similarly, there were no differences in the frequency of use of antimalarials since 68% of ANA negative patients and 64% of ANA positive patients were taking these agents.

Since ICs containing ANAs and their cognate antigens can drive IFN production, we investigated the relationship between serologic findings and the IFN signature. The results presented in Table 3 indicate that those patients who were ANA negative as assessed by either the Kallestad or Immuno Concepts kits

Table 3. Baseline demographic and clinical characteristics of the patients with systemic lupus erythematosus by ANA positivity*

	Central laboratory		Duke laboratory		Immuno Concepts		
	ANA+ (n = 138)	ANA− (n = 44)	ANA+ (n = 156)	ANA− (n = 25)	ANA+ (n = 131)	ANA− (n = 50)	Total population (n = 183)
Age, mean years	38.8	45.4	40	43.9	39.8	42.5	40.4
Disease duration in years, mean	8.15	8.55	8.41	7.89	8.25	8.58	8.1
SLEDAI score, mean	9.87†	8.52	9.72†	8.32	9.82†	8.76	9.5
SLEDAI score ≥10	72 (52.2)†	13 (29.6)	76 (48.7)	8 (32.0)	68 (51.9)†	16 (32)	85 (46.4)
Immunosuppressive agent use‡	60 (43.5)	22 (50.0)	–	–	–	–	–
Oral glucocorticoid use >7.5 mg/day	59 (42.8)	20 (45.5)	–	–	–	–	–
anti-dsDNA, mean	116.2†	36.4	106.8†	39.3	111.6†	60.5	97
anti-low C3, no. (%)	43 (31.2)†	5 (11.4)	45 (28.9)†	2 (8.0)	41 (31.3)†	6 (12.0)	48 (26.4)
anti-SSA, no. (%)	57 (41.3)†	11 (25.0)	60 (38.5)	6 (24.0)	55 (42.0)†	11 (22.0)	68 (37.4)
anti-SSB, no. (%)	25 (18.1)†	1 (2.3)	23 (14.7)	3 (12.0)	24 (18.3)	2 (4.0)	26 (14.2)
anti-RNP, no. (%)	35 (25.4)†	1 (2.3)	35 (22.4)†	1 (4.0)	31 (23.7)	5 (10.0)	36 (19.7)
anti-Sm, no. (%)	32 (23.2)†	1 (2.3)	31 (19.9)	2 (8.0)	31 (23.7)	2 (4.0)	33 (18.1)
IFN high, no./no. assessed (%)	91/127 (71.7)†	10/40 (25.0)	93/144 (64.6)†	9/23 (39.1)	82/118 (69.5)	20/49 (40.8)	101/167 (60.5)

* Baseline demographic and clinical characteristics by antinuclear antibody (ANA) status were not shown for the EuroImmun, Nova Lite, and Zeus assays as the majority of subjects (>94%) were ANA+. The *t*-test was used to compare mean baseline values to determine significance, and Fisher's exact test was used for comparison of all other values. SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; anti-dsDNA = anti–double-stranded DNA; IFN = interferon.

† Significantly different versus ANA– group ($P \leq 0.05$).

‡ Immunosuppressive agents included methotrexate, azathioprine, leflunomide, and mycophenolate mofetil.

were less likely to have high IFN expression. Taken together, these results indicate that IFA determinations, depending on the kit, can be related to disease activity and presence of high IFN expression.

DISCUSSION

This study provides new information on the use of ANA testing for screening in clinical trials and the potential influence of serologic status on treatment response. We showed that different IFA kits produce varying frequencies of positivity among patients with sufficient disease activity for clinical trial eligibility. Further, our findings indicate that patients with negative ANA values in certain kits may differ from those with positive responses in immunologic findings, including high IFN expression. Together, these findings suggest that the performance characteristics of assays may impact the value of this testing to determine trial eligibility and possibly eventual prescription of medications approved for active, autoantibody-positive disease.

While ANA testing represents an essential element in patient evaluation, the assays ("kits") used for such testing can differ in performance characteristics. These differences can relate to factors such as properties of the fluorescent detection antibodies, conditions for cell fixation and properties of the cell lines themselves (8). Observer differences can also affect IFA determinations. In routine clinical care, the major problem with most IFA kits has been the high frequency of positive results in otherwise healthy individuals. The issue of false-negatives as well as variability of assay results in SLE patients has received less attention.

The use of ANA testing in the trial setting differs from that of the routine management of patients with SLE. Differences include disease duration and possible changes in serologic findings that arise from the natural history of the disease or the effects of immunosuppressive therapy, especially with agents that can affect B cells such as cyclophosphamide or mycophenolate mofetil. In routine care, assessment of ANAs and anti-RBPs tends to be performed at the initial evaluation only. Thus, there is only limited information on their evolution over time and longitudinal expression.

The patient cohort in this study consisted of patients enrolled in a clinical trial to evaluate the efficacy and safety of a monoclonal anti–IL-6 antibody in the treatment of active SLE. Nevertheless, the frequency of ANA negativity assessed at the central laboratory was 23.8%. Review by experts, however, substantiated the diagnosis of SLE based on prior ANA or anti-dsDNA positivity as well as clinical and laboratory features. Consistent with a prior study of another patient population (9), these results indicate that ANA determinations can vary depending on the kit, with observer differences also affecting testing results.

The IFA is often viewed as the gold standard for ANA assessment since it allows for the detection of antibodies to a potentially large number of antigens (13,14). This ability distinguishes the IFA from other ANA assays such as bead-based or line immunoassays that involve only a limited number of antigens (8). Although the IFA assay should allow broad ANA detection, the kits in our study differed in the frequency of positive and negative results.

The results of this study have important implications for the use of serology in the clinical trial setting. If ANA positivity is considered an essential feature of SLE, ANA positivity at the time of screening can provide some assurance as to diagnosis. Conversely, ANA negativity, while not excluding the diagnosis of SLE, may reflect immunologic findings as well as disease activity (as indicated by the data presented in Table 3). For both the Kallestad and Immuno Concepts kits, ANA-negative patients differed from ANA-positive patients in disease activity, levels of anti-dsDNA and complement C3 and C4, and frequency of anti-RBPs and high IFN expression. A positive value with these kits may therefore signify greater disease activity and the possible likelihood of response to certain agents.

Since the IFN signature may reflect immune cell activation by ICs with anti-dsDNA or anti-RBPs, a lower frequency of IFN expression would be expected in patients who are ANA negative. The role of IFN as a marker of disease activity is not yet clear, however (15,16). In this regard, ANA titers may also be relevant to the immunologic profile of a patient in view of data indicating that the extent of in vitro IFN generation may be related to the total amount of anti-RBPs, as reflected in a composite index created by summing levels of antibodies to SSA, SSB, Sm, and RNP (3).

Together, these findings highlight the challenges of ANA testing in the trial setting to screen for serologic activity. This setting can be considered "post-autoimmunity" (i.e., after diagnosis and treatment) in contrast to a setting of "pre-autoimmunity" (i.e., before disease is clinically evident). With established disease, the frequency of ANA positivity may, in fact, be lower than the 95–99% positivity rate usually considered in the diagnosis of SLE. However, our data suggest that a frequency of ANA positivity of essentially 100% can be achieved by the utilization of certain kits or the combination of kits.

Thus, our results suggest that, in the clinical trial setting, ANA assays, depending on their performance characteristics, not only may affect assessment of trial eligibility but also could identify immunologic features potentially relevant to treatment response. In this regard, in the development of belimumab, the use of ANA and anti-dsDNA positivity as a criterion for entry likely influenced the success of the trials, allowing detection of a response that may have been obscured by either enrolling all patients irrespective of ANA status or screening with an ANA assay with 95–99% positive results. The variations in IFA determinations and their association with certain clinical and immunologic features highlight the need to better understand the use of this biomarker for screening and its potential impact on treatment responses.

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 submitted for publication. Dr. Pisetsky 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. Pisetsky, Thompson, Diehl, Sridharan.

Acquisition of data. Thompson, Diehl, Sridharan.

Analysis and interpretation of data. Pisetsky, Thompson, Wajdula, Diehl, Sridharan.

ROLE OF THE STUDY SPONSOR

The study was a collaboration between the laboratory of Dr. Pisetsky at Duke University Medical Center and Pfizer. The co-authors from Pfizer contributed to the design of the study as well as 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 required approval by Pfizer.

ADDITIONAL DISCLOSURE

Author Sridharan is an employee of PPD, Inc.

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BRIEF REPORT

Enhanced Programmed Death 1 and Diminished Programmed Death Ligand 1 Up-Regulation Capacity of Post-Activated Lupus B Cells

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Objective. To assess the expression of programmed death 1 (PD-1), PD ligand 1 (PD-L1), and PD-L2 by B cells from patients with systemic lupus erythematosus (SLE) at baseline and after in vitro stimulation and to analyze their functional relationship to B cell proliferation.

Methods. Peripheral blood mononuclear cells obtained from 29 SLE patients and 27 healthy donors were stimulated with interleukin-2 (IL-2)/IL-10, anti–B cell receptor (anti-BCR), CpG, and CD40L alone or in combination. Expression of PD-1, PD-L1, and PD-L2 on defined B cell subsets as well as on CD3+ T cells was analyzed by flow cytometry at baseline and after 48 hours of stimulation. Additionally, after 48 hours of stimulation, CD71 was evaluated as a proliferation marker on CD19+CD20+ B cells.

Results. Increased PD-1 expression was characteristic of unstimulated lupus B cells and T cells. Upon stimulation of B cells with IL-2/IL-10, anti-BCR, CpG, and CD40L for 48 hours, the capacity of SLE B cells to up-regulate PD-L1 expression was substantially diminished ($P = 0.0006$) along with reduced B cell proliferation ($P = 0.0039$). Reduced PD-L1 expression was inversely correlated with the presence of the interferon signature ($r = –0.8571$, $P < 0.0001$) and the clinical SLE Disease Activity Index score ($r = –0.5696$, $P = 0.0087$).

Conclusion. Post-activated, hyporesponsive lupus B cells are characterized by a phenotype of increased PD-1, functionally diminished PD-L1 up-regulation capacity, and reduced proliferation upon stimulation.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multiorgan autoimmune disease with underlying abnormalities of cellular and humoral immunity. B cells are considered key players in SLE as precursors of autoantibody-producing plasma cells, antigen-presenting cells, and cytokine and chemokine producers. Recent studies have shown that SLE patients present with anergic or post-activated B cells, characterized by hyporesponsiveness upon B cell receptor (BCR) and Toll-like receptor 9 (TLR-9) stimulation as well as indications of impaired T cell and B cell interaction (1).

In this context, costimulatory and coinhibitory signals regulate the interaction between T and B cells during an inflammatory response, which is crucial for an appropriate host reaction and maintenance of self tolerance. Programmed death 1 (PD-1) is a major inhibitory receptor expressed by activated lymphocytes. Engagement of its 2 ligands (PD ligand 1 [PD-L1] and PD-L2) play an important regulatory role during immune responses against viruses, chronic infections, and tumors (2). Cancer therapy has been revolutionized in recent years by the revitalization of exhausted T cells via immune checkpoint inhibitors, such as blockers of the PD-1 pathway. This therapeutic concept is also associated with immune-related side effects mimicking certain autoimmune features (3). The resulting clinical implications, together with known risk alleles of PD-1 and related susceptibility for SLE (4) as well as observations that PD-1–knockout mice develop a lupus-like pathology (5), suggest potential involvement of the PD-1 pathway in SLE pathogenesis. However, results from studies of mice with experimental lupus-like disease in which the PD-1 pathway has been blocked or enhanced are a subject of controversy (6,7). In addition, little is known about the expression and kinetics of PD-1, PD-L1, and PD-L2 by B lymphocytes from SLE patients. Thus, the current study was undertaken to address

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

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the hypothesis that abnormalities of these particular checkpoint molecules might be involved in the pathology of SLE B cells and their post-activated, hyporesponsive status.

PATIENTS AND METHODS

Study participants. Whole blood samples were collected from 29 patients who met the American College of Rheumatology criteria for SLE (8) (see Supplementary Table 1, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40897/abstract). Exclusion criteria included treatment with prednisolone (≥20 mg/day) at the time of enrollment, previous rituximab therapy, or any prior experimental B cell–depleting therapy. Twenty-seven healthy donors served as controls. The study was approved by the Local Ethics Committee at Charité University Hospitals Berlin, and all participants provided written informed consent.

Culture conditions. Peripheral blood mononuclear cells (PBMCs) were prepared by density-gradient centrifugation. One million PBMCs were cultured for 48 hours in 96-well, round-bottomed plates (Greiner Bio-One) in medium alone or in medium containing goat $F(ab)_2$ fragments against human IgG, IgM, and IgA (anti-BCR; 0.5 μg/ml) (Jackson ImmunoResearch Laboratories); recombinant human interleukin-2 (IL-2) and IL-10 (both 20 ng/ml) (ImmunoKontact); CD40L (0.5 μg/ml) (Miltenyi Biotec); and CpG-containing oligodeoxynucleotide (0.5 mg/ml) (sequence 59-TsCsg sTsCsg sTsTsT sTsgsT sCsgsT sTsTsTsgsTsC sgsTsT-39; TIB MolBiol) alone or in combination.

Flow cytometry. For baseline screening and B cell stimulation studies, PBMCs were stained with phycoerythrin (PE)–Cy7–conjugated CD19 (clone SJ25C1), Pacific Blue–conjugated CD3 (clone UCHT1), PerCP–Cy5.5–conjugated PD-1 (clone EH12.1), allophycocyanin (APC)–H7–conjugated CD14 (clone MOP9), and fluorescein isothiocyanate (FITC)–conjugated CD27 (clone L128) (all from BD Biosciences) as well as BV510-conjugated CD20 (clone 2H7), PE-conjugated PD-L1 (clone 29E.2A3), and APC-conjugated PD-L2 (clone 24F.10C12) (all from BioLegend). For B cell proliferation studies, PBMCs were stained with FITC-conjugated CD71 (clone OKT9; eBioscience).

For examination of B cell subsets at baseline, PBMCs were stained with BV711-conjugated CD19 (clone SJ25C1), BUV395-conjugated CD3 (clone UCHT1), BUV395-conjugated CD14 (clone M5E2), BV786-conjugated CD27 (clone L128), PerCP–Cy5.5–conjugated PD-1, and PE-conjugated CD11c (clone Blys6) (all from BD Biosciences) as well as BV510-conjugated CD20, PE-CF594–conjugated IgD (clone IA6-2), APC-conjugated PD-L1, and APC-conjugated PD-L2 (all from BioLegend).

For the detection of sialic acid–binding Ig-like lectin 1 (Siglec-1) (CD169) on monocytes, whole blood samples were lysed first (PharmLyse; Becton Dickinson) for 15 minutes at room temperature. Staining was performed with anti–Siglec-1–AF647 (7–239) (BioLegend). Prior to all membrane staining, the Fc receptor (FcR) was blocked for 5 minutes at 4°C using FcR blocking reagent (Miltenyi Biotec). Cells were stained for 15 minutes at 4°C.

Prior to measurement, 1 μl of 300 n*M* DAPI (Invitrogen) was added to exclude dead cells. The stained samples were assessed by flow cytometry using a FACS Canto II flow cytometer (Becton Dickinson) for baseline screening and B cell stimulation studies and a FACS LSR Fortessa flow cytometer (Becton Dickinson) for detailed evaluation of B cell subsets at baseline. Results were analyzed with FlowJo software, version 10.

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 6. Differences between findings of stimulation experiments in cells from healthy donors compared to SLE patients were assessed by Mann-Whitney U test. Correlations were calculated using Spearman's rank coefficient. *P* values less than 0.05 were considered significant.

RESULTS

Increased PD-1 expression on unstimulated T cells and B cells in SLE. First, we investigated the expression of PD-1 and its corresponding ligands PD-L1 and PD-L2 at baseline on CD27– and CD27+ B cells as well as on $CD20^{low}$CD27++ plasmablasts from control subjects and SLE patients (Figure 1A). Expression of PD-1, PD-L1, and PD-L2 was higher in CD27+ B cells compared to CD27– B cells in SLE patients as well as controls. The highest expression of PD-L2 was found on $CD20^{low}$CD27++ plasmablasts.

Of particular note, PD-1 expression on unstimulated CD27– and CD27+ B cells from SLE patients was significantly higher compared to cells from control subjects, while there was no difference in the expression of the corresponding ligands. To assess which B cell subsets express higher PD-1, we monitored CD27–IgD+ (naive), CD27–IgD– (double-negative), CD27+IgD+ (nonswitched memory), CD27+IgD– (switched memory), and CD11c+ (age-associated) B cells (gating strategy and distribution of subsets shown in Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40897/abstract). Naive and switched memory B cells from SLE patients exhibited significantly higher PD-1 expression compared to healthy donors. PD-L1 and PD-L2 were most highly expressed on nonswitched memory B cells in healthy donors and SLE patients (Figure 1B). Control subjects and SLE patients expressed similar levels of PD-1 and its ligands in CD11c+ age-associated B cells (see Supplementary Figure 2, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40897/abstract).

A significant increase in PD-1 expression on CD3+ T cells from SLE patients was found compared to healthy donors at baseline (Figure 1C), whereas there was no difference between the 2 groups in expression of either ligand on T cells. Interestingly, a positive correlation was found between the expression of PD-1 on T cells and its expression on B cells ($r = 0.6274$, $P = 0.0108$).

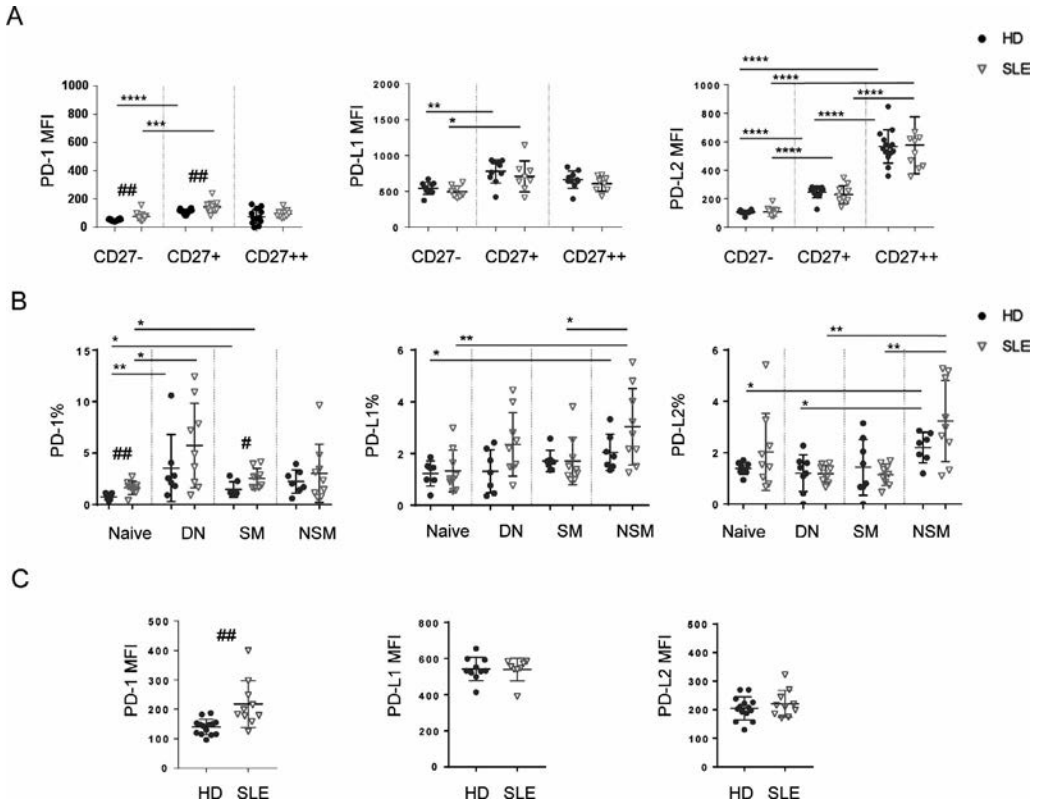

Figure 1. Unstimulated B cells and T cells from systemic lupus erythematosus (SLE) patients exhibit increased expression of programmed death 1 (PD-1). PD-1, PD ligand 1 (PD-L1), and PD-L2 expression at baseline in cell subsets from 7–10 healthy donors (HDs) and 9–10 SLE patients is shown. **A**, PD-1, PD-L1, and PD-L2 expression by CD20+CD27– and CD20+CD27+ B cells and CD20–CD27++ plasmablasts. **B**, PD-1, PD-L1, and PD-L2 expression by naive (CD27–IgD+), double-negative (DN) (CD27–IgD–), switched memory (SM) (CD27+IgD–), and nonswitched memory (NSM) (CD27+IgD+) B cells. **C**, PD-1, PD-L1, and PD-L2 expression by CD3+ T cells. For gating strategies, see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40897/abstract. Symbols represent individual subjects; bars show the mean ± SD. * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P < 0.0001$. # = $P \leq 0.05$; ## = $P \leq 0.01$, SLE patients versus healthy donors. MFI = mean fluorescence intensity.

For comparison, we studied the expression of 2 other costimulatory molecules on B cells: CD40, crucial for T cell–dependent B cell activation (via the CD40/CD40L axis), and CD86, a marker of activated B cells and modulating T cell signaling. At baseline, CD40 was highly expressed on CD27– and CD27+ B cells. In contrast, CD86 was highly expressed on CD27++ plasmablasts compared to other subsets. Nevertheless, B cells from SLE patients and healthy controls showed a similar expression pattern of CD40 and CD86 (see Supplementary Figures 3A and B, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40897/abstract).

Diminished PD-L1 up-regulation capacity by CD19+CD20+ lupus B cells upon stimulation. PBMCs obtained from healthy donors and SLE patients were stimulated for 48 hours under different conditions (anti-BCR, CpG, CD40L, and IL-2/IL-10 alone or in combinations) (Figures 2A–C). Stimulation of B cells with anti-BCR alone (data not shown) or IL-2/IL-10 alone did not result in increased expression of any PD-1 family members. However, the highest expression of PD-1, PD-L1, and PD-L2 on CD19+CD20+ B cells from healthy donors and SLE patients was found after stimulation with anti-BCR together with CpG and CD40L (Figures 2A–C). Under these conditions, only the up-regulation of PD-L1 was significantly diminished on SLE B cells compared to controls (Figure 2B). There was no difference in PD-1 expression (Figure 2A) or PD-L2 expression (Figure 2C). We also observed diminished PD-L1 up-regulation by SLE B cells upon stimulation with CpG alone, while stimulation with CD40L alone induced similar expression of PD-1, PD-L1, and PD-L2 in healthy controls and SLE patients.

Overall expression patterns of CD86 and CD40 on stimulated SLE B cells were similar (Figure 2D and Supplementary Figure 4 at http://onlinelibrary.wiley.com/doi/10.1002/art.40897/abstract, respectively), including diminished responsiveness to CpG stimulation but preserved response to CD40 stimulation. Of particular note, reduced expression of CD86 and CD40 correlated with diminished PD-L1 up-regulation upon stimulation of SLE B cells ($P = 0.0280$ and $P = 0.0266$, respectively).

Inverse correlation of CD169 (Siglec-1) expression levels and PD-L1 levels in activated CD19+CD20+ lupus B cells. We subsequently addressed whether there was any relationship between lupus activity and the expression profile of analyzed checkpoint molecules. In this context, it has been previously

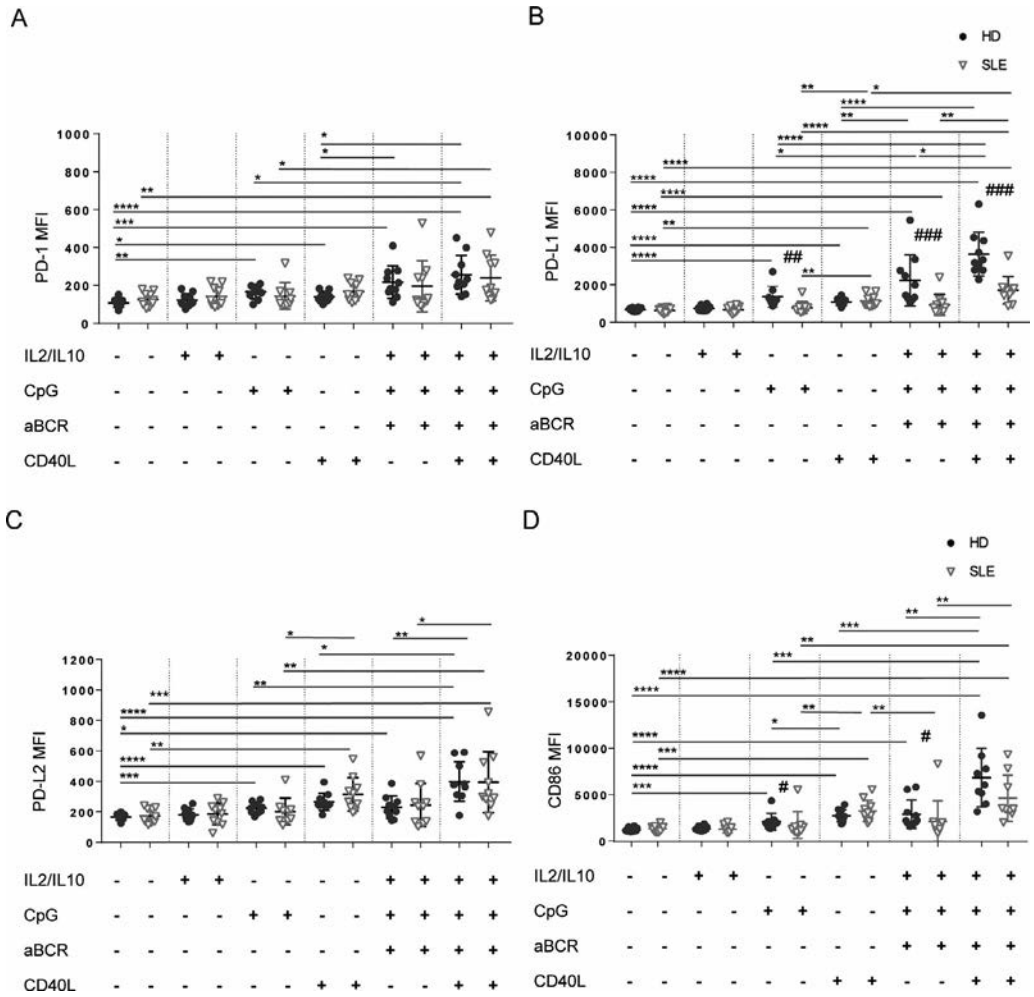

Figure 2. Reduced PD-L1 and CD86 expression by stimulated B cells from 10 patients with SLE compared to 10 healthy donors. Expression of PD-1 (**A**), PD-L1 (**B**), PD-L2 (**C**), and CD86 (**D**) by CD19+CD20+ B cells after stimulation with interleukin-2 (IL-2)/IL-10, CpG, anti–B cell receptor (anti-BCR), and CD40L is shown. Symbols represent individual subjects; bars show the mean ± SD. * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P < 0.0001$. # = $P \leq 0.05$; ## = $P \leq 0.01$; ### = $P \leq 0.001$, SLE patients versus healthy donors. See Figure 1 for other definitions.

demonstrated that expression of Siglec-1 on monocytes can serve as a surrogate marker of the type I interferon (IFN) signature in SLE (9). In the present study, we found that PD-L1 expression on B cells was inversely correlated with Siglec-1 expression (Figure 3A) and with SLE disease activity measured by the clinical SLE Disease Activity Index score (10) (Figure 3B). Regarding other costimulatory molecules, we also observed an inverse correlation between Siglec-1 and CD86 expression (Figure 3C).

