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The Number of Elevated Cytokines and Chemokines in Preclinical Seropositive Rheumatoid Arthritis Predicts Time to Diagnosis in an Age-Dependent Manner

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Objective. **To evaluate levels of biomarkers in preclinical rheumatoid arthritis (RA) and to use elevated biomarkers to develop a model for the prediction of time to future diagnosis of seropositive RA.**

Methods. **Stored samples obtained from 73 military cases with seropositive RA prior to RA diagnosis and from controls (mean 2.9 samples per case; samples collected a mean of 6.6 years prior to diagnosis) were tested for rheumatoid factor (RF) isotypes, anti–cyclic citrullinated peptide (anti-CCP) antibodies, 14 cytokines and chemokines (by bead-based assay), and C-reactive protein (CRP).**

Results. **Preclinical positivity for anti-CCP and/or ≥2 RF isotypes was >96% specific for future RA. In preclinical RA, levels of the following were positive in a significantly greater proportion of RA cases versus controls: interleukin-1α (IL-1α), IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, IL-15, fibroblast growth factor 2, flt-3 ligand, tumor necrosis factor α, interferon-γ–inducible 10-kd protein, granulocyte–macrophage colony-stimulating factor, and CRP. Also, increasing numbers of elevated cytokines/chemokines were present in cases nearer to the time of diagnosis. RA patients who were ≥40 years old at diagnosis had a higher proportion of samples positive for cytokines/chemokines 5–10 years prior to diagnosis than did patients who were <40 years old at diagnosis ($P < 0.01$). In regression modeling using only case samples positive for autoantibodies highly specific for future RA, increasing numbers of cytokines/chemokines were predictive of decreased time to diagnosis, and the predicted time to diagnosis based on cytokines/chemokines was longer in older compared with younger cases.**

Conclusion. **Levels of autoantibodies, cytokines/chemokines, and CRP are elevated in the preclinical period of RA development. In preclinical autoantibody-positive cases, the number of elevated cytokines/chemokines is predictive of the time of diagnosis of future RA in an age-dependent manner.**

Multiple studies have demonstrated that levels of disease-related biomarkers may be elevated prior to the onset of symptomatic rheumatoid arthritis (RA). These biomarkers include rheumatoid factor (RF) and antibodies to citrullinated protein antigens, as well as secre-

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A patent application that includes Drs. Deane, Hueber, Robinson, and Holers has been filed for the use of biomarkers to predict clinically actionable events in rheumatoid arthritis, and royalties have been received for this patent. In addition, licensing agreements regarding the use of biomarkers have been established.

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tory phospholipase A_2, multiple cytokines/chemokines, and variably, C-reactive protein (CRP) (1–17). These findings suggest that there is a substantial "preclinical" period of RA, during which detectable immunologic and inflammatory changes are occurring that are related to disease development. Since elevated levels of RA-related autoantibodies in preclinical RA may be highly specific for future RA (7,9), there is hope that these autoantibodies may be used to predict which currently asymptomatic individuals are at sufficiently high risk for future RA that they may be targeted with preventive therapies.

However, while RA-related autoantibodies are likely highly predictive of future symptomatic disease, they may be present for many years prior to the onset of articular symptoms, and are therefore perhaps less useful in isolation for prediction of imminent symptomatic disease (7,9). As such, assessment of additional biomarkers in the preclinical period of RA may aid in the development of models to predict accurately the timing of the onset of symptomatic disease. Additionally, as demonstrated by our prior findings (13) and those of Bos et al (18) showing that individuals with an older age at diagnosis of RA have a longer duration of preclinical autoantibody positivity, age-related duration of other preclinical elevations in biomarkers may influence the development of models to predict the timing of the onset of symptomatic future RA.

In this study we used stored preclinical samples from members of the military with RA to evaluate the following: 1) levels of autoantibodies, cytokines/chemokines, and CRP during the preclinical phase of RA development, and their diagnostic accuracy for future disease, 2) age-related differences in the timing of elevations of these biomarkers, and 3) biomarker testing to predict the timing of the onset of symptomatic RA in subjects at high risk for future disease.

PATIENTS AND METHODS

Study population. Eighty-three patients with RA were identified at the Walter Reed Army Medical Center (WRAMC) Rheumatology Clinic. These patients had been clinically evaluated between 1989 and 2003, had their date of diagnosis of RA established by chart review, and all had stored serum samples (290 total samples, including pre– and post–RA diagnosis samples) available through the Department of Defense Serum Repository (DoDSR). This repository was created to improve the health of the members of the US Armed Services, and serum samples are collected at enlistment, deployment, and at regular intervals during military service and stored at −30°C. Additionally, 83 military subjects without RA were identified in the DoDSR to serve as controls. Controls were matched to cases for age (case age at diagnosis), sex, race, number of samples available, duration of sample storage, and enlistment region.

Selection of seropositive RA cases. Due to the specificity of autoantibodies for established and future RA demonstrated in prior studies (7,9,19,20), and to concerns that seronegative RA represents a disease distinct from seropositive RA (21), of the 83 cases the 73 (88%) who had seropositive RA were selected for analyses. Patients were classified as having seropositive RA if postdiagnosis samples were determined to be RF positive by WRAMC testing (nephelometry or latex agglutination; n = 67) and/or postdiagnosis or immediate prediagnosis samples (obtained within 1 year prior to diagnosis) were determined to be RF positive (by enzyme-linked immunosorbent assay [ELISA]) or anti–cyclic citrullinated peptide (anti-CCP) antibody positive (n = 6). All 73 seropositive RA cases met ≥4 of the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 revised criteria for RA (22).

Biomarker analysis. *Autoantibody and CRP testing.* All samples were tested for the RF isotypes IgM, IgA, and IgG (measured in IU/ml) using ELISA (Quanta Lite) kits according to the recommendations of the manufacturer (Inova Diagnostics). Anti-CCP was measured (in units/ml) using anti-CCP2 ELISA (Diastat; Axis-Shield Diagnostics). CRP was measured (in mg/liter) using a high-sensitivity nephelometric assay (BN II Nephelomter; Dade-Behring).

