Anti-carbamylated Protein Antibodies Are Present Prior to Rheumatoid Arthritis and Are Associated with Its Future Diagnosis

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ABSTRACT. ***Objective.*** Anti-carbamylated protein (anti-CarP) antibodies could further elucidate early rheumatoid arthritis (RA) pathogenesis and predict clinical disease. We compared the diagnostic accuracy of anti-CarP antibodies for future RA to other RA-related antibodies in military personnel.

Methods. Stored pre-RA diagnosis serum samples from 76 RA cases were tested for anti-CarP fetal calf serum (FCS), anti-CarP fibrinogen (Fib), anticyclic citrullinated peptide antibodies version 2 (anti-CCP2), rheumatoid factor-nephelometry (RF-Neph), and RF isotypes [immunoglobulin M (IgM), IgG, and IgA]. Positivity for all antibodies was determined as ≥ 2 SD of log-transformed means from controls. Relationships between autoantibodies and future RA were assessed in pre-diagnosis serum for all RA cases compared to controls using sensitivity, specificity, and logistic regression. Differences in diagnostic accuracy between antibody combinations were assessed using comparisons of area under the curves (AUC).

Results. Anti-CarP-FCS was 26% sensitive and 95% specific for future RA, whereas anti-CarP-Fib was 16% sensitive and 95% specific for future RA. Anti-CarP-FCS positivity was associated with future RA, while anti-CarP-Fib trended toward association. The antibody combination of anti-CCP2 and/or ≥ 2 RF (RF-Neph and/or RF-isotypes) resulted in an AUC of 0.72 for future RA, where the AUC was 0.71 with the addition of anti-CarP-FCS to this prior combination.

Conclusion. Adding anti-CarP-FCS to antibody combinations did not improve AUC. However, anti-CarP-FCS was associated with future onset of RA, and was present in prediagnosis serum in ~10% of RA cases negative for anti-CCP2 but positive for RF. (First Release Jan 15 2015; J Rheumatol 2015;42:572–9; doi:10.3899/jrheum.140767)

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Discovery of antibodies to citrullinated protein antigens (ACPA) has improved our understanding of the seropositive subset of rheumatoid arthritis (RA). ACPA and rheumatoid factor (RF) are present in the serum of patients with RA years before the clinical diagnosis of RA[1,2,3,4,5,6]. While these autoantibodies, particularly anticyclic citrullinated peptide (anti-CCP), are highly specific (~95–99%) for RA, the sensitivity in those who later develop RA is notably lower (< 70%)[1,3,5,6]. Testing for RF and anti-CCP simultaneously can improve sensitivity by ~4–7% while maintaining high specificity (~90–98%)[3,5]. Yet sensitivity of these combinations is still limited. Additional autoantibodies that improve sensitivity for RA while maintaining high specificity would be useful diagnostic and prediction tools[7].

Shi, *et al* reported the discovery of anti-carbamylated protein antibodies (anti-CarP) in patients with RA[8,9], including anti-CCP–negative patients as well[8,9]. Presence of anti-CarP in early RA was associated with increased disease

severity, manifested by future joint destruction[8], and they were detectable in some children with juvenile idiopathic arthritis[10]. Additionally, in subjects without current RA but positive for anti-CCP version 2 (anti-CCP2) and/or RF-immunoglobulin M (IgM) with a history of arthralgia, anti-CarP was 57% sensitive and 94% specific in identifying individuals who later developed classified RA [2010 American College of Rheumatology (ACR)/European League Against Rheumatism criteria][11]. Further, in stored samples collected prior to RA onset, anti-CarP was present prior to RA diagnosis in 5/79 subjects who were otherwise negative for anti-CCP2 and RF-IgM[12].

Anti-CarP alone or in combination with other clinically available RA-related autoantibodies could be useful in predicting the future onset of RA. Our study evaluated the timing of appearance and diagnostic accuracy for future RA of anti-CarP compared to other RA-related autoantibodies in US military personnel.