Up-regulated PD-L1 expression as a marker of B cell proliferation in SLE patients and healthy controls. To investigate the impact of PD-1 on proliferation as an indicator of potential post-activated, hyporesponsive cells, we used transferrin receptor 1 (CD71) membrane expression as a marker of B cell proliferation and its relationship to PD-L1 expression. Up-regulation of CD71 has been shown to correlate with Ki-67 expression (11). After 48 hours of stimulation with anti-BCR, CD40L, and CpG, B cells from SLE patients proliferated less then B cells from healthy controls (Figure 3D). A direct correlation was found between B cell proliferation and PD-L1 expression, both in healthy donors and SLE patients (Figure 3E).

DISCUSSION

Immune checkpoints are vital regulatory pathways that maintain the homeostasis and tolerance of the immune system. The PD-1 pathway is one of the comprehensively investigated immune checkpoints in SLE, with a focus on T cells and most studies being conducted with preclinical mouse models, and with conflicting results. The expression and kinetics of PD-1 family members on human B cells have not yet been delineated in detail.

Herein, we show that PD-1 expression is increased in naive and switched memory SLE B cells at baseline, which is consistent with a previous report describing increased PD-1 expression on CD19+ and in CD3+ lymphocytes (12). Recent studies have demonstrated higher PD-1 transcript levels in CD27–IgD–CXCR5–CD11c+ (13) and $CD11c^{high}Tbet+CD27^{low}CD38^{low}$ B cell subsets

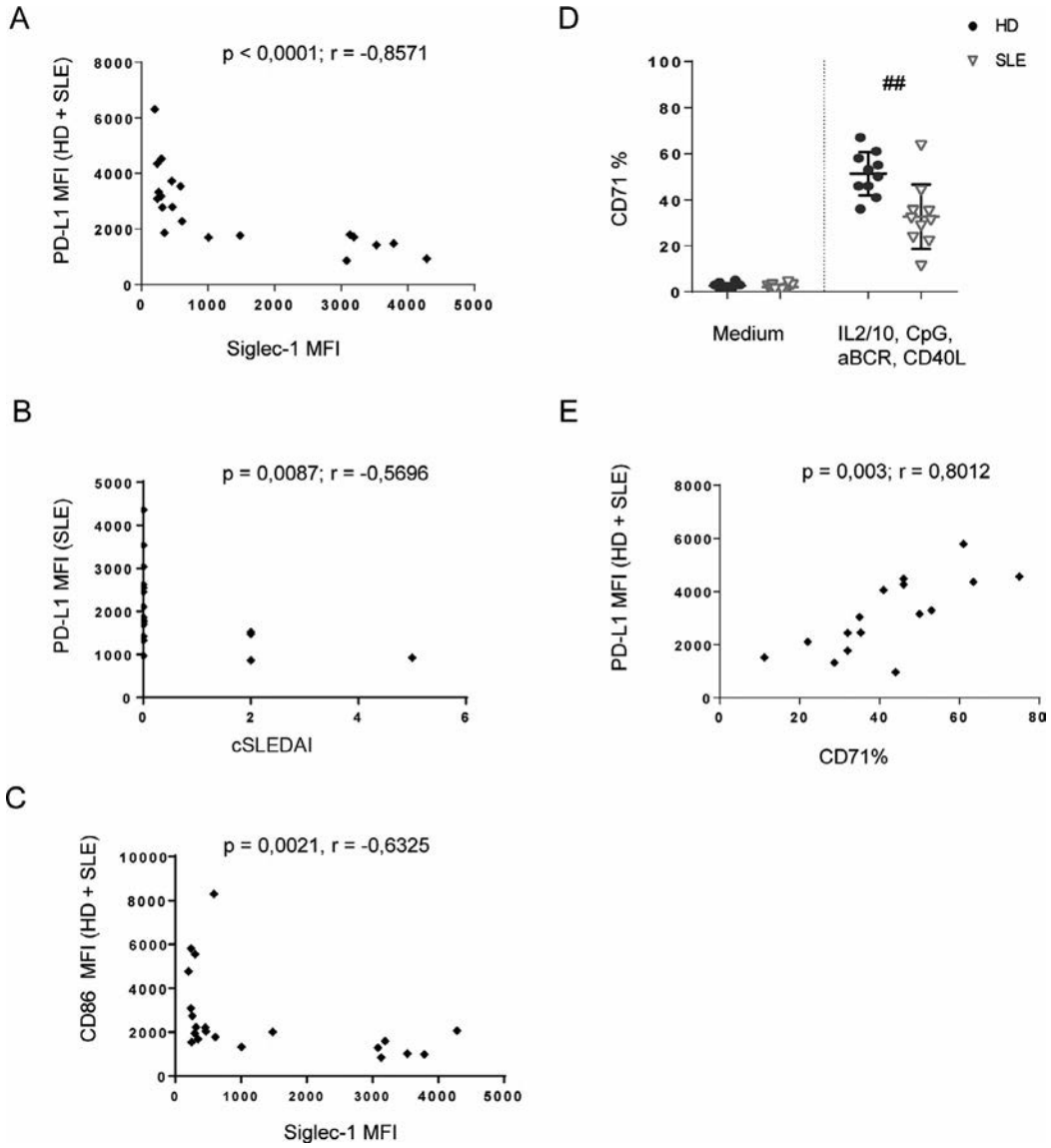

Figure 3. Correlation between PD-L1 expression after stimulation and type I interferon signature and proliferation. **A** and **C**, Inverse correlation between expression of sialic acid–binding Ig-like lectin 1 (Siglec-1) (CD169) at baseline and PD-L1 (**A**) or CD86 (**C**) on CD19+CD20+ B cells from 10 patients with SLE and 10 healthy donors after 48 hours of stimulation with interleukin-2 (IL-2)/IL-10, CpG, anti–B cell receptor (anti-BCR), and CD40L. **B**, Inverse correlation between clinical SLE Disease Activity Index (cSLEDAI) score and PD-L1 expression on CD19+CD20+ B cells after 48 hours of stimulation with IL-2/IL-10, CpG, anti-BCR, and CD40L. **D**, Frequency of CD71-expressing CD19+CD20+ B cells, as a marker of proliferation, with and without stimulation for 48 hours with IL-2/IL-10, CpG, anti-BCR, and CD40. ## = $P \leq 0.01$, SLE patients versus healthy donors. Symbols represent individual subjects; bars show the mean ± SD. **E**, Correlation between CD71 and PD-L1 expression on CD19+CD20+ B cells after 48 hours of stimulation with IL-2/IL-10, CpG, anti-BCR, and CD40L. See Figure 1 for other definitions.

(14) in SLE patients. In our cohort, we also observed increased PD-1 expression in CD27–IgD– double-negative B cells from SLE patients compared to healthy controls. The low frequency of reported CD27– B cell subsets makes appropriate monitoring of the surface expression of PD-1 molecules challenging.

Interestingly, a positive correlation was found between higher expression of PD-1 on SLE T cells and higher expression on B cells, suggesting a shared regulation of PD-1 membrane expression on these cells in SLE. Since it is known that expression of PD-1 and its ligands depends on cellular activation, we addressed the question if and how certain stimulation conditions are able to modulate/induce membrane expression of these molecules on B lymphocytes. As members of the adaptive immune system, B cells require at least 2 signals for proper activation. The first signal occurs with antigen recognition through the BCR. The second signal occurs by engagement of activating coreceptors during interaction with T cells (via the CD40/CD40L axis) or by activation of TLR-9 via CpG sequences. We showed that PD-L1 upregulation was significantly diminished in B cells from SLE patients compared to those from healthy controls upon CpG stimulation alone or in combination with anti-BCR and CD40L. PD-L1 expression correlated inversely with the type I IFN signature as well as

lupus disease activity. B cell stimulation via anti-BCR, CpG, and CD40L up-regulated PD-L1 membrane expression, especially by proliferating B cells, in healthy donors or SLE patients. These data are consistent with the conclusion that proliferation seems to be a key mechanism to enhance PD-L1 expression on B cells, control ensuing immune responses, and restore homeostasis.

Prior studies on B cells in SLE have shown an altered responsiveness to TLR-9 stimulation, such as cytokine production, activation, and proliferation (1). In this context, our data support the idea that post-activated, hyporesponsive SLE B cells are marked by significantly enhanced PD-1 expression at baseline but show functionally reduced responses upon TLR-9 and anti-BCR stimulation, with diminished proliferation along with reduced PD-L1 up-regulation capacity. TLR-9 stimulation via CpG also resulted in diminished expression of the costimulatory molecule CD40 and a lower frequency of activated CD86+ SLE B cells compared to cells from healthy donors. Thus, the reduced up-regulation of PD-L1 by SLE B cells also affects expression of coinhibitory and costimulatory molecules, suggesting a more general hyporesponsiveness and impaired regulatory functions. In this regard, the current results provide insight into the crucial role of T cell–dependent activation of SLE B cells via CD40/CD40L interaction. While TLR-9 engagement in SLE B cells appeared to be abnormal and possibly related to their post-activation status, activation of CD40 resulted in consistent up-regulation of inhibitory molecules PD-1, PD-L1, and PD-L2 and stimulatory molecules CD86 and CD40. However, the functional impact of the observed hyporesponsive status is still a matter of debate. For example, other than in chronic infections, exhausted CD8+ T cells seem to predict a favorable prognosis in autoimmune diseases such as SLE (15).

The study has some limitations. First, we cannot exclude the possibility that the differences seen after stimulation are related to the differential expansion of B cell subsets between SLE patients and healthy donors (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40897/abstract). Second, all SLE patients included in the study were receiving medication, and most had low disease activity (Supplementary Table 1, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40897/abstract). The influences of treatment on PD-1 expression by SLE B cells are still a matter of debate. We cannot exclude the possibility that higher disease activity could affect the PD-1 axis at baseline in a more pronounced way.

While the PD-1 axis in SLE remains to be further characterized, it was recently shown that PD-1/PD-L1 interactions play a crucial role in the stringency of germinal center affinity selection (16). Moreover, PD-L1–deficient B cells in this mouse model exhibited subsequent outgrowth of low-affinity or irrelevant antibodies (16), suggesting initiation and perpetuation of autoimmunity.

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 submitted for publication. Dr. Stefanski 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. Stefanski, Dörner.
Acquisition of data. Reiter, Hiepe, Stefanski.
Analysis and interpretation of data. Wiedemann, Lino, Stefanski, Dörner.

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The Epidemiology of Antiphospholipid Syndrome: A Population-Based Study

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Objective. To estimate the annual incidence and prevalence of and frequency of mortality associated with antiphospholipid syndrome (APS).

Methods. An inception cohort of patients with incident APS in 2000–2015 from a geographically well-defined population was identified based on comprehensive individual medical records review. All cases met the 2006 Sydney criteria for APS (primary definition) or had a diagnosis of APS confirmed by physician consensus (secondary definition). Levels of lupus anticoagulant, IgM and IgG anticardiolipin antibodies, and anti–β2-glycoprotein I antibodies were tested in a centralized laboratory. Incidence rates were age- and sex-adjusted to the 2010 US white population. Prevalence estimates were obtained from the incidence rates, assuming that there was no increased mortality associated with APS and that migration in or out of the area was independent of disease status.

Results. Among this cohort in 2000–2015, 33 cases of incident APS, as defined by the Sydney criteria, were identified (mean age of patients 54.2 years; 55% female, 97% white). The annual incidence of APS in adults ages ≥18 years was 2.1 (95% confidence interval [95% CI] 1.4–2.8) per 100,000 population. Incidence rates were similar in both sexes. The estimated prevalence of APS was 50 (95% CI 42–58) per 100,000 population, and was similar in both sexes. Six patients (18%) had a concurrent diagnosis of systemic lupus erythematosus. The most frequent clinical manifestation was deep vein thrombosis. The overall frequency of mortality among patients with APS was not significantly different from that in the general population (standardized mortality ratio 1.61, 95% CI 0.74–3.05).

Conclusion. APS occurred in ~2 persons per 100,000 population per year. The estimated prevalence was 50 per 100,000 population. Overall mortality was not notably different from that observed in the general population.

INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by vascular (arterial and/or venous) thrombosis and/or pregnancy morbidity in the presence of antiphospholipid antibodies (aPL) (1). APS occurs alone or in association with other autoimmune diseases, particularly systemic lupus erythematosus (SLE).

APS is defined according to the 2006 Sydney international consensus criteria for the classification of APS (2). It requires the presence of a clinical criterion, either a vascular (venous or arterial) thrombosis or pregnancy morbidity, and a laboratory criterion, based on measurements of persistent aPL on 2 or more occasions, at least 12 weeks apart. The types of aPL accepted for the laboratory criterion include the lupus anticoagulant (LAC), anticardiolipin (aCL) IgG and IgM, and anti–β_2-glycoprotein I (anti-β_2GPI) IgG and IgM antibodies.

It has been speculated that APS is a leading cause of thrombosis and pregnancy morbidity particularly in the young. However, the incidence and prevalence of APS is unknown. Epidemiologic characteristics of the disease have been described in specific disease cohorts, such as patients with SLE or those with stroke, but the burden of the disease in the general population remains unknown (3).

Estimating the frequency of APS in the general population has been identified as an urgent need in order to understand the magnitude of the disease burden (4). In a systematic review, the authors concluded that there was a lack of robust scientific data to estimate the frequency of APS in the population (5). The most common methodologic barriers identified were that included patients were

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individuals with a single positive finding for aPL with no confirmation at least 12 weeks later, as well as inclusion of low-titer aCL or anti-β_2GPI antibodies when detected by enzyme-linked immunosorbent assay at a recommended cutoff level of 40 units. The available studies have been mostly retrospective in nature and performed at major referral medical centers, and none were population-based (5).

The aim of this study was to characterize the annual incidence and prevalence of APS and ascertain the frequency of mortality associated with APS in a population-based cohort of patients with APS in 2000–2015 from Olmsted County, Minnesota.

PATIENTS AND METHODS

Study design. Through the resources of the Rochester Epidemiology Project (REP), a record linkage system, the population of Olmsted County, Minnesota is well suited for investigation of the epidemiology of APS because comprehensive medical records for all residents seeking medical care are available. The REP allows ready access to the medical records from all health care providers for the local population, including the Mayo Clinic, the Olmsted Medical Center and their affiliated hospitals, local nursing homes, and the few private practitioners. Data about dates and causes of death are routinely tracked and readily available. This system ensures virtually complete ascertainment of all clinically recognized cases of APS among the residents of Olmsted County, Minnesota (6). The demographics, distribution of morbidity, and death rates in Olmsted County are similar to those in the state of Minnesota and the upper Midwest. The characteristics and strengths of the REP, as well as its generalizability, have been described elsewhere (7,8).

The population size of Olmsted County in 2010 was 144,248, with 74.7% of individuals being age ≥18 years. Furthermore, the ethnic distribution of the population in 2010 was 85.7% white, 4.2% Hispanic, 4.8% African American, 5.5% Asian/Native Hawaiian/Pacific Islander, and 0.2% American Indian/Alaska Native. The study was approved by the institutional review boards of the Mayo Clinic and Olmsted Medical Center.

Case finding and ascertainment. Potential cases of APS were identified based on laboratory reports. The case-finding strategy was designed to be highly sensitive and comprehensive. We queried the REP patient database for any individuals who were tested for aPL, either anti-β_2GPI IgG or IgM antibodies or LAC IgG or IgM antibodies (by dilute Russell viper venom time [DRVVT], DRVVT mix, DRVVT confirmation, or STACLOT), for whom the test result was reported as out of range or abnormal. Those with at least 2 abnormal APS antibody test results at any point in time between January 1, 2000 and December 31, 2015 were selected for extensive chart review. All of the APS-related laboratory studies in Olmsted County, regardless of provider, were performed at the Special Coagulation Laboratory (Mayo Clinic, Rochester, MN), and were interpreted and standardized based on international consensus criteria.

Identification of the cohort of patients with APS meeting the 2006 Sydney criteria was performed by rigorous application of the classification criteria, including the following features: 1) all cases had to have a recorded venous or arterial thrombotic event or pregnancy morbidity; 2) the time interval between the initial and repeated laboratory testing had to be at least 12 weeks apart; and 3) aCL and anti-β_2GPI antibodies were considered positive only if they had a value of ≥40 IgG phospholipid (GPL)/IgM phospholipid (MPL) units. Although in the Sydney classification criteria for APS there is no clear threshold for anti-β_2GPI antibody positivity, we used the cutoff of ≥40 GPL/MPL units because it is easier to replicate and apply elsewhere. For those cases where the laboratory test was performed before the implementation of GPL or MPL units as a measure of anti-β_2GPI antibodies, an elevated serum titer was considered positive, if reported as such by the laboratory.

The Sydney criteria are often used in clinical research as the diagnostic classification criteria for APS and commonly used in clinical practice as a framework for diagnosis. However, they do not include all of the manifestations of the disease, such as thrombocytopenia or heart valvulopathies. Furthermore, patients whose confirmatory laboratory testing is done fewer than 12 weeks apart will not fulfill the classification criteria. Thus, we used 2 definitions of an APS case, with 1 being based on the Sydney criteria and 1 being augmented by physician diagnosis. Physician-diagnosed cases of APS were those not meeting the Sydney criteria. Patients not classified as having APS by the Sydney criteria but who had 1 of the following characteristics were considered to be probable cases: 1) presence of clinical features of APS not included in the updated Sydney criteria, such as thrombocytopenia or Libman-Sacks endocarditis or chorea, among others (2); 2) diagnosed as having APS by a physician; or 3) meeting clinical criteria but not laboratory criteria based on borderline results (i.e., <40 GPL/MPL units) for aPL antibody positivity or timing of the laboratory criterion confirmation. These potential cases were independently evaluated by 2 rheumatologists (MMP and KGM) and 1 hematologist (RKP), and patients with physician consensus–defined APS were included under the physician definition if at least 2 of 3 of the evaluating physicians agreed.

The cases identified by the Sydney criteria definition were used for the primary analysis. The expanded cohort, including those who met the Sydney criteria and those who met the physician diagnosis definition, was used for secondary analysis. The incidence date of APS was defined as the earliest date of criteria fulfillment (i.e., date of the laboratory confirmation) or the date of the laboratory tests closest to 12 weeks apart for the additional patients who did not fulfill the criteria. Patients needed to be Olmsted County residents on the APS incidence date to be included in the inception cohort.

The review of all medical records and data extraction were performed using a standardized data extraction form by 2 investigators (ADG and MMP) and verified separately by 1 of them (ADG). Data regarding age, sex, self-reported race and ethnicity, date of diagnosis and date of last follow-up, vital status, clinical characteristics, and laboratory findings were recorded.

Testing for aPL. All reagents were from US sources, unless stated otherwise. Briefly, reagents for determination of prothrombin time included Dade Innovin (Siemens) and HemosIL RecombiPlasTin2G (Instrumentation Laboratory), and for determination of activated partial thromboplastin time, Platelin (BioMerieux) and HemosIL SynthASil (Instrumentation Laboratory) were used. The source of normal pooled plasma and factor-deficient plasma was PrecisioBioLogic. Over the study period, assays were performed on an MDA-180 (Organon Teknika), followed by use of a Sta-R (Stago) and, more recently, an ACL TOP 700 (Instrumentation Laboratory). All assays were performed in accordance with the manufacturers' instructions. LAC testing was performed using DRVVT (CRYOcheck, LA CHECK, and LA SURE; PrecisionBioLogic) and STACLOT-LA (Diagnostica Stago) on an ACL TOP 700. Testing for aCL and anti-β_2GPI was performed using a VarelisA kit performed on an Alisei platform. Information about all of the kits and reagents used for antibody testing before 2005 was not available.

Statistical analysis. Age- and sex-specific incidence rates of APS were calculated both in the Sydney criteria–defined case cohort and in the expanded cohorts that also included physician consensus–defined cases, using the number of incident cases as the numerator and population estimates based on decennial census counts as the denominator, with linear interpolation used to estimate population size for intercensal years. Overall incidence rates were age- and/or sex-adjusted to the estimated 2010 white population of the US. In order to compute 95% confidence intervals (95% CIs) for incidence rates, it was assumed that the number of incident cases followed a Poisson distribution. Trends in incidence rates were examined using Poisson regression methods with smoothing splines for age and calendar year. Survival rates following the diagnosis of APS were estimated using Kaplan-Meier methods, and were compared to the expected survival rates in the Minnesota white population.

Prevalence was calculated using a cohort method. Prevalence can be easily estimated from incidence when the following 3 conditions are met: 1) the disease is not associated with any excess mortality, 2) there are no important calendar time trends, and 3) migration in or out of the census population is independent of disease status. The method involves applying age-, sex-, and calendar year–specific incidence rates of disease and mortality rates from life tables to a hypothetical population, to yield estimates of prevalence (9). The CIs for the prevalence estimates were obtained using bootstrap methods. The estimated number of persons in the US with APS on January 1, 2015 was estimated by applying the age- and sex-specific US population counts from the US Census Bureau to the estimated prevalence rates. Analyses were performed using SAS software version 9.4 (SAS Institute) and R version 3.4.2 (R Foundation for Statistical Computing).

RESULTS

Demographic, clinical, and laboratory characteristics of the APS incident cohort and time of diagnosis. As illustrated in the flow diagram in Figure 1, 501 cases with at least 2 abnormal test results reported by the laboratory were screened. A total of 59 potential cases of APS were identified

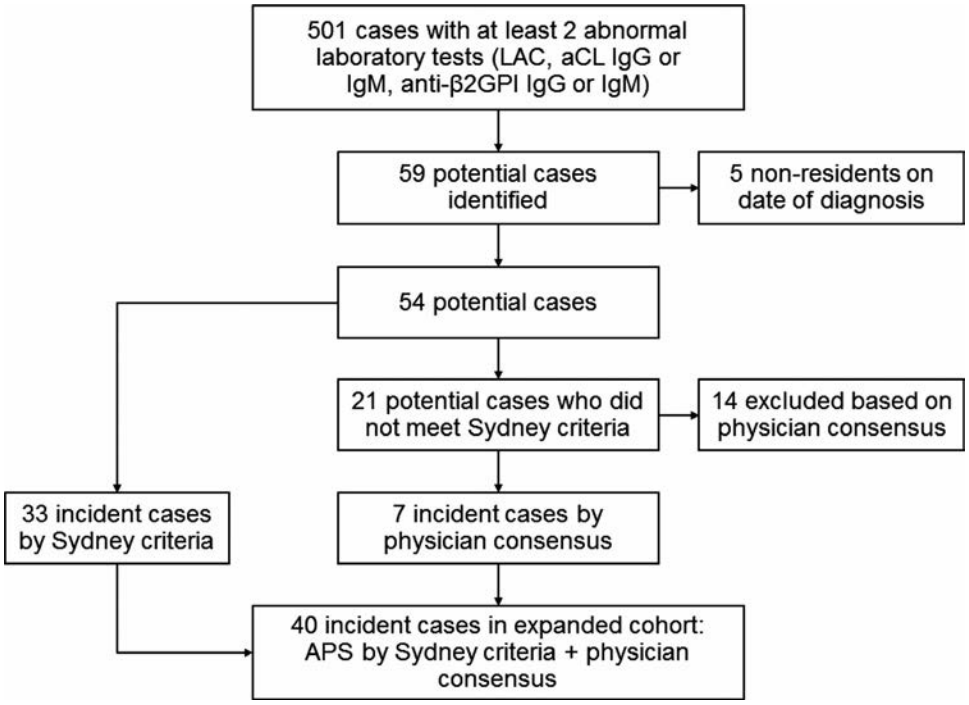

Figure 1. Flow chart describing the screening process for the identification of patients with antiphospholipid syndrome (APS) diagnosed in Olmsted County, Minnesota from 2000 to 2015. LAC = lupus anticoagulant; aCL = anticardiolipin; anti-β_2GPI = anti–β_2-glycoprotein I.

as meeting the eligibility criteria. Five cases were excluded since those individuals were not residents of Olmsted County at the time of diagnosis. The primary analysis included 33 subjects who met the Sydney criteria for APS. An additional 21 subjects were reviewed by 3 physician experts, and 7 cases of APS were identified; the rest (14 cases) were excluded. A total of 40 incident cases of APS were identified on the basis of the Sydney criteria or, for the secondary analysis, by physician diagnosis consensus.