Cytokine/chemokine testing. All samples were tested for the following 14 cytokines/chemokines: interleukin-1α (IL-1α), IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, IL-15, eotaxin (or CCL11), fibroblast growth factor 2 (FGF-2), flt-3 ligand, tumor necrosis factor α (TNFα), interferon-γ–inducible 10-kd protein (IP-10 or CXCL10), granulocyte–macrophage colony-stimulating factor (GM-CSF), and monocyte chemotactic protein 1 (MCP-1 or CCL2). Cytokines and chemokines were measured using a bead-based 14-plex assay (Beadlyte kit; Upstate) and the Luminex xMAP 200 System. Serum samples were thawed and incubated with cytokine-specific antibody-coupled beads, followed by incubation with indicator antibodies. After incubation, samples were read with the Luminex 200 System. Quantitative levels of cytokines/chemokines were determined by comparison to standard curves and were reported in picograms per milliliter. Additionally, since RF may interfere with cytokine assays, Heteroblock reagent (Omega Biologicals) was used in all samples (3 μg of Heteroblock per 1 ml of serum) to minimize the effect of RF (23). Prior studies have shown that the levels of cytokines/chemokines obtained using this bead-based assay are highly correlated with those obtained using ELISAs (23,24).

Dichotomous values for autoantibodies, cytokines/chemokines, and CRP. *Autoantibodies.* Since the 1987 ACR RA criteria specify that an RF level is considered to be positive if it is present in <5% of control subjects (22), we established a dichotomous cutoff level for each of the RF assays that was positive in <5% of 491 blood donor controls who were not members of the military. Anti-CCP was considered to be positive if levels were >5 units/ml (kit cutoff value).

Cytokines/chemokines. A dichotomous cutoff value for each cytokine/chemokine was determined using post–RA diagnosis serum samples that were obtained from military cases with seropositive RA within 2 years of diagnosis (n = 42

Table 1. Demographic and clinical characteristics of the military cases with RA and controls*

	All cases (n = 83)	Seronegative RA cases (n = 10)	Seropositive RA cases (n = 73)	Controls (for seropositive cases) (n = 73)†	Seropositive cases <40 years old at diagnosis of RA (n = 35)	Seropositive cases ≥40 years old at diagnosis of RA (n = 38)	P‡
Age at diagnosis, years							<0.01
Mean ± SD	39.9 ± 10.0	39.1 ± 8.7	40.0 ± 10.3	39.9 ± 10.3	31.3 ± 5.6	48.1 ± 6.2	
Range	20.9–66.1	26.4–53.5	20.9–66.1	20.9–66.1	20.9–39.7	40.0–66.1	
Male, no. (%)	49 (59.0)	6 (60.0)	43 (58.9)	43 (58.9)	17 (48.6)	26 (68.4)	0.10
Race, no. (%)							0.27
White	57 (68.7)	8 (80.0)	49 (67.1)	49 (67.1)	21 (60.0)	28 (73.7)	
Black	21 (25.3)	2 (20.0)	19 (26.0)	19 (26.0)	10 (28.6)	9 (23.7)	
Other§	5 (6.0)	0 (0.0)	5 (6.9)	5 (6.9)	4 (11.4)	1 (2.6)	
No. (%) with erosions	42 (50.6)	4 (40.0)	38 (52.0)	NA	20 (57.1)	18 (47.4)	0.48
Total number of samples per case							0.10
Mean ± SD	3.5 ± 1.2	3.6 ± 1.0	3.5 ± 1.3	3.5 ± 1.3	3.7 ± 1.2	3.2 ± 1.3	
Range	1–5	2–5	1–5	1–5	1–5	1–5	
Number of prediagnosis samples per case							0.41
Mean ± SD	2.9 ± 1.2	3.1 ± 1.0	2.9 ± 1.2	2.9 ± 1.2	3.0 ± 1.1	2.8 ± 1.4	
Range	1–4	1–4	1–4	1–4	1–4	1–4	
Years from first collection of samples to diagnosis							0.08
Mean ± SD	6.6 ± 3.7	6.4 ± 2.7	6.6 ± 3.9	6.6 ± 3.9	5.8 ± 3.5	7.3 ± 4.1	
Range	0.06–13.67	0.84–10.89	0.06–13.67	0.04–13.71	0.99–13.63	0.06–13.67	

* Patients were considered to have seropositive rheumatoid arthritis (RA) if they were rheumatoid factor (RF) positive, as determined by Walter Reed Army Medical Center testing (nephelometry or latex agglutination), and/or were RF or anti–cyclic citrullinated peptide positive, as determined by testing of samples obtained postdiagnosis or within 1 year prior to diagnosis.
† Controls were matched to cases by age, based on the age of the case at the time of diagnosis of RA. For example, if a case was 40 years old at the time of diagnosis of RA, the corresponding control would also be 40 years old.
‡ For cases <40 years old at diagnosis versus cases ≥40 years old at diagnosis, by chi-square test or *t*-test.
§ Asian, Native American, and multiracial subjects.

samples available in this time period) and controls. A receiver operating curve analysis was performed to establish a cutoff point for each cytokine/chemokine that was >90% specific for the diagnosis of RA (25). This 2-year period was selected since levels of cytokines and chemokines were likely to be elevated due to temporal proximity to the onset of symptomatic disease.

CRP. For CRP, 2 separate cutoff points for positivity were used: >5 mg/liter and >10 mg/liter.

Ethical considerations. The study protocol and analyses were approved by the respective Institutional Review Boards at the WRAMC, the University of Colorado, and Stanford University.

Statistical analysis. Proportions of biomarker positivity were compared between groups using the chi-square test (or Fisher's exact test when appropriate). The median number of cytokines/chemokines found to be positive for each prediagnosis time interval were compared between age-at-diagnosis groups using the Mann-Whitney U test, since due to small numbers, nonparametric analysis was most conservative. Sensitivity and specificity calculations were performed using 2×2 table analyses comparing military RA cases with military controls as well as with 200 blood donor controls (59% male; mean ± SD age 53 ± 15 years). These latter controls were separate from the military, and separate from the 491 controls used for establishing RF cutoff values. Regression analysis with random subject terms for intercept and slope was used to determine the prediagnosis time of separation of cytokine/chemokine counts in cases versus controls, using a series of approximate *t*-tests to compare case and control mean regression values at incremented prediagnosis times (26). Additionally, this analysis was controlled for multiple comparisons with a Scheffé adjusted critical value (27). There was no significant difference in residual distributions using Gaussian or Poisson modeling, suggesting that assumption of a normal distribution in this analysis is sufficient. The duration of time (in years) from a preclinical sample with biomarker positivity to time of diagnosis was modeled using a mixed effects linear regression with a random intercept term and a backward stepwise procedure, with final variables included in the model based on a likelihood ratio test. This approach additionally allowed for correlation of multiple measures within an individual. All analyses were performed using SAS, version 9.1.3 (SAS Institute).

