Relatives Without Rheumatoid Arthritis Show Reactivity to Anti–Citrullinated Protein/Peptide Antibodies That Are Associated With Arthritis-Related Traits

Studies of the Etiology of Rheumatoid Arthritis

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Objective. To examine reactivity to anti–citrullinated protein/peptide antibodies (ACPAs) and determine associations between ACPAs and other rheumatoid arthritis (RA)–related autoantibodies and clinically assessed swollen or tender joints in unaffected first-degree relatives of RA patients.

Methods. Serum samples were obtained from first-degree relatives without RA according to the 1987 American College of Rheumatology (ACR) and the 2010 ACR/European League Against Rheumatism classification criteria. A bead-based assay was used to measure 16 separate ACPAs in sera from 111 antibody-positive first-degree relatives who were positive on at least 1 visit for any of 5 RA-related autoantibodies (rheumatoid factor [RF], anti–cyclic citrullinated peptide 2 [anti–CCP-2], and RF isotypes), and sera from 99 antibody-negative first-degree relatives who were never autoantibody positive. Cutoffs for positivity for each ACPA were determined using receiver operating characteristic curves derived from data on 200 RA patients and 98 blood donor controls, in which positivity for ≥9 ACPAs had 92% specificity and 62% sensitivity for RA. In first-degree relatives, ACPA reactivity was assessed, and associations between ACPAs (number positive, and positivity for ≥9 ACPAs) and RA-related characteristics were examined.

Results. Fifty-seven percent of anti–CCP-2–positive first-degree relatives and 8% of anti–CCP-2–negative first-degree relatives were positive for ≥9 ACPAs. After adjusting for age, sex, ethnicity, and pack-years of smoking, an increasing number of ACPAs was directly associated with the presence of ≥1 tender joint on examination (odds ratio [OR] 1.18, 95% confidence interval [95% CI] 1.04–1.34), with the greatest risk of having ≥1 tender joint seen in first-degree relatives positive for ≥9 ACPAs (OR 5.00, 95% CI 1.37–18.18).

Conclusion. RA-free first-degree relatives (even

those negative for RF and anti–CCP-2) demonstrate reactivity to multiple ACPAs, and the presence of an increasing number of ACPAs may be associated with signs of joint inflammation. Prospective evaluation of the relationship between these findings and the progression of classifiable RA is warranted.

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease of unknown etiology that leads to joint damage, significant disability, and reduced life expectancy (1). Nearly 70% of cases of established RA are characterized by the presence of autoantibodies, either rheumatoid factor (RF) or antibodies to citrullinated protein antigens (ACPAs). ACPAs can be detected with an anti–cyclic citrullinated peptide (anti-CCP) antibody test, the most specific clinical test currently available. RF and anti-CCP antibodies are routine targets of testing in RA patients and can aid in making the diagnosis of RA; however, the prospective sensitivity and specificity of these tests are still uncertain in clinically unaffected populations (2,3). In addition, ACPAs recognize many citrullinated epitopes, thereby limiting the ability to make inferences about the type and expansion of unique ACPA responses (4,5).

Development of RA has not been associated with recognition of a specific citrullinated epitope, although seropositive patients with arthralgia who display an expanded ACPA repertoire have a higher risk of developing arthritis (6), and a recent study indicated that specific patterns prior to symptom onset may exist (7). While the full extent of reactivity is unknown, ACPAs have been shown to bind to citrullinated epitopes on fibrinogen, α-enolase, vimentin, type II collagen, histones, and biglycan (4,7–16).

ACPAs likely play a role in the pathogenesis of RA. In murine models of arthritis, ACPAs induce disease (17), increase disease severity (18), and enhance tissue injury (5). ACPAs have been shown to activate complement through both the classical and alternative pathways (19), are found in circulating immune complexes (20), and stimulate macrophage production of tumor necrosis factor α through Toll-like receptor 4 and Fcγ receptor (21,22). ACPAs are highly specific for the diagnosis of RA and are present in the blood for a significant period of time prior to symptom onset, as has been demonstrated by previous biobank studies examining ACPAs in stored samples obtained from individuals who subsequently developed signs and symptoms and were diagnosed as having RA (23–25). In addition, spreading of ACPAs to additional citrullinated epitopes can occur years prior to the diagnosis of RA (8,9,11),

with RF and anti–CCP-2 titers increasing nearer the time of disease onset (8,23,24), suggesting that there is an expansion of autoimmunity in early RA development that, if fully understood, may provide insight into the earliest antigenic targets important in disease pathogenesis.