MATERIALS AND METHODS

Study population. The study population had been previously described[2,3,4]. Subjects were military personnel, consisting of 83 RA cases and 82 controls with stored pre- and postdiagnosis serum samples. RA cases were identified at the Walter Reed Army Medical Center Rheumatology Clinic, and evaluated in clinic from 1989 to 2003. RA cases had the date of RA diagnosis, age at diagnosis, and race determined by chart review. RA cases had both pre- and postdiagnosis serum samples (n = 290, mean samples/subject = 3.5) available through the Department of Defense Serum Repository (DoDSR). A subset of RA cases was determined as seropositive based on the postdiagnosis RF positivity identified on chart review, or if pre- or postdiagnosis samples tested positive for RF (any assay) and/or anti-CCP2 within 1 year of their diagnosis, as described[2,3]. Controls were also derived from the DoDSR and matched to cases based on age (case age at diagnosis), sex, race, number of samples available (n = 290, mean samples/subject = 3.5), duration of sample storage, and enlistment region.

Biomarker analyses. Samples were tested in the Rheumatology Clinical Research Laboratory at the University of Colorado for clinically available RA-related autoantibodies, including RF by several methods and anti-CCP2. RF was measured by nephelometry (RF-Neph, Dade Behring), and RF isotypes IgM, IgG, and IgA were measured using ELISA kits (INOVA Quanta Lite). Anti-CCP2 was measured by the Diastat kit (Axis-Shield).

Generation of carbamylated antigens. Carbamylated proteins were generated as described by Shi, *et al*[8]. Fetal calf serum (FCS; Bodinco) was carbamylated or left untreated. For generating carbamylated FCS (Ca-FCS), FCS was diluted in H_2O to 4 mg/ml and potassium cyanate (Sigma) was added to a concentration of 1 mol/l. Following incubation at 37°C for 12 h, the sample was extensively dialyzed against H_2O using 10K MWCO SnakeSkin Dialysis Tubing (Thermo Scientific). Protein concentration was measured by both NanoDrop (Thermo Scientific) and BCA Protein Assay (Thermo Scientific). Carbamylated fibrinogen (Ca-Fib) was generated by incubating 5 mg/ml human Fib (Sigma) with 0.5 m potassium cyanate at 4°C for 3 days, followed by dialysis against phosphate buffered saline (PBS).

Detection of anti-CarP-FCS by ELISA. Nonmodified FCS and Ca-FCS were coated at 10 μg/ml in 50 μl (diluted in pH 9.6, 0.1 m carbonate-bicarbonate buffer) on Nunc MaxiSorp plates overnight (Thermo Scientific). After washing in PBS containing 0.05% tween (PT; Sigma), plates were blocked by incubating 100 μl PBS/1% bovine serum albumin (BSA; Sigma) for 6 h at 4°C. Following additional washing, wells were incubated with 50 µl serum at a 1/50 dilution in PBS/0.05% tween/1% BSA buffer (PTB) on ice overnight. All subsequent incubations were performed in PTB. As a standard, serial dilutions of a pool of positive sera were used. Human IgG was detected using rabbit anti-human IgG antibody (DAKO) incubated on ice for 3.5 h. After washing, wells were incubated on ice for 3.5 h with horseradish peroxidase (HRP)-labeled goat antirabbit IgG antibody (DAKO). The final wash was followed by visualization of HRP enzyme activity using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid; ABTS) as substrate. We transformed the absorbance on both Ca-FCS and FCS to aU/ml and subtracted the background signal of FCS (aU/ml) from the signal of CarP-FCS (aU/ml) to analyze the specific anti-CarP-FCS reactivity.

Detection of anti-CarP-Fib by ELISA. Nonmodified Fib and Ca-Fib were coated at 20 μg/ml in 50 μl (diluted in pH 9.0 PBS) on Nunc MaxiSorp plates overnight (Thermo Scientific). Following washing in PT, plates were blocked by incubating 200 μl pH 9.0 PBS/2% BSA for 2 h at 4°C. Following additional washing, wells were incubated with 50 μl serum at a 1/50 dilution in radioimmunoassay (RIA) buffer on ice for 3 h (10 mM Tris pH 7.6; 350 mM NaCl; 1% TritonX; 0.5% Na-deoxycholate; 0.1% sodium dodecyl sulfate; Sigma). All subsequent incubations were performed in RIA buffer. As a standard, serial dilutions of a pool of positive sera were used. Human IgG was detected using HRP-labeled rabbit anti-human IgG antibody (DAKO) incubated on ice for 2 h. Following the last washings, HRP enzyme activity was visualized using ABTS.