RESULTS

Patient demographics and autoantibody testing. Subject demographics are presented in Table 1. Anti-

Table 2. Proportions of positivity, and sensitivity and specificity for future RA diagnosis, of autoantibodies tested at any point in the pre–RA diagnosis period in cases with seropositive RA and controls*

	Cases (n = 73)	Controls (n = 73)	Sensitivity, %	Specificity (compared with matched military controls), %	Specificity (compared with blood donor controls), %†
Anti-CCP (>5 units/ml)‡	51 (69.9)	0 (0)	69.9	100	99.0
IgA-RF (>10.5 units)	40 (54.8)	3 (4.1)	54.8	95.9	98.0
IgG-RF (>10.9 units)	24 (32.9)	5 (6.8)	32.9	93.2	94.0
IgM-RF (>13.6 units)	41 (56.2)	3 (4.1)	56.2	95.9	94.0
Anti-CCP and/or ≥2 RF isotypes	54 (74.0)	1 (1.4)	74.0	98.6	96.5

* Values are the number (%) of subjects. In addition to rheumatoid factor (RF) testing by enzyme-linked immunosorbent assay for isotypes, RF was assessed by nephelometry, according to the recommendations of the manufacturer (Dade-Behring) with a cutoff value of 24.4 units yielding a sensitivity and specificity for future rheumatoid arthritis (RA) diagnosis of 53.4% and 93.2%, respectively, when compared with military controls.
† The blood donor controls were randomly selected blood donors (n = 200) (mean age 53 years; 59% male [not significantly different from the military population]), and some may have had a diagnosis of RA. As such, specificity using this group as a comparison may be underestimated.
‡ When the cutoff value for anti–cyclic citrullinated peptide (anti-CCP) positivity was lowered from >5 units/ml to >2 units/ml, sensitivity and specificity in any preclinical sample for future RA diagnosis were 75.3% and 100%, respectively, compared with the military controls, and specificity was 96.5% when compared with the 200 nonmilitary blood donor controls.

CCP positivity at any point prediagnosis had a sensitivity of 69.6% for future seropositive RA, with a specificity of 100% compared with military controls, and 99% compared with 200 nonmilitary blood donor controls (Table 2). Prediagnosis positivity for anti-CCP and/or ≥2 RF isotypes (IgM, IgG, or IgA) had an overall sensitivity of 74.0% and specificity of 98.6% for future seropositive RA compared with matched military controls, and a specificity of 96.5% compared with blood donor controls, although the sensitivity of autoantibodies for fu-

Table 3. Sensitivity and specificity of autoantibodies for future diagnosis of RA by prediagnosis time interval and age at diagnosis*

	Anti-CCP	IgA-RF	IgG-RF	IgM-RF	Anti-CCP and/or ≥2 RF isotypes
Time interval ≥0 to ≤1 year prior to RA diagnosis					
Age at diagnosis <40 years					
Sensitivity	77.8	72.2	22.2	55.6	83.3
Specificity	100	100	100	100	100
Age at diagnosis ≥40 years					
Sensitivity	77.8	77.8	55.6	77.8	77.8
Specificity	100	80.0	90.0	100	90.0
Time interval >1 to ≤5 years prior to RA diagnosis					
Age at diagnosis <40 years					
Sensitivity	61.3	32.3	16.1	32.3	64.5
Specificity	100	96.8	93.6	100.0	100.0
Age at diagnosis ≥40 years					
Sensitivity	64.0	44.0	36.0	52.0	68.0
Specificity	100	96.0	92.0	96.0	96.0
Time interval >5 to ≤10 years prior to RA diagnosis					
Age at diagnosis <40 years					
Sensitivity	17.6	17.6	11.8	17.6	23.5
Specificity	100	100.0	100.0	100	100
Age at diagnosis ≥40 years					
Sensitivity	43.5	30.4	21.7	52.2	43.5
Specificity	100	100.0	95.6	91.3	100
Time interval ≥10 years prior to RA diagnosis					
Age at diagnosis <40 years					
Sensitivity	0	0	0	0	0
Specificity	100	100	100	100	100
Age at diagnosis ≥40 years					
Sensitivity	18.2	18.2	0	27.3	27.3
Specificity	100	100	100	100.0	100

* Values are the percent. Sensitivity and specificity for future rheumatoid arthritis (RA) diagnosis were calculated comparing RA case and military control samples for each age-at-diagnosis group and time interval. Anti-CCP = anti–cyclic citrullinated peptide; RF = rheumatoid factor.

Table 4. Positivity of cytokines/chemokines and C-reactive protein in cases with seropositive RA and controls, and sensitivity and specificity of biomarkers for future diagnosis of seropositive RA*