First-degree relatives of patients with RA are at increased risk of developing RA (26). As these individuals do not have clinically apparent disease but are at increased risk of future RA, they are an informative population in which to study relationships between RA-related autoantibodies, epidemiologic exposures, and potential etiologies of RA (27–34). Previous ACPA studies in unaffected family members have indicated an increased prevalence of positivity for ACPAs in this group compared to healthy control subjects (27,35). When characterization of the ACPA epitope response was performed in a subset of the subjects studied, few unaffected relatives showed any reaction to the 8 citrullinated epitopes studied (35), although abnormal reactivity was found in patients with established RA, suggesting that evolution of ACPA reactivity is an important part of a transition from asymptomatic autoimmunity to symptomatic inflammatory arthritis.

The goals of these analyses were to examine whether ACPA array testing detects autoimmunity in individuals at risk of RA, beyond the tests for anti–CCP-2 and RF, and whether this autoimmunity is biologically relevant, as indicated by clinical findings in the joints, such as tenderness and swelling, that may be indicative of early inflammatory arthritis. Specifically, in our well-characterized cohort of first-degree relatives without classified RA, we examined evidence of reactivity to a panel of ACPAs that has been shown to measure epitope spreading prior to diagnosis of RA (8). In addition, we examined the association of ACPAs with RA-related characteristics, including positivity for RA-related autoantibodies identified through standard clinical testing (i.e., detection of RF by several methods and detection of anti–CCP-2) and independently assessed swollen or tender joint counts on clinical examination.
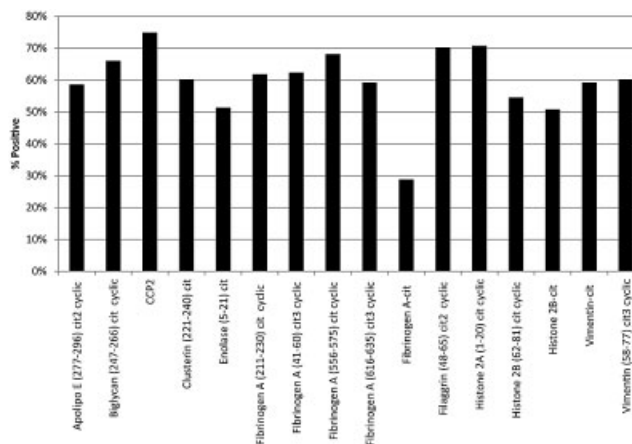
SUBJECTS AND METHODS

Study population. Studies of the Etiology of Rheumatoid Arthritis (SERA) is designed to examine the role of environmental and genetic factors in the development and progression of RA-related autoimmunity and to explore preclinical immunologic changes and other relevant phenotypes (32). Probands with RA are identified from academic centers, Department of Veterans Affairs hospitals, and private and public sector rheumatology clinics at sites based in Denver,

New York, Chicago, Omaha (as the center of the Rheumatoid Arthritis Investigational Network), Seattle, and Los Angeles. Probands must meet ≥4 of the 1987 American College of Rheumatology (ACR) classification criteria for RA (36) based on chart review, or have a diagnosis of RA from a board-certified rheumatologist.

First-degree relatives of the probands with RA are also recruited into the SERA, as their risk for RA is estimated to be increased 3–9-fold compared to that in the general population (26). The definition of first-degree relative includes parent, full sibling, or offspring of a proband. Recruitment of first-degree relatives occurs through their probands or responses to advertising. First-degree relatives are eligible to participate in the study if they do not have a diagnosis of RA and are age ≥18 years. At the initial research visit, first-degree relatives complete disease and exposure assessment questionnaires, undergo a standardized interview and 68–joint count examination by a trained study physician or nurse, and have blood withdrawn. First-degree relatives determined to have a diagnosis of RA based on the 1987 ACR criteria (36) at the time of their initial visit are excluded from the SERA longitudinal cohort. All eligible first-degree relatives are invited to attend longitudinal followup visits, which include blood withdrawal, joint examination, interview, and questionnaires; first-degree relatives who are found to be positive for any RA-related autoantibody at any visit are seen annually, and autoantibody-negative first-degree relatives are seen every other year.