Defining positivity for antibodies. Because no established cutoff levels for positivity existed for anti-CarP, we determined cutoff values for anti-CarP in serum. Anti-CarP were measured in 82 healthy controls. Binary cutoffs were determined for anti-CarP by randomly splitting the 82 healthy controls into 2 groups containing 41 subjects each. The first group was used to determine the binary cutoff values for anti-CarP-FCS and anti-CarP-Fib (cutoff controls), and the second group (controls) was reserved for comparison analyses with the cases.

Because we had multiple serum samples per subject, we defined anti-CarP positivity using a single sample from each cutoff control to retain statistical independence. We selected the single serum sample from each of the 41 cutoff controls with measured anti-CarP-FCS and anti-CarP-Fib closest in time to their matched RA case's diagnosis date. Anti-CarP measures were natural log–transformed because they were not normally distributed. Positivity for anti-CarP-FCS and anti-CarP-Fib was defined as ≥ 2 SD above the natural log–transformed mean. After back-transforming these values, positivity was determined to be > 427.4 aU/ml for anti-CarP-FCS and > 233.8 aU/ml for anti-CarP-Fib.

To allow for comparable results across antibodies, positivity for RF-Neph, RF isotypes, and anti-CCP2 were also defined as ≥ 2 SD above the natural log–transformed mean using the same single serum sample from the 41 cutoff controls. After back-transforming these values, positivity was determined as follows: RF-Neph > 24.0 units/ml; RF-IgM > 13.5 units/ml; RF-IgG > 25.0 units/ml; RF-IgA > 17.5 units/ml; and anti-CCP2 > 0.6 units/ml.

Additionally, we considered cutoffs based on clinical recommendations for anti-CCP2 and the RF antibodies. Clinical positivity for RF and RF isotypes were determined using the ACR classification criteria for RA specificities as being present in < 5% of 491 healthy blood bank donor controls[13]. Clinical positivity for RF antibodies were defined as follows: RF > 24.4 units/ml; RF-IgM > 13.6 units/ml; RF-IgG > 10.9 units/ml; and RF-IgA > 10.5 units/ml. Clinical positivity for anti-CCP2 was based on manufacturer specification of > 5 units/ml.

Once we defined antibody positivity using the single serum sample in the cutoff controls, we applied these positivity cutoffs to all the prediagnosis serum of RA cases and to all available serum for the remaining 41 controls reserved for comparison against the RA cases.

Diagnostic accuracy and association of antibodies for future RA. Using 2 × 2 tables, we determined diagnostic accuracy, measured by sensitivity and specificity, of each antibody or various combinations of antibodies ever testing positive at any point in the prediagnosis period for our RA cases,

and at any point for our controls. Cases (76 of 83 total) with sufficient prediagnosis serum sample volumes (n = 210 samples) were tested for all autoantibodies (anti-CarP-FCS, anti-CarP-Fib, anti-CCP2, and RF assays), as were comparator controls (n = 41). We characterized the diagnostic accuracy of antibody positivity, first based on the ≥ 2 SD above the mean cutoff, and then using clinical test-based cutoffs for RF and anti-CCP2 defined as ever testing positive in any sample any time before RA diagnosis for RA cases and any time for controls. We then characterized diagnostic accuracy of antibody positivity in seropositive RA cases; however, we did not present those results in a table because they were similar to results in all RA cases.

Diagnostic accuracy was assessed at the following time periods before RA diagnosis: ≥ 0 to ≤ 1 year, > 1 to ≤ 5 years, and > 5 years. Of note was the period ≥ 0 to ≤ 1 year, where for RA cases, serum was limited strictly to this time period, whereas for controls, we evaluated samples ≤ 1 year and any time after their matched RA cases' diagnosis dates. All other time periods were as specified for both RA cases and controls.

The discriminative abilities between antibodies and combinations of antibodies between the 76 RA cases and 41 controls were assessed through comparisons of area under the curve (AUC) based on the binary cutoffs. Using a binary cutoff, the AUC was the average of sensitivity and specificity. This property allowed us to compare the combined improvement of diagnostic accuracy of both sensitivity and specificity. All AUC analyses accounted for comparisons of antibodies in the same individuals.

To complement diagnostic accuracy results, we used logistic regression analyses to characterize the relative association (OR) between RA case status and presence of these autoantibodies in prediagnosis serum in both seropositive and seronegative RA cases compared to controls.