A summary of the demographic and clinical characteristics of the patients at the time of diagnosis in the Sydney criteria–defined APS cohort and the expanded cohort including physician diagnosis is shown in Table 1. The mean age in the Sydney criteria–defined APS cohort was 54.2 years, and 55% were female. In both cohorts, the majority of the patients were white; 1 patient was African American. Around one-fifth of each cohort had a diagnosis of SLE according to the American College of

Table 1. Demographic and clinical characteristics of the patients with incident APS by cohort*

	Sydney criteria cohort (n = 33)	Expanded cohort (n = 40)
Age, mean ± SD years	54.2 ± 18.5	55.7 ± 19.0
Sex, female	18 (55)	20 (50)
Race, white	32 (97)	39 (98)
Length of follow-up, mean ± SD years	8.3 ± 4.9	8.2 ± 4.7
Smoker (current or former)	13 (39)	16 (40)
SLE diagnosis	6 (18)	7 (18)
Thrombosis	33 (100)	40 (100)
DVT	14 (42)	19 (48)
PE	13 (39)	15 (38)
TIA	4 (12)	4 (10)
Stroke	11 (33)	14 (35)
MI	0	0
Peripheral arterial thrombosis	2 (6)	3 (8)
Biopsy-proven microvascular thrombosis	2 (6)	2 (5)
Pregnancy morbidity	3/18 (17)	3/20 (15)
Noncriteria manifestations	13 (39)	16 (40)
Livedo	3 (9)	3 (8)
Superficial thrombophlebitis	1 (3)	3 (8)
Chronic cutaneous ulcers	2 (6)	2 (5)
Cardiac valve disease	1 (3)	1 (3)
Pulmonary hypertension	4 (12)	5 (13)
aPL nephropathy	0	0
Thrombocytopenia (<100,000)	5 (15)	5 (13)
Other neurologic manifestations†	2 (6)	2 (5)
APS antibody profile		
Positive for LAC	24/32 (75)	27/39 (69)
Positive for aCL	23/33 (70)	24/40 (60)
IgG	13/33 (39)	13/40 (33)
IgM	14/33 (42)	15/38 (38)
Positive for anti-β_2GPI	5/9 (56)	5/12 (42)
IgG	2/9 (22)	2/12 (17)
IgM	1/9 (11)	2/12 (8)

* Patients were diagnosed as having antiphospholipid syndrome (APS) in Olmsted County, Minnesota from 2000 to 2015 and grouped according to those who met the Sydney criteria for classification of APS or those in the expanded cohort, in which 7 patients were included based on physician consensus diagnosis. Except where indicated otherwise, values are the number/total number assessed (%) of patients. SLE = systemic lupus erythematosus; DVT = deep vein thrombosis; PE = pulmonary embolism; TIA = transient ischemic attack; MI = myocardial infarction; aPL = antiphospholipid antibody; LAC = lupus anticoagulant; aCL = anticardiolipin; anti-β_2GPI = anti–β2-glycoprotein I.
† Includes seizures and cognitive dysfunction.

Rheumatology classification criteria (10). The demographic characteristics of subjects in the expanded cohort were similar.

Thrombotic events were significantly more frequent than obstetric events or other noncriteria manifestations of the disease. In the population-based, Sydney criteria–defined APS cohort, deep vein thrombosis (42%), followed closely by pulmonary embolism (39%), were the most frequent thrombotic manifestations of APS, while the most frequent arterial manifestations were ischemic stroke and peripheral arterial thrombosis. No myocardial infarctions were recorded at the time of diagnosis. Pregnancy morbidity was identified in 3 (17%) of 18 female patients in the Sydney criteria–defined APS cohort. These 3 women had 3 or more embryonic losses before week 10 of pregnancy, while 2 of these patients also experienced at least 1 fetal death after week 10, and 1 gave birth to a premature baby before week 34. Forty percent of the patients had at least 1 noncriteria manifestation: thrombocytopenia was the most frequently observed (15%), while chronic cutaneous ulcers and cardiac valve disease were observed in 2 patients (6%) and 1 patient (3%), respectively.

LAC and aCL IgG and IgM antibodies were assessed in the majority of patients, but only 9 of 33 patients had anti-β_2GPI IgG and IgM antibodies tested. Overall, three-fourths of the cases were positive for LAC and either IgG or IgM aCL, while ≤20% of patients who were tested for anti-β_2GPI antibodies were positive.

The expanded cohort had similar proportions of clinical manifestations except with regard to the antibody profiles. In this cohort that included physician consensus–diagnosed cases, the proportion of patients considered to be positive for APS antibodies (≥40 GPL/MPL units) was lower, as expected. The characteristics of the 7 patients in the expanded cohort are detailed in Supplementary Table 1 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40901/abstract). Three patients did not meet the criteria for APS because the laboratory confirmation was performed in <12 weeks, while 4

Table 2. Annual incidence of antiphospholipid syndrome in Olmsted County, Minnesota 2000–2015 by sex and age*

	Sydney criteria cohort		Expanded cohort	
	No. of incident cases	Incidence rate (95% CI)	No. of incident cases	Incidence rate (95% CI)
Sex				
Female				
By age				
18–44 years	6	1.4	6	1.4
45–54 years	3	1.8	3	1.8
55–64 years	2	1.7	2	1.7
65–74 years	1	1.3	1	1.3
>75 years	6	7.7	8	10.2
Total†	18	2.1 (1.1–3.1)	20	2.4 (1.3–3.4)
Male				
By age				
18–44 years	7	1.7	8	2.0
45–54 years	1	0.6	1	0.6
55–64 years	6	5.4	8	7.3
65–74 years	0	0.0	1	1.5
>75 years	1	2.0	2	4.0
Total†	15	2.0 (1.0–3.0)	20	2.7 (1.5–3.9)
Overall				
By age				
18–44 years	13	1.6	14	1.7
45–54 years	4	1.2	4	1.2
55–64 years	8	3.5	10	4.4
65–74 years	1	0.7	2	1.4
>75 years	7	5.5	10	7.8
Total‡	33	2.1 (1.4–2.8)	40	2.6 (1.8–3.4)

* Subjects were divided into cohorts according to those who met the Sydney classification criteria for antiphospholipid syndrome or the expanded cohort that included cases by physician consensus diagnosis. 95% CI = 95% confidence interval.
† Age-adjusted to the 2010 US white population.
‡ Age- and sex-adjusted to the 2010 US white population.

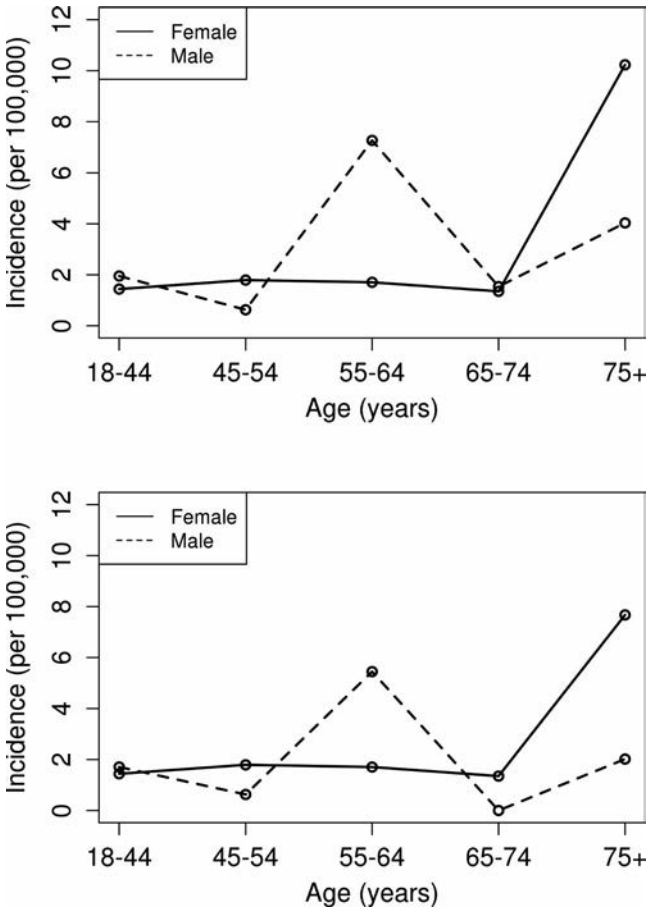

Figure 2. Age- and sex-specific incidence rates of antiphospholipid syndrome in subjects considered to be cases by the Sydney criteria definition (top) and in subjects in the expanded cohort that included case definition by physician consensus (bottom).

patients did not meet the criteria because they had titers of APS antibodies lower than the cutoff value.

Incidence and prevalence of APS in the study population. Using the Sydney criteria definition, 33 cases of APS were diagnosed during 2000–2015. Annual incidence rates stratified by age and sex are detailed in Table 2. The overall annual incidence rate of APS in adults ages ≥18 years (age- and sex-adjusted to the 2010 US white population) was 2.1 (95% CI 1.4–2.8) per 100,000 population. In the female population, the age-adjusted incidence rate was 2.1 (95% CI 1.1–3.1), and in the male population, it was 2.0 (95% CI 1.0–3.0). Age-specific incidence rates of APS peaked in those who were age 75 years or older (Figure 2); the incidence of APS increased significantly with age ($P = 0.007$). There was no evidence of a difference in APS incidence rates by sex, and no evidence of a differential age effect on APS incidence rates in men versus women. The APS incidence rates were slightly higher in the expanded cohort. We did not observe trends in the incidence rates over time.

The estimated prevalence of APS (adjusted to the 2010 US white population) was 50 (95% CI 42–58) per 100,000 population. The prevalence was 51 (95% CI 31–72) per 100,000 population among women, and 48 (95% CI 29–68) per 100,000 population among men. Based on these findings and the US Census data, an estimated 119,300 persons in the US were affected by APS in 2015. The prevalence of APS overall and by sex in the expanded cohort was somewhat higher than that in the Sydney criteria–defined case cohort (Table 3).

Mortality rates among APS cases compared to the general population of the geographic region. During a median follow-up of 8.3 years, there were 9 deaths in the Sydney criteria–defined incident APS cohort. Based on the Minnesota life tables, 5.6 deaths were expected. The standardized mortality ratio (SMR) of APS in patients who met the Sydney criteria for APS was 1.61 (95% CI 0.74–3.05). In this population after the incidence of APS, the 10-year survival rate was 80% (95% CI 66–100%). Results with regard to mortality and survival rates were similar in the expanded cohort (Table 4).

DISCUSSION

In recent literature, Erkan and Lockshin stated that "Although there are many speculations, epidemiology of APS is yet to be elucidated" (11). Previous studies have assessed the incidence and prevalence of APS in particular disease cohorts (e.g., SLE), and the available APS studies are based on data from referral centers and rheumatology practices (12,13). The present population-based study of clinically identified APS is the first to describe basic and fundamental epidemiologic data on incidence, prevalence, and mortality rates, which are needed to understand the population burden of APS. Overall, the age- and sex-adjusted annual incidence of APS was 2.1 per 100,000 population, and the prevalence was 50 per 100,000 population. The incidence was similar between women and men, and mortality was not different from that in the general population.

Table 3. Estimated age- and sex-adjusted prevalence rates of antiphospholipid syndrome in Olmsted County, Minnesota 2000–2015*

	Sydney criteria cohort	Expanded cohort
Overall	50 (42–58)	59 (52–67)
Females	51 (31–72)	54 (41–70)
Males	48 (29–68)	64 (44–86)

* Subjects were divided into cohorts according to those who met the Sydney classification criteria for antiphospholipid syndrome or the expanded cohort that included cases by physician consensus diagnosis. Values are the prevalence rate (per 100,000 population) (95% confidence interval).

Table 4. Mortality and survival rates in patients with incident antiphospholipid syndrome among residents of Olmsted County, Minnesota 2000–2015*

	Sydney criteria cohort	Expanded cohort
No. of patients	33	40
Observed no. of deaths	9	10
Expected no. of deaths	5.6	7.6
SMR (95% CI)	1.61 (0.74–3.05)	1.32 (0.63–2.42)
Survival rate, % (95% CI)		
2 years	97 (91–100)	98 (93–100)
5 years	90 (79–100)	91 (82–100)
10 years	80 (66–100)	83 (70–98)

* Subjects were divided into cohorts according to those who met the Sydney classification criteria for antiphospholipid syndrome or the expanded cohort that included cases by physician consensus diagnosis. SMR = standardized mortality ratio; 95% CI = 95% confidence interval.

In general, the incidence of autoimmune diseases, such as Sjögren's syndrome, rheumatoid arthritis, and in particular SLE, tends to be more common in women than in men (14–16). In this incident APS cohort, the disease rates were very similar between men and women. In a large European APS cohort, the female-to-male ratio among patients with primary APS was 5; however, after exclusion of patients with SLE, the female-to-male ratio decreased to 3.5, and decreased to 1.0 after the obstetric APS cases were excluded. This estimate is similar to our findings and to those reported in the APS Alliance for Clinical Trials and International Networking (APS ACTION) clinical database (11).

The frequency of aPL antibodies reportedly increases with age, but it remains unclear whether these antibodies are pathogenic or an epiphenomenon of a malignancy or other underlying process (17). Furthermore, the frequency of thrombotic events increases in relation to aging as well. These factors are limitations of the current definition of APS. In accordance with current classification standards, the incidence rates observed in the current study population peaked late in life, with a notably higher incidence of APS in subjects after the age of 55 years, and especially in those over the age of 75 years.

Although the mortality rate was not significantly different between cases with APS and the general Minnesota population, these findings do not exclude the possibility of increased mortality among those with APS. In the Euro-phospholipid cohort, the unadjusted SMR was 1.8 (18), which is consistent with our findings. However, the 5- and 10-year survival rates were lower in our inception cohort, likely due to differences in the age of the patients in our study compared to the Euro-phospholipid study (18).

The clinical manifestations observed in patients in the current cohort were consistent with those reported by others. Deep vein thrombosis was the most frequent manifestation, occurring in 42% of the cases, while stroke was the most common arterial event (33%), and 17% of the women in the study had pregnancy morbidity. Although the Sydney criteria only includes thrombotic events and pregnancy morbidity, 40% of the cases had at least 1 noncriteria manifestation of the disease, highlighting the importance of noncriteria manifestations and the systemic nature of the disease. However, other manifestations, such as livedo reticularis and renal disease, were not as frequent. Since these data were recorded in routine healthcare encounters, the lack of systematic assessment may explain some of the discrepancies.

This study has several limitations, inherent to all retrospective study designs. First, the study was based on medical records review of clinically identified cases of APS and not on a serologic survey; thus, case ascertainment depended on the completeness of evaluation and documentation by the providers. Consequently, the burden of understudied and undiagnosed cases remains unknown. We rigorously applied the Sydney criteria; the GPL and MPL cutoff values had to be ≥40 GPL/MPL units, and the aPL testing had to be confirmed 12 weeks later. While almost all of the cases were tested for aCL and LAC antibodies, only 9 of 33 patients were evaluated for anti-β_2GPI. This may be attributable to the wide variety of specialties involved in the care of these patients, and the lack of familiarity with current assessment standards. Although the APS literature is published in the fields of rheumatology and hematology, patients in this cohort were assessed by a wide variety of clinicians, including neurologists, primary care physicians, and cardiologists, among others.

Second, our results can be generalized to subjects whose demographic profile is similar to that of the upper Midwest. Furthermore, APS is more common in patients with SLE, and therefore it is possible that the burden of APS may be higher in those ethnic groups in whom SLE is more common. Our results may not be generalizable to more diverse populations with other ethnic or racial compositions.

Third, our study population was too small to provide stable incidence rates. This limitation is inherent to the population covered by the records-linkage system of the REP, which, in this study, included only residents of Olmsted County.

The major strengths of our study are that the case-finding strategy relied on laboratory data that were obtained in the same laboratory throughout the span of the study. Furthermore, the records linkage system of the REP facilitated our population-based study, since it allowed us to identify all cases of clinically detected APS in the community and to verify the cases by comprehensive medical records review, thereby minimizing referral bias and misclassification.

In conclusion, the results from this population-based study revealed that definite APS occurred in ~2 persons per 100,000 population per year. The estimated prevalence was 50 per 100,000 population. The overall frequency of mortality was not different from that in the general population. The incidence and

prevalence of APS in the same population was at least as common as SLE (15).

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. Duarte-García 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. Duarte-García, Crowson, Amin, Warrington, Matteson.

Acquisition of data. Duarte-García, Pham, Moder, Pruthi, Matteson.

Analysis and interpretation of data. Duarte-García, Crowson, Warrington, Matteson.

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Phenotypes Determined by Cluster Analysis and Their Survival in the Prospective European Scleroderma Trials and Research Cohort of Patients With Systemic Sclerosis

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Objective. Systemic sclerosis (SSc) is a heterogeneous connective tissue disease that is typically subdivided into limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc) depending on the extent of skin involvement. This subclassification may not capture the entire variability of clinical phenotypes. The European Scleroderma Trials and Research (EUSTAR) database includes data on a prospective cohort of SSc patients from 122 European referral centers. This study was undertaken to perform a cluster analysis of EUSTAR data to distinguish and characterize homogeneous phenotypes without any a priori assumptions, and to examine survival among the clusters obtained.

Methods. A total of 11,318 patients were registered in the EUSTAR database, and 6,927 were included in the study. Twenty-four clinical and serologic variables were used for clustering.

Results. Clustering analyses provided a first delineation of 2 clusters showing moderate stability. In an exploratory attempt, we further characterized 6 homogeneous groups that differed with regard to their clinical features, autoantibody profile, and mortality. Some groups resembled usual dcSSc or lcSSc prototypes, but others exhibited unique features, such as a majority of lcSSc patients with a high rate of visceral damage and antitopoisomerase antibodies. Prognosis varied among groups and the presence of organ damage markedly impacted survival regardless of cutaneous involvement.

Conclusion. Our findings suggest that restricting subsets of SSc patients to only those based on cutaneous involvement may not capture the complete heterogeneity of the disease. Organ damage and antibody profile should be taken into consideration when individuating homogeneous groups of patients with a distinct prognosis.

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Drs. Sobanski and Giovannelli contributed equally to this work. Drs. Launay and Hachulla contributed equally to this work.

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Data are available from the European Scleroderma Trials and Research database upon request.

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INTRODUCTION

Systemic sclerosis (SSc) is a chronic disease that affects connective tissue and is characterized by vascular damage, autoimmunity, and fibrosis. The European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) have recently developed new classification criteria for SSc (1). To date, the subclassification of SSc patients mainly relies on the cutaneous involvement subsets proposed by LeRoy et al in 1988 (2–4). It separates patients into 2 main groups: diffuse cutaneous SSc (dcSSc) associated with early skin changes affecting the trunk and proximal limbs, and limited cutaneous SSc (lcSSc), in which skin fibrosis is limited to the hands, face, feet, and forearms. Organ damage can vary between the 2 subsets, with an early and significant incidence of organ damage (lung fibrosis, gastrointestinal [GI] involvement, heart disease, and renal crisis) in dcSSc and pulmonary hypertension (PH) in lcSSc (4). The 2 subsets also differ in autoantibody profile, with a high prevalence (70–80%) of anticentromere antibodies (ACAs) in lcSSc, and a predominant presence of antibodies against topoisomerase I (anti–topo I) in dcSSc (30%) compared to lcSSc in the study by LeRoy et al (4). In addition, mortality is higher in patients with dcSSc than in patients with lcSSc (5,6). Overall, previous studies suggest that lcSSc and dcSSc are 2 clearly differentiated phenotypes with regard to clinical characteristics, serologic profiles, and prognosis (7).

Yet, past and recent studies of large cohorts have challenged this distinction by highlighting an often-neglected heterogeneity among clinical subsets (8–12), as suggested by, for example, lcSSc patients with anti–topo I antibodies and severe interstitial lung disease (ILD). One method of dealing with heterogeneity is to conduct a cluster analysis in order to organize data from a heterogeneous population into a fairly small number of homogeneous groups. Cluster analysis has been applied to various conditions, such as gout (13), chronic heart failure (14), asthma (15), mixed connective tissue diseases (16), and antineutrophil cytoplasmic antibody–associated vasculitis (17). Cluster analyses have also been carried out in 2 SSc studies, to our knowledge (18,19). One of them included patients from the EULAR European Scleroderma Trials and Research (EUSTAR) cohort but was centered on capillaroscopy patterns (18). Another recent study took into account a limited number of cluster variables and a limited number of patients (19). The aim of this study was to distinguish and characterize homogeneous groups of SSc patients using cluster analysis within the large EUSTAR cohort, and analyze survival between the clusters obtained.

PATIENTS AND METHODS

Patient population. SSc patients were included in the prospective, open, multinational SSc EUSTAR cohort beginning in June 2004 (20–22). For the present study, the EUSTAR database was locked in April 2014. Eligible patients were age ≥18 years, fulfilled the ACR criteria for SSc (23), and had a calculable SSc disease duration, i.e., a date of disease onset (defined as the onset of the first non–Raynaud's phenomenon symptom) and at least one date of study visit.

All patients agreed to participate in the EUSTAR cohort by signing informed consent forms approved by the local ethics committees. The study was conducted in accordance with the principles of the Declaration of Helsinki, local laws, and Guidelines for Good Clinical Practice (21,22). See Appendix A for a list of the EUSTAR Collaborators.

Definition and selection of variables. The EUSTAR database contains data on demographic characteristics, disease features, organ damage, laboratory parameters, capillaroscopy, echocardiography, pulmonary function tests (PFTs), and medication. In order to harmonize clinical practices and ensure reliable evaluation of parameters among centers, EUSTAR arranges regular training courses and edits SSc management guidelines (24,25).

Autoantibodies were identified and characterized according to the local center's guidelines (21,22). Clustering variables were selected in order to ensure a global phenotype of SSc patients by considering clinical relevance and representativeness of disease features, eliminating redundant variables providing analogous information, and dismissing variables with a high rate of missing values. We retained 24 variables, including symptoms or organ involvement observed at least once among visits (Raynaud's phenomenon, esophageal, stomach, and intestinal symptoms, digital ulcers, joint synovitis, joint contractures, tendon friction rubs, muscle weakness, muscle atrophy, arterial hypertension, palpitations, and renal crisis), laboratory values (creatine kinase elevation, proteinuria, antinuclear antibody, ACA, and anti–topo I antibody positivity), results of other tests (restrictive defect on PFTs, lung fibrosis on plain radiography, conduction blocks, abnormal diastolic function, suspected PH on cardiac echography), and the peak modified Rodnan skin thickness score (MRSS) observed during follow-up (Table 1 and Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40906/abstract). Each variable included for symptoms or organ involvement, laboratory values, and results of other tests was considered positive for a specific patient if "yes" was recorded at least once for that variable at any of the visits included.