As of October 2009, 1,421 first-degree relatives had been evaluated at least once in the SERA. In this cohort, 236 first-degree relatives were positive for any of 5 RA-associated autoantibodies (RF, the RF isotypes IgM, IgG, and IgA, or anti–CCP-2 autoantibodies) on at least 1 of their visits, while 1,185 first-degree relatives were negative for these autoantibodies. We selected 113 first-degree relatives who had been both autoantibody positive and seen for at least 2 visits (antibody-positive first-degree relatives), for a total of 297 visits; the ACPA assay in 1 of these first-degree relatives had errors, leaving 112 antibody-positive first-degree relatives for analysis. In addition, 100 first-degree relatives who had never been autoantibody positive at any visit (antibody-negative first-degree relatives) were selected, using the data from 1 visit. These subjects were frequency-matched to the 113 autoantibody-positive first-degree relatives according to age, sex, and ethnicity. This method of first-degree relative selection allowed us to maximize the number of visits in which subjects were positive for either autoantibody phenotype analyzed. Although first-degree relatives who meet the 1987 classification criteria for RA at the initial visit are not included in the SERA, 4 of the first-degree relatives were positive for RA using the ACR/European League Against Rheumatism (EULAR) 2010 criteria (37) on at least 1 of their visits. Therefore, to ensure that the population examined was truly RA-free, we removed all visits where the first-degree relative met the ACR/EULAR 2010 classification criteria. This removed 5 visits from our analyses, which left 111 antibody-positive first-degree relatives (with data from 292 visits) and 99 antibody-negative first-degree relatives (with data from 1 visit) available for analysis.

Autoantibody studies. All samples were tested for the presence of RF, RF isotypes (IgM, IgG, and IgA), and anti–CCP autoantibodies. RF (in IU/ml) was measured by

Figure 1. Percentage of rheumatoid arthritis (RA) probands positive for each anti–citrullinated protein antibody. The prevalence ranges from 29% for citrullinated (cit) fibrinogen A to 75% for cyclic citrullinated peptide 2 (CCP-2), with the majority of probands with RA being positive for >50% of each of the epitopes. Apolipo E = apolipoprotein E.

nephelometry using a Dade Behring BN 100 system. The RF isotypes IgM, IgG, and IgA (in IU/ml) were measured using enzyme-linked immunosorbent assay (ELISA) kits (Quanta Lite; Inova Diagnostics) in accordance with the manufacturer's specifications. Anti-CCP (in units/ml) was measured using an anti–CCP-2 ELISA kit (Diastat; Axis-Shield Diagnostics). We established a dichotomous cutoff level for each of the RF assays according to the 1987 ACR RA criteria, specifying a positive RF level if RF was present in <5% of 491 blood donor controls, separate from the SERA first-degree relative population. Anti-CCP was considered positive at a level of >5 units/ml, in accordance with the manufacturer's recommendation. Positivity for the high-risk autoantibody profile, which has been determined to have 96% specificity for future RA, as demonstrated in a prior study by our group (38) and confirmed in other studies (23,24,39–41), was defined as being positive for anti–CCP-2 autoantibodies and/or 2 of the RF isotypes (IgM, IgG, or IgA).

To identify autoantibody reactivities to specific citrullinated antigens, a novel multiplex platform was developed (42) using 16 citrullinated autoantigens, as well as 3 native proteins that are not targeted in RA as background controls (8). This array uses a custom Bio-Plex bead-based autoantibody assay (Bio-Rad), in which antigens are conjugated to spectrally distinct beads. Protein antigens were coupled to beads using N-hydroxysuccinimide ester chemistry, and peptide antigens were synthesized with C-terminal biotin (using 9-fluorenylmethoxycarbonyl chemistry) and coupled to avidin-coated beads. Pooled beads were mixed with serum samples and diluents and incubated at room temperature. After washing, an anti-human IgG antibody conjugated to phycoerythrin (PE) was added to the dyed beads and incubated at room temperature. After another wash, the bead mixture was passed through a laser detector (Luminex 200) that identifies beads based on the fluorescence of the dyes. The amount of antibody bound to each bead was determined by the fluorescence of PE.

Table 1. Characteristics of antibody-positive and antibody-negative first-degree relatives at the first visit*