Assessing the timing of antibody appearance. Timing of antibody appearance in prediagnosis serum was assessed in seropositive RA cases that were ever positive for more than 1 antibody during the prediagnosis period. We determined whether anti-CCP2 was present in serum before the appearance of anti-CarP-FCS or vice versa, and then whether anti-CCP2 was present in serum before the appearance of anti-CarP-Fib or vice versa. The appearance of RF-Neph in relation to anti-CarP-FCS and anti-CarP-Fib was also addressed. A small proportion of cases were already positive for these autoantibodies, representing left-censorship, likely underrepresenting the true mean duration of autoantibody positivity. Because of the small proportion of autoantibody positivity and left-censoring, the nonparametric Wilcoxon signed-rank test was used instead of survival analysis to determine which autoantibody preceded the other based on the mean time of appearance for those with both antibodies.

Antibody levels and variability of positivity in the prediagnosis period. Linear mixed models of natural log-transformed antibody levels characterized trends in mean anti-CarP-FCS, anti-CarP-Fib, RF-Neph, and anti-CCP2 for RA cases and controls up to 10 years before the clinical diagnosis of RA. To characterize mean trends of anti-CarP-FCS and anti-CarP-Fib, we used all RA cases (n = 76) and controls (n = 76) who had measures of anti-CarP-FCS and anti-CarP-Fib because these analyses did not depend on positivity cutoffs, giving an observation sample size of n = 360. In our assessment of anti-CCP2 and RF-Neph trends, all RA cases (n = 82) and controls (n = 82) had complete measures of these antibodies, resulting in a larger observation sample size (n = 441). We determined the best model fit for both linear and squared trends, and identified the time before RA diagnosis when mean levels of antibodies began to differ between RA cases and controls. To account for multiple comparisons at each timepoint within each model, we used a Scheffe p value correction for comparisons in linear combinations[14].

Additionally, we characterized the variability of positivity in multiple samples over time for anti-CarP-FCS, anti-CarP-Fib, anti-CCP2, and RF-Neph in the prediagnosis period for seropositive RA cases and controls by determining the proportion of those who tested positive for an antibody, but had subsequent levels decreased below the cutoff.

All statistical analyses were performed using SAS version 9.3 (SAS Institute Inc.).

Ethical considerations. The study protocol and analyses were approved by the respective institutional review boards at the Walter Reed Army Medical Center and the University of Colorado.

RESULTS

Study population demographics. Demographic characteristics of RA cases, controls, and cutoff controls were not statistically different (Table 1).

Diagnostic accuracy and associations in RA cases. The antibody systems anti-CCP2, RF, and anti-CarP were all detected prior to RA diagnosis, as evidenced by a proportion of RA cases positive for these antibodies in the prediagnosis period. The sensitivity, specificity, and relative associations for future RA based on ever being antibody positive in serum at any time before clinical diagnosis of RA in our 76 seropositive and seronegative RA cases and 41 controls are presented in Table 2. Results are presented to allow the assessment of diagnostic accuracy with the addition of a positive test for anti-CarP-FCS compared to the more established RA-related autoantibodies, either as a single test or a combination of results. As single tests, anti-CCP2 demonstrated the highest sensitivity (52%) for future RA, whereas RF-Neph, RF-IgG, RF-IgA, anti-CarP-FCS, and anti-CarP-Fib had the highest specificities (~95–97%).

Diagnostic accuracy statistics and relative associations for future RA based on clinically relevant cutoffs for RF and anti-CCP2 are presented in Table 3, and were qualitatively similar to Table 2.

Diagnostic accuracy statistics and relative associations for future RA at different time intervals in our 76 seropositive and seronegative RA cases and 41 controls are presented in Table 4. Similar trends in sensitivity and specificity were observed across time periods.

Of the 76 RA cases with anti-CarP tested, 67 (88.1%) were defined as having seropositive RA (RF and/or anti-CCP2), as described in Materials and Methods. Overall, the diagnostic accuracy statistics for future seropositive RA were similar to those found in all RA cases (data not shown). Among the 28 seropositive RA cases positive for ≥ 1 RF, but never positive for anti-CCP2 at any time prior to RA diagnosis, anti-CarP-FCS was present in 3 of these individuals (10.7%). None of the 9 RA cases classified as seronegative RA for both RF and anti-CCP2 were positive for anti-CarP-FCS or anti-CarP-Fib based on our defined cutoffs.

