

# Combinations of Anticyclic Citrullinated Protein Antibody, Rheumatoid Factor, and Serum Calprotectin Positivity Are Associated With the Diagnosis of Rheumatoid Arthritis Within 3 Years

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**Objective.** To evaluate the prevalence of elevations of anti-cyclic citrullinated peptide-3 (anti-CCP3) antibody, rheumatoid factor IgM (RF-IgM) and serum calprotectin (sCP) in pre-rheumatoid arthritis (RA) as well as the diagnostic accuracies of these biomarkers for the timing of diagnosis of future RA.

**Methods.** A total of 215 RA cases, each with approximately three pre-RA diagnoses and one post-RA diagnosis serum sample, and controls were identified from the Department of Defense Serum Repository. All case samples and a single sample from each control subject were tested for anti-CCP3 (IgG), RF-IgM, and sCP. The diagnostic accuracies of biomarkers for future RA were evaluated.

**Results.** Anti-CCP3, RF-IgM, and sCP were elevated in pre-RA, with anti-CCP3 and sCP significantly elevated compared with RF-IgM at the earliest time points. Within the cases, the combination of anti-CCP3 and RF-IgM positivity had a positive predictive value (PPV) of 35.6% for a diagnosis of RA in 3 years or less, which is significantly higher than the PPV of 18.7% for anti-CCP3 positivity alone ( $P < 0.001$ ). A combination of anti-CCP3, RF-IgM, and sCP had the highest PPV (53.0%) for a diagnosis of RA in 3 years or less; however, this was not significantly higher than the PPV for anti-CCP3 and RF-IgM positivity ( $P = 0.248$ ).

**Conclusion.** Anti-CCP3, RF-IgM, and sCP are elevated in pre-RA; furthermore, combinations of elevations of these biomarkers are more commonly seen in the period of less than or equal to 3 years to diagnosis. This may be considered in creating inclusion criteria in prevention trials in RA. In addition, the biologic relationships of these biomarkers in pre-RA need exploration.

## INTRODUCTION

Serum elevations of antibodies to citrullinated protein antigens (ACPA) and rheumatoid factor (RF) are associated with the future development of rheumatoid arthritis (RA) (1). Based on this,

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Drs. Deane, Mikuls, and Holers, and Ms. Feser and Ms. Moss's work was supported by the Department of Defense Congressionally Directed Medical Research Program (PR120839 and PR191079). Dr. Kelmenson was supported by National Institutes of Health (grant T32 AR007534) and National Institutes of Health/National Center for Advancing Translational Sciences Colorado (grant UL1 TR001082).

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these markers are a key part of the inclusion criteria for several completed or ongoing prevention trials (1–4). However, because ACPA and/or RF may be present for more than 10 years prior to a diagnosis of RA, using additional biomarkers to improve the prediction of a transition from pre-RA to a clinically apparent disease

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Inova Diagnostics provided calprotectin kits for testing. Dr. Deane has served as a consultant for Inova Diagnostics. No other disclosures relevant to this article were reported.

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Submitted for publication March 7, 2021; accepted in revised form June 15, 2021.

within a defined time frame would be especially useful to identify individuals who would benefit most from participation in clinical trials designed to delay or prevent disease onset within a defined time period (1–3).

Calprotectin is a heterodimer of two proteins (S100A8 and S100A9) produced by monocytes and neutrophils in circulation and tissue in response to inflammation (5). Serum calprotectin (sCP) levels in patients with established RA have been shown to correlate with disease activity scores and with ultrasound synovitis scores (6–8); however, the role of sCP in pre-RA is not well studied. As such, we sought to evaluate the prevalence of elevations and the diagnostic accuracies of ACPA, RF-IgM, and sCP in pre-RA and, in particular, the potential ability of sCP to improve the prediction of the timing of a future diagnosis of RA.

**METHODS**

**Study subjects.** We evaluated pre- and post-RA diagnosis serum samples from 215 cases with RA (213 meeting the 1987 criteria and two diagnosed with RA by a board-certified rheumatologist) as well as a single sample from each subject within a set of controls without RA who were matched to cases on age, sex, race, and duration of sample storage. These samples were obtained from the Department of Defense Serum Repository (DoDSR), which was established to monitor the health of United States Uniformed Services personnel and can be used to evaluate biomarkers before and after disease diagnosis. Full details of the development of this DoDSR RA cohort are published (9).