Statistical analysis. *Cluster analysis*. Cluster analysis determines the distances between individuals using the combined values of their measured features to obtain groups of individuals who have a greater resemblance to each other than to those in the other groups. Cluster analysis was carried out by ascendant hierarchical clustering of the 24 selected

Table 1. Characteristics of the EUSTAR patients analyzed and not analyzed and characteristics of the patients in the present study by cutaneous subset*

	EUSTAR population			Study population		
	Patients analyzed (n = 6,927)	Patients not analyzed (n = 1,505)	*P*†	dcSSc	lcSSc	*P*†
% of patients	–	–	–	42	58	–
Demographic characteristics						
Sex, female	86 (6,924)	83 (1,505)	<0.001	80	91	<0.001
Ethnicity			<0.001			<0.001
White	95 (3,973)	87 (1,176)		92	97	
Asian	3 (3,973)	11 (1,176)		5	2	
Black	2 (3,973)	2 (1,176)		3	1	
Age, mean ± SD years (n)	58.7 ± 13.2 (6,927)	56.3 ± 13.9 (1,505)	<0.001	55.6 ± 13.0	60.9 ± 13.0	<0.001
Age at first non–Raynaud's phenomenon symptom, mean ± SD years (n)	47.3 ± 13.3 (6,927)	47.6 ± 14.1 (1,505)	0.474	45.6 ± 13.2	48.5 ± 13.3	<0.001
Disease duration, mean ± SD years (n)‡	11.4 ± 8.1 (6,927)	8.7 ± 8.1 (1,505)	<0.001	10.0 ± 7.4	12.4 ± 8.5	<0.001
Time from onset of Raynaud's phenomenon to first non–Raynaud's phenomenon symptom, mean ± SD years (n)	3.9 ± 8.0 (5,868)	3.4 ± 8.1 (1,351)	<0.001	2.0 ± 5.6	5.2 ± 9.2	<0.001
Time from first non–Raynaud's phenomenon symptom to EUSTAR enrollment, mean ± SD years (n)	9.4 ± 7.8 (4,875)	7.8 ± 7.8 (1,271)	<0.001	8.0 ± 7.3	10.3 ± 8.1	<0.001
Time from EUSTAR enrollment to last visit, mean ± SD years (n)	2.6 ± 2.5 (4,875)	0.8 ± 1.7 (1,271)	<0.001	2.7 ± 2.6	2.5 ± 2.5	0.031
Body mass index, mean ± SD kg/m^2 (n)	23.6 ± 4.3 (2,483)	24.4 ± 4.8 (889)	<0.001	22.9 ± 4.0	24.1 ± 4.4	<0.001
SSc characteristics						
Autoantibody status						
Antinuclear antibody positive§	96 (6,927)	94 (1,412)	<0.001	97	96	0.400
Anticentromere antibody positive§	37 (6,927)	36 (1,264)	0.751	14	54	<0.001
Anti–topoisomerase I antibody positive§	39 (6,927)	36 (1,270)	0.028	61	23	<0.001
Anti–U1 RNP antibody positive	5 (4,054)	7 (807)	0.006	5	5	0.770
Anti-PM/Scl antibody positive	3 (3,335)	4 (648)	0.278	5	2	<0.001
Anti–RNA polymerase III antibody positive	4 (3,163)	6 (563)	0.025	6	3	<0.001
Cutaneous involvement						
dcSSc	42 (6,913)	38 (1,437)	0.011	–	–	–
Peak MRSS value, mean ± SD (n)§	12.0 ± 9.2 (6,927)	10.9 ± 9.7 (1,170)	<0.001	18.3 ± 9.8	7.5 ± 5.2	<0.001

(Continued)

Table 1. *(Cont'd)*

	EUSTAR population			Study population		
	Patients analyzed (n = 6,927)	Patients not analyzed (n = 1,505)	*P*†	dcSSc	lcSSc	*P*†
Gastrointestinal involvement¶						
Esophageal symptoms§	81 (6,927)	69 (1,498)	<0.001	84	79	<0.001
Stomach symptoms§	42 (6,927)	27 (1,491)	<0.001	47	38	<0.001
Intestinal symptoms§	43 (6,927)	33 (1,497)	<0.001	44	42	0.027
Joint involvement						
Joint contractures§	48 (6,927)	35 (1,492)	<0.001	64	36	<0.001
Joint synovitis§	26 (6,927)	18 (1,496)	<0.001	32	22	<0.001
Tendon friction rubs§	17 (6,927)	8 (1,477)	<0.001	28	9	<0.001
Vascular involvement						
Raynaud's phenomenon§	98 (6,927)	97 (1,500)	<0.001	98	98	0.340
History of or current digital ulcers§	49 (6,927)	35 (1,491)	<0.001	58	42	<0.001
Muscular involvement						
Muscle weakness§	39 (6,927)	24 (1,488)	<0.001	47	33	<0.001
Muscle atrophy§	22 (6,927)	12 (1,484)	<0.001	30	16	<0.001
CK elevation§	13 (6,927)	13 (1,231)	0.711	18	9	<0.001
Cardiac involvement						
Systemic arterial hypertension§	34 (6,927)	27 (1,492)	<0.001	33	35	0.150
Palpitations§	39 (6,927)	26 (1,483)	<0.001	41	38	0.014
Conduction blocks§	22 (6,927)	14 (1,152)	<0.001	24	20	<0.001
LVEF <50%	5 (4,239)	5 (879)	0.799	6	4	<0.001
Abnormal diastolic function§	33 (6,927)	22 (1,116)	<0.001	34	33	0.588
Pericardial effusion	11 (4,442)	8 (920)	0.042	13	9	<0.001
Pulmonary hypertension						
Pulmonary hypertension on echocardiography§	31 (6,927)	22 (1,173)	<0.001	33	29	<0.001
Systolic PAP measured by echocardiography, mean ± SD mm Hg (n)	34.5 ± 15.3 (3,983)	34.2 ± 15.1 (727)	0.041	34.8 ± 16.4	34.2 ± 14.5	0.013
Interstitial lung disease						
Lung fibrosis on plain radiography§	49 (6,927)	39 (1,033)	<0.001	63	39	<0.001
Lung fibrosis on HRCT	57 (3,424)	53 (816)	0.023	68	48	<0.001
Restrictive defect on PFTs§	43 (6,927)	33 (1,083)	<0.001	57	32	<0.001
FVC, mean ± SD % predicted (n)	89.3 ± 21.7 (4,349)	90.0 ± 21.8 (903)	0.437	81.4 ± 21.1	94.9 ± 20.3	<0.001
DL_{CO}, mean ± SD % predicted (n)	61.8 ± 20.1 (6,196)	66.1 ± 21.1 (1,026)	<0.001	57.4 ± 19.9	64.9 ± 19.7	<0.001
6-minute walking distance, mean ± SD meters (n)	392 ± 134 (1,179)	411 ± 145 (338)	0.007	394 ± 137	391 ± 131	0.872

(Continued)

Table 1. *(Cont'd)*

	EUSTAR population			Study population		
	Patients analyzed (n = 6,927)	Patients not analyzed (n = 1,505)	*P*†	dcSSc	lcSSc	*P*†
Renal involvement						
History of renal crisis§	3 (6,927)	3 (1,497)	0.626	5	2	<0.001
Proteinuria§	12 (6,927)	10 (1,308)	0.082	15	9	<0.001
Blood tests						
CRP elevation	36 (4,736)	31 (1,100)	<0.001	44	30	<0.001
Hypocomplementemia	11 (4,469)	10 (860)	0.409	12	11	0.504
Treatment						
Past or current steroids	43 (4,647)	38 (1,081)	0.006	55	34	<0.001
Prednisone, mean ± SD mg/day (n)	4.4 ± 7.5 (4,644)	5.1 ± 9.7 (1,080)	0.081	6.0 ± 8.7	3.3 ± 6.1	<0.001
Past or current immunosuppressive drugs	42 (4,631)	44 (1,085)	0.162	60	28	<0.001

* Except where indicated otherwise, values are the percent (number with data available). EUSTAR = European Scleroderma Trials and Research; dcSSc = diffuse cutaneous systemic sclerosis; lcSSc = limited cutaneous systemic sclerosis; MRSS = modified Rodnan skin thickness score; CK = creatine kinase; LVEF = left ventricular ejection fraction; PAP = pulmonary artery pressure; HRCT = high-resolution computed tomography; PFTs = pulmonary function tests; FVC = forced vital capacity; DLco = diffusing capacity for carbon monoxide; CRP = C-reactive protein.
† By Student's t-test for continuous variables and Fisher's exact test for categorical variables.
‡ Time between the first non–Raynaud's phenomenon symptom and the last visit.
§ Clustering variables.
¶ Esophageal symptoms included dysphagia and/or reflux, stomach symptoms included early satiety and/or vomiting, and intestinal symptoms included diarrhea, bloating, and/or constipation.

variables using Ward's minimum variance method. Results were graphically represented in a dendrogram. We estimated the number of clusters using the visual distance criterion of the horizontal intersection at the highest dissimilarity level on the dendrogram (i.e., where the vertical branches were the longest). In an exploratory approach, we increased the number of clusters considered in the suboptimal visual distance criterion by cutting the dendrogram horizontally at the second highest level of dissimilarity (26).

Evaluation of clusterwise stability and reproducibility is a major issue in cluster analysis (27). To assess stability and reproducibility, we conducted 100 iterations of the clustering process (with the number of clusters in the primary analysis) in randomly selected subsets of up to 50% of the original data set, and estimated the clusterwise stability by computing the Jaccard coefficient (which is a measure of similarity between data sets) between every cluster of the primary analysis and the most comparable cluster retrieved in each iteration (27). A Jaccard similarity index of ≤0.5 indicates a weakly stable and reproducible cluster (28).

The main cluster analysis was carried out in patients without missing data for the 24 selected variables. In order to estimate the impact of late complications on the cluster analysis, we performed a sensitivity analysis by selecting patients with a disease duration of >10 years (adequate time for the occurrence of organ damage). In order to study the possible impact of rare antibodies on the clustering process, we performed a second sensitivity analysis by adding in the clustering variables anti–RNA polymerase III, anti-PM/Scl, and anti–U1 RNP antibodies. Finally, a third sensitivity analysis was conducted to evaluate the potential survival bias, and was restricted to patients with a disease duration at the enrollment visit of <5 years. The descriptive words used to refer to disease features or severity in the Results section (low/mild/moderate/severe) were not used during the clustering process but were used to describe and interpret the groups of patients in accordance with established practice (13,14).

Survival analysis. Survival was assessed using disease duration (the time from disease onset to the most recent date data were obtained). We found that a high percentage (52%) of patients were lost to follow-up (i.e., data last obtained prior to January 2012), which was responsible for a significant overestimation of survival. Because we could not update data with actual vital status, we chose to exclude those patients from the survival analysis. A sensitivity analysis that included those patients was therefore performed. We also performed a sensitivity analysis using onset of Raynaud's phenomenon as the definition of disease onset.

Survival rates were examined using several Cox proportional hazards models: unadjusted, adjusted for age at disease onset, adjusted for age at disease onset and sex, and adjusted for age at disease onset, sex, and immunosuppressive treatment. The proportional hazards assumption for Cox regression models was assessed by the graphical study of Schonfeld's residues,

and the log linearity assumption for quantitative predictors was assessed using cubic spline functions. Finally, we calculated the C-index for each Cox regression model (i.e., the estimation of the probability of concordance, which is equivalent to the area under the receiver operating characteristic curve for logistic regression models). Statistical analyses were carried out using

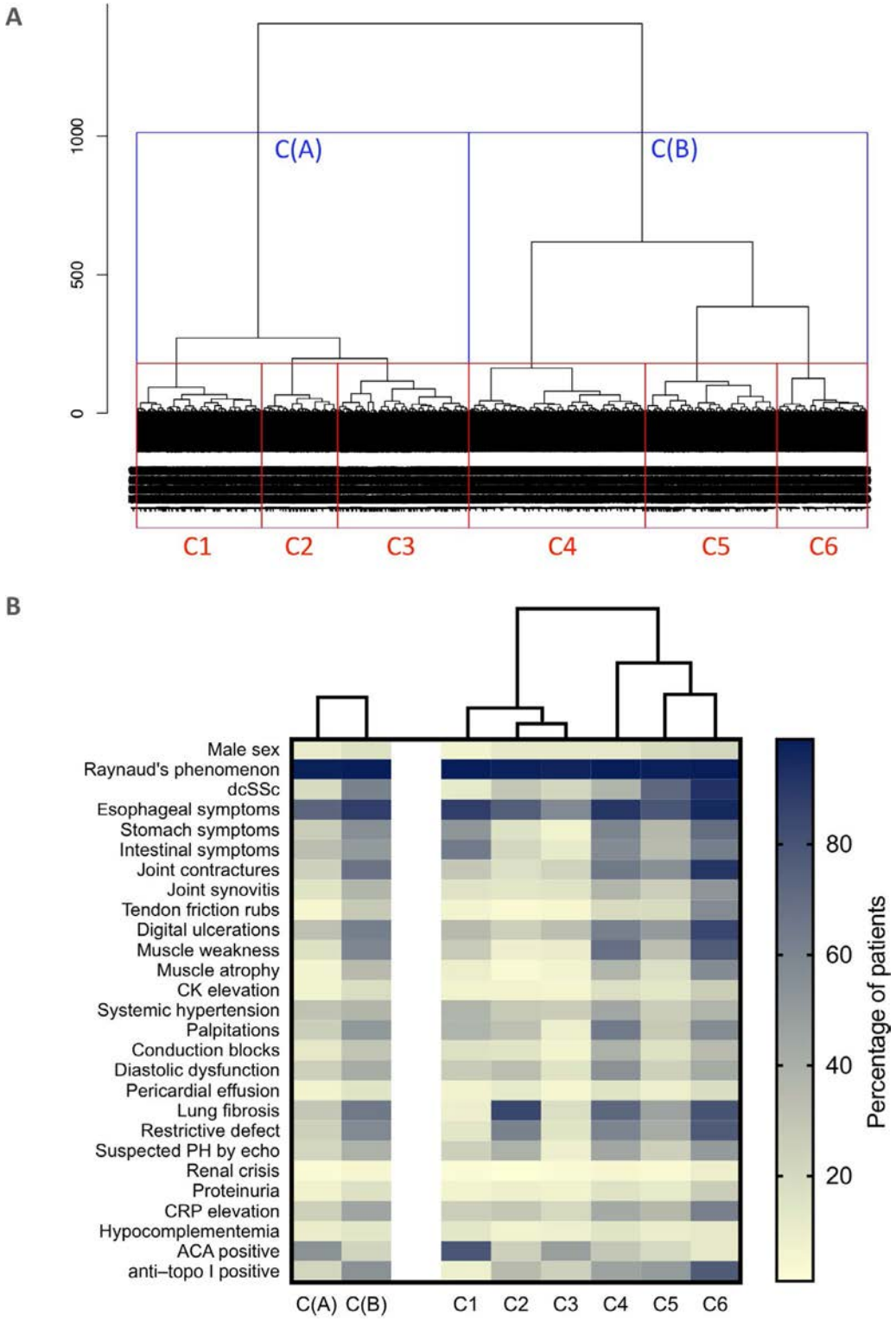

Figure 1. **A**, Dendrogram of the 6,927 patients with systemic sclerosis (SSc) included in the cluster analysis. The length of the vertical lines represents the degree of similarity between patients. Patients were divided into 2 clusters (cluster A and B) and into 6 clusters (clusters 1–6). **B**, Heatmap showing the clinical characteristics in each cluster. dcSSc = diffuse cutaneous SSc; CK = creatine kinase; PH = pulmonary hypertension; CRP = C-reactive protein; ACA = anticentromere antibody; anti–topo I = anti–topoisomerase I.

the "survival" and "fastcluster" packages in R software, version 2.14 (29). *P* values less than 0.05 were considered significant.

RESULTS

Patient characteristics. A total of 11,318 patients (from 122 centers) were registered in the EUSTAR database as of April 2014, and 34,066 visits were recorded. Of these patients, 2,886 were excluded and 1,505 were not analyzed (due to ≥1 missing value for the variables used for clustering). Therefore 6,927 patients (from 120 centers) were incorporated in the cluster analysis (Supplementary Figure 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40906/abstract). Compared to patients who were not included in the analysis, patients who were included were slightly older (mean ± SD age 58.7 ± 13.2 versus 56.3 ± 13.9 years; $P < 0.001$), had a longer disease duration (mean ± SD 11.4 ± 8.1 versus 8.7 ± 8.1 years; $P < 0.001$), had a higher rate of dcSSc (42% versus 38%, $P = 0.011$), and had generally more severe disease as indicated by proportions of organ damage (Table 1). The median number of visits per patient was 3 (interquartile range 4).

Of the patients included, 42% had dcSSc and 58% had lcSSc. Patients with dcSSc were significantly younger than those with lcSSc, and had more severe disease. Of the patients with dcSSc, 14% had ACAs and 61% had anti–topo I antibodies, and of the patients with lcSSc, 54% had ACAs and 23% had anti–topo I antibodies (Table 1).

Primary cluster analysis. Clustering of individuals on the basis of the 24 selected variables yielded an optimal number of 2 clusters: cluster A and cluster B (Figure 1A). Jaccard indexes showed moderate stability: 0.64 for cluster A and 0.66 for cluster B. The characteristics of the 2 clusters are summarized in Table 2, Supplementary Table 1 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40906/abstract), and Figures 1B and 2. Contingency tables (Supplementary Tables 2 and 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40906/abstract) show the proportions of patients with ACAs and anti–topo I antibodies in the different subsets of SSc according to skin involvement (lcSSc or dcSSc).

*Cluster A (n = 3,149; 45.*5%). Cluster A contained principally patients with lcSSc (81%). Less than a third of the patients in this cluster had severe organ damage (digital ulcers, intestinal symptoms, or muscle, joint, cardiac, or lung involvement). ACAs were present in 54% of the patients, and anti–topo I antibodies were present in 21%.

*Cluster B (n = 3,778; 54.*5%). Patients in cluster B were a little younger than those in cluster A, with a younger age at disease onset. In cluster B, 61% of the patients had dcSSc. A majority of the patients presented with digital ulcers, joint contractures, intestinal involvement, and ILD. The autoantibody profile was the opposite of that seen in cluster A; 54% of the patients were positive for anti–topo I antibodies and 22% were positive for ACAs.

Exploratory cluster analysis. In an exploratory attempt to decipher the heterogeneity of the disease, we then increased the number of clusters. Graphical observation of the dendrogram determined that a suboptimal number of clusters was 6 (Figure 1A). As a consequence, we observed a decrease in Jaccard coefficients (ranging from 0.32 to 0.68). The characteristics of clusters 1–6 are summarized in Table 2, Figure 1B, and Figure 3.

Cluster 1 (n = 1,186; 17%). A majority of the patients in cluster 1 (89%) had lcSSc, and most were female. They were older at disease onset, had a high prevalence of GI involvement, and had a low proportion of patients with ILD. Most of the patients in cluster 1 (79%) were ACA positive.

Cluster 2 (n = 720; 10%). Cluster 2 was composed mainly of lcSSc patients (71%), with increased frequencies of suspected PH by echocardiography (39%), ILD (85%), and restrictive defect (61%). Anti–topo I antibodies were present in 35% of the patients, and ACAs were present in 24%.

Cluster 3 (n = 1,243; 18%). Cluster 3 included mainly patients with lcSSc (79%) characterized by low prevalence of GI involvement and ILD. ACAs were twice as frequent as anti–topo I antibodies (48% versus 24%, respectively).

Cluster 4 (n = 1,673; 24%). Patients in cluster 4 were mainly lcSSc patients (63%) with severe disease as demonstrated by high proportions of cardiac and lung, muscular, joint, and GI involvement and digital ulcers. Anti–topo I antibodies were present in 46% of the patients and ACAs in 29%.

Cluster 5 (n = 1,249; 18%). Cluster 5 consisted mainly of patients with dcSSc (72%), with a notable proportion of male patients (19%), and GI, joint, and cardiac disease and moderate lung involvement. Half of the patients in cluster 5 were anti–topo I antibody positive and 20% were ACA positive.

Cluster 6 (n = 856; 12%). Cluster 6 was characterized by the highest proportion of patients with dcSSc (92%) and men (21%), the highest mean peak MRSS (27.2), and severe disease as shown by high frequencies of GI, joint, muscular, renal, lung, and cardiac disease. Anti–topo I antibodies were present in 77% of the patients and ACAs in 12% of the patients.

Sensitivity cluster analyses. Three sensitivity cluster analyses were conducted. The first included only patients with a disease duration of >10 years (Supplementary Table 4, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40906/abstract), the second included anti–U1 RNP, anti–RNA polymerase III, and anti-PM/Scl antibodies as clustering variables (Supplementary Table 5, available on the *Arthritis & Rheumatology* web site at

Table 2. Characteristics of the patients in the 2 and 6 clusters found in the cluster analysis (n = 6,927)*

	2 clusters		6 clusters					
	Cluster A	Cluster B	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
Jaccard index	0.64	0.66	0.39	0.32	0.57	0.38	0.68	0.43
No. of patients	3,149	3,778	1,186	720	1,243	1,673	1,249	856
Demographic characteristics								
Sex, female	90	84	94	88	88	88	81	79
Ethnicity								
White	94	96	97	88	94	96	94	96
Asian	5	2	2	10	4	2	3	2
Black	2	2	1	2	2	2	3	2
Age, mean ± SD years	59.2 ± 13.3	58.2 ± 13.2	61.3 ± 12.9	60 ± 12.8	56.6 ± 13.5	61.2 ± 12.6	55.8 ± 13.2	55.9 ± 13.2
Age at first non-Raynaud's symptom, mean ± SD years	47.9 ± 13.3	46.7 ± 13.3	48.9 ± 13.1	48.3 ± 12.8	46.7 ± 13.6	48.1 ± 13.1	46 ± 13.4	45.1 ± 13.4
Disease duration, mean ± SD years†	11.3 ± 8.2	11.5 ± 8.1	12.4 ± 8.1	11.8 ± 8.3	9.9 ± 7.9	13.2 ± 8.4	9.8 ± 7.6	10.8 ± 7.5
Time from onset of Raynaud's phenomenon to first non–Raynaud's phenomenon symptom, mean ± SD years	4.8 ± 8.7	3.1 ± 7.3	5.4 ± 8.7	4.4 ± 9.1	4.4 ± 8.5	3.9 ± 8.2	2.8 ± 6.6	2.2 ± 6.1
Time from first non–Raynaud's phenomenon symptom to EUSTAR enrollment, mean ± SD years	9.4 ± 7.9	9.3 ± 7.8	10.3 ± 7.9	9.8 ± 8.2	8.2 ± 7.4	10.5 ± 8.1	8.1 ± 7.4	8.6 ± 7.4
Time from EUSTAR enrollment to last visit, mean ± SD years	2.2 ± 2.3	2.8 ± 2.6	2.5 ± 2.3	2.3 ± 2.5	1.8 ± 2.2	3 ± 2.7	2.4 ± 2.5	2.9 ± 2.5
Body mass index, mean ± SD kg/m^2	24.1 ± 4.3	23.2 ± 4.2	24.3 ± 4.4	24.5 ± 4.6	23.6 ± 4	23.6 ± 4.4	23.3 ± 3.9	22.1 ± 4.2
SSc characteristics								
Autoantibody status								
Antinuclear antibody positive‡	96	97	98	94	95	97	95	98
Anticentromere antibody positive‡	54	22	79	24	48	29	20	12
Anti–topoisomerase I antibody positive‡	21	54	8	35	24	46	50	77
Anti–U1 RNP antibody positive	5	5	3	8	5	7	3	4
Anti-PM/Scl antibody positive	2	4	1	3	1	4	4	6
Anti–RNA polymerase III antibody positive	3	5	2	3	4	3	6	6

(Continued)

Table 2. *(Cont'd)*

	2 clusters		6 clusters					
	Cluster A	Cluster B	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
Cutaneous involvement								
dcSSc	19	61	11	29	21	37	72	92
Peak MRSS, mean ± SD‡	6.6 ± 4.3	16.5 ± 9.8	6.6 ± 4.2	7.2 ± 4.6	6.3 ± 4.1	9.2 ± 5.3	19 ± 6.7	27.2 ± 8.7
Gastrointestinal involvement§								
Esophageal symptoms‡	73	88	88	76	58	91	79	95
Stomach symptoms‡	26	55	52	16	7	60	36	70
Intestinal symptoms‡	33	50	64	21	11	57	34	63
Joint involvement								
Joint contractures‡	24	67	29	17	23	65	55	91
Joint synovitis‡	14	37	15	13	15	37	25	53
Tendon friction rubs‡	4	28	6	3	4	19	19	57
Vascular involvement								
Raynaud's phenomenon‡	98	99	99	98	97	99	98	99
History of or current digital ulcers‡	32	63	35	24	33	62	50	85
Muscular involvement								
Muscle weakness‡	16	59	27	8	10	69	33	77
Muscle atrophy‡	6	35	9	3	6	38	17	57
CK elevation‡	6	18	7	7	5	17	13	26
Cardiac involvement								
Systemic arterial hypertension‡	31	37	38	28	26	44	26	38
Palpitations‡	25	51	38	32	9	64	28	57
Conduction blocks‡	12	30	16	14	6	39	16	34
LVEF <50%	3	7	3	3	2	6	5	10
Abnormal diastolic function‡	24	42	27	33	15	54	24	43
Pericardial effusion	7	14	7	11	4	15	9	18
Pulmonary hypertension								
Pulmonary hypertension on echocardiography‡	21	39	24	39	8	44	24	50
Systolic PAP measured by echocardiography, mean ± SD mm Hg	32.5 ± 13.7	36 ± 16.2	33 ± 14.3	36.7 ± 14.1	29.4 ± 12	37.2 ± 14.6	32.4 ± 12	38.1 ± 22.1

(Continued)

Table 2. *(Cont'd)*

	2 clusters		6 clusters					
	Cluster A	Cluster B	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
Interstitial lung disease								
Lung fibrosis on plain radiography‡	29	65	8	85	17	72	46	80
Lung fibrosis on HRCT	38	70	22	78	29	73	56	82
Restrictive defect on PFTs‡	24	58	13	61	14	60	42	77
FVC, mean ± SD % predicted	97.8 ± 19.3	82.7 ± 21.1	101.2 ± 17.4	86.7 ± 21.9	99.9 ± 17.7	84.4 ± 20.8	87.5 ± 19.8	72.8 ± 20.3
DLco, mean ± SD % predicted	68 ± 18.9	56.6 ± 19.7	69.8 ± 17.2	57.7 ± 19.3	72.3 ± 18	55.2 ± 18.8	62.5 ± 20.3	50.6 ± 18.1
6-minute walking distance, mean ± SD meters	411 ± 129	381 ± 136	400 ± 135	405 ± 130	427 ± 121	366 ± 133	418 ± 130	362 ± 138
Renal involvement								
History of renal crisis‡	2	4	2	1	2	4	3	8
Proteinuria‡	7	16	6	8	7	15	11	26
Blood tests								
CRP elevation	24	45	25	29	20	43	36	62
Hypocomplementemia	10	13	13	7	8	14	10	12
Treatment								
Past or current steroids	27	55	22	45	24	57	44	65
Prednisone, mean ± SD mg/day	2.8 ± 6.4	5.7 ± 7.9	2 ± 4.9	5.5 ± 9.3	2.3 ± 5.6	5.6 ± 7.6	4.6 ± 7.6	7.3 ± 8.8
Past or current immunosuppressive drugs	27	54	17	44	27	48	54	66
Mortality								
Number of deaths per 1,000 patient-years	10.3	22.6	7.5	17.3	9.7	19.1	20.8	31.9

* Except where indicated otherwise, values are the percent of patients. See Table 1 for definitions.
† Time between the first non–Raynaud's phenomenon symptom and the last visit.
‡ Clustering variables.
§ Esophageal symptoms included dysphagia and/or reflux, stomach symptoms included early satiety and/or vomiting, and intestinal symptoms included diarrhea, bloating, and/or constipation.

http://onlinelibrary.wiley.com/doi/10.1002/art.40906/abstract), and the third included only patients with a disease duration of <5 years at the enrollment visit (Supplementary Table 6, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40906/abstract). Results of the sensitivity analyses were similar to those of the main cluster analysis.

Survival analyses. Kaplan-Meier curves are shown in Figures 2 and 3 and Supplementary Figures 3 and 4 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40906/abstract). Survival rates are presented in Supplementary Table 7 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40906/abstract), and the results of Cox regression analyses are shown in Table 3.