The combination of antibodies, anti-CCP2, and/or ≥ 1 RF (Neph or isotypes) demonstrated 67% sensitivity and 73% specificity for future RA, with an AUC of 0.70; the addition of anti-CarP-FCS increased sensitivity to 68% while decreasing specificity to 68%, resulting in an AUC of 0.68, which was not significantly different from the AUC for anti-CCP2 and/or ≥ 1 RF (p = 0.33). The profile anti-CCP2 and/or ≥ 2 RF (Neph or isotypes) demonstrated 58% sensitivity and 85% specificity with an AUC of 0.72; the addition

Table 1. Descriptive statistics for RA cases, comparison control group, and the control group used to define the cutoff. Values are mean ± SD or n (%) unless otherwise specified.

Characteristic	RA Cases, n = 76; 210 Samples	Controls, n = 41; 136 Samples	Cutoff Controls†, n = 41; 121 Samples	p*
No. samples	2.8 ± 1.1	3.3 ± 1.3	3.0 ± 1.2	0.07
Age at diagnosis, yrs	39.8 ± 9.9	40.6 ± 10.2	39.1 ± 9.6	0.79
Male sex	45 (59.2)	25 (61.0)	23 (56.1)	0.90
Race				0.58
White	51 (67.1)	31 (75.6)	25 (61.0)	
Black	21 (27.6)	9 (22.0)	12 (29.3)	
Other	4 (5.3)	1 (2.4)	4 (9.7)	

* Reported p value is testing the difference across all 3 groups. † Control group used only to determine positivity cutoff for anti-CarP-FCS and anti-CarP-Fib. RA: rheumatoid arthritis; anti-CarP: anti-carbamylated protein; FCS: fetal calf serum; Fib: fibrinogen.

Table 2. Diagnostic accuracy† and OR of RA-related antibodies in prediagnosis serum samples for future RA. Positivity defined as ≥ 2 SD above the mean in healthy controls reserved to define cutoff: RF-Neph > 24.0 units/ml; RF-IgM > 13.5 units/ml; RF-IgG > 25.0 units/ml; RF-IgA > 17.5 units/ml; anti-CCP2 > 0.6 units/ml; anti-CarP-FCS > 427.4 units/ml; and anti-CarP-Fib > 233.9 units/ml.

Antibody	RA, +/76	Control, +/41	SN, %	SP, %	OR	95% CI	p
Anti-CCP2	40/76	4/41	52.6	90.2	10.28	3.34–31.68	< 0.01
RF-Neph	27/76	1/41	35.5	97.6	22.04	2.87–169.36	< 0.01
RF IgM	32/76	5/41	42.1	87.8	5.24	1.85–14.82	< 0.01
RF IgG	8/76	2/41	10.5	95.1	2.29	0.46–11.35	0.31
RF IgA	24/76	2/41	31.6	95.1	9.00	2.01–40.38	< 0.01
≥ 1 RF*	42/76	8/41	55.3	80.5	5.10	2.08–12.47	< 0.01
≥ 2 RF*	24/76	2/41	31.4	95.1	9.00	2.01–40.38	< 0.01
Anti-CarP-FCS	20/76	2/41	26.3	95.1	6.96	1.54–31.52	0.01
Anti-CarP-Fib	12/76	2/41	15.8	95.1	3.66	0.78–17.21	0.10
Anti-CCP2 and/or ≥ 1 RF*	51/76	11/41	67.1	73.2	5.56	2.40–12.89	< 0.01
Anti-CCP2 and/or ≥ 1 RF* and/or anti-CarP-FCS	52/76	13/41	68.4	68.3	4.68	2.06–10.56	< 0.01
Anti-CCP2 and/or ≥ 2 RF*	44/76	6/41	57.9	85.4	8.02	3.02–21.34	< 0.01
Anti-CCP2 and/or ≥ 2 RF* and/or anti-CarP-FCS	46/76	8/41	60.5	80.5	6.32	2.57–15.54	< 0.01