**Biomarker testing.** For cases with RA, an average of three samples from the pre-RA diagnosis period and one sample from the post-RA diagnosis period were tested using enzyme-linked immunoabsorbent assays (ELISAs) for anticyclic citrullinated peptide-3 (anti-CCP3; IgG detection) and RF-IgM, as well as sCP using a research-only ELISA. All assays (CCP3, RF, and sCP) were based on the QUANTA Lite platform (Inova Diagnostics). In addition, the newest sample from each control subject was tested for anti-CCP3, RF-IgM, and sCP. All testing was performed at the University of Colorado in the Exsera Biolabs, with the technician blinded to the case-control status of samples.

Anti-CCP3 positivity was evaluated on the basis of the manufacturer-established cutoff of 20 units or greater, which is reported per the package insert to be approximately 96% specific

for RA. In the absence of established cutoffs for RF isotypes, RF-IgM positivity was determined on the basis of a previously determined cutoff level of greater than 26.59 units, which was present in less than 2% of a subset of 156 DoDSR control subjects (9). sCP positivity was determined by creating a cutoff level of greater than 13.27 units, which was present in less than 2% of an additional subset of 96 DoDSR controls. This subset was selected because they had adequate serum volumes available for testing. A subset of samples underwent duplicate well testing for sCP, with a mean difference between wells of less than 6%.

**Statistical analyses.** We determined the prevalence of positivity for each biomarker for a single sample from each case at given time points before and after RA diagnosis and compared the prevalence of positivity for each biomarker at each of the three pre-RA and one post-RA diagnosis time points using  $\chi^2$  and Fisher's exact testing when appropriate. We also determined the diagnostic accuracy of anti-CCP3, RF-IgM, and sCP in cases compared with a single time point in a subset of 48 controls who were kept separate from the subjects used to set the cutoffs for RF-IgM and sCP. Within cases, we stratified subjects into two groups on the basis of whether the subject ever had sCP positivity to determine whether sCP-positive subjects were different in terms of their age at diagnosis of RA, sex, smoking status, or body mass index (BMI) category (Centers for Disease Control classification of normal = 18.5 to <25; overweight or obese =  $\geq 25$ ), using Fisher's exact tests or t tests as appropriate.

In addition, to address how combinations of biomarkers could be used to estimate a time to future diagnosis of RA, we calculated within cases the diagnostic accuracy, including sensitivity, specificity, positive predictive values (PPVs) and negative predictive values (NPVs), and likelihood ratios (LRs) of whether a sample was within 3 years of or more from a future diagnosis of RA. This differs from the classical definition of diagnostic accuracy studied in case-control studies but it is still clinically useful to identify how accurately these tests confer an imminent (within 3 years) RA diagnosis. The rationale for evaluating a 3-year interval was based on the assumption that 3 years before a diagnosis of clinically apparent RA would represent a reasonable interval to consider the initiation of an intervention for RA prevention such as through enrollment in a clinical prevention trial.

For these analyses, because all subjects in the study were seen more than one time in the pre-RA period, each observation

**Table 1.** Characteristics of RA cases and controls

	Cases (N = 215)	Controls for Comparison With Cases (N = 48)	Controls for sCP Test Cutoff Determination (N = 96)
Female, sex, %	48.0	47.9	45.8
Age at diagnosis of RA (or age at sample collection for controls), mean (SD), years	37.0 (7.9)	33.8 (8.2)	35.2 (7.8)
Non-Hispanic white, %	56.3	56.3	56.3

Abbreviation: RA, rheumatoid arthritis; sCP, serum calprotectin.

is not independent; for example, a single individual may have two samples in the window of 3 years or less prior to diagnosis. As such, traditional methods of calculating diagnostic accuracy were unavailable. Therefore, we used a bootstrap sampling approach to calculate diagnostic accuracy, proceeding as follows: First, we took a bootstrap sample of the data on cases from all visits before RA diagnosis. Then, for each subject, we randomly sampled a single sample from that subject's set of samples, which could be either within 3 years of RA diagnosis or earlier. With this approach, because each case is included only once, the filtered data set can then be used to estimate diagnostic accuracy statistics. The bootstrap resampling was repeated 10 000 times, after which our estimated diagnostic accuracy components are averaged over all bootstrap iterations. We additionally used the bootstrap resamples of our measures of interest to infer whether significant differences existed between different combinations of biomarker positivity, and these were used as the basis for all *P* values comparing diagnostic accuracy results. Diagnostic accuracies (eg, PPV and NPV) were calculated assuming that the prevalence of getting RA within 3 years is constant over the course of the age range of the population (in this population, each case had approximately a 7.9% chance of being diagnosed with RA in any given 3-year period).