The risk of death was increased for patients with dcSSc compared to patients with lcSSc, with a hazard ratio (HR) of 2.03 (95% confidence interval [95% CI] 1.61–2.56) in the most-adjusted model. An increased risk of death was also present in

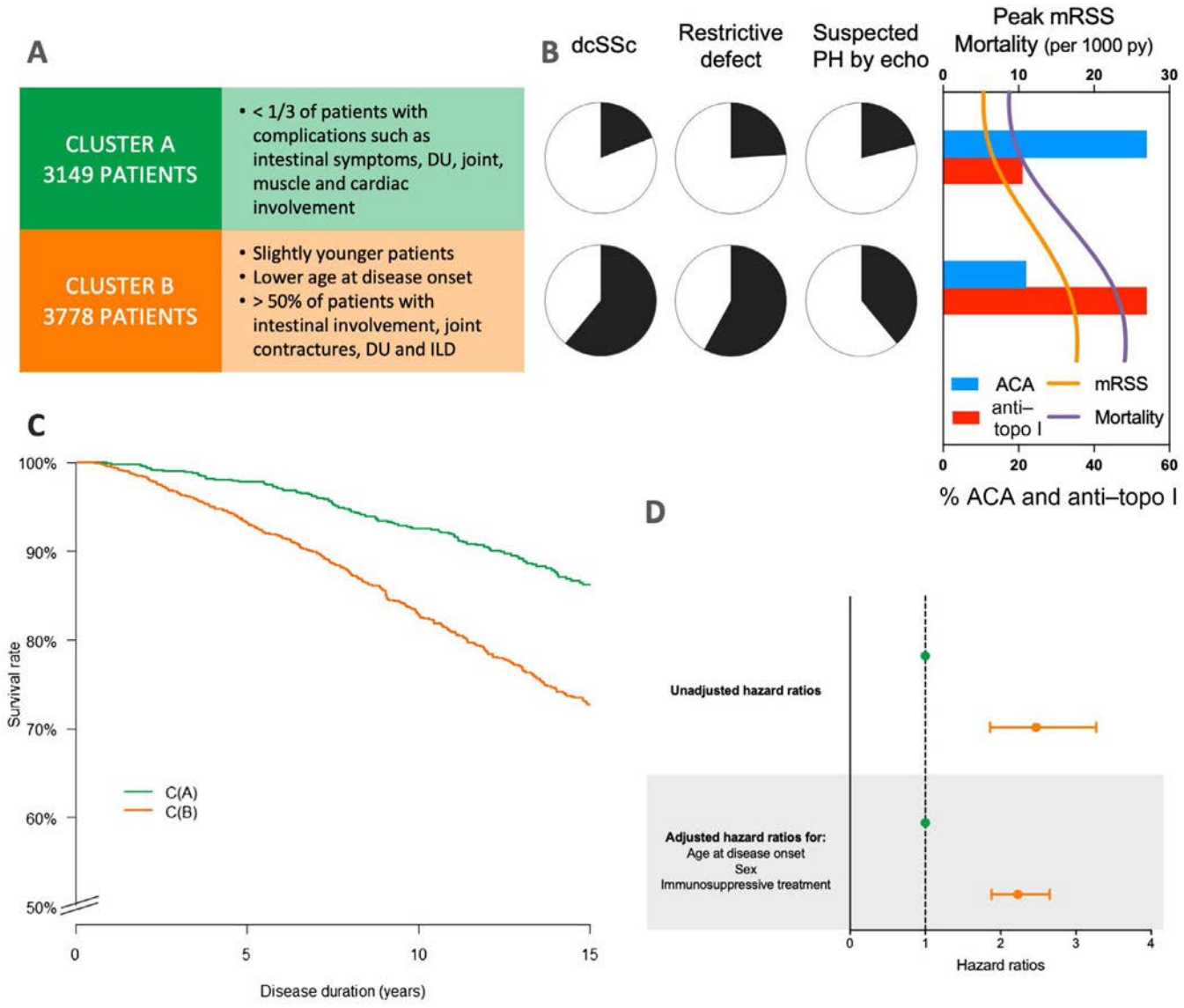

Figure 2. **A**, Main characteristics of the 2 clusters (cluster A and cluster B) of patients with systemic sclerosis (SSc). **B**, Left, Proportions of each cluster with the main clinical characteristics of diffuse cutaneous SSc (dcSSc), restrictive defect, and suspected pulmonary hypertension (PH) on echocardiography (echo). Right, Peak modified Rodnan skin thickness score (MRSS), mortality (per 1,000 patient-years [py]), and percentages of patients with anticentromere antibodies (ACAs) and anti–topoisomerase I (anti–topo I) antibodies in each cluster. **C**, Kaplan-Meier survival curves for the 2 clusters. **D**, Forest plot showing mortality hazard ratios and 95% confidence intervals for the 2 clusters. Broken line shows the hazard ratio for the reference group. Green symbols represent cluster A; orange symbols represent cluster B. DU = digital ulcer; ILD = interstitial lung disease.

cluster B compared to cluster A (HR 2.47 [95% CI 1.86–3.27]). When analyzing 6 clusters, we noticed a continuous increasing mortality from cluster 1 to cluster 6 in the most-adjusted model. The risk of death had a magnitude superior to those noted in the 2 previous analyses (i.e., HR 6.14 [95% CI 3.81–9.89] for cluster 6 compared to cluster 1). C-indexes were similar for the most-adjusted models: lcSSc versus dcSSc, cluster A versus cluster B, and for the 6 clusters (mean ± SEM 0.78 ± 0.02, 0.78 ± 0.02, and 0.79 ± 0.02, respectively).

The sensitivity analysis taking into account patients who were lost to follow-up yielded comparable HRs when we examined survival in clusters A and B and clusters 1–6 (data not shown). We also performed a sensitivity analysis using the onset of Raynaud's phenomenon as the date of disease onset (Supplementary Table 8, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40906/abstract), which yielded similar results, albeit the number of patients with available data was lower.

DISCUSSION

This study aimed to distinguish homogeneous groups in a substantial population of ~7,000 SSc patients using a clus-

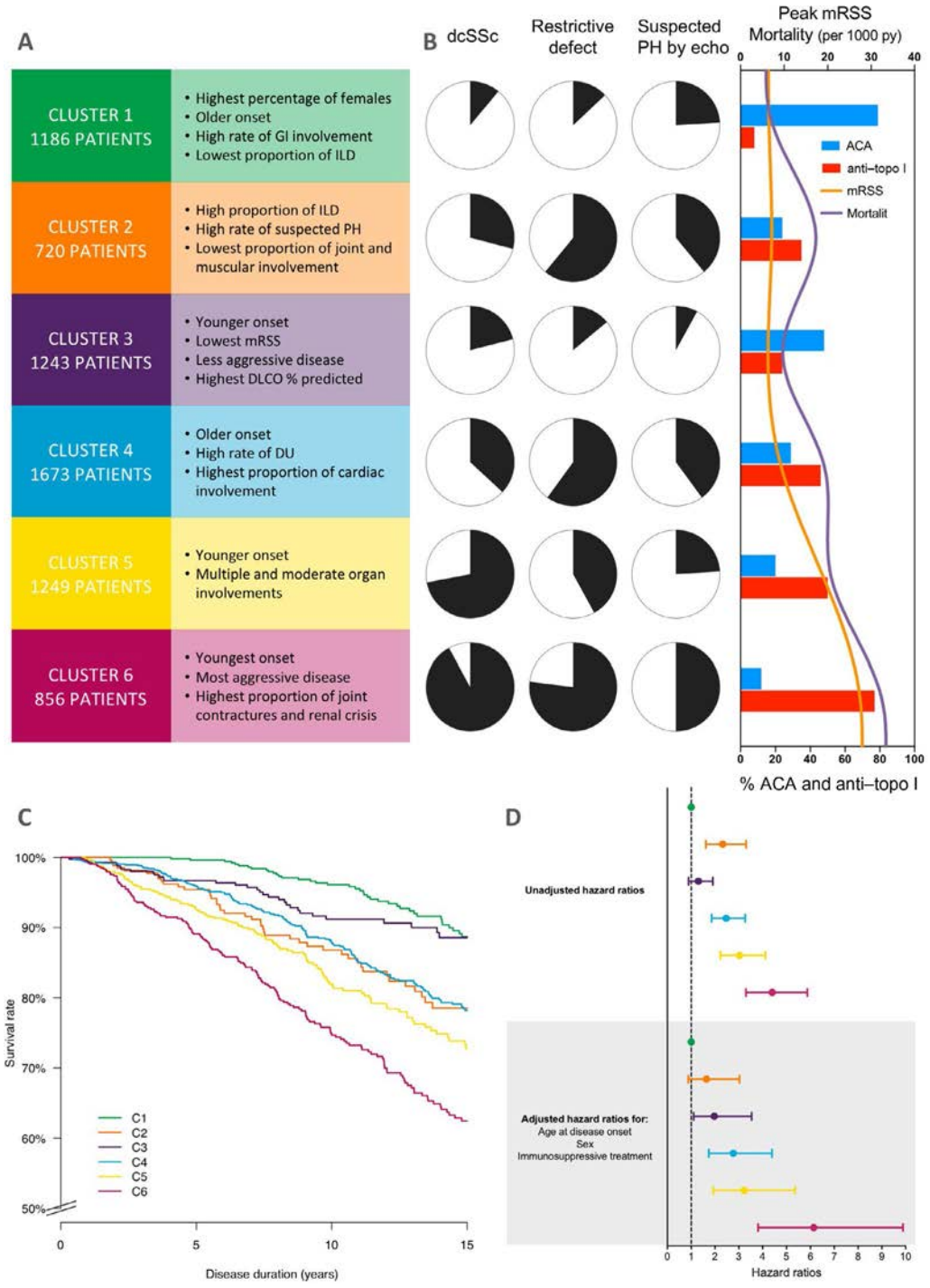

Figure 3. **A**, Main characteristics of the 6 clusters (clusters 1–6) of patients with systemic sclerosis (SSc). **B**, Left, Proportions of each cluster with the main clinical characteristics of diffuse cutaneous SSc (dcSSc), restrictive defect, and suspected pulmonary hypertension (PH) on echocardiography (echo). Right, Peak modified Rodnan skin thickness score (MRSS), mortality (per 1,000 patient-years [py]), and percentages of patients with anticentromere antibodies (ACAs) and anti–topoisomerase I (anti–topo I) antibodies in each cluster. **C**, Kaplan-Meier survival curves for the 6 clusters. **D**, Forest plot showing mortality hazard ratios and 95% confidence intervals for the 6 clusters. Broken line shows the hazard ratio for the reference group. Colors represent the different clusters as indicated in **C**. GI = gastrointestinal; ILD = interstitial lung disease; DL_{CO} = diffusing capacity for carbon monoxide; DU = digital ulcer.

ter analysis. The study had 2 main findings. First, the optimal clustering divided patients into 2 distinct groups according to their clinical and serologic features and disease severity and prognosis; these 2 categories partially overlapped with the classifications dcSSc and lcSSc. Second, an exploratory analysis yielded 6 homogeneous subsets of individuals that broadly dif-

Table 3. Cox regression analyses*

	Univariable analysis (n = 3,352)		Multivariable analysis					
			Adjusted for age at disease onset (n = 3,352)		Adjusted for age at disease onset and sex (n = 3,352)		Adjusted for age at disease onset, sex, and immunosuppressive treatment (n = 2,887)	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Cutaneous involvement								
lcSSc	Reference		Reference		Reference		Reference	
dcSSc	1.90 (1.64–2.19)	<0.001	2.39 (2.07–2.77)	<0.001	2.14 (1.85–2.48)	<0.001	2.03 (1.61–2.56)	<0.001
C-index†	0.60 ± 0.01		0.73 ± 0.01		0.75 ± 0.01		0.78 ± 0.02	
2 clusters								
Cluster A	Reference		Reference		Reference		Reference	
Cluster B	2.23 (1.88–2.65)	<0.001	2.40 (2.02–2.85)	<0.001	2.26 (1.91–2.69)	<0.001	2.47 (1.86–3.27)	<0.001
C-index†	0.59 ± 0.01		0.72 ± 0.01		0.74 ± 0.01		0.78 ± 0.02	
6 clusters								
Cluster 1	Reference		Reference		Reference		Reference	
Cluster 2	2.32 (1.62–3.31)	<0.001	2.10 (1.46–3.00)	<0.001	1.97 (1.38–2.82)	<0.001	1.64 (0.88–3.03)	0.119
Cluster 3	1.30 (0.89–1.91)	0.172	1.63 (1.11–2.38)	0.012	1.62 (1.11–2.37)	0.013	1.97 (1.10–3.54)	0.023
Cluster 4	2.47 (1.86–3.27)	<0.001	2.49 (1.88–3.30)	<0.001	2.40 (1.81–3.19)	<0.001	2.77 (1.74–4.39)	<0.001
Cluster 5	3.03 (2.23–4.11)	<0.001	3.77 (2.77–5.12)	<0.001	3.37 (2.47–4.58)	<0.001	3.22 (1.93–5.36)	<0.001
Cluster 6	4.40 (3.30–5.87)	<0.001	5.85 (4.38–7.81)	<0.001	5.20 (3.89–6.95)	<0.001	6.14 (3.81–9.89)	<0.001
C-index†	0.63 ± 0.01		0.75 ± 0.01		0.76 ± 0.01		0.79 ± 0.02	

* Disease onset was defined as the first non–Raynaud's phenomenon symptom (see Supplementary Table 8, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40906/abstract, for sensitivity analysis using the onset of Raynaud's phenomenon as the definition of disease onset). HR = hazard ratio; 95% CI = 95% confidence interval; lcSSc = limited cutaneous systemic sclerosis; dcSSc = diffuse cutaneous systemic sclerosis.

† The C-index was calculated for each Cox regression model, and corresponds to the estimation of the probability of concordance, equivalent to the area under the receiver operating characteristic curve for logistic regression models. A value of 1 indicates perfect agreement and 0.5 indicates an agreement that is no better than chance. Values for the C-index are the mean ± SEM.

fered with regard to clinical features, autoantibody profiles, and survival.

The fact that 2 clusters were found could be considered a validation of the expected dichotomy between dcSSc and lcSSc. However, 19% of the patients in cluster A had dcSSc and 21% had anti–topo I antibodies. In cluster B, 39% of the patients had lcSSc and 22% had ACAs. No clear parallels between the severity of organ damage and the cutaneous extent of SSc were observed. This finding is consistent with the results of recent studies. For example, Nihtyanova et al demonstrated that the presence of significant organ involvement was a strong predictor of prognosis, in both lcSSc and dcSSc, in a study of nearly 400 consecutive patients followed up for up to 15 years. Notably, survival curves were close for the 2 cutaneous subsets when organ damage was present (30). Taken together, these results suggest that, while there is consensus on the relevance and practicality of subdividing SSc into lcSSc and dcSSc (31), this binary classification may be too restrictive as a separation within a continuous spectrum of varying severity primarily driven by organ damage and subsequent prognosis (12).

In an exploratory attempt to study the heterogeneity of SSc more in depth, we found 6 additional clusters. Some of the 6 clusters obtained were expected, since they were consistent with the historical descriptions of lcSSc and dcSSc. Indeed, cluster 1 included patients with the classic presentation of lcSSc, i.e., older female patients with a low rate of severe organ damage, a high frequency of ACA positivity, and a generally favorable prognosis. Cluster 6 resembled the classic description of dcSSc, with a high rate of male patients, the highest frequency of anti–topo I antibody–positive patients, and a high rate of severe organ damage and poor prognosis. Intriguingly, we observed clusters of patients that seemed to be grouped together based on characteristics other than the degree of skin involvement. Cluster 2 was composed principally of patients with lcSSc but with a rather high frequency of anti–topo I antibody–positivity and high rates of ILD and suspected PH. Of note, the prognosis for patients in cluster 2 was significantly worse than that for patients in cluster 1. Similarly, cluster 4 consisted of predominantly patients with lcSSc, often with visceral complication. Cluster 5 comprised, for the most

part, patients with dcSSc, but we noted lower frequencies of ILD and suspected PH in this group than in clusters 2, 4, or 6. These findings indicate that subclassifications established solely on the extent of skin involvement might not be entirely representative of the severity of organ damage and prognosis.

Furthermore, this work highlighted some groups of patients in which the classic relationships between lcSSc and ACAs and between dcSSc and anti–topo I antibodies were not obvious. For example, in cluster 2, 71% of the patients were classified as having lcSSc, although 85% had lung fibrosis. Moreover, we found a relatively small proportion of ACA-positive patients (24%) and a notable rate of anti–topo I antibody positivity (35%), which was unexpected in a group in which the majority of the patients had lcSSc. The prognosis for the patients in this group was worse than that for the patients in cluster 1, which included mainly patients with lcSSc and few with organ damage, which supports the findings of Nihtyanova et al (30). Likewise, a Canadian Scleroderma Research Group study examined the clinical features and mortality of anti–topo I antibody–positive lcSSc and ACA-positive dcSSc patients. The autoantibody profile seemed to be more strongly associated with demographic characteristics and visceral damage than with the skin subgroup. Mortality was related to both skin and serologic profile (9). Kranenburg et al also demonstrated that lcSSc patients who were positive for anti–topo I antibodies contrasted with lcSSc patients who were negative for anti–topo I antibodies and dcSSc patients who were positive for anti–topo I antibodies in terms of survival and organ involvement (32). Taken together, those studies suggest that subclassification combining antibody profile and skin involvement might predict clinical outcomes more accurately than skin or serologic features alone (9,32).

The heterogeneity of SSc has been discussed over a long period, and many studies were published both before and after the work of LeRoy et al describing the limited and diffuse subsets (2–4,33,34). The significance of serologic profile has also been highlighted by Patterson et al, who characterized 5 groups of patients with homogeneous clinical and organ involvement (11,12). Significant efforts to classify patients into subsets on the basis of common clinical phenotypes, rather than through a predetermined decision process, have proposed to classify individuals using changes in MRSS over time (34,35), changes in the forced vital capacity percent predicted value (36,37), or gene expression patterns in the skin (38,39). Each of these attempts has resulted in a small number of subsets that define the range of phenotypes captured by the stratification characteristics (12). There is growing interest in a new subclassification of SSc that combines patterns of underlying pathogenesis, organ damage, and prognosis in order to personalize disease management and ameliorate outcomes (12,31).

This study has strengths and limitations. The principal strengths are the number of patients included in this large, prospective, multicenter cohort, and the lack of any a priori assumptions. The main weakness is that several clinically relevant variables were lacking or were disregarded due to the proportion of missing data being too high (e.g., autoantibodies other than ACAs/anti–topo I antibodies, extent of ILD on high-resolution computed tomography [HRCT] scan, detailed skin involvement, and overlap syndromes). In addition, 1,505 of 8,432 patients were excluded from the cluster analysis because of missing data for any of the selected clustering variables. Since those excluded patients had slightly less severe disease than the included ones, it could affect the extrapolation of our results. Imputation of missing data by model-based clustering was not performed because we could not assume that these data were missing at random (40,41). Moreover, several definitions of variables lacked precision (e.g., ILD was defined as lung fibrosis on radiography whereas HRCT scan is now widely used, and PH was defined as suspicion on echocardiography without invasive confirmation).

We also acknowledge that a thorough analysis of treatment regimens was not possible due to missing data. Nevertheless, for a majority of the patients we were able to determine whether or not they had been taking an immunosuppressive drug. To account for the potential effect of these drugs on survival, survival analyses were adjusted for immunosuppressive treatment. A potentially important bias is the influence of disease duration on the clustering process, since the frequency of organ damage tends to increase as the disorder progresses. Also, disease duration at the enrollment visit was relatively long, raising the possibility that study results were influenced by survival bias. Yet, the sensitivity analyses that included only patients with a long disease duration and those that included only patients with a short disease duration yielded similar results.

Another limitation is that a significant number of patients were excluded from the survival analysis because of loss to follow-up. Nevertheless, this exclusion did not alter the survival differences between clusters in a sensitivity analysis. The primary aim of our study was not to assess the prognosis factors for survival in SSc, but to decipher the heterogeneity of SSc by a cluster analysis and describe the survival rate in the clusters obtained, allowing us to validate this approach post hoc. In studies assessing the prognosis factors of survival, baseline data are most often used. In our study, we had to include follow-up data in order to identify the occurrence of organ involvement. Therefore, we considered an organ complication to be present if the corresponding variable was described as "positive" at least once among all the visits included for a specific patient. We did not describe the progression of organ involvement in the whole population or in the different clusters because the limited number of follow-up visits precluded us from performing a precise temporal description. In the end, the weak reproducibility of the exploratory analysis with 6 clusters precludes translating these results to a new subclassification (e.g., to allocate an individual to a designated group on the basis of their features). Moreover, previous studies have shown differences between distinct geographical

cohorts (42). Of note, 95% of the patients included in this study were white. It is likely that inclusion of a higher proportion of Asian or black patients could have modified the results.

In conclusion, this study shows that SSc is a very heterogeneous condition. While there is consensus regarding the relevance and practicality of the subclassification of SSc into lcSSc and dcSSc, this binary system might omit a wider spectrum of clinical phenotypes characterized not only by skin involvement but also by organ damage, serologic profile, and subsequent prognosis. There is an increasing demand for a future SSc classification that combines these different patterns, in order to personalize approaches to diagnosis and clinical management.

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. Sobanski 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. Sobanski, Giovannelli, Allanore, Launay, Hachulla.

Acquisition of data. Sobanski, Giovannelli, Allanore, Riemekasten, Airò, Vettori, Cozzi, Distler, Matucci-Cerinic, Denton, Launay, Hachulla.

Analysis and interpretation of data. Sobanski, Giovannelli, Allanore, Distler, Matucci-Cerinic, Denton, Launay, Hachulla.

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APPENDIX A: THE EUSTAR COLLABORATORS

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Protective Effect Against Cancer of Antibodies to the Large Subunits of Both RNA Polymerases I and III in Scleroderma

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Objective. While compelling data suggest a cancer-induced autoimmunity model in scleroderma patients with anti–RNA polymerase III large subunit (anti-RPC155) antibodies, ~85% of these patients do not manifest cancer. This study was undertaken to determine whether additional autoantigens are targeted in anti-RPC155–positive scleroderma patients without detectable cancer.

Methods. The study included 168 scleroderma patients with anti-RPC155 antibodies (80 with a history of cancer and 88 with no cancer diagnosis after >5 years of follow-up). Thirty-five sera (17 from patients with cancer and 18 from patients without cancer) were randomly selected for autoantibody discovery using immunoprecipitation (IP). An ~194-kd band was enriched in the subgroup without cancer; this was identified as RNA polymerase I large subunit (RPA194).

Results. RPA194 generated by in vitro transcription/translation was used for IPs performed on the entire cohort to test whether anti-RPA194 was enriched among anti-RPC155–positive patients without cancer. Anti-RPA194 antibodies were significantly more common in the group without cancer (16 [18.2%] of 88) than in the group with cancer (3 [3.8%] of 80) ($P = 0.003$). Patients with both anti-RPA194 and anti-RPC155 were significantly less likely to have severe gastrointestinal disease than patients with anti-RPC155 only (26.3% versus 51.0%; $P = 0.043$).

Conclusion. Anti-RPA194 antibodies are enriched in anti-RPC155–positive scleroderma patients without cancer. Since somatic mutations in the gene encoding *RPC155* in cancer in scleroderma patients appears to play a role in immune response initiation against RPC155 in those patients, these data raise the possibility that the development of immune responses to both RPC155 and RPA194 may influence clinical cancer emergence. Further study is required to define whether different autoantibody combinations have utility as tools for cancer risk stratification in scleroderma.

INTRODUCTION

Emerging data suggest that subsets of patients with systemic sclerosis (scleroderma) may have cancer-induced autoimmunity (1). This relationship between cancer and scleroderma emergence has been most notable among scleroderma patients with antibodies against the large subunit of RNA polymerase III (RPC155). Scleroderma patients with these autoantibodies have a significantly higher risk of developing cancer within a short interval of scleroderma onset compared to scleroderma patients without anti-RPC155 antibodies (2–7). Furthermore, recent data demonstrate that this translates to a 2.8-fold increased risk of cancer within 3 years of scleroderma onset when compared to the expected cancer incidence in the general population (8). Mechanistic studies have demonstrated that genetic alterations (somatic mutations and/or loss of heterozygosity) are present in the gene (*POLR3A* locus) that encodes for RPC155 in some of these patients' cancers, with development of both mutation-specific and cross-reactive immune responses (9).

While these data strongly suggest a model of cancer-induced autoimmunity, it is notable that ~85% of scleroderma patients with anti-RPC155 antibodies do not manifest cancer clinically over an extensive follow-up period (8). These data raise the tantalizing possibility that cancer may be an underlying trigger for scleroderma

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Drs. Shah, Laiho, Rosen, and Casciola-Rosen have submitted a patent application for materials and methods used in the assessment of cancer risk and the treatment of cancer. Dr. Laiho holds a patent on small-molecule RNA polymerase I inhibitors managed by Johns Hopkins University, for which she receives licensing fees.

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in most patients with anti-RPC155 antibodies, with the antitumor immune response being variably successful in eliminating the cancer or maintaining it in equilibrium such that it does not emerge (10). In this context, an important relevant property of the immune response is its ability to diversify to additional epitopes within the primary target (intramolecular spreading) and also to additional proteins that bind to the primary target at some stage in its functional cycle (intermolecular spreading) (11). It is noteworthy that many targets of the autoimmune response in scleroderma (e.g., RNA polymerases, the minor spliceosome, and the centromere) are multicomponent complexes. Furthermore, multiple components of these complexes are recognized by autoantibodies, suggesting antigenic spreading (12).

We hypothesized that the immune response in anti-RPC155–positive scleroderma patients in whom cancer does not emerge might target additional autoantigens. To address this hypothesis, we initially studied a small group of patients with anti-RPC155 antibodies with cancer and patients with anti-RPC155 antibodies without cancer, and compared the autoantibody specificities in these 2 groups by immunoprecipitation (IP). Interestingly, in anti-RPC155 antibody–positive patients without cancer, a 194-kd protein was enriched. Noting the molecular weight, the prior description of RNA polymerase I as an autoantigen in scleroderma (13), and the observation that an inhibitor inducing destruction of the catalytic subunit of RNA polymerase I (RPA194) is itself an effective anticancer agent (14), we pursued whether, and then rapidly confirmed that, the 194-kd protein was RPA194. When the frequency of RPA194 antibodies was assayed in a large cohort of anti-RPC155–positive scleroderma patients with and those without cancer, we confirmed that anti-RPA194 antibodies were enriched among anti-RPC155–positive patients without cancer.

These data strongly suggest that in scleroderma patients, targeting of the catalytic components of both RNA polymerase I and III complexes (that is, RPA194 and RPC155, respectively) is associated with a decreased emergence of cancer, raising the possibility that the combined immune responses may affect cancer survival and fitness. These observations have important implications for understanding the mechanisms underlying the association of cancer and scleroderma, as well as control of cancer by the immune system. Knowing the RPA194 antibody status in anti-RPC155–positive patients may also enable improved precision in cancer prediction in this subgroup.