RA cases sample n = 210, avg. samples/subject = 2.8; controls sample n = 136, avg. sample/subject = 3.3. OR, 95% CI, and p cannot be calculated because no controls met positivity criteria. † Contains the following statistics: SN and SP. * Count of RF by Neph and RF isotypes (IgM, IgG, IgA). RA: rheumatoid arthritis; RF: rheumatoid factor; Neph: nephelometry; anti-CCP2: anticyclic citrullinated peptide antibodies version 2; IgM: immunoglobulin M; anti-CarP: anti-carbamylated protein; FCS: fetal calf serum; Fib: fibrinogen; +/n: n positive over the case/control group size; SN: sensitivity; SP: specificity.

of anti-CarP-FCS increased sensitivity to 61% while decreasing specificity to 81%, resulting in an AUC of 0.71, which was not significantly different from the AUC for anti-CCP2 and/or ≥ 2 RF (p = 0.56).

Timing of antibody appearance. Table 5 presents the order of antibody appearance in seropositive RA cases testing positive in the prediagnosis period for both antibodies. Overall, when able to assess, anti-CCP2 was present prior to anti-CarP-FCS and anti-CarP-Fib, where RF-Neph was present after anti-CarP-FCS and anti-CarP-Fib. However, these results were not significantly different.

Trends in antibody levels during the prediagnosis period. Figure 1 characterizes mean levels of antibodies, back-transformed from the log scale, over the 10-year period preceding RA case diagnosis. There was a general trend for increasing mean levels of antibodies over time for anti-CarP-FCS, anti-CarP-Fib, anti-CCP2, and RF-Neph in RA cases where the levels in controls remained stable. The mean levels of anti-CarP-FCS were significantly higher for RA cases compared to controls 4 years before RA diagnosis. Mean anti-CarP-Fib levels were higher in RA cases compared to controls, but this trend was not statistically significant. For anti-CCP2 and RF-Neph antibodies, mean levels exponentially increased in RA cases shortly before RA diagnosis, and mean levels for controls remained consistently low. Anti-CCP2 levels were significantly higher in RA cases compared to controls 10 years before RA diagnosis. Mean levels of RF-Neph were significantly higher in RA cases compared to controls 6 years before RA diagnosis.

Autoantibody fluctuations in positivity over time. In the 20

Table 3. Diagnostic accuracy† and OR of RA-related antibodies in prediagnosis serum samples for future RA. Positivity for RF defined on clinical recommendations as present in < 5% of 491 healthy blood donors: RF > 24.4 units/ml; RF-IgM > 13.6 units/ml; RF-IgG > 10.9 units/ml; and RF-IgA > 10.5 units/ml. Positivity for anti-CCP2 based on manufacturer specifications at > 5 units/ml. Positivity for anti-CarP defined as ≥ 2 SD above the mean in healthy controls reserved to define cutoff: anti-CarP-FCS > 427.4 units/ml and anti-CarP-Fib > 233.9 units/ml.

Antibody	RA, +/76	Control, +/41	SN, %	SP, %	OR	95% CI	p
Anti-CCP2	34/76	0/41	44.7	100.0	NA	NA	NA
RF	27/76	1/41	35.5	97.6	22.04	2.87–169.36	< 0.01
RF IgM	32/76	5/41	42.1	87.8	5.24	1.85–14.82	< 0.01
RF IgG	11/76	3/41	14.5	92.7	2.14	0.56–8.17	0.26
RF IgA	30/76	3/41	39.5	92.7	8.26	2.34–29.19	< 0.01
≥ 1 RF*	43/76	9/41	56.6	78.1	4.63	1.95–11.03	< 0.01
≥ 2 RF*	29/76	3/41	38.2	92.7	7.82	2.21–27.64	< 0.01
Anti-CarP-FCS	20/76	2/41	26.3	95.1	6.96	1.54–31.50	< 0.01
Anti-CarP-Fib	12/76	2/41	15.8	95.1	3.66	0.78–17.23	0.08
Anti-CCP2 and/or ≥ 1 RF*	48/76	9/41	63.2	78.1	6.10	2.54–14.61	< 0.01
Anti-CCP2 and/or ≥ 1 RF* and/or anti-CarP-FCS	49/76	11/41	64.5	73.2	4.95	2.15–11.41	< 0.01
Anti-CCP2 and/or ≥ 2 RF*	41/76	3/41	53.9	92.7	14.84	4.21–52.25	< 0.01
Anti-CCP2 and/or ≥ 2 RF* and/or anti-CarP-FCS	43/76	5/41	56.6	87.8	9.38	3.32–26.53	< 0.01