Biomarker	Cases (n = 73)	Controls (n = 73)	P	Sensitivity, %	Specificity, %
Eotaxin	10 (13.7)	12 (16.4)	0.64	13.7	86.3
FGF-2	28 (38.4)	12 (16.4)	<0.01	38.4	83.6
flt-3 ligand	42 (57.5)	29 (39.7)	0.03	57.5	60.3
GM-CSF	33 (45.2)	15 (20.6)	<0.01	45.2	53.4
IL-1α	39 (53.4)	26 (35.6)	0.03	53.4	64.4
IL-1β	28 (38.4)	15 (20.6)	0.02	38.4	79.4
IL-6	25 (34.2)	14 (19.2)	0.04	34.2	80.8
IL-10	27 (37.0)	11 (15.0)	<0.01	37.0	84.9
IL-12p40	41 (56.2)	17 (23.3)	<0.01	56.2	76.7
IL-12p70	29 (39.7)	17 (23.3)	0.03	39.7	76.7
IL-15	31 (42.5)	13 (17.8)	<0.01	42.5	82.2
IP-10	41 (56.2)	25 (34.2)	0.01	56.2	65.8
MCP-1	27 (37.0)	24 (32.9)	0.60	37.0	67.1
TNFα	33 (45.2)	14 (19.2)	<0.01	45.2	80.8
>5 cytokines/chemokines (any type)	29 (39.7)	16 (21.9)	0.02	39.7	78.1
>10 cytokines/chemokines (any type)	12 (16.4)	3 (4.1)	0.03	16.4	95.9
Anti-CCP	51 (69.9)	0 (0)	<0.01	69.9	100
Anti-CCP and/or >5 cytokines/chemokines	57 (78.1)	16 (21.9)	<0.01	78.1	78.1
Anti-CCP and/or >10 cytokines/chemokines	53 (72.6)	3 (4.1)	<0.01	72.6	95.9
Any sample positive for a single RF isotype (any isotype)	11 (15.1)	9 (12.3)	0.81	15.1	87.7
Single RF isotype and/or >5 cytokines/chemokines	39 (53.4)	21 (28.8)	<0.01	53.4	71.2
Single RF isotype AND >5 cytokines/chemokines	1 (1.4)	4 (5.5)	0.34	1.4	94.5
Single RF isotype and/or >10 cytokines/chemokines	23 (31.5)	10 (13.7)	0.02	31.5	86.3
Single RF isotype AND >10 cytokines/chemokines	0 (0)	2 (2.7)	0.5	0.0	97.3
Anti-CCP and/or ≥2 RF isotypes	54 (74.0)	1 (1.4)	<0.01	74.0	98.6
Anti-CCP and/or ≥2 RF isotypes and/or >5 cytokines/chemokines	59 (80.8)	17 (23.3)	<0.01	80.8	76.7
Anti-CCP and/or ≥2 RF isotypes AND >5 cytokines/chemokines	24 (32.9)	0 (0)	<0.01	32.9	100
Anti-CCP and/or ≥2 RF isotypes and/or >10 cytokines/chemokines	55 (75.3)	4 (5.5)	<0.01	74.3	94.5
Anti-CCP and/or ≥2 RF isotypes AND >10 cytokines/chemokines	11 (15.1)	0 (0)	<0.01	15.1	100

* Values are the number (%) of subjects with at least 1 sample found to be positive for the indicated biomarker at any time prior to diagnosis of rheumatoid arthritis (RA) in the cases. Cases were individuals who developed seropositive RA (rheumatoid factor [RF] and/or anti–cyclic citrullinated peptide [anti-CCP] positive); controls were members of the military who did not have RA and were matched to cases for age, race, sex, and duration of serum sample storage. Sensitivity and specificity for future diagnosis of seropositive RA were determined in a case–control analysis. FGF-2 = fibroblast growth factor 2; GM-CSF = granulocyte–macrophage colony-stimulating factor; IL-1α = interleukin-1α; IP-10 = interferon-γ–inducible 10-kd protein; MCP-1 = monocyte chemotactic protein 1; TNFα = tumor necrosis factor α.

ture disease increased closer to diagnosis (Table 3). The proportions of seropositive RA cases with preclinical autoantibody positivity (any type) did not significantly differ with age at diagnosis (<40 or ≥40 years) (data not shown).

Cytokine, chemokine, and CRP testing. CRP and all cytokines/chemokines, except eotaxin and MCP-1, were positive in ≥1 prediagnosis sample in significantly more cases than controls (Table 4). Between age at diagnosis groups (<40 or ≥40 years), there were no differences in the proportion of cases with prediagnosis positivity for cytokines/chemokines (either individual cytokines/chemokines or cytokine/chemokine counts) or CRP, with the exception that IL-10 was positive prediagnosis in 51.4% of the cases who were younger than 40 years old at diagnosis versus 23.7% of the cases who were 40 years old or older (P = 0.02). In cases, the number of elevated prediagnosis cytokines/chemokines increased over time, becoming significantly elevated compared with controls ~7.2 years prior to the diagnosis of RA (Figure 1).

The sensitivity and specificity for future RA of preclinical cytokine/chemokine positivity combined with autoantibody positivity are presented in Table 4, and data regarding the diagnostic accuracy for future RA of cytokine/chemokine counts in seropositive and seronegative RA are available online at http://medschool.ucdenver.edu/rheumatology (Supplemental Figures A and B under Journal Supplements). Of note, there was a lower proportion of seronegative RA cases (n = 10) with preclinical positivity for cytokines/chemokines than of seropositive cases (data not shown). Although not statistically significant, these results suggest that seronegative RA has fewer prediagnosis cytokine/chemokine elevations than seropositive RA. Also, in regression analyses (data not shown), there was no association of

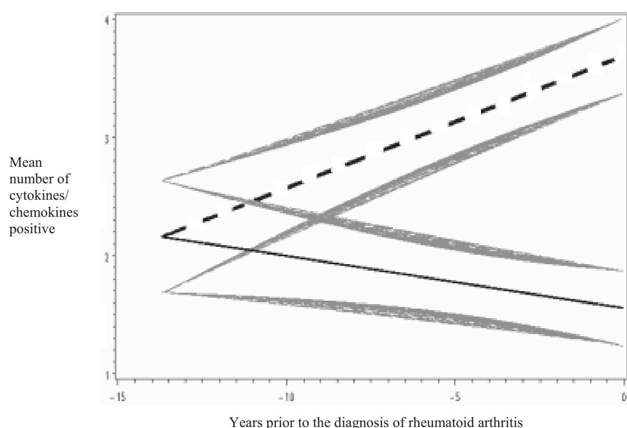

Figure 1. Mixed model regression lines of the mean number of cytokines or chemokines found to be positive, as determined with an established cutoff value (cytokine/chemokine counts), in seropositive rheumatoid arthritis (RA) cases (n = 73 cases with 212 prediagnosis samples) versus controls over time in the preclinical period of RA development. The broken line shows the mean values for cases; the solid line shows the mean values for controls. The curved lines represent the SEM for each regression line. In this mixed model analysis RA cases had significantly elevated cytokine/chemokine counts compared with controls ~7.2 years prior to diagnosis of RA.

individual cytokine/chemokine or CRP positivity with a specific autoantibody (any type) in seropositive or seronegative RA cases.

Additionally, to determine whether cytokine/chemokine positivity preceded autoantibody positivity, 58 samples collected a mean of 5.6 years prior to diagnosis were identified from 16 cases that were initially anti-CCP negative but would later develop prediagnosis anti-CCP positivity. In these samples, the cytokines IL-1α, IL-6, and IP-10 were positive in a higher proportion of cases than their controls prior to anti-CCP positivity ($P < 0.05$ by chi-square test). In 33 pre–anti-CCP–positive samples from 12 cases that were also negative for RF isotype(s), IL-1α and IP-10 were still elevated to a statistically significant degree in cases versus their controls ($P < 0.05$).