	Antibody-positive first-degree relatives (n = 111)	Antibody-negative first-degree relatives (n = 99)	P
Age, mean ± SD years	49.3 ± 17.3	49.8 ± 17.8	0.83
Female	83 (74.7)	74 (74.8)	1.0
Non-Hispanic white ethnicity	91 (82.0)	82 (82.8)	0.68
Ever smoker	35 (31.5)	38 (38.1)	0.32
Current smoker	10 (9.1)	9 (9.3)	0.96
RF positive by nepholometry	34 (30.6)	0 (0.0)	NA
RF-isotype positive			
IgM	27 (24.3)	0 (0.0)	NA
IgG	37 (33.3)	0 (0.0)	NA
IgA	21 (18.9)	0 (0.0)	NA
Anti–CCP-2 positive	7 (6.3)	0 (0.0)	NA
High-risk profile positive	19 (17.1)	0 (0.0)	NA
Number of ACPAs, mean ± SD	2.3 ± 4.0	1.4 ± 2.8	0.08
Positive for ≥9 ACPAs	14 (12.6)	7 (7.1)	0.18
≥1 swollen joint on examination	11 (9.9)	4 (4.0)	0.10
≥1 tender joint on examination	7 (6.3)	1 (1.0)	0.045

* Except where indicated otherwise, values are the number (%) of subjects. RF = rheumatoid factor; NA = not applicable; anti-CCP = anti–cyclic citrullinated peptide 2; ACPAs = anti–citrullinated protein antibodies.

Determination of cutoffs for ACPA positivity. Cutoffs for positivity for each of the ACPAs individually were developed using receiver operating characteristic (ROC) curves of data from 200 RA probands and 98 blood donor controls. Values that provided >90% specificity for RA were used as cutoffs to define positivity for individual ACPAs. Positivity for the ACPA epitopes in the RA probands ranged from 29% to 75% (Figure 1). We also used a ROC curve to determine a cutoff for the total number of positive ACPAs (e.g., 0, 1, 2, etc.) that would provide >90% specificity for RA.

Statistical analysis. All analyses were conducted in SAS (version 9.3). Associations were examined in a model in which the predictor variables were an ordinal measure, defined as the number of positive ACPAs (range 0–16), and a dichotomous measure, defined as positivity for ≥9 ACPAs, an ACPA count that has been demonstrated to have 92% specificity and 62% sensitivity for RA in ROC curve analysis. The study outcomes were 1) the presence of RF by nephelometry, 2) the presence of the individual RF isotypes IgG, IgM, or IgA, 3) the presence of the high-risk profile (positive for anti–CCP-2 and/or 2 RF isotypes), and 4) the presence of swollen or tender joints on examination (≥1 affected joint). Patients with swollen or tender joints on examination were excluded if the features could be attributed to injury, degenerative joint disease, or findings in the first metatarsophalangeal joint, which is more commonly affected by osteoarthritis rather than RA. These exclusions were considered negative for swollen or tender joints. As there were only a few anti–CCP-2–positive first-degree relatives (n = 7), we did not analyze anti–CCP-2 positivity as an independent outcome.

Relationships between ACPAs and our dichotomous outcomes of RF positivity, RF-isotype positivity, high-risk profile positivity, presence of ≥1 swollen joint, and presence of ≥1 tender joint were calculated using a nonlinear mixed model, accounting for multiple visits per subject (1 visit per autoantibody-negative first-degree relative, and an average of 2.62 visits per autoantibody-positive first-degree relative). This logistic regression model with random subject effects assumed a logit link function and an outcome with a Bernoulli distribution, conditional on subjects. Mixed models account for multiple visits per subject by distinguishing variability between subjects from variability between repeated measurements from the same subject. These models estimate these 2 components separately, and then use functions of these variance estimates as weights to provide the best statistically available method to adjust for differing numbers of visits per subject. Results are reported as the odds ratio (OR) and 95% confidence interval (95% CI). All multivariate analyses were adjusted for age, sex, ethnicity, and pack-years of smoking.
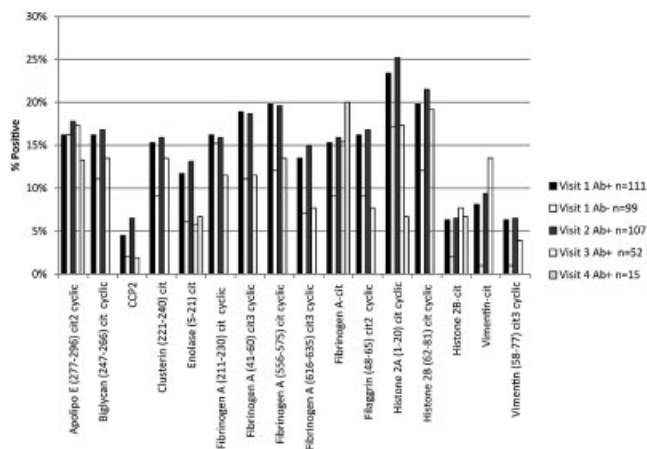
RESULTS