RA cases positive for RF and/or CCP2 sample n = 166, avg. sample/subject = 2.5; controls sample n = 135, avg. sample/subject = 3.3. † Contains the following statistics: SN and SP.* Count of RF by Neph and RF isotypes (IgM, IgG, IgA). RA: rheumatoid arthritis; RF: rheumatoid factor; Neph: nephelometry; IgM: immunoglobulin M; anti-CCP2: anticyclic citrullinated peptide antibodies version 2; anti-CarP: anti-carbamylated protein; FCS: fetal calf serum; Fib: fibrinogen; SN: sensitivity; SP: specificity; +/n: n positive over the case/control group size; NA: OR, 95% CI, and p cannot be calculated because no controls met positivity criteria.

seropositive RA cases positive for anti-CarP-FCS, 6 (30%) had anti-CarP-FCS levels that decreased below the cutoff in subsequent prediagnosis samples, and 1 of 2 controls that tested positive had levels decrease below the cutoff. Of the 12 anti-CarP-Fib positive cases, 2 (17%) had anti-CarP-Fib levels decrease below the cutoff, while the 2 controls that tested positive remained positive. Further, among the 39 seropositive RA cases positive for anti-CCP2, 1 (3%) had anti-CCP2 levels that decreased below the cutoff, while 2 of the 4 controls that tested positive for anti-CCP2 had levels that decreased below the cutoff. Among the 27 seropositive RA cases positive for RF-Neph, 5 (19%) had RF-Neph levels that decreased below the cutoff, and 4 of the 8 controls had RF-Neph levels that decreased below the cutoff.

DISCUSSION

Our results indicate that anti-CarP-FCS and anti-CarP-Fib are present in prediagnosis serum of RA cases. Both anti-CarP-FCS and anti-CarP-Fib exhibited lower sensitivity (< 30%) than anti-CCP2 or RF, although the specificity for anti-CarP was comparatively high (> 95%). Anti-CarP-FCS exhibited a greater sensitivity and the same specificity as anti-CarP-Fib. Further, anti-CarP-FCS was significantly associated with future RA, while anti-CarP-Fib only trended toward a significant association, which influenced our decision to consider calculations of diagnostic accuracy for future RA using only anti-CarP-FCS in antibody combinations. While we did not observe significant differences in AUC with the addition of anti-CarP-FCS to combinations of anti-CCP and/or RF, we did observe a modestly increased sensitivity and decreased specificity for future RA. This could suggest utility of anti-CarP in assays that test for multiple antibodies at once, or for assessment of risk of future erosive disease in individuals who exhibit anti-CarP[8].

Notably, as no recommended cutoff for anti-CarP exists, we randomly divided the controls into 2 groups to define cutoff levels, reserving 1 set of controls as an independent comparison group. This split may have introduced bias because of unequal groups, although similar demographic characteristics between our control groups (Table 1) suggested such bias was minimal. Additionally, the smaller control groups may have allowed outlier values to influence cutoff values, decreasing our ability to detect significant associations. However, the ≥ 2 SD above the mean cutoff for anti-CarP-FCS in our reduced sample size of 41 was > 427, whereas if we used all 82 controls, our cutoff would have been > 472; ANOVA indicated that splitting the controls did not result in statistically different cutoff levels (p = 0.64). Both these cutoff levels are higher than Shi, *et al*'s initial work on the anti-CarP system, where the positivity cutoff level was > 348[8]. Therefore, our higher cutoff levels for anti-CarP may be less sensitive for future RA than previously reported studies.

Our higher cutoff levels, in addition to small case numbers, could explain why we did not observe anti-CarP in any seronegative RA cases. However, anti-CarP-FCS was still present in prediagnosis serum samples in 10.7% of RA cases who never tested positive for anti-CCP2, which is

Table 4. Diagnostic accuracy† and OR of RA-related antibodies in serum samples at different time intervals during the prediagnosis period for future RA. Positivity defined as ≥ 2 SD above the mean in healthy controls reserved to define cutoff: RF-Neph > 24.0 units/ml; RF-IgM > 13.5 units/ml; RF-IgG > 25.0 units/ml; RF-IgA > 17.5 units/ml; anti-CCP2 > 0.6 units/ml; anti-CarP-FCS > 427.4 units/ml; and anti-CarP-Fib > 233.9 units/ml.