Finally, we investigated whether sCP positivity associates with the positivity of other biomarkers at each time point using Fisher's exact tests, adjusting for multiplicity using Bonferroni's method.

**Ethical considerations.** All studies were approved by institutional review boards at the participating institutions.

## RESULTS

The characteristics of the cases and controls, including autoantibody and sCP positivity before and after RA diagnosis, are presented in Tables 1 and 2. Overall, anti-CCP3, RF-IgM, and

sCP, as single biomarkers or in combination, were highly specific for RA when compared with those of the control subjects. In addition, in the earliest samples (collected a mean of 11.5 years before RA diagnosis), anti-CCP3, and sCP were positive in a higher number of subjects compared with RF-IgM (anti-CCP3 22.3% versus RF-IgM 8.8%; *P* < 0.001; sCP 17.7% versus RF-IgM 8.8%; *P* = 0.010). However, in a pairwise comparison, the difference between anti-CCP3 and sCP positivity at this earliest time point was not statistically significant (*P* = 0.278). Furthermore, the number of subjects testing positive for anti-CCP3, RF-IgM, and sCP increased as a diagnosis of RA approached, with the exception that the prevalence of sCP positivity had a non-significant decrease from the immediate pre-RA sample compared with the post-RA diagnosis sample (29.1% versus 24.8%; *P* = 0.383).

In analyses of cases only, positivity for sCP in any sample was more common in men than women (61.4% versus 38.6%; *P* = 0.002); in addition, positivity for sCP was more common in subjects whose BMI was classified as overweight or obese compared with those with normal BMI (79.4% versus 25.4%; *P* = 0.006). There was no association of sCP positivity with smoking or age (data not shown).

When the biomarkers were evaluated in combinations, positivity for both anti-CCP3 and RF-IgM had a significantly higher PPV for an RA diagnosis in 3 years or less than anti-CCP3 alone (35.6% versus 18.7%; *P* < 0.001) (Table 3). Moreover, "triple" positivity for anti-CCP3, RF-IgM, and sCP had the highest PPV for a diagnosis of RA within 3 years; however, the difference compared with the combination of anti-CCP3 and RF-IgM positivity did not achieve statistical significance (53.0% versus 35.6%; *P* = 0.248). Furthermore, the combination of anti-CCP3 and sCP positivity within cases who were never positive for RF-IgM had a PPV of 16.4%.

In addition, because there were significant associations between male sex and obese/overweight status and sCP positivity,

**Table 2.** Biomarker positivity before and after RA diagnosis in RA cases and in controls

	RA Cases: Earliest Sample Before RA Diagnosis	RA Cases: Intermediate Sample Before RA Diagnosis	RA Cases: Immediate Sample Before RA Diagnosis	RA Cases: Postdiagnosis Sample	Controls <sup>a</sup>
Subject samples available per time interval, <sup>a</sup> n	215	214	213	214	48
Time sample collected in relationship to RA diagnosis, mean (SD), years	-11.5 (5.2)	-2.7 (1.5)	-0.9 (0.8)	1.3 (0.9)	N/A
Anti-CCP3 positive, n (%)	48 (22.3)	127 (59.3)	157 (73.7)	161 (75.2)	0 (0.0)
RF-IgM positive, n (%)	19 (8.8)	75 (35.0)	112 (52.6)	112 (52.3)	0 (0.0)
sCP positive, n (%)	38 (17.7)	50 (23.4)	62 (29.1)	53 (24.8)	2 (4.2)
Anti-CCP3 and RF-IgM positive, n (%)	10 (4.7)	67 (31.3)	105 (49.3)	100 (46.7)	0 (0.0)
Anti-CCP3, RF-IgM, and sCP positive, n (%)	3 (1.4)	14 (6.5)	38 (17.8)	27 (12.6)	0 (0.0)
Anti-CCP3 and sCP positive, n (%)	8 (3.7)	23 (10.7)	51 (23.9)	39 (18.2)	0 (0.0)

Abbreviation: CCP, cyclic citrullinated peptide; N/A, not applicable; RA, rheumatoid arthritis; RF-IgM, rheumatoid factor immunoglobulin M; sCP, serum calprotectin.

There were a total of 215 RA cases, and each time interval contains a single sample from each case; however, not all cases had a sample from each time interval.

<sup>a</sup> The control samples are from 48 individuals and a single time point matched in the duration of collection and storage to RA case postdiagnosis sample.