PATIENTS AND METHODS

Study population. Patients who had scleroderma, as defined by the American College of Rheumatology (ACR)/European League Against Rheumatism 2013 classification criteria or ACR 1980 criteria or the presence of at least 3 of 5 CREST syndrome criteria (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia), and a banked serum sample were included in the study (15,16). RPC155 antibody status was determined by clinically obtained assays, and 168 scleroderma patients with anti-RPC155 antibodies were identified for this study, including 80 with a history of cancer and 88 who had no history of cancer after at least 5 years of follow-up. Anti-RPC155 antibody status was subsequently confirmed in all 168 patients by enzyme-linked immunosorbent assay (ELISA; Inova Diagnostics), using 20 units as the cut-off value for assigning antibody positivity, as recommended by the manufacturer. From this overall study population, 35 sera were randomly selected for initial autoantibody discovery, 17 from anti-RPC155–positive scleroderma patients with cancer and 18 from anti-RPC155–positive scleroderma patients without cancer. The RPC155 antibody status in this subset was validated by a second assay method: IP using ^{35}S-methionine–labeled RPC155 generated by in vitro transcription/translation (IVTT), as described below. For the cancer patients, the serum sample obtained closest to the date of cancer diagnosis was studied. For patients without cancer, the most recent available serum sample was studied.

Exposure and outcome assessment. Demographic data, scleroderma onset dates, clinical characteristics, and cardiopulmonary testing data were abstracted from the cohort database. Age at scleroderma onset was defined as age at the first scleroderma symptom, either Raynaud's phenomenon or non–Raynaud's phenomenon manifestation. Scleroderma subtype was classified as limited cutaneous if skin thickening was distal to the elbows and/or knees, and as diffuse cutaneous if skin thickening involved the upper arms, thighs, chest, or abdomen (17). Measurements of forced vital capacity (FVC) and diffusing capacity for carbon monoxide were adjusted for age and sex (18,19). A restrictive ventilatory defect suggestive of interstitial lung disease (ILD) was defined as an FVC of <70% predicted ever. Echocardiographic evidence suggestive of pulmonary hypertension was defined as a right ventricular systolic pressure (RVSP) of ≥45 mm Hg ever (20). Myopathy was defined as a history of abnormal muscle enzymes or by electromyography, muscle magnetic resonance imaging, and/or muscle biopsy findings. Severe Raynaud's phenomenon was defined as a maximum Medsger peripheral vascular severity score of ≥2 (digital pitting scars, ulcers, or gangrene), and severe gastrointestinal (GI) disease was defined as a modified maximum Medsger GI severity score of ≥2 (high-dose antireflux medications or antibiotics required for small intestinal bacterial overgrowth, malabsorption syndrome, episodes of pseudo-obstruction, or hyperalimentation required) (21). Cancer diagnosis site, histology, and date were reviewed for all patients and confirmed by cancer pathology reports, oncology records, and other physicians' notes. The interval between cancer diagnosis and onset of scleroderma was calculated as the difference between the cancer diagnosis date and the date of the first scleroderma symptom.

IP using ^{35}S-methionine–labeled proteins generated by IVTT. Complementary DNAs (cDNAs) encoding full-length human RPC155 (purchased from OriGene) and RPA194 (human RPA194 cloned into pCMV6-HA-His vector from OriGene) were used to generate ^{35}S-methionine–labeled proteins by IVTT, according to the manufacturer's protocol (Promega). IP using these products was performed (22), and the immunoprecipitates were electrophoresed on 10% sodium

Table 1. Clinical characteristics of the anti-RPC155–positive scleroderma patients with and those without cancer*

Variable	Patients with cancer (n = 80)	Patients with no known cancer (n = 88)	*P*
Age at scleroderma onset, years†	51.7 ± 14.0	45.3 ± 13.6	0.0035
Age at first non–Raynaud's phenomenon symptom, years	55.1 ± 11.3	47.4 ± 12.2	<0.0001
Disease duration at first visit, years	6.4 ± 10.6	6.2 ± 7.8	0.9331
Interval between cancer diagnosis and scleroderma onset, years	4.6 ± 14.9	NA	NA
Interval between cancer diagnosis and first non–Raynaud's phenomenon symptom, years	1.1 ± 11.3	NA	NA
Male sex, no. (%)	19 (23.8)	12 (13.6)	0.091
Race, no. (%)			0.519
White	77 (97.5)	82 (93.2)	
Black	2 (2.5)	4 (4.6)	
Asian	0 (0)	2 (2.3)	
Subtype, no. (%)			0.374
Limited cutaneous	21 (26.3)	18 (20.5)	
Diffuse cutaneous	59 (73.8)	70 (79.6)	
Ever smoked, no. (%)	44 (55.0)	39 (44.3)	0.167
MRSS at first visit to center	18.7 ± 12.9	19.1 ± 13.8	0.8359
Maximum ever MRSS	22.5 ± 13.5	22.4 ± 14.9	0.9563
Renal crisis, no. (%)	10 (12.5)	11 (12.5)	1.000
Myopathy, no. (%)	13 (16.3)	14 (15.9)	0.952
ILD, no. (%)‡	28 (37.3)	43 (50.6)	0.092
Baseline pulmonary function			
FVC, % predicted	85.2 ± 15.2	83.0 ± 17.1	0.4052
DLco, % predicted	82.3 ± 21.2	83.7 ± 25.2	0.7213
Pulmonary hypertension, no. (%)§	18 (23.4)	31 (37.4)	0.055
Baseline RVSP, mm Hg	32.3 ± 10.0	33.0 ± 9.4	0.7330
Baseline ejection fraction, %	63.0 ± 6.8	59.9 ± 7.4	0.0112
Severe Raynaud's phenomenon, no. (%)¶	39 (48.8)	54 (61.4)	0.100
Severe GI disease, no. (%)¶	38 (47.5)	43 (48.9)	0.860
Calcinosis, no. (%)	27 (33.8)	51 (58.0)	0.002
Telangiectasia, no. (%)	78 (97.5)	86 (97.7)	1.000
Tendon friction rubs, no. (%)	36 (45.0)	41 (46.6)	0.836

* In the group of patients with cancer, data were available for 79 patients for age at first non–Raynaud's phenomenon symptom, race, modified Rodnan skin thickness score (MRSS) at first visit, and maximum MRSS, 75 patients for interstitial lung disease (ILD), 73 patients for forced vital capacity (FVC) and baseline ejection fraction, 65 patients for diffusing capacity for carbon monoxide (DLco), 77 patients for pulmonary hypertension, and 45 patients for baseline right ventricular systolic pressure (RVSP). In the group with no known cancer, data were available for 87 patients for age at first non–Raynaud's phenomenon symptom, 86 patients for MRSS at first visit, 85 patients for ILD, 79 patients for FVC, 70 patients for DLco, 83 patients for pulmonary hypertension, 51 patients for baseline RVSP, and 73 patients for baseline ejection fraction. Except where indicated otherwise, values are the mean ± SD. NA = not applicable; GI = gastrointestinal.
† Scleroderma onset was defined as the first symptom, either Raynaud's phenomenon or non–Raynaud's phenomenon.
‡ Defined as an FVC of <70% predicted ever.
§ Defined as an RVSP of ≥45 mm Hg ever.
¶ Defined as an organ-specific severity score of ≥2 at any time during the disease course.

dodecyl sulfate (SDS)–polyacrylamide gels and visualized by fluorography. As we reported previously, IVTT IP and ELISA gave identical readouts for RPC155 antibody status (12). For the RPA194 IP, experiments were normalized by including a reference IP performed with the same strongly positive anti-RPA194 serum in each set (and electrophoresed on each gel). Densitometry was performed on all autoradiograms, and values were normalized to the reference IP. Antibody positivity was defined as a normalized densitometry value of >1. Results of IP performed with IVTT RPA194 using sera from 36 healthy controls were all negative.

IP from radiolabeled HeLa and 624 melanocyte cells. HeLa cells (purchased from ATCC) and 624 melanocyte cells were cultured using standard protocols before plating and radiolabeling for 2 hours with ^{35}S-methionine/cysteine. Cells were lysed in radioimmunoprecipitation assay buffer (50 m*M* Tris [pH 7.4], 150 m*M* NaCl, 5 m*M* EDTA, 0.5% Nonidet P40, 0.5% sodium deoxycholate, and 0.1% SDS), and precleared with immobilized protein A–agarose (Thermo Scientific). IPs were performed by adding 1 μl of patient serum to the lysate (for 1 hour at 4°C), followed by protein A–agarose (for 25 minutes at 4°C). After washing, the immunoprecipitates were electrophoresed on 10% SDS–polyacrylamide gels and visualized by fluorography. Of note, IPs performed using radiolabeled cell lysates detected all antibody specificities in the serum, whereas IPs performed with IVTT protein detected only antibodies against the input protein.

Statistical analysis. Demographic, clinical, and autoantibody characteristics were compared between 1) anti-RPC155–positive patients with cancer and anti-RPC155–positive patients without cancer and 2) anti-RPA194–positive patients and anti-RPA194–negative patients using Student's *t*-test for continuous variables and the chi-square test or Fisher's exact test for dichotomous or categorical variables where appropriate. The Mann-Whitney U test was used to assess differences in ordinal variables. Statistical analyses were performed using Stata version 13.1 (StataCorp). *P* values less than 0.05 (2-sided) were considered significant.

RESULTS

Clinical characteristics of the anti-RPC155–positive scleroderma cohorts. This study was performed using sera from a cohort of 168 well-characterized scleroderma patients with anti-RPC155 antibodies evaluated at the Johns Hopkins Scleroderma Center. Eighty sera were from patients with a history of cancer, and 88 were from randomly selected anti-RPC155 antibody–positive patients with no known malignancy after ≥5 years of follow-up. The clinical characteristics of these patients were examined by cancer status (Table 1). Scleroderma patients with anti-RPC155 and cancer had a short interval between cancer diagnosis and scleroderma onset (mean ± SD 1.1 ± 11.3 years from the first non–Raynaud's phenomenon symptom and 4.6 ± 14.9 years from the first symptom, either Raynaud's phenomenon or non–Raynaud's phenomenon manifestation). Scleroderma patients with cancer were older at scleroderma onset (mean ± SD age 51.7 ± 14.0 years versus 45.3 ± 13.6 years; $P = 0.0035$) and less likely to have calcinosis than scleroderma patients without cancer (33.8% versus 58.0%; $P = 0.002$). Both groups had a comparable disease duration at presentation to our center and were similar with regard to sex, race, scleroderma subtype, and smoking history. Clinical characteristics, including skin severity, baseline pulmonary function, baseline RVSP, and frequency of renal crisis, myopathy, telangiectasia, and tendon friction rubs, were not significantly different between groups. The mean baseline left ventricular ejection fraction was statistically, but not clinically, significantly higher in the cancer group. Cancer sites observed are available upon request from the corresponding author.

Discovery and identification of anti-RPA194 antibodies in scleroderma patients with RPC155 antibodies, using cancer status as a filter. To address whether the immune response differed in anti-RPC155–positive scleroderma patients with and those without cancer, we initially used an IP approach on a subset of 35 sera randomly selected from the cohorts described above. That is, sera from 17 anti-RPC155–positive scleroderma patients with cancer and 18 anti-RPC155–positive scleroderma patients without cancer were used for discovery. These 35 sera were used to immunoprecipitate proteins from radiolabeled HeLa cell lysates, and the profiles were compared. Representative results using serum from a patient without cancer (FW-1170) and a patient with cancer (FW-1088) are shown in Figure 1A. A subset of the discovery sera was also tested by IP using radiolabeled 624 melanocyte lysates (Figures 1A and B), and similar data were obtained.

In 7 (38.9%) of the 18 patients without cancer, a ~194-kd band was detected; this was only seen in 1 (6.3%) of 16 of the patients with cancer (see representative examples in Figure 1A). Additional bands (at 110, 40, 34, and 28 kd, which were possibly other protein components of the polymerase complexes) were variably immunoprecipitated by some of the sera. This finding is consistent with elegant work published by Kuwana et al (23), who used an IP approach with radiolabeled cell extracts to show that anti-RNA polymerase antibodies in scleroderma patient sera recognize multiple subunits of the RNA polymerases. Of note, the additional immunoprecipitated bands we detected were observed with similar frequencies in both the group with and the group without cancer. Because we were seeking specificities that could distinguish these groups, we focused on the 194-kd specificity that was enriched in patients without cancer.

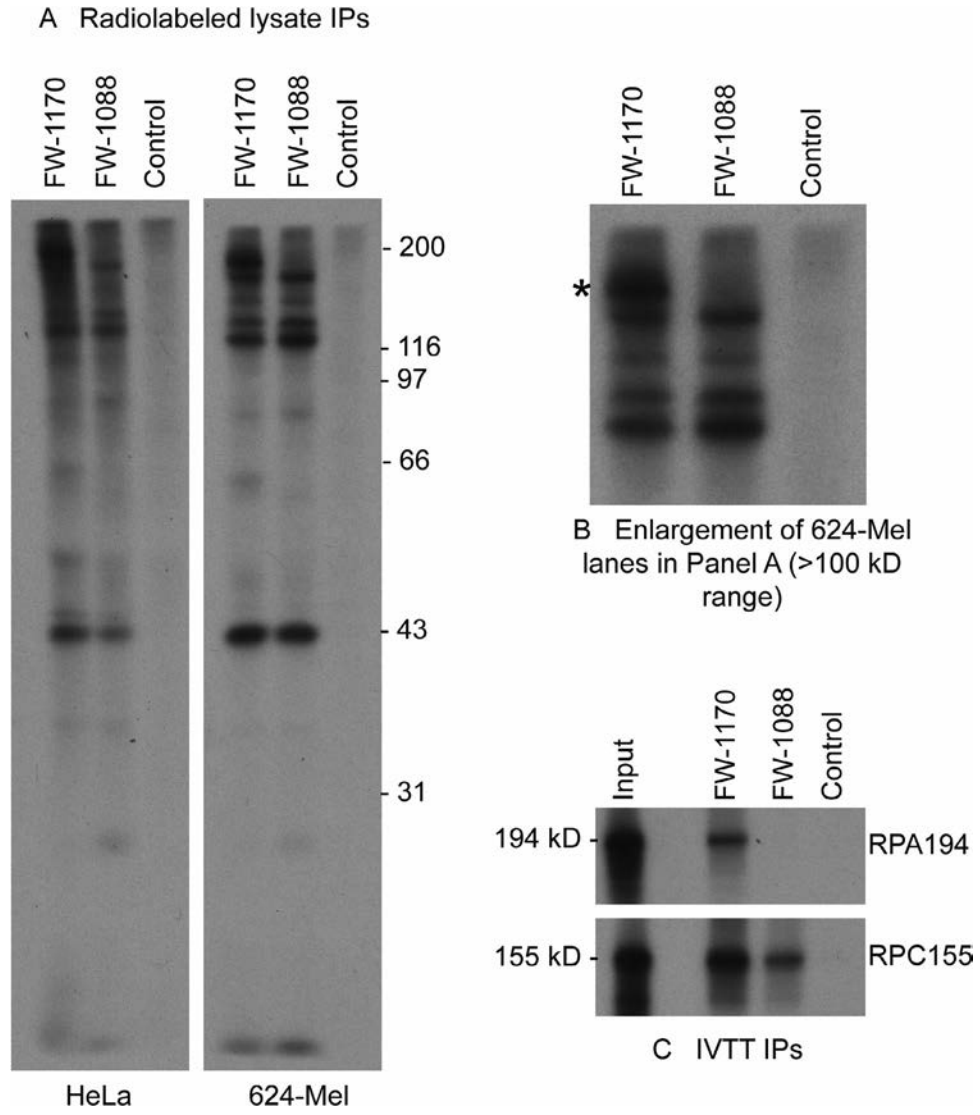

Figure 1. Discovery and identification of anti-RPA194 antibodies in anti-RPC155–positive scleroderma patients. **A**, Immunoprecipitation (IP) using radiolabeled lysates made from HeLa cells (left) or 624 melanocytes (624 Mel) (right). Sera from all patients included in the study were assayed for anti-RPC155 antibody status as described in Patients and Methods. Representative results using sera from an anti-RPC155–positive patient without cancer (FW-1170) and an anti-RPC155–positive patient with cancer (FW-1088) are shown. Serum from a healthy donor was used as a control. Migration of molecular weight marker standards is shown on the right. **B**, Enlarged view of the upper section of the 624 melanocyte lanes (>100 kd) shown in **A**. The asterisk indicates the 194-kd band detected in the IP with serum FW-1170 (and enriched in the group without cancer). **C**, Representative results of in vitro transcription/translation (IVTT) IP, which was used to confirm the IP results for the 35 sera in the discovery cohort. IP was performed with the same sera used in **A**. Input material (no IP) was ^{35}S-methionine–labeled RPA194 (top) or RPC155 (bottom) generated by IVTT, as described in Patients and Methods.

Since 1) RNA polymerases I and III share some common subunits which can enable intermolecular spreading, 2) RNA polymerase I is a known scleroderma autoantigen (13,24), and 3) the large subunit of RNA polymerase I (RPA194) is 194 kd, we tested whether the 194-kd band might be RPA194. We generated radiolabeled RPA194 by IVTT from cDNA encoding full-length RPA194, and performed IP with the putative anti-RPA194–positive serum (FW-1170) and an RPA194–negative serum (FW-1088) (Figure 1C). RPA194 pulldown was observed with the FW-1170 patient serum only, confirming that RPA194 antibodies were present in the FW-1170 serum and absent in the FW-1088 serum.

Enrichment of anti-RPA194 antibodies in anti-RPC155–positive scleroderma patients without cancer. Using IPs performed using RPA194 generated by IVTT, the full study cohort of 168 anti-RPC155–positive sera were tested for anti-RPA194 antibodies. Anti-RPA194 antibodies were significantly more common in the group without cancer (16 [18.2%] of 88 patients) than in the group with cancer

Table 2. Clinical characteristics of the anti-RPA194–positive and anti-RPA194–negative scleroderma patients*

Variable	Anti-RPA194–positive patients (n = 19)	Anti-RPA194–negative patients (n = 149)
Age at scleroderma onset, years†	44.5 ± 15.6	48.8 ± 13.9
Age at first non–Raynaud's phenomenon symptom, years	50.3 ± 11.3	51.1 ± 12.5
Disease duration at first visit, years	6.8 ± 10.1	6.2 ± 9.1
Male sex, no. (%)	1 (5.3)	30 (20.1)
Race, no. (%)		
White	19 (100)	140 (94.6)
Black	0 (0)	6 (4.1)
Asian	0 (0)	2 (1.4)
Subtype, no. (%)		
Limited cutaneous	2 (10.5)	37 (24.8)
Diffuse cutaneous	17 (89.5)	112 (75.2)
Ever smoked, no. (%)	10 (52.6)	73 (49.0)
MRSS at first visit to center	23.2 ± 14.2	18.3 ± 13.2
Maximum ever MRSS	27.5 ± 15.0	21.8 ± 14.0
Renal crisis, no. (%)	0 (0)	21 (14.1)
Myopathy, no. (%)	3 (15.8)	24 (16.1)
ILD, no. (%)‡	7 (38.9)	64 (45.1)
Baseline pulmonary function		
FVC, % predicted	85.2 ± 16.1	83.9 ± 16.2
DLco, % predicted	86.3 ± 22.6	82.6 ± 23.4
Pulmonary hypertension, no. (%)§	6 (33.3)	43 (30.3)
Baseline RVSP, mm Hg	33.6 ± 9.0	32.5 ± 9.8
Baseline ejection fraction, %	60.3 ± 9.1	61.6 ± 7.0
Severe Raynaud's phenomenon, no. (%)¶	12 (63.2)	81 (54.4)
Severe GI disease, no. (%)¶	5 (26.3)	76 (51.0)#
Calcinosis, no. (%)	11 (57.9)	67 (45.0)
Telangiectasia, no. (%)	18 (94.7)	146 (98.0)
Tendon friction rubs, no. (%)	10 (52.6)	67 (45.0)

* All patients were anti-RPC155 positive. In the anti-RPA194–positive group, data were available for 18 patients for age at first non–Raynaud's phenomenon symptom, ILD, and pulmonary hypertension, 16 patients for FVC, 14 patients for DLco, 10 patients for RVSP, and 17 patients for baseline ejection fraction. In the anti-RPA194–negative group, data were available for 148 patients for age at first non–Raynaud's phenomenon symptom, race, and maximum ever MRSS, 146 patients for MRSS at first visit, 142 patients for ILD and pulmonary hypertension, 136 patients for FVC, 121 patients for DLco, 86 patients for baseline RVSP, and 129 patients for baseline ejection fraction. Except where indicated otherwise, values are the mean ± SD. See Table 1 for definitions.

† Scleroderma onset was defined as the first symptom, either Raynaud's phenomenon or non–Raynaud's phenomenon.

‡ Defined as an FVC of <70% predicted ever.

§ Defined as an RVSP of ≥45 mm Hg ever

¶ Defined as an organ-specific severity score of ≥2 at any time during the disease course.

$P = 0.043$ versus anti-RPA194–positive patients.

(3 [3.8%] of 80 patients; $P = 0.003$). Even when secondary analyses were performed defining anti-RPC155 positivity by a more stringent cutoff of ≥40 units, the finding that anti-RPA194 antibodies were more common in the group without cancer remained unchanged (18.4% in the group without cancer versus 4.05% in the group with cancer; $P = 0.008$). Of the 3 scleroderma patients with cancer who had both anti-RPC155 and anti-RPA194, 1 patient had a basal cell skin cancer 25 years prior to scleroderma onset, 1 had prostate cancer detected 1.25 years prior to scleroderma onset, and 1 had a uterine

cancer diagnosed 5 years after scleroderma onset. Of the 18 sera from anti-RPC155–positive scleroderma patients without cancer that were used for discovery (as described above), 7 were confirmed to have anti-RPA194 antibodies using the IVTT IP assay, consistent with the initial screening frequency. Thirty-four sera from healthy controls were also tested by IVTT IP assay; none immunoprecipitated IVTT RPA194.

We next assessed whether the clinical phenotype differed between anti-RPC155–positive patients with and those without anti-RPA194 antibodies (Table 2). There were no differences in age at scleroderma onset, race, smoking status, or the frequency of ILD, pulmonary hypertension, myopathy, severe Raynaud's phenomenon, calcinosis, telangiectasia, or tendon friction rubs. While the differences were not significant, anti-RPA194–positive patients were more likely to be women (94.7% versus 79.9%; $P = 0.205$), have diffuse cutaneous scleroderma (89.5% versus 75.2%; $P = 0.249$), and have a higher baseline modified Rodnan skin thickness score (MRSS) (mean ± SD 23.2 ± 14.2 versus 18.1 ± 13.2; $P = 0.1358$) and a higher maximum MRSS (27.5 ± 15.0 versus 21.8 ± 14.0; $P = 0.0990$) than anti-RPA194–negative patients. Anti-RPA194–positive patients were also less likely to have a history of renal crisis (0% versus 14.1%; $P = 0.134$) and were significantly less likely to have severe GI disease (26.3% versus 51.0%; $P = 0.043$). Interestingly, this group was significantly more likely to have a nucleolar pattern on antinuclear antibody (ANA) staining (44.4% versus 12.5%; $P = 0.001$) than anti-RPA194–negative patients, although ANA titers did not differ between groups. This suggests that detection of a nucleolar staining pattern is more likely to capture the anti-RPA194 antibody–positive group. However, the clinical utility of these findings will be greatly enhanced by the development of a commercial assay that specifically detects anti-RPA194 antibodies.

DISCUSSION

The existence of antibodies against the RNA polymerases has been recognized for many years in scleroderma patients (23). The recent observation that autoantibodies to RPC155 in scleroderma define a group of patients with a higher risk of cancer (standardized incidence ratio 2.84 [95% confidence interval 1.89–4.10]; odds ratio ranging from 3.85 to 5.83) occurring within a short interval of scleroderma onset has been confirmed in multiple different cohorts (2–8). Mechanistic studies in scleroderma patients with cancer have demonstrated that somatic mutations at the *POLR3A* locus are present in the cancers of some anti-RPC155–positive patients, in whom mutation-specific and cross-reactive T cell immune responses develop (9). These findings strongly suggest that somatic mutations in RPC155 in scleroderma patients' cancers initiate the anti-RPC155 immune response, which spreads to the wild-type protein, exerting both anticancer and autoimmune effects.

The finding that only 15–20% of patients with anti-RPC155 ever manifest a cancer suggests several possibilities. In a first scenario, different mechanisms may underlie the development of anti-RPC155 immune responses and scleroderma phenotype in patients with and those without cancer. Alternately, it is possible that similar mechanisms (cancer with changes in RPC155 structure due to mutation) drive this immune response and scleroderma phenotype in all anti-RPC155–positive patients, but that the group with cancer and the group without cancer represent differences in the efficacy of the anticancer effect of the immune response, perhaps marked by additional immune responses in patients without cancer, or differential sensitivity of the cancer to the induced immune response. In this study, we sought evidence of the possibility that additional immune specificities are found in anti-RPC155–positive scleroderma patients without a detectable cancer.

The initial screen from radiolabeled cells demonstrated striking enrichment of a 194-kd protein immunoprecipitated by antibodies from anti-RPC155–positive patients without cancer. The migration of this protein, coupled with prior descriptions of antibodies to RNA polymerase I in scleroderma (13), and recent evidence that inhibition of RNA polymerase I activity has potent anticancer effects, rapidly led us to confirm that RPA194 was the undefined band recognized by the index sera without cancer. Subsequent analysis demonstrated that RPA194 antibodies were found in 18.2% of anti-RPC155–positive patients without cancer, while they were infrequent (3.8%) in anti-RPC155–positive patients with cancer. It is of interest that one of the patients with cancer and antibodies to RPA194 had a basal cell cancer that occurred 25 years prior to scleroderma, suggesting that they might be unrelated. Regardless, anti-RPA194 autoantibodies are enriched in anti-RPC155–positive patients without cancer. That immune responses to RPA194 are associated with the subgroup of anti-RPC155–positive scleroderma patients without cancer is particularly interesting, as recent studies have shown that a small molecule (BMH21) that inhibits RNA polymerase I activity has broad, potent, and selective antitumor activities across multiple cancer cell lines in vitro, and represses tumor growth in vivo in mouse models (14). It is of interest that 6 polymerase subunits are shared among RNA polymerases I and III, providing an important opportunity for intermolecular spreading. Understanding the targeting of these shared subunits in patients with and those without cancer might shed additional mechanistic insights into the origin of the broader immune responses in scleroderma patients without cancer.