Characteristics of the study population at the first visit are presented in Table 1. Among autoantibody-positive first-degree relatives (n = 111), the mean ± SD age was 49.3 ± 17.3 years, while among autoantibody-negative first-degree relatives (n = 99), the mean ± SD age was 49.8 ± 17.8 years (P = 0.83). In both groups, 75% of the subjects were female and ~82% were non-Hispanic white (P not significant). Thirty-two percent of autoantibody-positive first-degree relatives and 38% of autoantibody-negative first-degree relatives had ever smoked (P = 0.32), while ~9% of both groups were current smokers. Among autoantibody-positive first-degree relatives, 9.9% had at least 1 swollen joint and 6.3% had at least 1 tender joint; among autoantibody-
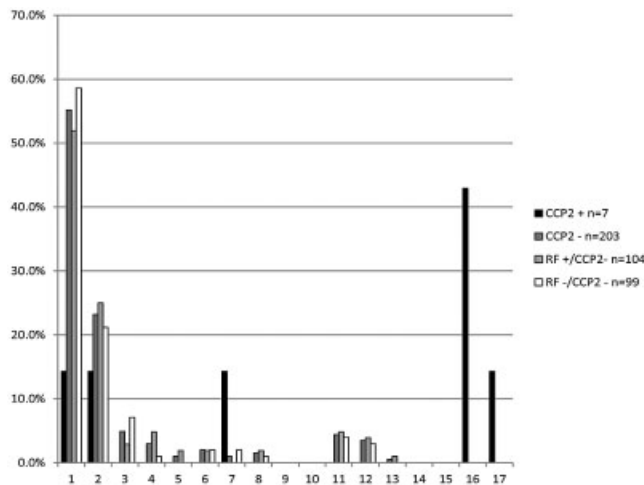
negative first-degree relatives, 4.0% had at least 1 swollen joint and 1.0% had at least 1 tender joint. Of the autoantibody-positive first-degree relatives, 30.6% were positive for RF by nephelometry, 17.1% were positive for the high-risk autoantibody profile, and 6.3% were positive for anti–CCP-2 autoantibodies.

Fourteen antibody-positive first-degree relatives (12.6%) and 7 antibody-negative first-degree relatives (7.1%) were positive for at least 9 ACPAs ($P = 0.18$) (Table 1). As an entire group, antibody-negative first-degree relatives were positive for a mean ± SD 1.4 ± 2.8 ACPAs, only 1 fewer than in antibody-positive first-degree relatives (mean ± SD 2.3 ± 4.0 ACPAs) ($P = 0.08$). A greater proportion of antibody-positive first-degree relatives than antibody-negative first-degree relatives were positive for citrullinated cyclic vimentin (aa 58–77) ($P = 0.045$). The distribution of first-degree relatives positive for each ACPA at visits 1–4 is shown in Figure 2.

We then examined ACPA positivity in relation to positivity for RF (as determined by all assays), as well as positivity for anti–CCP-2 (as measured by ELISA). First-degree relatives in our study who were anti–CCP-2 positive were positive for a mean ± SD 9.7 ± 7.2 ACPAs, while those who were anti–CCP-2 negative were positive for a mean ± SD 1.6 ± 3.0 ACPAs ($P < 0.0001$). Of the 7 anti–CCP-2–positive first-degree relatives, 57.1% were positive for at least 9 ACPAs (Figure 3). While 55% of anti–CCP-2–negative first-degree rela-

Figure 3. Proportion of first-degree relatives positive for each number of different anti–citrullinated protein antibodies (ACPAs) according to cyclic citrullinated peptide 2 (CCP-2) and rheumatoid factor (RF) status at the first visit. Among individuals negative for both anti–CCP-2 and RF, 41.4% were positive for at least 1 ACPA, while 57.1% of anti–CCP-2–positive first-degree relatives and 8.4% of anti–CCP-2–negative first-degree relatives were positive for ≥9 ACPAs. Of the anti–CCP-2–negative first-degree relatives, 9.7% of RF-positive first-degree relatives and 7.1% of RF-negative first-degree relatives were positive for at least 9 ACPAs.

tives were not positive for any ACPA, it is notable that ~8% of anti–CCP-2–negative first-degree relatives were positive for 9 or more ACPAs. Within the anti–CCP-2–negative group, first-degree relatives who were RF positive had a mean ± SD 1.8 ± 3.2 ACPAs, while first-degree relatives who were RF negative were positive for a mean ± SD 1.4 ± 2.8 ACPAs ($P = 0.39$). Seven percent of RF-negative individuals were positive for at least 9 ACPAs, while 9.7% of RF-positive individuals were positive for at least 9 ACPAs (Figure 3).