Biomarker positivity by prediagnosis intervals and age at diagnosis. Overall, biomarkers were positive preclinically in greater proportions of subjects nearer the time of diagnosis (Table 5). For the time interval <10 to ≥5 years prior to diagnosis, the median cytokine/chemokine count in subjects who were ≥40 years old at diagnosis was greater when compared with those who were younger than 40 years old at diagnosis (median counts 6.5 versus 3.0; $P < 0.01$), while younger cases were more likely to have elevated biomarker levels immediately prior to diagnosis, indicating an age-related effect on cytokine/chemokine positivity in cases (Table 5). Additional data regarding proportions of cases with biomarker positivity by prediagnosis time interval and age group are presented in Table 5.

Cytokine/chemokine stability. To evaluate the possible degradation of cytokines/chemokines over time due to storage, cytokine/chemokine counts in samples from a 2-year prediagnosis period in 8 cases who were diagnosed as having seropositive RA in 1991 were compared with counts in prediagnosis samples from a 2-year period from 8 cases diagnosed in 2002. In this comparison, there was no significant difference in the mean cytokine/chemokine count (5.3 versus 5.6; $P = 0.84$), suggesting that cytokine/chemokine degradation over the ~11 year increase in storage time did not affect the results.

We also investigated the fluctuations of biomarker positivity in prediagnosis seropositive RA. For autoantibodies, reversion from positive to negative occurred in <7% of cases, regardless of autoantibody type. For cytokines and chemokines, in 212 prediagnosis samples from 73 seropositive cases, there were 271 conversions from negative to positive for at least 1 cytokine/chemokine. Additionally, in the same 212 samples, there were 184 reversions from positive to negative for at least 1 cytokine/chemokine. However, 75% of case cytokine/chemokine conversions to positive occurred ≤5 years prior to diagnosis, and more cases had persistent cytokine/chemokine positivity once positive, leading to the overall increase in cytokine/chemokine counts demonstrated in Figure 1. Additional biomarker stability data are available online at http://medschool.ucdenver.edu/rheumatology (Supplemental Table A under Journal Supplements).

Prediction of time to diagnosis of future RA. To develop a regression model that would be clinically useful in prospective studies for prediction of the time of onset of future seropositive RA in subjects highly likely to develop future RA, we used only prediagnosis case samples that were positive for anti-CCP and/or ≥2 RF isotypes, since this autoantibody profile was reasonably sensitive (74%) and highly specific (>96%) for future seropositive RA (Table 2). Fifty-four seropositive RA cases (61% male; mean age at diagnosis 39.1 years) had 101 prediagnosis samples that were positive for this autoantibody profile. (These samples were collected a mean of 3.6 years prior to diagnosis.) The outcome for this analysis was the duration of time between preclinical sample and the diagnosis of RA, and the initial predictor variables evaluated were sex, race, autoantibody levels, CRP level, individual levels and counts of positive

Table 5. Proportions of samples from cases with seropositive RA that were positive for biomarkers during defined prediagnosis time intervals, by age at diagnosis*

	Age at diagnosis of RA <40 years old					Age at diagnosis of RA ≥40 years old				
	Time interval prior to diagnosis, years				Total positive samples (cases)†	Time interval prior to diagnosis, years				Total positive samples (cases)†
	≥10	<10≥5	<5≥1	<1≥0		≥10	<10≥5	<5≥1	<1≥0	
Anti-CCP	0.00 (0)	0.08 (3)	0.53 (19)	0.39 (14)	36 (26)	0.06 (2)	0.29 (10)	0.46 (16)	0.20 (7)	35 (25)
IgA-RF	0.00 (0)	0.12 (3)	0.38 (10)	0.50 (13)	26 (20)	0.07 (2)	0.26 (7)	0.41 (11)	0.26 (7)	27 (20)
IgG-RF	0.00 (0)	0.18 (2)	0.46 (5)	0.36 (4)	11 (9)	0.00 (0)	0.26 (5)	0.47 (9)	0.26 (5)	19 (15)
IgM-RF	0.00 (0)	0.13 (3)	0.44 (10)	0.44 (10)	23 (18)	0.08 (3)	0.34 (12)	0.37 (13)	0.20 (7)	35 (23)
Anti-CCP and/or ≥2 RF isotypes	0.00 (0)	0.10 (4)	0.51 (20)	0.38 (15)	39 (27)	0.08 (3)	0.27 (10)	0.46 (17)	0.19 (7)	37 (27)
Eotaxin	0.00 (0)	0.17 (1)	0.33 (2)	0.50 (3)	6 (5)	0.00 (0)	0.80 (4)	0.20 (1)	0.00 (0)	5 (5)
FGF-2	0.00 (0)	0.19 (4)	0.52 (11)	0.29 (6)	21 (17)	0.00 (0)	0.31 (4)	0.38 (5)	0.31 (4)	13 (11)
flt-3	0.07 (2)	0.15 (4)	0.52 (14)	0.26 (7)	27 (22)	0.00 (0)	0.46 (11)	0.42 (10)	0.12 (3)	24 (20)
GM-CSF	0.11 (3)	0.18 (5)	0.41 (11)	0.30 (8)	27 (20)	0.6 (1)	0.35 (6)	0.53 (9)	0.06 (1)	17 (13)
IL-1α	0.08 (2)	0.08 (2)	0.54 (14)	0.31 (8)	26 (17)	0.03 (1)	0.38 (11)	0.41 (12)	0.17 (5)	29 (22)
IL-1β	0.00 (0)	0.11 (2)	0.50 (9)	0.39 (7)	18 (15)	0.00 (0)	0.41 (7)	0.29 (5)	0.29 (5)	17 (13)
IL-6	0.00 (0)	0.07 (1)	0.47 (7)	0.47 (7)	15 (13)	0.07 (1)	0.40 (6)	0.40 (6)	0.13 (2)	15 (12)
IL-10	0.10 (2)	0.15 (3)	0.50 (10)	0.25 (5)	20 (18)	0.10 (1)	0.40 (4)	0.50 (5)	0.00 (0)	10 (9)
IL-12p40	0.07 (2)	0.11 (3)	0.59 (16)	0.22 (6)	27 (21)	0.04 (1)	0.36 (10)	0.46 (13)	0.14 (4)	28 (20)
IL-12p70	0.10 (2)	0.10 (2)	0.47 (9)	0.32 (6)	19 (16)	0.00 (0)	0.38 (5)	0.54 (7)	0.08 (1)	13 (13)
IL-15	0.04 (1)	0.18 (4)	0.50 (11)	0.27 (6)	22 (18)	0.00 (0)	0.27 (4)	0.47 (7)	0.27 (4)	15 (13)
IP-10	0.00 (0)	0.11 (3)	0.56 (15)	0.22 (6)	27 (19)	0.11 (3)	0.38 (10)	0.38 (10)	0.12 (3)	26 (22)
MCP	0.00 (0)	0.24 (4)	0.47 (8)	0.29 (5)	17 (13)	0.17 (3)	0.39 (7)	0.33 (6)	0.11 (2)	18 (14)
TNFα	0.00 (0)	0.19 (4)	0.48 (10)	0.33 (7)	21 (17)	0.10 (2)	0.47 (9)	0.31 (6)	0.10 (2)	19 (16)
CRP >5 mg/liter	0.00 (0)	0.07 (1)	0.36 (5)	0.57 (8)	14 (13)	0.09 (2)	0.32 (7)	0.41 (9)	0.18 (4)	22 (16)
CRP >10 mg/liter	0.00 (0)	0.12 (1)	0.12 (1)	0.75 (6)	8 (8)	0.00 (0)	0.50 (4)	0.38 (3)	0.12 (1)	8 (8)
0–4 cytokines/chemokines positive	0.09 (4)	0.28 (13)	0.39 (18)	0.24 (11)	46 (26)	0.22 (11)	0.32 (16)	0.36 (18)	0.10 (5)	50 (33)
5–9 cytokines/chemokines positive	0.08 (1)	0.23 (3)	0.54 (7)	0.15 (2)	13 (12)	0.00 (0)	0.22 (2)	0.44 (4)	0.33 (3)	9 (9)
≥10 cytokines/chemokines positive	0.00 (0)	0.08 (1)	0.50 (6)	0.42 (5)	12 (11)	0.00 (0)	0.56 (5)	0.33 (3)	0.11 (1)	9 (9)
Median samples positive for ≥1 of the 14 cytokines/chemokines‡	0.5	3.0	10.5	6.0	–	1.0	6.5	6.5	3.0	–
Total samples (total cases)	5 (5)	27 (17)	56 (31)	18 (18)	–	14 (11)	40 (23)	42 (25)	10 (9)	–