Taken together, the observations that many cancers have increased RNA polymerase I activity, that inhibition of this activity has anticancer effects, and that immune responses to the catalytic subunit of RNA polymerase I (RPA194) are associated

with a lower frequency of cancer in the anti-RPC155–positive scleroderma group raise the possibility that the RPA194 immune response plays a mechanistic role in the immune-mediated control of cancer in scleroderma. Like most autoantigens in the systemic rheumatic diseases, RPA194 is intracellular, raising important questions about how such an effect might be mediated. Several scenarios are possible. First, since RNA polymerase I expression and activity is increased in many cancers, it is possible that an anticancer effect is exerted by cell-mediated immunity, which is selectively directed against cancer cells expressing high levels of RPA194. Second, it is possible that autoantibodies recognizing intracellular antigens may have direct anticancer effects (25). For example, a series of recent studies showed that anti-DNA antibodies induced the death of cancer cells in vitro and in vivo, particularly in cancer cell lines with defects in various DNA repair pathways (25). It is similarly possible that autoantibodies recognizing RPA194 might inhibit RPA194 inside cells, thereby exerting anticancer effects. Third, a different set of initiating events (other than cancer) might be responsible for initiating the combined immune responses against both RPC155 and RPA194. Confirming these findings in other scleroderma cohorts, and pursuing these mechanistic questions, remain important priorities.

If the association of anti-RPA194 antibodies with decreased cancer incidence in a subgroup of RPC155-positive scleroderma patients is confirmed in other studies, then detecting anti-RPA194 at diagnosis might have clinical utility in identifying the subset of anti-RPC155–positive patients who do not require extensive malignancy evaluation. The observation that only ~20% of anti-RPC155–positive patients without cancer have anti-RPA194 antibodies suggests that additional mechanisms may be associated with a lower cancer prevalence in the remaining patients. One possibility is that additional immune specificities (other than RPA194) occur in the context of RPC155 immune responses. Finding such specificities would further support the hypothesis that orthogonal immune responses targeting additional cellular machines are associated with decreased cancer frequencies in the high-risk anti-RPC155–positive group.

The subgroup of scleroderma patients with both RPC155 and RPA194 antibodies was not large enough to determine with certainty whether they manifested a more severe form of scleroderma than those with anti-RPC155 alone, but the available data does not suggest this. Indeed, none of the anti-RPA194–positive patients manifested scleroderma renal crisis (present in 14% of the group with anti-RPC155 only), and severe GI disease was present significantly less frequently in the subgroup with both anti-RPC155 and anti-RPA194 than in the subgroup with anti-RPC155 only (26% versus 51%). In future studies with larger numbers of RPA194 antibody–positive patients, it will be important to define the phenotype and mortality in the anti-RPC155 groups with or without RPA194 antibodies.

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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. Casciola-Rosen 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. Shah, Laiho, Rosen, Casciola-Rosen.
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Effect of Dietary and Supplemental Omega-3 Polyunsaturated Fatty Acids on Risk of Recurrent Gout Flares

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Objective. To determine the relationship between omega-3 polyunsaturated fatty acid (n-3 PUFA) consumption (dietary or supplemental) and risk of gout flares.

Methods. We used data from the Boston University Online Gout Study, an internet-based case-crossover study conducted from February 2003 to January 2012. At the times of gout flares (hazard period) and during gout flare–free periods (control periods), participants completed questionnaires regarding exposures, including supplements and diet, during the preceding 48 hours. We examined the relationship of self-reported n-3 PUFA–rich supplements and fish intake with the risk of recurrent gout flares using conditional logistic regression, adjusting for total purine intake, diuretic use, and other urate-lowering or flare prophylactic medications (allopurinol, nonsteroidal antiinflammatory drugs, or colchicine).

Results. Of the 724 participants, 85% met the 1977 American College of Rheumatology preliminary criteria for the classification of the acute arthritis of primary gout. Twenty-two percent of the participants reported some form of n-3 PUFA consumption (supplements, 4.6%; dietary fatty fish, 19%) in the 48 hours preceding a gout flare or flare-free period. The adjusted odds ratios were 1.01 (95% confidence interval [95% CI] 0.63–1.60; $P = 0.98$) for all 3 supplements combined and 0.74 (95% CI 0.54–0.99; $P = 0.04$) for consumption of ≥2 n-3 PUFA–rich fish servings.

Conclusion. Dietary n-3 PUFA–rich fish consumption, when adjusted for total purine intake, was associated with lower risk of recurrent gout flares, whereas n-3 PUFA supplementation alone, as taken in a self-directed manner, was not. Consumption of specific sources and adequate doses of n-3 PUFA for gout flare prevention warrants further study in an adequately powered clinical trial.

INTRODUCTION

Gout, an inflammatory crystal-induced arthritis caused by monosodium urate deposition within joints, affects approximately 8.3 million adults in the US (1,2). Although the pathophysiology of this disease is well understood and urate-lowering therapies are widely available, recurrence of gout flares remains high (2,3). Identifying additional options for gout flare management could alleviate the burden of this common inflammatory disorder.

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) have recently garnered interest for their potential antiinflammatory effects. Fatty fish such as tuna, mackerel, and salmon are a rich natural source of the biologically active n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (4). Although another formulation of n-3 PUFA called α-linolenic acid can also be found in plant-based sources such as flax, canola, soybeans, and walnuts, α-linolenic acid is biologically inactive and must be consumed in large quantities to achieve the same level of EPA and DHA available in fatty fish (4,5). With respect to gout, certain n-3 PUFAs have been shown to have multiple antiinflammatory effects through rapid and selective inhibition of the NLRP3 inflammasome via G protein–coupled receptors (GPRs), specifically GPR120 and GPR140, through β-arrestin (6–9). Additional downstream effects include suppression of Toll-like receptor activation, neutrophil

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chemotaxis, NF-κB activity, and prostaglandin synthesis (8–11). These in vitro findings have been supported by the results of in vivo experiments in which rats that were fed a diet of oil-rich fish and plant seeds developed less inflammation when injected with monosodium urate crystals than rats fed a standard diet (12). Importantly, these antiinflammatory effects occur independently of enzymatic product generation, a process that relies on the slow integration of n-3 PUFAs into the leukocyte plasma membrane (9). By bypassing this slower pathway, it is possible that n-3 PUFAs have acute benefits even when consumed over short intervals.

Clinically, n-3 PUFA supplementation has demonstrated antiinflammatory effects in several arthritic conditions. Multiple randomized controlled trials, systematic reviews, and meta-analyses have noted reduced disease activity with n-3 PUFA consumption in patients with rheumatoid arthritis and psoriatic arthritis (13–20). Fewer studies have explored the impact of n-3 PUFA consumption on gout, however. Abhishek et al showed that low serum n-3 PUFA levels were associated with increased frequency of gout flares, although they were not able to adjust for other dietary confounders (21). Interestingly, n-3 PUFA–rich diets have also been linked to reduced inflammation in the gut microbiome, with clinical implications for metabolic syndrome, cancer, and nonalcoholic fatty liver disease (22–25). Whether this is the same mechanism by which n-3 PUFA can affect gout remains to be elucidated.

In contrast to n-3 PUFAs, n-6 PUFAs such as arachidonic acid and linoleic acid, which are commonly found in vegetable oils and animal sources, possess greater proinflammatory properties mediated by generation of prostaglandins, leukotrienes, cyclooxygenase, and thromboxanes (5). There is evidence that the ratio of n-3 to n-6 PUFA consumption plays a critical role in the development of inflammation (8,26). Diets rich in n-6 PUFA but low in n-3 PUFA have been associated with increased inflammation in rheumatoid arthritis, cardiovascular disease, cancer, and even osteoarthritis (27–29). No studies to date have examined the effect of n-6 PUFA on gout flare development. Understanding the benefits and potential harms of n-3 and n-6 PUFA consumption with regard to gout may have broad health implications, given that approximately half of American adults report using 1 or more dietary supplements, with approximately 12% using supplements for joint health (30).

In this study, we sought to determine the relationship between n-3 PUFA consumption (dietary or supplemental) and risk of gout flares in a case-crossover investigation. We hypothesized that subjects taking n-3 PUFA supplements or consuming n-3 PUFA–rich diets would have lower risk of gout flare recurrence compared with subjects who did not consume n-3 PUFA. We also sought to examine the impact of the ratio of n-3 to n-6 levels on gout flare risk.

PATIENTS AND METHODS

Study design. The Boston University Online Gout Study was an internet-based, case-crossover study conducted from 2003 to 2012 and originally aimed at identifying risk factors for recurrent gout flares (31). Each subject served as his or her own control, eliminating between-person differences (e.g., genetics, sex, education, and race) to minimize time-invariant confounding (32). Such an approach allowed for comparison of the relative frequency of risk factors prior to gout flares (hazard periods) and during gout flare–free periods (control periods) within the same subject. Several nutritional factors, including consumption of purine-rich foods, use of alcohol, and consumption of cherries, in relation to gout flares have been examined in this study (33–35). The Institutional Review Board of Boston University Medical Campus approved this study.

Study sample. Potential subjects were recruited through a Google advertisement linked to the search term "gout," which then directed them to the study web site (https://dcc2.bumc.bu.edu/GOUT) on an independent secure server within the Boston University Medical Center domain. At baseline, subjects provided information on sociodemographic characteristics, medical history, past medications, and gout-related history (i.e., diagnosis of initial gout attack, age at onset, medication used for the treatment of attacks, and number of gout attacks in the last 12 months).

Eligibility criteria included the following: 1) age ≥18 years, 2) resident of US, 3) physician diagnosis of gout, 4) experienced a gout flare within the preceding 12 months, 5) agree to the release of medical records pertaining to gout diagnosis, and 6) electronically provide informed consent. Gout diagnoses were confirmed through chart review of participant medical records and/or a checklist, completed by the subject's physician, of the features in the 1977 American College of Rheumatology (ACR) preliminary criteria for the classification of the acute arthritis of primary gout (36). All medical records and checklists were separately reviewed by 2 rheumatologists to ensure accurate diagnosis of gout according to the ACR criteria.

Gout flare assessment. At the time of a gout flare, subjects were asked to complete online questionnaires regarding the attack, including date of onset, anatomic location of the flare, clinical signs and symptoms (e.g., erythema, swelling, or pain within 24 hours), and treatment (e.g., colchicine, nonsteroidal antiinflammatory drugs [NSAIDs], systemic glucocorticoids, and intraarticular glucocorticoid injections). This method has been advocated by the ACR/European League Against Rheumatism and utilized in gout trials for defining gout flares based on patient-reported features (37,38). To ensure more stringent inclusion criteria, definite flares were further limited to the following: 1) presenting with erythema, podagra, and maximal pain within 24 hours of onset, 2) requiring treatment with at least 1 antiinflammatory medication (listed above), and 3) a combination of any of those features (e.g., cases with 2, 3, or all 4 features). Of the subjects' reported gout flares, 95% met these criteria.

Diet and supplement intake assessment. Participants completed questionnaires every 3 months over a 12-month period

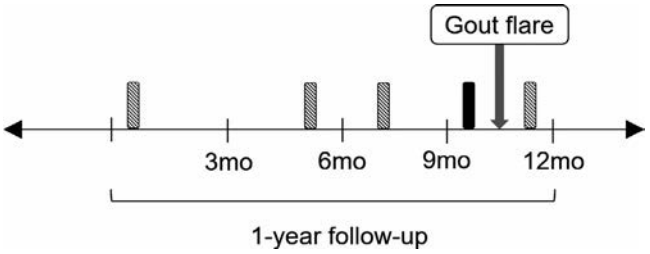

Figure 1. Case-crossover study design and timing of hazard periods (solid bar) and control periods (hatched bars) with respect to gout flares for an individual participant. Participants were evaluated during control and hazard periods for 1 year. Hazard periods were defined as the 48 hours preceding a gout attack. Control periods, which were defined as the 48 hours preceding gout flare–free periods, were assessed every 3 months during the 1-year follow-up for each participant. Exposure to supplemental and dietary omega-3 polyunsaturated fatty acid was compared between control and hazard periods.

during intercritical periods and additionally at times of gout flares, at which a series of potential exposures over the prior 24 and 48 hours were assessed (Figure 1). Exposures assessed included dietary factors, medications, trauma/physical activity, and geographic location. Via free text, subjects could enter information on additional medications or supplements not specifically queried. N-3 PUFA–rich supplements were identified from among the free-text entries as "fish oil," "cod liver oil," and "omega-3 fatty acids." Doses were not consistently reported. N-3 PUFA consumption related to fatty fish, including anchovies, mackerel, salmon, sardines, trout, and herring, was identified and quantified as the total number of servings in the preceding 24 and 48 hours, based on standard serving sizes for cooked fish (5 ounces).

Statistical analysis. We categorized n-3 PUFA–rich supplements as "any" versus "none," taken in the 48 hours preceding a gout flare or flare-free period, for each type individually (fish oil, cod liver oil, and n-3 PUFA supplements) as well as for any of the 3 types combined. For n-3 PUFA consumption related to fatty fish in the preceding 48 hours, we categorized the number of servings as 0, 1, or ≥2 servings. Because these fatty fish have relatively high n-3 to n-6 PUFA ratios, we also examined foods with differing ratios to further explore the potentially beneficial effects of n-3 PUFA versus the potentially detrimental effects of n-6 PUFA. We used spinach as an example of a food with a relatively equivalent n-3 to n-6 PUFA ratio, and eggs as an example of a food with a relatively low n-3 to n-6 PUFA ratio. We examined the relationship of n-3 PUFA consumption, as defined by these various types of supplements and food sources, with the risk of recurrent gout flares using conditional logistic regression, which takes into account the matching of each subject's own hazard and control periods (39). In multivariable regression models, we adjusted for diuretic use, alcohol intake, total purine intake, and gout-related medication use (allopurinol, colchicine, NSAIDs, and other urate-lowering therapies). In a sensitivity analysis, we adjusted for purine intake related to foods other than fatty fish, instead of total purine intake, to examine the full effects of fatty fish intake on the risk of gout flare.

RESULTS

Between February 2003 and January 2012, 724 participants completed online questionnaires during control and hazard periods. Participants were recruited from 48 states and Washington, DC. The mean age of the participants was 54.5 years, and the study population was predominantly white (88.7%), male (78.5%), and obese, with a mean body mass index of 32.1 kg/m^2 (Table 1). Approximately 85% of the participants met the 1977 ACR preliminary criteria for the classification of the acute arthritis of primary gout, and 48% were taking urate-lowering medications or prophylactics against flares (NSAIDs, 38.0%; colchicine, 25.4%; and allopurinol, 43.8%). During the 1-year follow-up period, a total

Table 1. Baseline characteristics of the study sample (n = 724)*

Age, mean ± SD years	54.5 ± 12.5
Male sex	568 (78.5)
BMI, mean ± SD kg/m^2	32.1 ± 6.9
Race	
White	642 (88.7)
African American	20 (2.7)
Other	53 (7.3)
Refused to answer	9 (1.2)
Education	
Did not complete high school	14 (1.9)
Completed high school	66 (9.1)
Some college/technical school	223 (30.8)
Completed college/technical school	175 (24.2)
Some professional/graduate school	78 (10.8)
Completed professional/graduate school	168 (23.2)
Annual household income, $	
<25,000	58 (8.0)
25,000–49,999	141 (19.5)
50,000–74,999	133 (18.4)
75,000–99,999	106 (14.6)
≥100,000	186 (25.7)
Refused to answer	100 (13.8)
Disease duration, mean ± SD years	8.0 ± 9.3
Use of alcohol	316 (43.7)
Treatments	
NSAIDs	275 (38.0)
Colchicine	184 (25.4)
Allopurinol	317 (43.8)

* Except where indicated otherwise, values are the number (%) of patients. BMI = body mass index; NSAIDs = nonsteroidal antiinflammatory drugs.

of 1,434 gout attacks occurred, 89% of which were managed with NSAIDS, colchicine, and/or intraarticular/systemic glucocorticoids.

Of the 724 participants, 22% reported consuming n-3 PUFAs within the 48 hours preceding a gout flare or flare-free period; of these, 4.6% reported consuming n-3 PUFAs in a supplemental form (self-reported as fish oil, cod liver oil, or n-3 PUFA) and 19% reported consuming dietary fatty fish (self-reported as anchovies, mackerel, salmon, sardines, trout, or herring). Among the participants who consumed a supplemental form of n-3 PUFA, none of the 3 supplements individually significantly reduced recurrent gout flare risk. After adjustment for dietary factors (use of alcohol and total purine intake), diuretic use, and use of other urate-lowering medications or prophylactics against flares (allopurinol, NSAIDs, and colchicine), the adjusted odds ratio (OR_{adj}) for any consumption of the 3 supplement types combined in the preceding 48 hours was 1.01 (95% confidence interval [95% CI] 0.63–1.60; $P = 0.98$) compared with no consumption (Table 2).

In contrast, participants who consumed n-3 PUFA–rich fish experienced a significant reduction in recurrent gout flare risk. Consuming any amount of fatty fish in the 48 hours preceding a gout flare period was associated with a 23% lower risk of gout flare compared with no fatty fish consumption (OR_{adj} 0.77 [95% CI 0.61–0.96]; $P = 0.02$), after adjustment for potential confounders. Moreover, a dose-response relationship was noted. For 1 serving of n-3 PUFA–rich fish consumed, the OR_{adj} was 0.79 (95% CI 0.61–1.03; $P = 0.08$), and for ≥2 servings consumed, the OR_{adj} was 0.74 (95% CI 0.54–0.99; $P = 0.04$) compared with no consumption in the preceding 48 hours (Table 3). After adjustment for purine content other than that derived from fatty fish consumption, the occurrence of any intake of n-3 PUFA–rich fish in the preceding 48 hours was no longer associated with risk of gout flares (OR_{adj} 1.09 [95% CI 0.88–1.36]).

Because we hypothesized that n-6 PUFAs may counteract beneficial effects of n-3 PUFAs in relation to gout flares, we further evaluated the effects of various foods on risk of gout flare based on their n-3 to n-6 PUFA ratios. In contrast to the findings for fatty fish, which possess higher n-3 to n-6 ratios, foods with more neutral n-3 to n-6 ratios, such as spinach, did not confer any risk reduction (OR_{adj} 1.19 [95% CI 0.85–1.66], for ≥2 servings consumed) (Table 4). Foods with low n-3 to n-6 ratios, such as eggs, significantly increased the risk of gout flare (OR_{adj} 1.34 [95% CI 1.10–1.63], for ≥2 servings consumed).

DISCUSSION

In this large cohort of US adults with preexisting gout, n-3 PUFA–rich fish consumption was significantly associated with lower risk of recurrent gout attacks. Participants who consumed n-3-PUFA–rich fish in the 48 hours preceding a gout flare period had a 23% lower risk of recurrent gout flare compared with those who did not. Risk reduction also occurred in

Table 2. Relationship of n-3 PUFA–rich supplement consumption with risk of recurrent gout flares*

Supplement	Control periods, no.	Case periods, no.	Adjusted OR (95% CI)†
Fish oil			
Not consumed	1,870	1,381	1.0 (referent)
Consumed	77	53	1.04 (0.58–1.87)
N-3 PUFA			
Not consumed	1,914	1,417	1.0 (referent)
Consumed	33	17	1.33 (0.43–4.10)
Cod liver oil			
Not consumed	1,940	1,432	1.0 (referent)
Consumed	7	2	0.48 (0.03–7.65)
All 3 supplements			
Not consumed	1,830	1,362	1.0 (referent)
Consumed	117	72	1.01 (0.63–1.60)

* N-3 PUFA = omega-3 polyunsaturated fatty acid; OR = odds ratio; 95% CI = 95% confidence interval.
† Adjusted for diet (use of alcohol and total purine intake), diuretic use, and use of other urate-lowering or prophylactic medications against flares (allopurinol, nonsteroidal antiinflammatory drugs, colchicine).

Table 3. Relationship of n-3 PUFA–rich fish consumption with risk of recurrent gout flares*

Exposure	Control periods, no.	Case periods, no.	Adjusted OR (95% CI)†
N-3 PUFA–rich fish:			
0 servings	1,564	1,123	1.0 (referent)
1 serving	214	165	0.79 (0.61–1.03)
≥2 servings	166	146	0.74 (0.54–0.99)‡

* N-3 PUFA = omega-3 polyunsaturated fatty acid; OR = odds ratio; 95% CI = 95% confidence interval.
† Adjusted for diet (use of alcohol and purine intake), diuretic use, and use of other urate-lowering medications or prophylactics against flares (allopurinol, nonsteroidal antiinflammatory drugs, colchicine).
‡ $P = 0.04$.

a dose-dependent manner, whereby increases in the number of servings were associated with lower risk of gout flares.

In contrast, n-3 PUFA supplements such as fish oil and cod liver oil, as used in the community with varying regimens and doses, did not appear to have a protective effect against flare occurrence. One possible explanation for this discrepancy between dietary and supplemental n-3 PUFA is insufficient dosing. Prior population studies have shown that median total daily intake of fish oil in the community is approximately 0.3 gm of EPA and DHA (40). In past rheumatoid arthritis studies, antiinflammatory effects of n-3 PUFA consumption were observed at daily doses of 3.4 gm and higher, which is approximately 10 times greater than the typical over-the-counter formulation (14). Depending on the type of fish and method of preparation, EPA and DHA levels in fatty fish sources can range from 0.7 gm to 5.6 gm per serving size (41). As such, patients taking supplemental n-3 PUFA are more likely to be consuming a level of EPA and DHA far below that which is required for a significant antiinflammatory effect. The results of our study suggest that n-3 PUFA supplements, when administered at adequate doses, may provide a prophylactic effect. In addition to this potential therapeutic benefit, high-dose n-3 PUFA has a relatively benign side-effect profile. Although increased bleeding times have been observed in Greenland Inuits due to their n-3 PUFA–rich diet, no studies have shown significant bleeding risk with high-dose fish oil supplementation (42,43). Gastrointestinal side effects such as nausea, eructation, and loose stools are much more common with increased dosing of supplements, but whether this is the case with dietary fatty fish is not clear.

Table 4. Risk of recurrent gout flare based on different n-3 to n-6 PUFA ratios*

Exposure	Adjusted OR (95% CI)†
N-3 > n-6 (fatty fish)	–
1 serving	0.79 (0.61–1.03)
≥2 servings	0.74 (0.54–0.99)
N-3 = n-6 (spinach)	–
1 serving	0.83 (0.62–1.10)
≥2 servings	1.19 (0.85–1.66)
N-3 < n-6 (eggs)	–
1 serving	0.85 (0.66–1.08)
≥2 servings	1.34 (1.10–1.63)

* Referent group was zero servings. N-3 PUFA = omega-3 polyunsaturated fatty acid; OR = odds ratio; 95% CI = 95% confidence interval.
† Adjusted for diet (use of alcohol and purine intake), diuretic use, and use of other urate-lowering medications or prophylactics against flares (allopurinol, nonsteroidal antiinflammatory drugs, colchicine).

The risk reduction observed in this study remained after adjustment for total purine content consumed, including that contributed by fatty fish consumption. It is well established that purine-rich diets, which include most seafood, lead to increased risk of gout flares (44). Our study demonstrated that isolation of dietary n-3 PUFA independent of purine content led to protective antiinflammatory effects. However, when we evaluated the association of n-3 PUFA–rich fish consumption with adjustment for purine consumption not related to the fish intake, there was no longer an association noted. Thus, this study provides a proof-of-concept finding regarding the potential for n-3 PUFA to provide a beneficial effect in reducing gout flare risk in the absence of concomitant purines. While isolation of n-3 PUFA is difficult to achieve with dietary fatty fish since it has concomitant purines, supplements would bypass this obstacle and offer a pharmacologic benefit without the gout-inducing features of purines.

We also observed an association between relative levels of n-3 and n-6 PUFA and gout flare risk. Foods with a higher n-3 to n-6 ratio, such as fatty fish, were associated with lower risk of gout flares, foods with a more neutral n-3 to n-6 ratio, such as spinach, had no effect on gout flare risk, and foods with a lower n-3 to n-6 ratio, such as eggs, increased the risk of gout flare. Although our study did not assess whether the beneficial effects of dietary and supplemental n-3 PUFA were truly related to mechanisms affecting inflammation, our results align with those of prior studies suggesting that n-3 to n-6 levels can influence the degree of inflammation (27,29).

This study comprised a large adult cohort with extensive data on a wide range of triggers and confounding variables. The case-crossover design allowed for the analysis of multiple exposures and their acute effects while avoiding control selection bias through self-matching of subjects. Internet recruitment enabled access to a large participant population, and real-time self-reporting via online questionnaires reduced recall bias. Also, our results were fairly robust to unmeasured confounding, as the observed OR_{adj} of 0.77 for any fatty fish consumption in the 48 hours preceding a gout flare period compared with no such consumption could only be explained away by an unmeasured confounder that was associated with both the exposure and the outcome by a risk ratio of 1.92-fold each, above and beyond the measured confounders, while weaker confounding could not do so (45).

There were some limitations to our study. Crystal-proven diagnosis of gout was not common, reflecting the general community pattern of gout diagnosis; in the Health Professionals Follow-Up Study, only 7% of participants had a crystal-proven diagnosis of gout (46). Nonetheless, all patients in the current study met the 1977 ACR preliminary criteria for the classification of the acute arthritis of primary gout or had their gout diagnosis confirmed by chart review, and we implemented additional criteria to define "definite flare." Additionally, the study design required self-reporting of exposures, including dietary intake, although any misclassification was likely to be nondifferential, particularly since study participants were not apprised of study hypotheses. Also, the various types of fish evaluated do not have equal amounts of n-3 PUFA, and therefore this misclassification would likely have biased our results toward the null. Furthermore, our study was not able to assess supplementation dose or serum n-3 PUFA levels; therefore, we cannot rule out an effect of potentially adequately dosed supplements. Only a few subjects reported use of supplemental n-3 PUFA (4.6% of participants), which limited precision. Our study also did not assess serum urate levels, so it is unclear if n-3 PUFA effects were mediated through effects on urate level or through other mechanisms. Finally, it is possible that the beneficial effects of fatty fish intake may be, in part, related to not ingesting other potentially risk-inducing foods (e.g., red meat); we attempted to account for this with purine intake adjustment.