We next examined associations of ACPA positivity with positivity for RF (by nephelometry), positivity for the RF isotypes, and positivity for the high-risk profile. Using a nonlinear mixed model to account for multiple visits per person, and adjusting for age, sex, ethnicity, and pack-years of smoking, an increasing number of ACPAs was significantly associated with being positive for RF by nephelometry, being positive for IgA-RF, and being positive for the high-risk autoantibody profile (Table 2). Those individuals who were positive for at least 9 ACPAs had a 6-fold increased risk of having the high-risk profile compared to those with <9 ACPAs, and a 4.5-fold increased risk of being positive for IgM-RF. Removal of the 7 individuals who were anti–CCP-2 positive attenuated these results to-

Figure 2. Percentage of first-degree relatives positive for each anti–citrullinated protein antibody at visits 1–4. The percentage positive remains relatively stable over successive visits. Only 3 individuals had a fifth visit, and therefore are not included due to small numbers. Ab+ = rheumatoid arthritis–related autoantibody positive; Ab− = rheumatoid arthritis–related autoantibody negative (see Figure 1 for other definitions).

Table 2. Association between an increasing number of anti–citrullinated protein antibodies (ACPAs) and rheumatoid arthritis–related characteristics*

	Outcome variable						
	RF positivity	IgG-RF positivity	IgM-RF positivity	IgA-RF positivity	High-risk profile	≥1 swollen joint	≥1 tender joint
Model 1							
Number of ACPAs	1.13 (1.0001–1.27)	1.00 (0.90–1.11)	1.16 (0.98–1.37)	1.14 (1.01–1.29)	1.21 (1.08–1.35)	1.11 (0.96–1.28)	1.18 (1.04–1.34)
ACPAs ≥9	0.55 (0.03–8.83)	1.30 (0.44–3.82)	4.55 (1.09–18.91)	3.24 (0.89–11.76)	5.79 (1.83–18.35)	3.64 (0.90–14.63)	5.00 (1.37–18.18)
Model 2							
Number of ACPAs	1.01 (0.86–1.18)	0.98 (0.86–1.11)	1.14 (0.94–1.39)	0.98 (0.83–1.15)	1.02 (0.88–1.19)	1.13 (0.95–1.34)	1.22 (1.06–1.41)
ACPAs ≥9	0.10 (0.002–5.03)	1.23 (0.35–4.36)	4.01 (0.83–19.23)	0.79 (0.15–4.10)	1.63 (0.40–6.60)	3.94 (0.88–17.57)	5.32 (1.32–21.32)

* Values are the odds ratio (95% confidence interval). All models are adjusted for age, sex, ethnicity, and pack-years of smoking, and account for multiple visits per subject. Model 1 analyses include all individuals. Model 2 analyses were run after removal of the 7 anti–cyclic citrullinated peptide–positive individuals. RF = rheumatoid factor.

ward the null. The greatest effect of attenuation was on the high-risk profile, which includes anti–CCP-2 positivity in its definition (Table 2).

Last, we examined associations with possible signs of inflammation. After adjustment for age, sex, ethnicity, and pack-years of smoking, having an increasing number of ACPAs was associated with having at least 1 tender joint on examination (OR 1.18, 95% CI 1.04–1.34). Being positive for at least 9 ACPAs, as compared to <9 ACPAs, was associated with significantly greater odds of having at least 1 tender joint on examination (OR 5.00, 95% CI 1.37–18.18). These associations remained statistically significant after removal of the anti–CCP-2–positive individuals from the analysis (Table 2).

DISCUSSION

In first-degree relatives without a diagnosis of RA according to the 1987 ACR criteria (36) and the 2010 ACR/EULAR (37) criteria, reactivity to multiple ACPA epitopes was evident, and the presence of an increasing number of ACPAs was associated with having at least 1 tender joint. A trend toward association was observed for having an increasing number of ACPAs and having at least 1 swollen joint, although this was not significant. Being positive for a higher number of ACPAs was associated with being positive for RF in general, and with the IgA isotype specifically, and was also associated with being positive for the high-risk autoantibody profile (i.e., positive for anti–CCP-2 and/or 2 RF isotypes). Moreover, in patients who had at least 9 ACPAs, the associations with positivity for IgM-RF and the high-risk profile, as well as the presence of tenderness on joint examination, were even greater.