**Table 3.** Diagnostic accuracy of various tests within cases for a sample being present 3 years or less prior to RA diagnosis, averaged over bootstrap resamples with 95% CIs

Biomarker	Sensitivity <sup>a</sup> (95% CI)	Specificity <sup>a</sup> (95% CI)	PPV (95% CI)	P Value	NPV (95% CI)	Positive LR (95% CI)	Negative LR (95% CI)	OR (95% CI)
Anti-CCP3 positive	69.3% (63.9-74.8%)	73.7% (66.3-80.8%)	18.7% (14.6-24.0%)	-	96.5% (95.8-97.3%)	2.6 (2.0-3.7)	0.4 (0.3-0.5)	6.4 (4.0-10.8)
RF-IgM positive	46.6% (40.2-53.0%)	88.7% (83.1-94.0%)	27.2% (18.3-41.1%)	0.037 <sup>b</sup>	95.1% (94.4-95.7%)	4.2 (2.6-8.1)	0.6 (0.5-0.7)	7.0 (3.8-15.0)
sCP positive	27.0% (20.8-33.3%)	81.8% (75.0-88.3%)	11.6% (7.6-17.5%)	0.024 <sup>b</sup>	93.0% (92.0-93.7%)	1.5 (1.0-2.4)	0.9 (0.8-1.0)	1.7 (1.0-3.1)
Anti-CCP3 and RF-IgM positive	43.1% (36.9-49.6%)	92.8% (88.4-96.9%)	35.6% (23.2-55.5%)	<0.001 <sup>b</sup>	95.0% (94.4-95.6%)	6.1 (3.5-14.2)	0.6 (0.5-0.7)	10.0 (5.2-26.0)
Anti-CCP and sCP positive in RF-IgM-negative cases (n = 72)	7.3% (2.2-14.30%)	96.7% (90.9-100.0%)	16.4% (2.0-100.0%)	0.384 <sup>b</sup>	92.4% (91.7-93.1%)	2.3 (0.2-∞) <sup>d</sup>	1.0 (0.9-1.1)	2.4 (0.2-∞) <sup>d</sup>
Anti-CCP3, RF-IgM, and sCP positive	13.5% (8.7-18.4%)	98.5% (96.3-100.0%)	52.7% (20.8-100%)	0.240 <sup>c</sup>	93.0% (92.6-93.4%)	10.1 (3.1-∞) <sup>d</sup>	0.9 (0.8-0.9)	11.4 (3.3-∞) <sup>d</sup>

Abbreviation: CCP, cyclic citrullinated peptide; CI, confidence interval; LR, likelihood ratio; NPV, negative predictive value; OR, odds ratio; PPV, positive predictive value; RF-IgM, rheumatoid factor immunoglobulin M; sCP, serum calprotectin.

<sup>a</sup> In bootstrap analyses, on average, 120 subjects contributed samples to the interval <=3 years, and 84 subjects to the interval less than 3 years.  
<sup>b</sup> The P value from the comparison of PPVs between anti-CCP3 (as a referent) and the additional biomarker(s) (eg, RF-IgM, sCP, or combinations).  
<sup>c</sup> The P value from the comparison of PPVs between anti-CCP3 + RF-IgM positive and Anti-CCP3, RF-IgM, and sCP positive.  
<sup>d</sup> The median presented to account for a small number of bootstrap samples with infinite value.

we evaluated the PPVs of biomarkers in cases stratified by sex and by BMI (Supplementary Table 1). Similar to what was seen in the nonstratified analyses, overall, combinations of biomarkers had increasing PPVs for a sample being less than 3 years from a diagnosis of RA, with the highest PPVs seen in samples that were positive for anti-CCP3, RF-IgM, and sCP. In addition, the PPVs of biomarkers were similar in men and women, and there was a trend for lower PPVs in cases who were overweight or obese compared with those who were normal weight.

When evaluated as individual biomarkers, positive RF-IgM had a higher PPV for an RA diagnosis in 3 years or less than anti-CCP3 (27.2% versus 18.7%;  $P = 0.037$ ), although sCP alone had a lower PPV for an RA diagnosis in 3 years or less compared with anti-CCP3 (11.6% versus 18.7%;  $P = 0.024$ ). There were no significant associations of sCP positivity with anti-CCP3 and/or RF-IgM positivity (data not shown).

## DISCUSSION

Several important findings have been demonstrated herein, including the first description that sCP can be abnormal prior to a diagnosis of RA in a subset of individuals. In addition, within cases, an increasing number of positive biomarkers (anti-CCP3, RF-IgM, and sCP) in a sample yielded an increasing PPV for a diagnosis of RA within 3 years. Furthermore, these findings add to the understanding that anti-CCP3 can be present in pre-RA (10).