Our study highlights the potentially beneficial effect of n-3 PUFA for limiting the symptom burden from acute flares in gout. Although supplemental n-3 PUFA as taken in a self-directed manner was not found to be protective, and fatty fish is not the ideal source of n-3 PUFA due to its concomitant purines, these results provide support for future clinical trials examining n-3 PUFA supplementation at appropriate antiinflammatory doses for gout flare prevention.

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 submitted for publication. Dr. Neogi 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. M. Zhang, Y. Zhang, Terkeltaub, Neogi.
Acquisition of data. Neogi.
Analysis and interpretation of data. M. Zhang, Y. Zhang, Terkeltaub, Chen, Neogi.

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Empowering Rheumatology Professionals

LETTERS

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Could the levels of inflammatory biomarkers predict osteoarthritis? Comment on the article by Roemer et al

To the Editor:

We read with great interest the article by Dr. Roemer et al, in which they determined the role of inflammatory biomarkers at 2 years post–anterior cruciate ligament (ACL) injury in predicting the risk of radiographic knee osteoarthritis (ROA) and magnetic resonance imaging (MRI)–defined knee osteoarthritis (OA) at 5 years postinjury (1). The results confirmed that neither MRI findings nor the levels of inflammation biomarkers in the synovial fluid (SF) or serum at 2 years postinjury were predictive of the incidence of ROA or MRI-defined OA (MROA) at 5 years postinjury.

To our limited knowledge, ACL rupture is a common destructive injury associated with long-term sequelae, including meniscal tears, cartilage damage, and increased risk of knee OA. ACL reconstruction is recommended to prevent knee instability, reduce the possibility of meniscal tears and further surgery, and restore early physical activity; however, ACL reconstruction does not reduce the incidence of early-onset OA (2). It was confirmed that OA is a low-grade inflammatory disease of the synovial joints. High levels of inflammatory cytokines are induced following acute ACL injury, and these are associated with proteolysis of aggrecan and type II collagen. Cytokine levels often continue to increase up to 5 years after ACL injury (3). In a systematic review of patients with ACL injury who had undergone ACL reconstruction, overall levels of inflammatory cytokines were increased in the SF postoperatively as compared with preoperative values, and patients were found to have altered levels of other biomarkers that could be indicative of OA (4).

We have several concerns regarding the recent article by Roemer and colleagues (1). First, in most other studies, inflammatory cytokine levels at different time points were examined, and their relationship to the subsequent incidence of OA was analyzed. We question why the authors chose 2 years as the time point for cytokine measurements instead of other time points in assessing their capacity for predicting the incidence of OA at 5 years after ACL injury. The authors did not provide an explanation, and we were unclear as to why they did not choose to continuously monitor the levels of inflammatory cytokines (before and after the onset of the most severe synovitis) as an OA predictor. Second, surgical ACL reconstruction results in a second trauma to acutely injured joints, leading to long-term elevation in the levels of inflammatory cytokines that had already been found to be high in the SF of patients (5). Moreover, according to their article, the overall incidence of OA (including patellofemoral joint OA, temporomandibular joint OA, and whole joint OA) after ACL injury was not low. Because the patients with ACL injury who underwent ACL reconstruction received different treatments, the authors should have indicated whether there was a difference in the level of inflammatory factors and the incidence of OA after the different treatments. Third, the authors did not determine whether patients with ACL injury had a tibia fracture, especially a tibial plateau fracture, which can easily result in traumatic OA.

We respect the great contributions made by Roemer et al, and we anticipate the follow-up results of this study.

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Reply

To the Editor:

We thank Dr. Deng and colleagues for their interest in our article, and we are happy to answer the important questions raised. As explained in the Patients and Methods section, we chose to analyze the 2-year data (and not earlier time points) in regard to inflammatory activity based on MRI and SF biomarkers. Our goal was to observe patients that experienced prolonged or chronic inflammation, which may be regarded as a potential risk factor for OA development. Virtually all patients in an acute injury cohort (like the Knee ACL Nonsurgical versus Surgical Treatment [KANON] patients) will exhibit acute posttraumatic inflammation in their injured knee to a various extent as manifested by marked changes on imaging (i.e., effusion, traumatic synovitis, and hemarthrosis) or in biochemical parameters. In addition, knee surgery itself may impose a second "trauma" to the joint, resulting in secondary inflammation as previously reported (1). The specific time point of 2 years was chosen because we only had data available for a much smaller subset at the 1-year time point. Furthermore, inflammation at the 2-year time point can certainly be defined and understood as prolonged inflammation.

We fully agree that any surgical procedures performed in the knee over the study period could be of importance for the future risk of OA. However, to the best of our knowledge, there are very few reports supporting the notion that reconstruction of the ACL reduces structural damage or lowers the risk of long-term inflammation. Available reports on structural change over 5 years after ACL injury suggest no difference between treatment groups or, in some cases, increased structural change in ACL reconstructed knees as opposed to rehabilitation alone. Furthermore, we are not aware of any reports suggesting that knee surgery is of greater importance than other possible factors such as level of physical activity and weight change. Also, in the KANON patients, several types of surgeries (i.e., ACL reconstruction with and without meniscus surgery, arthroscopic meniscus partial resection, and fixation as well as synovectomies and diagnostic arthroscopies) were performed at different time points. Given the complexity described above, the limited sample size, and since the importance of type and/or timing of surgery is unknown, we decided not to include treatment as an OA predictor in our analyses. However, we will aim to disentangle the complex issue of potential covariates but this is preferably done using specifically designed approaches.

In addition, we acknowledge that the severity of baseline structural damage, including fractures, may play an important role in the development of subsequent OA. While we showed the frequencies of fracture in the KANON study in a previous publication (2), we did not consider tibial fractures in our current analyses as this was not the research question in focus.

The KANON study received funding from the Swedish Research Council, the Medical Faculty of Lund University, Region Skåne, the Thelma Zoegas Fund, the Stig & Ragna Gorthon Research Foundation, the Swedish National Center for Research in Sports, the Crafoord Foundation, the Tore Nilsson Research Fund, and Pfizer Global Research. Funding sources had no role in the design, collection, and interpretation of the data or the decision to submit for publication. Dr. Roemer has received consulting fees, speaking fees, and/or honoraria from Novartis (less than $10,000), owns stock or stock options in Boston Imaging Core Lab, LLC, and serves as Chief Medical Officer of Boston Imaging Core Lab, LLC. Dr. Guermazi has received consulting fees, speaking fees, and/or honoraria from Sanofi-Aventis, OrthoTrophix, AstraZeneca, GE Healthcare, Galapagos, and Roche (less than $10,000 each) and from Merck Serono, TissuGene, and Pfizer (more than $10,000 each), owns stock or stock options in Boston Imaging Core Lab, LLC, and serves as President of Boston Imaging Core Lab, LLC. No other disclosures relevant to this article were reported.

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Association of autoantibody quantification with systemic lupus erythematosus disease activity: comment on the article by Kim et al

To the Editor:

We read with great interest the recent article by Dr. Kim and colleagues regarding the association of blood concentrations of complement split product iC3b and serum C3 with systemic lupus erythematosus (SLE) disease activity (1). SLE is a prototypic autoimmune disease characterized by excess autoantibody production. Although there is an increasing number of publications on SLE biomarkers, there is no widely accepted biomarker for the evaluation of disease flare as of yet (2). Similar to that of com-

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Association of autoantibody quantification with systemic lupus erythematosus disease activity: comment on the article by Kim et al

To the Editor:

We read with great interest the recent article by Dr. Kim and colleagues regarding the association of blood concentrations of complement split product iC3b and serum C3 with systemic lupus erythematosus (SLE) disease activity (1). SLE is a prototypic autoimmune disease characterized by excess autoantibody production. Although there is an increasing number of publications on SLE biomarkers, there is no widely accepted biomarker for the evaluation of disease flare as of yet (2). Similar to that of com-

plement split products, the role of autoantibodies in evaluating disease activity remains a subject of controversy.

Autoantibody measurements based on multiplex bead technology have been proven to be accurate and convenient in patients with various connective tissue diseases (3). To clarify the role of autoantibodies in SLE disease progression, we quantified 15 routinely used IgG-class antibodies (anti–double-stranded DNA [anti-dsDNA], anti–SSA 52 kd [anti–Ro 52], anti–SSA 60 kd [anti–Ro 60], anti-SSB [La], anti-Sm, anti-RNP, anti–Scl-70, anti–Jo-1, anti–CENP-B, anti-PM/Scl, antinucleosome, anti–ribosomal P [anti-P], anti–proliferating cell nuclear antigen [anti-PCNA], antihistone, and anti–mitochondrial M2) simultaneously, using a multiplex technology–based test (AtheNA Multi-Lyte ANA Test System, REF A20001; Zeus Scientific). Ethics approval was obtained from the Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School (no. 2016-027-01), and each subject provided written informed consent.

In total, 163 hospitalized SLE patients were enrolled, all of whom fulfilled the 1997 updated American College of Rheumatology criteria for SLE (4). Disease activity, calculated according to the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score (5), was independently evaluated by one of the authors (YaZ), and Pearson correlation was used to identify the association between the SLEDAI score and antibody levels. Ninety-two percent of the patients were female, the mean ± SEM age was 36.6 ± 1.0 years (range 10–69 years), and the mean ± SEM disease duration was 6.6 ± 0.5 years. The average SLEDAI score was 5.7 ± 0.4 (range 0–20). In this cohort, 53.4% of the patients had renal involvement, and hypocomplementemia (based on C3 or C4 levels) occurred in 80.4%. Among the 15 autoantibodies measured, the levels of antinucleosome (r = 0.32, $P < 0.0001$), anti-dsDNA (r = 0.35, $P < 0.0001$), anti-Sm (r = 0.28, $P < 0.001$), anti-P (r = 0.24, $P < 0.01$), and antihistone (r = 0.30, $P < 0.0001$) (Figure 1A) were significantly correlated with SLEDAI scores.

To explore whether autoantibody levels varied along with the fluctuation of disease activity at different time points, we reexamined 21 of the patients after a mean ± SD of 3.0 ± 1.7 months. The average decline in the SLEDAI score was 2.7 ± 0.9 at the follow-up visit, and for 4 of the 5 above-mentioned

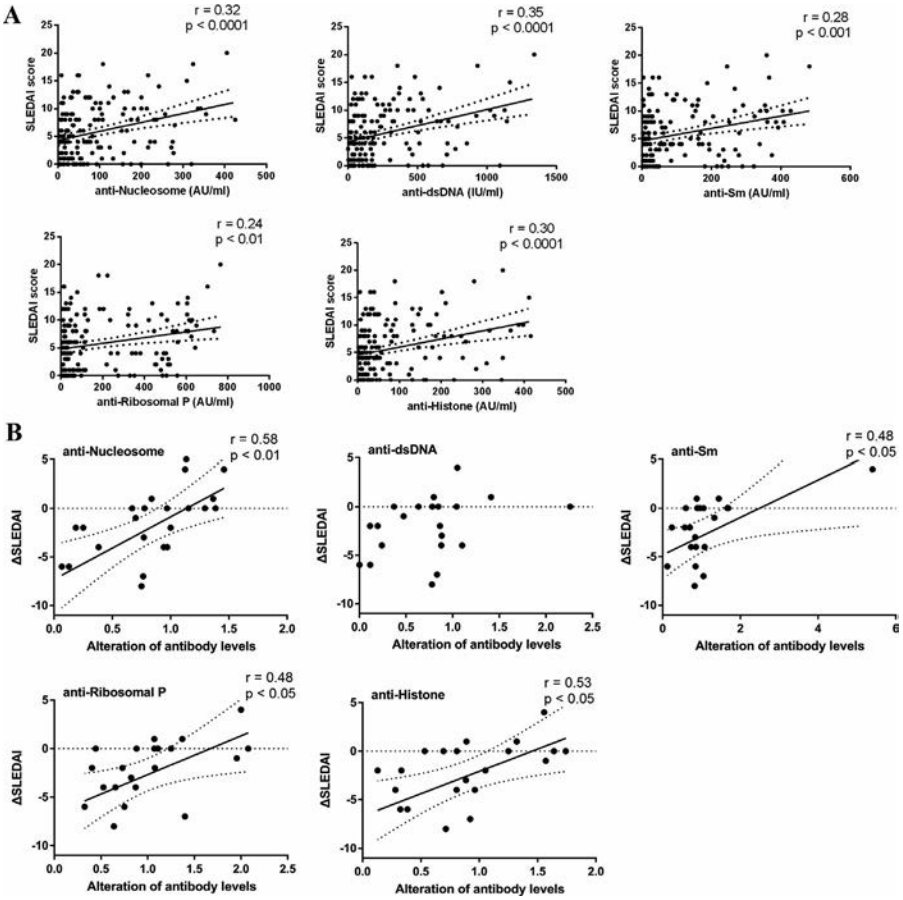

Figure 1. Associations of autoantibody levels with systemic lupus erythematosus (SLE) disease activity. **A**, The levels of antinucleosome, anti–double-stranded DNA (anti-dsDNA), anti-Sm, anti–ribosomal P, and antihistone were significantly correlated with the SLE Disease Activity Index (SLEDAI) score by Pearson correlation analysis. **B**, For 4 of the 5 autoantibodies in **A**, changes in levels over time were significantly correlated with changes in the SLEDAI score. Dotted lines above and below the correlation lines represent the 95% confidence intervals.

antibodies (antinucleosome [r = 0.58, $P < 0.01$], anti-P [r = 0.48, $P < 0.05$], anti-Sm [r = 0.48, $P < 0.05$], and antihistone [r = 0.53, $P < 0.05$]), alterations in levels were closely related to changes in the SLEDAI score (Figure 1B). Our data imply, for the first time, that the level of antihistone is relevant to changes in the SLEDAI score. Theoretically, antihistone may act together with other autoantibodies to influence complement-dependent phagocytosis by polymorphonuclear leukocytes (6), contributing to disease progression. Meanwhile, the expression of anti-dsDNA involves a mixture of antibodies that are all directed to DNA, among which the low-avidity antibodies are not specific for SLE or associated with disease activity (7). Similar to enzyme-linked immunosorbent assay, the laser bead immunoassays used in this study could not distinguish antibodies with different avidities; thus, it was a reasonable finding that anti-dsDNA levels did not significantly fluctuate with the changes in disease activity.

In conclusion, we demonstrated that the levels of antinucleosome, anti-P, anti-Sm, and antihistone were closely related to SLE disease activity at a specific time point, as well as to changes in disease activity at different time points. The detection of a variety of autoantibodies, and especially measurement of their levels, may help monitor disease activity in patients with lupus.

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Reply

To the Editor:

We thank Dr. Tang and colleagues for their interest in our article, and for sharing their data demonstrating the association of several autoantibodies with disease activity in a Chinese cohort of SLE patients. Autoantibodies in SLE patients arise years prior to clinical detectability (1), drive the formation of immune complexes that activate complement to induce target organ damage, and may serve as biomarkers of disease activity (2).

Tang et al used a multiplex bead platform to examine the relationship between the titers of 15 autoantibodies and SLE disease activity. In cross-sectional analyses, a correlation of antinucleosome, anti-dsDNA, anti-Sm, anti-P, and antihistone titers with SLEDAI scores (treated as a continuous variable) was observed in hospitalized patients. The correlation coefficients were modest (~0.3). This may be due to a substantial number of subjects in their cohort who had absent or low titers of autoantibodies, but were included in the correlation analysis. A longitudinal, post-hospitalization analysis was performed in a subset of these SLE patients (n = 21). It revealed a stronger association between these same autoantibodies and a change in SLEDAI scores (except with anti-dsDNA, which may be due to the detection of low-affinity antibodies as correctly noted by the authors) (3).

There are 2 limitations with the use of autoantibody titers as a surrogate for SLE disease activity. As mentioned above, not all patients may have autoreactivity with any given autoantigen. The other limitation is the variance observed between the assays used for autoantibody assessment. This is best described for anti-dsDNA (3), but this is also true for antinuclear antibody testing, where discordances using similar immunofluorescence assays have been demonstrated (4). A "gold standard platform" for autoantibody detection has not yet been resolved, which currently limits the biomarker potential of autoantibodies.

A single biomarker is likely insufficient to assess SLE disease activity. While our study demonstrated the independent association of iC3b:C3 ratios with SLE disease activity, not all subjects exhibited this association. For example, we have observed that only 14 of 27 subjects in our cohort had an increase in iC3b:C3

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There are 2 limitations with the use of autoantibody titers as a surrogate for SLE disease activity. As mentioned above, not all patients may have autoreactivity with any given autoantigen. The other limitation is the variance observed between the assays used for autoantibody assessment. This is best described for anti-dsDNA (3), but this is also true for antinuclear antibody testing, where discordances using similar immunofluorescence assays have been demonstrated (4). A "gold standard platform" for autoantibody detection has not yet been resolved, which currently limits the biomarker potential of autoantibodies.

A single biomarker is likely insufficient to assess SLE disease activity. While our study demonstrated the independent association of iC3b:C3 ratios with SLE disease activity, not all subjects exhibited this association. For example, we have observed that only 14 of 27 subjects in our cohort had an increase in iC3b:C3

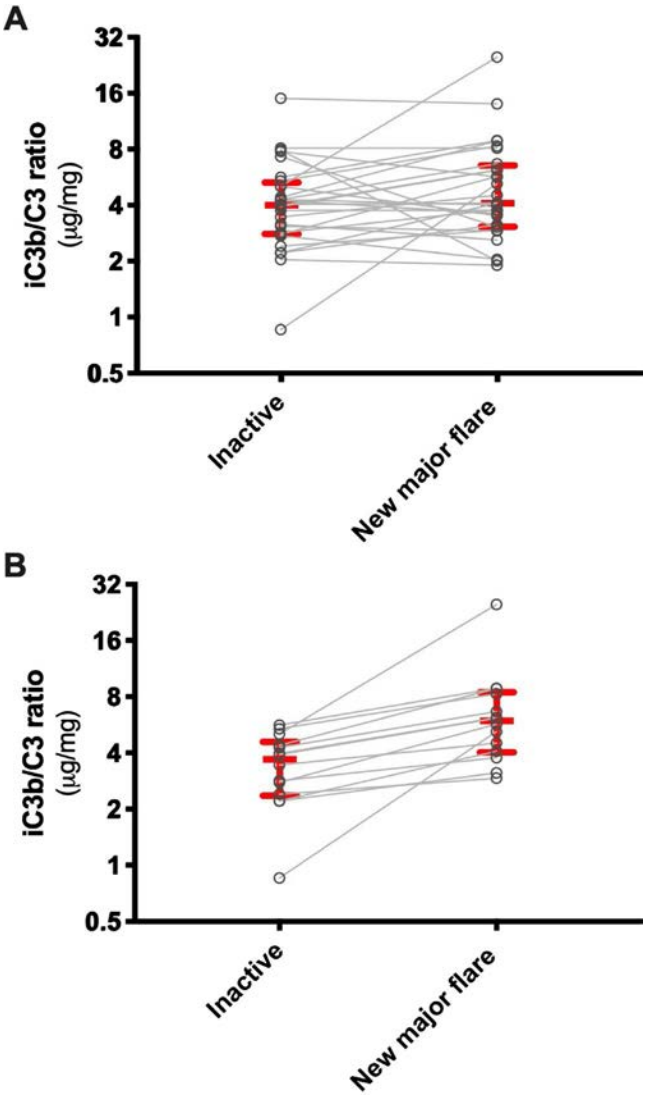

Figure 1. Association of iC3b:C3 ratios with new major flares in a proportion of subjects with systemic lupus erythematosus (SLE). **A**, Ratios during inactive disease and during major flare in each of 27 subjects assessed during both periods. In the overall group, iC3b:C3 ratios were only marginally lower during inactive disease (median 4.012 [interquartile range 2.803–5.307]) (red bars) compared to ratios during major flare (median 4.099 [interquartile range 3.068–6.544]) (P = 0.147). **B**, Ratios during inactive disease and during major flare in a subset of the overall group. In this subset consisting of 14 subjects, iC3b:C3 ratios did increase during major flare (major flare 5.938 [interquartile range 4.017–8.430]) compared to inactive disease (median 3.532 [interquartile range 2.359–4.565]) (P = 0.001).

ratios that correlated with a new major flare using the Fortin definition (5) (Figure 1). The assessment of additional complement species, such as hydrolyzed C3 (6) or other complement activation products (7,8), may be needed to fully categorize the complement activation signatures in patients with SLE. Furthermore, analysis of intraindividual changes of complement levels over time will likely have more clinical value than comparisons to the lower limit of normal cutoff defined in healthy controls. This is due to known alterations in complement metabolism in SLE. For example, C3 tickover is ongoing at low levels, even in patients with inactive disease (9). This significantly limits the appropriate interpretation of complement levels, if compared to healthy control reference values.

We envision that complement activation products could be combined with autoantibody profiles, clinical manifestations, and additional investigational biomarkers to distinguish clusters of SLE patients, each with a defined set of biomarkers that can be best associated with disease activity.

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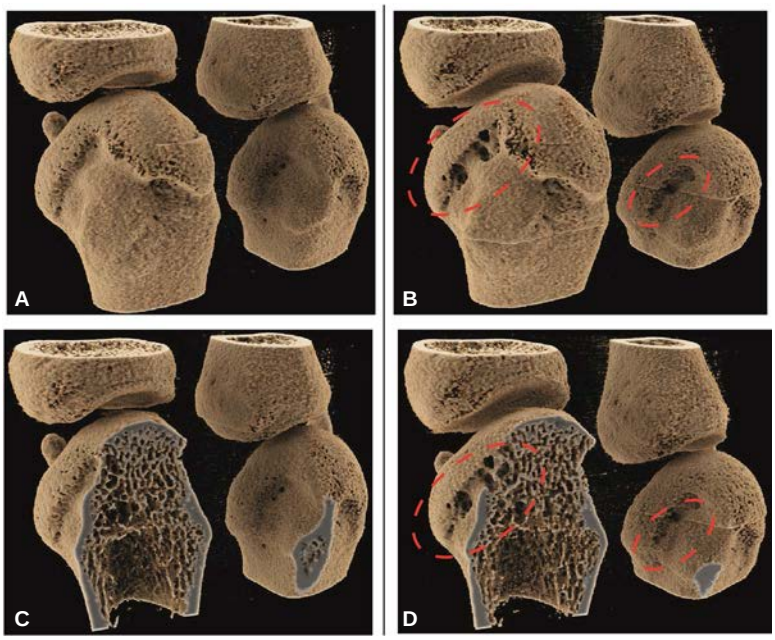
Clinical Images: Development of joint erosions in the preclinical phase of rheumatoid arthritis depicted by cinematic rendering



The patient, a 27-year-old man, was referred to our practice due to sporadic arthralgia. Morning stiffness and swelling were absent. No other medical conditions were reported. The patient was a current smoker. He was positive for anti–citrullinated peptide antibodies (750 units/ml); rheumatoid factor and C-reactive protein levels were within normal ranges. On clinical examination, no joint tenderness or swelling was detected, and ultrasound and magnetic resonance imaging did not show signs of synovitis. Cinematic rendering of high-resolution peripheral quantitative computed tomography (HR-pQCT) scans of the second and third metacarpophalangeal (MCP) joints revealed no abnormalities (**A** and **C**). Based on the autoantibody positivity without clinical or imaging signs of arthritis, he was considered to be in rheumatic arthritis (RA)–at-risk status (1). This status persisted over a period of 24 months with regular clinical follow-ups every 6 months. However, at the 24-month follow-up he reported that the arthralgia had recently worsened, and there were 4 swollen and 11 tender joints, including the MCP2 joints bilaterally. Ultrasound of an MCP2 joint revealed active synovitis. A second HR-pQCT scan with cinematic rendering showed clear signs of multiple erosions at the bare area of the joints (**circled areas** in **B** and **D**). Structural bone damage is often considered to be a late consequence of inflammation in RA (2). However, it is possible that it develops earlier, but has not been recognized due to insufficient imaging capability. The novel combination of HR-pQCT with cinematic rendering incorporates a more advanced lighting model for volume rendering, creating photorealistic, highly detailed 3-dimensional images of joint surfaces. HR-pQCT with cinematic rendering can reveal RA-related bone erosions at the onset of worsening symptoms, as in our patient, in whom the lesions formed in the preclinical phase of the disease. This finding provides possible evidence of a pathogenic role of autoimmunity in RA-related bone erosions.

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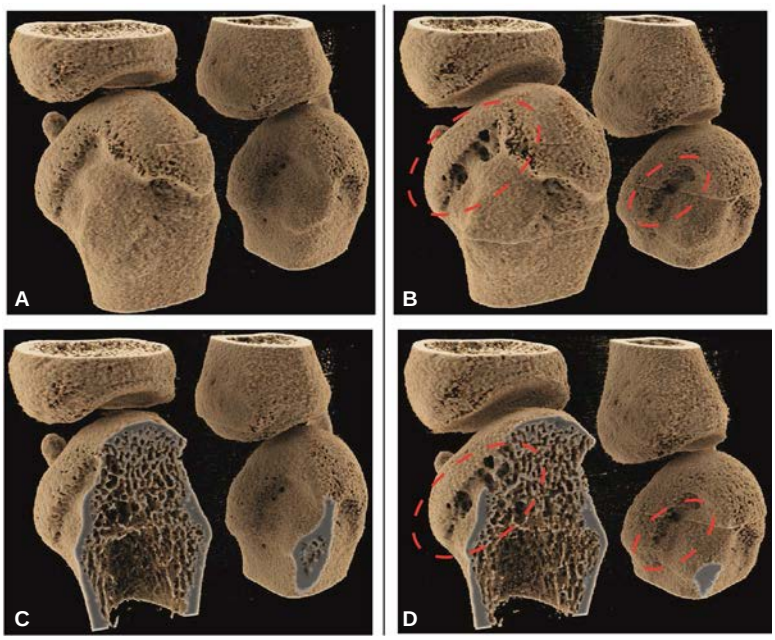

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