Of note, these results indicate that the ACPA

array utilized herein can detect autoantibodies in individuals who are negative for RF and negative for anti–CCP-2. Therefore, this method may be a more sensitive measure than the commonly used, clinically available tests for RF and CCP in detecting RA-related autoimmunity in at-risk individuals. Importantly, first-degree relatives without classifiable RA who demonstrate reactivity to a broad array of citrullinated epitopes with this ACPA testing may also have joints that are more severely affected, as indicated by the presence of joint tenderness on examination, supporting the notion that this ACPA array is associated with potentially biologically meaningful inflammatory processes, if indeed the joint tenderness indicates joint inflammation. Although the association with joint tenderness remained after removal of the anti–CCP-2–positive individuals, we did not have an appropriate number of antibody-negative first-degree relatives with ≥1 tender joint to determine whether this association could be seen in this group alone. Further testing in subsequent visits in this population will be informative to determine whether such an association exists.

The proportion of individuals positive for ≥9 ACPAs was similar between our antibody-negative population (7.1%) and the previous blood donor population (7.9%) whose data were used in the ROC curve analysis for determination of ACPA cutoffs. In addition, the blood donor population had a similar number of ACPAs (mean ± SD 1.5 ± 3.1) as that in the antibody-negative controls in the present study. This may indicate that the antibody-negative first-degree relatives are not exhibiting any expanded ACPA reactivity. However, it should be noted that we do not know the disease status of the blood donor population, and therefore we cannot rule out the possibility that some of the donors may have

been diagnosed as having RA, exhibited joint inflammation, or were themselves at high risk of RA. In fact, 2.3% of the blood donor population was positive for anti–CCP-2, of which 50% were positive for ≥9 ACPAs. If this blood donor population were limited to individuals who were antibody negative, 6.3% would be positive for ≥9 ACPAs. Efforts to test this assay on a truly disease-free, similarly clinically characterized healthy population without familial RA risk to determine what proportion is positive for ≥9 ACPAs should be pursued.

It is of interest that there were not greater differences in the mean number of ACPAs between our antibody-positive and antibody-negative first-degree relatives. The only significant differences were that a greater proportion of antibody-positive first-degree relatives were positive for citrullinated cyclic vimentin (aa 58–77), when compared to antibody-negative first-degree relatives. Since we had data from only 1 visit for the autoantibody-negative first-degree relatives for this analysis, it will be informative to observe the autoantibody-negative first-degree relatives over successive visits to determine whether the number of ACPAs and percent positive for each epitope would remain stable or whether a greater expansion of ACPAs might be found in comparison with autoantibody-positive first-degree relatives. Evaluating the number of ACPA-positive first-degree relatives may also be a way to identify those individuals who are anti–CCP-2 antibody negative and are more likely to progress toward the development of RA, although this has yet to be demonstrated. Both antibody-positive and antibody-negative first-degree relatives demonstrated reactivity to a wide range of citrullinated epitopes, raising the possibility that these individuals may already be showing epitope spreading, as has been seen in several studies (8,9,11).

Similar to the findings in previous studies (6,8,11), the ACPA response seems to be driven by the breadth of the response, as shown by the number of ACPAs, not by a specific epitope, which may vary between individuals. A specific citrullinated antigen that may be an initial target of autoimmunity may exist, but the correct epitope may not currently be included on this array. In addition, the possibility exists that there may be different initial epitopes for different subjects, and that it is the reactivity to any citrullinated antigen, rather than a specific citrullinated antigen, that is the important first step in RA-related autoimmunity. This is consistent with the findings in a recent study showing that the initial ACPA response was restricted but became more specific with higher antibody levels nearer symptom onset (7).

That being said, we observed several interesting trends in reactivity. In general, citrullinated cyclic histone 2A (aa 1–20) was among the most prevalent for both antibody-positive and antibody-negative first-degree relatives. Positivity for a few of the fibrinogen epitopes was high among the antibody-positive first-degree relatives, while citrullinated clusterin (aa 221–240) was prevalent among antibody-negative first-degree relatives. Histone 2A and some of the fibrinogen antigens were prevalent among the anti–CCP-2–positive individuals. Aside from CCP-2, the prevalent epitopes in the RA probands were citrullinated cyclic histone 2A (aa 1–20) and citrullinated cyclic fillagrin (aa 48–65).