* Except where indicated otherwise, the first value in each column represents the proportion of samples in that time interval that were positive for the given biomarker out of the total number of samples positive for that biomarker during the entire preclinical period. The number in parentheses in each column represents the total number of samples (not individuals) during that interval that were positive for a given biomarker. For example, for anti-CCP, for patients <40 years old at diagnosis and for the time interval <5 to ≥1 years prior to diagnosis, a proportion of 0.53 samples (19 of 36 total samples positive for anti-CCP at any point prediagnosis) were positive for anti-CCP for this interval. To calculate the proportion of samples that were positive for each biomarker out of the samples available during that time interval only, divide the number of positive samples by the total number of samples for a given biomarker and interval. (The total numbers of samples for each interval are shown in the final row.) CRP = C-reactive protein (see Table 4 for other definitions).

† Values are the total number of samples (total number of individual cases) in each age-at-diagnosis group that were positive for a given biomarker at any point prediagnosis.

‡ To determine whether the median number of prediagnosis samples that were positive for ≥1 cytokine/chemokine differed by age group during the prediagnosis period, these median values were compared between age-at-diagnosis groups (<40 years old versus ≥40 years old) and between each prediagnosis time interval, by nonparametric testing (Mann-Whitney U test). For age at diagnosis <40 years old versus ≥40 years old, $P = 0.43$ for the time period ≥10 years prior to diagnosis, $P < 0.01$ for the time period <10 to ≥5 years prior to diagnosis, $P = 0.01$ for the time period <5 to ≥1 years prior to diagnosis, and $P < 0.01$ for the time period <1 to ≥0 years prior to diagnosis. These analyses were not adjusted for length of time interval or number of samples or cases per interval.

cytokine/chemokines, and age at diagnosis (by decade). Of note, the variable age at diagnosis by decade (rather than <40 or ≥40 years) was created to allow for finer analysis of the relationship of age to the duration of prediagnosis biomarker positivity. Also, in a separate analysis, the medians of the durations from initial preclinical sample to diagnosis of RA between age at diagnosis group (by decade) showed no significant differences ($P = 0.46$).

Predictor variables for the time to diagnosis that were retained after backward elimination of covariates were cytokine/chemokine count ($\beta = 0.1782$, $P = 0.01$) and age at diagnosis (by decade) ($\beta = -1.0122$, $P = 0.02$). In this final model, the period of time from the preclinical sample to diagnosis of RA decreased as the number of cytokines/chemokines that were positive in the sample increased ($P < 0.01$ for model significance) (Figure 2). Also, as age at diagnosis (by decade) in-

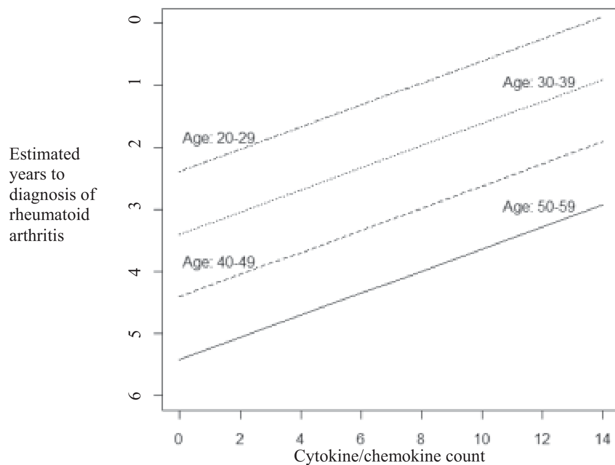

Figure 2. Estimation of time to diagnosis of future rheumatoid arthritis (RA) by age and cytokine/chemokine counts in subjects with autoantibody positivity highly specific for future RA. The results of regression modeling of the outcome time to diagnosis of future RA, based on the predictor variables age at diagnosis (by decade) and cytokine/chemokine count, are shown (P for model < 0.01). Prediagnosis RA case samples that were positive for autoantibodies that were highly specific (>96%) for RA (anti–cyclic citrullinated peptide and/or ≥2 or more rheumatoid factor isotypes) were used to develop this model (n = 54 cases with 101 pre–RA diagnosis samples). For example, using this model, in a case diagnosed as having RA between ages 50 and 59, a sample with a cytokine/chemokine count of 10 would be obtained ~4 years prior to diagnosis, while a sample with the same cytokine/chemokine count from an individual between ages 20 and 29 would be obtained ~1 year prior to diagnosis. The model is calculated as years to diagnosis $= -2.3861 - 1.0122 \times$ (decade of diagnosis) $+ 0.1782 \times$ (cytokine/chemokine count), where decade 20–29 is coded as 0, 30–39 is coded as 1, etc.