In particular, the addition of sCP to anti-CCP3 and RF-IgM provided the highest predictive values for a sample being within 3 years of a diagnosis of RA. Although this triple positivity was not a significant increase from the positivity of anti-CCP3 and RF-IgM, the trend is intriguing. Notably, herein, anti-CCP and RF positivity were highly specific for RA (Table 2); furthermore, these autoantibodies have already been demonstrated in multiple case-control and prospective studies to have a high PPV for future RA and are used as inclusion criteria in multiple prevention studies in RA (1–4,11). As such, although more studies are needed, these new findings could be used to support a stepwise approach in which anti-CCP and RF testing are used to identify individuals at high overall risk for future RA and sCP positivity is further used to help optimize the inclusion of individuals who will have an imminent onset of clinically apparent RA.

The elevation of sCP may also indicate that processes such as neutrophil activation and neutrophil extracellular trap formation may be increased in some individuals in the pre-RA period and be part of processes that lead to a transition to the development of clinically apparent RA (12). Of further interest was the finding that anti-CCP3 and sCP were elevated in a significantly higher proportion of subjects than RF-IgM at the earliest pre-RA serum sample available for the subjects (a mean of 11.3 years prior to RA diagnosis). This could indicate a potential role for processes that drive sCP in a subset of individuals in the earlier

steps in the evolution of RA. Furthermore, male sex and a higher BMI were associated herein with increased rates of sCP positivity within cases. Although published findings of sCP positivity and sex are mixed, previous studies have consistently identified that sCP can be higher in individuals who have obesity (13). As obesity has also been identified as a risk factor for future RA (14), these relationships will need future investigation. In addition, when we evaluated cases stratified by BMI, there was a tendency for decreased PPVs of biomarkers in individuals who are overweight/have obesity. We will avoid inferring too much from this finding given the small numbers of subjects in some comparisons; however, this could indicate that a higher BMI status itself is a risk factor for more imminent-onset RA and/or that sCP may have a stronger association with imminent RA in normal-weight individuals. This will need future evaluation.

Notably, we have not compared the performance of sCP with that of other measures of inflammation such as C-reactive protein (CRP). However, in another pre-RA cohort study, CRP did not significantly improve prediction for the timing of onset of RA (15). Furthermore, even in early clinically apparent RA, inflammatory markers such as CRP or the erythrocyte sedimentation rate may only be elevated in a subset of subjects (16), and indeed herein only approximately 24% of subjects with RA had sCP positivity in samples collected a mean of approximately 1.3 years after RA diagnosis. In addition, there was a decrease in the prevalence of positivity of sCP in post-RA diagnosis samples that, based on published work in patients with established RA (7), may be explained by treatment effect as approximately 90% of the cases in this DoDSR cohort were on disease-modifying RA-related therapy at this postdiagnosis time point. Going forward, sCP and other biomarkers should be explored to further understand their roles as well as measures of severity of disease, response to therapy, and long-term outcomes.

Finally, RF-IgM had a higher PPV than anti-CCP3 for an RA diagnosis within 3 years; in addition, RF-IgM positivity was less prevalent than anti-CCP3 positivity earlier in the pre-RA period. In aggregate, these findings suggest that RF-IgM may appear later in the evolution of RA than anti-CCP3; furthermore, RF-IgM may play a role in the transition from pre-RA to clinically apparent disease (9,17). These relationships will need exploration in future studies.

In conclusion, anti-CCP3, RF-IgM, and sCP are elevated in pre-RA, and combinations of these biomarkers provide modest PPVs for RA onset within 3 years. In particular, a clinically meaningful improvement of sCP on prediction of future RA is plausible, but further investigation is required to confirm this hypothesis as well as to understand the role of sCP in the pathophysiology of RA development.

## ACKNOWLEDGMENTS

The authors thank the Department of Defense Serum Repository as well as members of the United States Uniformed Services for the data and biospecimens that were used in this project.



## 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. Kevin D. 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.** Bettner, Peterson, Bergstedt, Kelmenson, Demoruelle, Mikuls, Edison, Parish, Feser, Frazer-Abel, Moss, Mahler, Holers, Deane.

**Acquisition of data.** Bettner, Peterson, Bergstedt, Kelmenson, Demoruelle, Mikuls, Edison, Parish, Feser, Frazer-Abel, Moss, Mahler, Holers, Deane.

**Analysis and interpretation of data.** Bettner, Peterson, Bergstedt, Kelmenson, Deane.

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