This panel included antigens that have been implicated in the literature (6,9,11,12,14–16,35,43–46) and those identified through proteomic screens (47) as targets in RA. While CCP-2 and citrullinated fillagrin may act as surrogate antigens for other RA autoantibodies, and antibodies targeting citrullinated fillagrin may cross-react with citrullinated fibrinogen (14), we chose to examine epitopes that have been associated with RA or that could potentially act as early antigenic targets. Although cross-reactivity among epitopes may occur, we excluded, using a matrix approach, any markers with close correlation to each other. The majority of ACPAs in this array appear to display distinct specificities, as has been noted in other studies (4,48). Antibodies targeting citrullinated proteins may also target uncitrullinated epitopes; however, marker-optimization studies in the development of this assay demonstrated minimal reactivity of the arginine-containing version of most peptides relative to the citrulline versions, indicating little cross-reactivity.

We found reactivity to multiple specific citrullinated proteins in the first-degree relatives we studied, whereas a study in North American Natives found that unaffected family members with positivity for anti-CCP did not show positivity for the limited number of ACPA epitopes tested using peptides derived from vimentin, fibrinogen, and enolase (35). Potential reasons for these differences include the fact that the ACPA testing in our study used a greater number of antigens and epitopes derived from these antigens, including whole proteins representing, and peptides derived from, vimentin, fibrinogen, and enolase, which may have resulted in greater autoantibody detection. In addition, we used a microarray that enabled measurement of multiple ACPAs, whereas the North American Natives study used an ELISA that measured antibodies against a restricted set of ACPAs, which likely provided our study with a greater sensitivity for ACPAs. It should be noted

that while the array used in the current study has been previously studied (8,42), the ELISA and ACPAs used in the North American Natives study have also been well characterized and validated, and may exhibit greater specificity for RA. These issues will need to be evaluated in further comparative studies.

Although ~50% of the first-degree relatives who were anti–CCP-2 negative were not positive for any ACPA, ~8% were positive for 9 or more ACPAs. This is consistent with studies that found specific ACPAs to be positive in individuals with CCP-negative RA (43,48–51). When the anti–CCP-2–positive individuals were removed from the analyses, the number of ACPAs and the presence of at least 9 ACPAs were no longer significantly associated with the high-risk profile, RF positivity by nephelometry, and positivity for the RF isotypes IgM and IgA, indicating that the presence of anti–CCP-2 antibodies may be driving the association with being autoantibody positive.

To our knowledge, this is the first study to demonstrate an association between an increasing number of ACPAs and the presence of at least 1 swollen or tender joint in RA-free first-degree relatives of patients with RA. Although none of the first-degree relatives had 1987 ACR or 2010 ACR/EULAR criteria–classifiable RA at the time of this study, these results could indicate that some individuals within our cohort were showing early signs of RA, and may have been further in the preclinical state than has been observed in previous studies of unaffected family members.

Similar to another study showing that seropositive arthralgia patients with an extended ACPA repertoire had a higher risk of developing arthritis (6), the first-degree relatives in our study, in whom increased reactivity to multiple ACPA epitopes was seen, may have already been progressing to RA. While no individuals in the cohort exhibited RA at the first visit, followup SERA visits found that 4 individuals met the ACR/EULAR 2010 classification criteria for RA (37) on at least 1 of their subsequent visits; unfortunately, there were not enough cases to further analyze this outcome. To ensure our population was free of RA, we removed the visits where the first-degree relatives met the ACR/EULAR 2010 criteria and all subsequent visits. Of these 4 individuals, 3 were antibody positive, 1 was anti–CCP-2 positive, and 1 was positive for at least 9 ACPAs. All 4 had at least 1 swollen joint, and 3 had at least 1 tender joint. Removal from the cohort of the visits of these 4 individuals did not significantly alter our inference with regard to tender joints on examination. While a previous study indicated no difference in joint swelling or tender-

ness between anti-CCP–positive and anti-CCP–negative individuals with RA (52), another study found that in those with early, untreated RA, ACPA positivity was a good predictor of joint damage (53); therefore, the expanded ACPA profile in our study may indicate differences in the predisease state.

In conclusion, in this study of first-degree relatives without 1987 ACR or 2010 ACR/EULAR criteria–classifiable RA, an increasing number of ACPAs was associated with RA-related disease characteristics, including positivity for RF, positivity for the RF isotypes, and positivity for the high-risk autoantibody profile (i.e., positive for anti–CCP-2 and/or 2 RF isotypes), as well as the presence of swollen or tender joints on clinical examination. In addition, 8% of the individuals who were negative for all other antibodies (RF and CCP-2) were positive for ≥9 ACPAs, providing evidence that expansion of ACPA testing may be warranted for the examination of autoimmunity in those individuals who may be presenting with preclinical RA.

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