creased, there was a corresponding increase in the duration of time from sample collection to the diagnosis of RA. Sex, race, and positivity for individual cytokines/chemokines or CRP did not contribute significantly to this model. Finally, while there was a trend toward increased levels of autoantibodies (as continuous variables) and CRP (dichotomous or continuous) closer to diagnosis, these levels did not contribute significantly to this model (data not shown).

Biomarker positivity in relation to symptom onset. Limited data regarding the duration of prediagnosis joint symptoms were available from chart review for 56 (~77%) of the 73 seropositive RA cases. The median time of onset of symptoms prior to diagnosis in these 56 cases was 0.5 years, with no difference in median time of onset of symptoms between age at diagnosis groups (symptom onset median 0.5 years prior to diagnosis for groups <40 and ≥40 years old; $P = 0.75$). To evaluate cytokine/chemokine positivity prior to symptom onset in these 56 cases, samples from 0–6 months prior to diagnosis were removed from the analysis. After removal of these samples (28 case–control samples, including removal of 4 case–control pairs since their only prediagnosis samples were from this period), the differences in proportions of cases versus controls who were positive for the following biomarkers were lost: flt-3 ligand, IL-1β, IL-6, IL-12p70, and CRP >5 mg/liter (but not >10 mg/liter). These findings indicate that there is a substantial number of cases with elevated levels of these cytokines/chemokines during the 0–6–month period prior to formal RA diagnosis, although notably these same cytokines/chemokines were positive in a subset of cases prior to 6 months prediagnosis (Table 5).

In addition, we repeated the predictive model for time of onset of future RA in case samples that were positive for anti-CCP and/or ≥2 RF isotypes, but with movement of the outcome from time of diagnosis to 6 months before diagnosis, leading to a smaller sample set of 48 cases with 89 samples (since 6 cases and 12 samples were present in this 0–6 months prediagnosis window). Compared with the original analysis, this analysis showed similar results in that increasing age at diagnosis was associated with longer time to diagnosis for a given cytokine/chemokine count ($P = 0.01$). Also, consistent with the removal of samples with increasing cytokine/chemokine counts from the 0–6 months prior to diagnosis (Figure 1), the y-intercepts were moved earlier in the prediagnosis period, the rate of progression to the end point was lower (decreased slope), and cytokine/chemokine count as a predictors of the outcome was marginally significant ($P = 0.06$). Additional results from this analysis are provided online at http://medschool.ucdenver.edu/rheumatology (Supplemental Figure C under Journal Supplements).

DISCUSSION

Consistent with the findings of prior studies, in this cohort of military patients with RA we have identified that autoantibody, circulating cytokine/chemokine, and CRP levels are elevated in preclinical RA, and that autoantibody positivity is highly specific for future seropositive RA (10–12,14,16,17). We have additionally shown that there is an increased duration of preclinical biomarker positivity in individuals with an older age at diagnosis, and we have developed a regression model that uses cytokine/chemokine counts and age at diagnosis to estimate the time from preclinical sample to the

time of RA diagnosis. Importantly, since this model was developed using prediagnosis case samples positive for autoantibodies that are highly specific for RA (anti-CCP and/or ≥2 RF isotypes), application of this model in prospective studies of individuals without symptomatic RA who are positive for these autoantibodies will allow for the prediction of age-related timing of the onset of symptomatic disease as well as the identification of subjects who may be ideal candidates for prevention trials due to their likely imminent onset of symptomatic disease.

Of note, while the RA-specific autoantibody profile used in the model is most likely to be positive within 5 years prior to diagnosis, it may be present up to 10 years before diagnosis (Table 5). As such, cytokine/chemokine analyses in these high-risk subjects allows for finer specification of time to diagnosis than does autoantibody testing alone. Also, while this model used age at diagnosis (by decade) to predict the time to diagnosis in RA cases, it can be applied to estimate the timing of the onset of future symptomatic RA in asymptomatic subjects who are followed up prospectively, with current age replacing age at diagnosis, since the 10-year intervals allow for adequate bracketing of subjects into age groups where biologic and other factors influencing the duration of preclinical biomarker positivity are appropriately accounted for.

The relationship between age and the increased duration of pre–RA diagnosis biomarker positivity identified here and in prior studies (13,18) may be due to differing genetic and environmental influences on disease development in younger versus older cases, or may be due to factors related to senescence of the effector mechanisms of the immune system in older subjects (13,28). Nonbiologic factors may also influence the duration of prediagnosis autoimmunity and inflammation in military RA subjects. For example, older military subjects may be less likely to present with medical complaints, since they wish to protect their work or retirement status, or they may be more likely to be engaged in sedentary tasks where joint symptoms may be less debilitating. In this study, however, there was no difference in the time of onset of prediagnosis symptoms by age at diagnosis. Furthermore, younger military subjects may appear to have a comparatively shorter preclinical duration of biomarker positivity because they have a smaller temporal span of preclinical samples available than older subjects. However, since the median duration from first preclinical sample to diagnosis was not significantly different between age groups (by decade) used in the prediction model, this was likely not a significant issue in the present study.

There may be an unidentifiable duration of preclinical biomarker positivity if subjects' earliest available prediagnosis samples were biomarker positive. We investigated this issue and found that there was no significant difference between age at diagnosis groups (<40 or ≥40 years old) in the number of initial preclinical samples that were positive for a biomarker, with the exception that GM-CSF was positive in the initial sample in a higher proportion of cases who were younger than 40 years at diagnosis. Additionally, for all autoantibodies and for IL-1α, IL-6, IL-15, IP-10, MCP-1, and CRP, there was a non–statistically significant trend for the patients who were 40 years old or older to have an increased proportion of first-sample biomarker positivity, suggesting that the longer duration of biomarker positivity pre–RA diagnosis in older cases may actually be underestimated. In sum, age-related duration of pre–RA diagnosis biomarker positivity in this population is likely a real phenomenon, important for understanding the evolution of RA as well as developing models to predict the timing of the onset of future disease.

The elevated levels of the cytokines/chemokines assessed here likely reflect various underlying processes, including general inflammation (IL-1α, IL-1β, TNFα, IL-6, and CRP), Th1-related processes (IL-12), Th2-related processes (eotaxin), T cell regulation (IL-10 and IL-15), or cellular signaling/growth factors (FGF-2, flt-3 ligand, and GM-CSF). Due to limitations in the size of our sample set and the number/type of cytokines/chemokines assessed in the present study, inferences that can be made regarding the biology and timing of specific immune responses in preclinical RA are likewise limited; however, there are several findings of particular interest. First, the presence of elevated levels of IL-6 in a subset of subjects prior to anti-CCP positivity is of interest since this cytokine is associated with the development of Th17 cells, which are thought to be important in RA pathogenesis (29). IL-17 and IL-23 are also important factors in this pathway, and although we did not assess these cytokines, Kokkonen et al (16) demonstrated elevated levels of IL-17 pre–RA diagnosis, and, taken together, their findings and the findings of the present study suggest that the Th17 pathway is important in preclinical RA. Second, the presence of elevated IP-10 levels prior to anti-CCP positivity is of interest in terms of disease pathogenesis, since this chemokine is an interferon-γ–induced protein promoting chemoattraction for macrophages, dendritic cells, and T cells, as well

as in terms of therapeutics, since blockade of IP-10 has been shown to reduce the severity of collagen-induced arthritis in mice (30). Third, the increased proportion of IL-10 positivity in cases who were younger than 40 years at diagnosis is also of interest, with the T cell regulatory aspects of this cytokine perhaps playing a role in the age at onset of disease (31). Fourth, the preclinical fluctuations in positivity of individual cytokines/chemokines likely reflects that inflammation, due to evolving immune reactions and/or level of tissue injury, builds and eventually reaches a threshold state when an individual transitions from asymptomatic autoimmunity/inflammation to clinically apparent disease (although the exact anatomic site of these early inflammatory/autoimmune processes in preclinical RA are unknown).

Furthermore, the loss of significant differences in preclinical positivity for a subset of biomarkers when samples from 0–6 months prior to diagnosis were removed from the analyses indicates that the immediate prediagnosis period is a time of increasing systemic inflammation, when early RA symptoms may be present. However, statistical power issues due to loss of samples and possible inaccuracies in patient recall of symptom onset may be factors here (32). Of note, because of overlap between increasing levels of cytokines/chemokines and symptoms during this immediate prediagnosis period, these military cases may be similar to the Dutch patients described by Bos et al (33) and van Baarsen et al (34) who have autoantibody positivity and arthralgia as well as increased gene expression for cytokines/chemokines but no clinical synovitis and who may later progress to having definable RA. Finally, while CRP levels did not predict the time of onset of RA, the highest proportion of cases with CRP positivity was observed in samples obtained <1 year prediagnosis, suggesting that CRP elevation in an at-risk individual may indicate the impending onset of symptomatic RA. All of these latter issues will need to be explored prospectively in additional sample sets, where the shortcomings of ascertainment of prediagnosis symptoms in retrospectively assembled cohorts can be addressed.

There are other caveats to our findings. There are no standardized cutoff values for positivity for cytokines/chemokines in the methodology used in the present study (Luminex), and our cutoff values for positivity may not be applicable to other populations. Also, because our cutoff levels were established using post–RA diagnosis samples, treatment factors may influence these levels, although it is likely that these levels were higher than may be expected in the preclinical period, leading to conservative estimates of prediagnosis cytokine/chemokine positivity.

Notably, there were differences in the diagnostic accuracy of cytokines/chemokines for future RA in our study compared with that by Kokkonen et al (16). On average we found that the 14 individual cytokines/chemokines studied had a sensitivity of ~42% and a specificity of ~74% for future RA, compared with an average sensitivity of ~17% and a set specificity of ~95% for any of the 15 cytokines studied by Kokkonen and colleagues (16). These differences may be due to differences in RA case ascertainment, methodologies of biomarker testing, and methodologies used to determine cutoff values and the sensitivity/specificity of the biomarkers for future RA. Additionally, compared with testing a single prediagnosis sample, our testing of multiple prediagnosis samples per individual likely allows for greater sensitivity to detect elevated levels, especially given the fluctuations in cytokine/chemokine positivity demonstrated in this study. In the future, standardization of cytokine/chemokine assessment and cutoff values for positivity will overcome many of these issues.

There may also be factors not related to RA that affected cytokine/chemokine levels, including methodologies of sample collection and sample storage (35). And, while military subjects were to have blood sampled for the DoDSR at defined times, it is possible that they preferentially had sampling during times of illness. However, since samples from carefully matched military controls were used for comparisons, we believe that such effects were accounted for.

Regarding the wider applicability of these findings, this military cohort may not represent typical RA in the general population. It was predominantly male, and the mean age at onset of disease was earlier than seen in the general population. There also may be biases in terms of which military subjects are referred for rheumatology evaluation, and these cases may represent more severe RA, evidenced by a relatively high proportion of patients with seropositive disease (~88%). These concerns, as well as the role of genetic and environmental factors (such as smoking) in predicting RA, need to be addressed by application of this model in additional populations.

In conclusion, the presence of elevated levels of autoantibodies and multiple cytokines/chemokines can be used, in autoantibody-positive subjects at high risk for future RA, to predict the timing of diagnosis. Going forward, validation of this model should be performed using existing biorepositories as well as ongoing prospec-

tive studies of individuals at risk for future RA (36,37), with the goals of understanding the biology of RA development and identifying subjects that can be considered for preventive interventions.

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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. Deane 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. Deane, Lazar, Derber, Norris, Robinson, Holers.

Acquisition of data. Deane, Hueber, Majka, Derber, Gilliland, Edison, Robinson.

Analysis and interpretation of data. Deane, O'Donnell, Lazar, Derber, Norris, Robinson, Holers.

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