Antibody	RA, +/n	Control, +/n	SN, %	SP, %	OR	95% CI	p
≥ 0 to ≤ 1 yr before RA diagnosis, RA cases sample n = 23, avg. sample/subject = 1; controls sample n = 37, avg. sample/subject = 1.3							
Anti-CCP2	14/23	2/28	60.9	92.9	20.22	3.83–106.81	< 0.01
≥ 1 RF*	15/23	4/28	65.2	85.7	11.25	2.88–43.95	< 0.01
≥ 2 RF*	8/23	2/28	34.8	92.9	6.93	1.30–37.01	0.02
Anti-CarP-FCS	7/23	2/28	30.4	92.9	5.69	1.05–30.85	0.04
Anti-CarP-Fib	5/23	1/28	21.7	96.4	7.49	0.81–69.62	0.08
Anti-CCP2 and/or ≥ 1 RF*	17/23	5/28	73.9	82.1	13.03	3.41–49.88	< 0.01
Anti-CCP2 and/or ≥ 1 RF* and/or anti-CarP-FCS	17/23	7/28	73.9	75.0	8.50	2.40–30.09	< 0.01
Anti-CCP2 and/or ≥ 2 RF*	16/23	4/28	69.6	85.7	13.71	3.44–54.61	< 0.01
Anti-CCP2 and/or ≥ 2 RF* and/or anti-CarP-FCS	16/23	6/28	69.6	78.6	8.38	2.36–29.74	< 0.01
> 1 to ≤ 5 yrs before RA diagnosis, RA cases sample n = 81, avg. sample/subject = 1.7; controls sample n = 40, avg. sample/subject = 1.4							
Anti-CCP2	25/49	1/29	51.0	96.6	29.17	3.67–231.56	< 0.01
≥ 1 RF*	22/49	4/29	44.9	86.2	5.09	1.54–16.84	< 0.01
≥ 2 RF*	13/49	1/29	26.5	96.6	10.11	1.25–81.99	0.03
Anti-CarP-FCS	9/49	0/29	18.4	100.0	NA	NA	NA
Anti-CarP-Fib	6/49	1/29	12.2	96.6	3.91	0.45-34.24	0.22
Anti-CCP2 and/or ≥ 1 RF*	32/49	5/29	65.3	82.8	9.04	2.92-27.94	< 0.01
Anti-CCP2 and/or ≥ 1 RF* and/or anti-CarP-FCS	32/49	5/29	65.3	82.8	9.04	2.92-27.94	< 0.01
Anti-CCP2 and/or ≥ 2 RF*	28/49	2/29	57.1	93.1	18.00	3.84-84.26	< 0.01
Anti-CCP2 and/or ≥ 2 RF* and/or anti-CarP-FCS	29/49	2/29	59.2	93.1	19.57	4.18–91.76	< 0.01
> 5 yrs before RA diagnosis, RA cases sample n = 89, avg. sample/subject = 1.9; controls sample n = 57, avg. sample/subject = 2.1							
Anti-CCP2	15/48	1/27	31.3	96.3	11.82	1.46–95.37	0.02
≥ 1 RF*	20/48	3/27	41.7	88.9	5.71	1.51–21.60	0.01
≥ 2 RF*	8/48	0/27	16.7	100.0	NA	NA	NA
Anti-CarP-FCS	8/48	1/27	16.7	96.3	5.20	0.61–44.05	0.14
Anti-CarP-Fib	5/48	1/27	10.4	96.3	3.02	0.33–27.33	0.32
Anti-CCP2 and/or ≥ 1 RF*	23/48	4/27	47.9	85.2	5.29	1.59–17.62	< 0.01
Anti-CCP2 and/or ≥ 1 RF* and/or anti-CarP-FCS	24/48	5/27	50.0	81.5	4.40	1.43–13.54	< 0.01
Anti-CCP2 and/or ≥ 2 RF*	16/48	1/27	33.3	96.3	13.00	1.62–104.58	0.02
Anti-CCP2 and/or ≥ 2 RF* and/or anti-CarP-FCS	17/48	2/27	35.4	92.6	6.86	1.45–32.52	0.02

† Contains the following statistics: SN and SP. * Count of RF by Neph and RF isotypes (IgM, IgG, IgA); individual RF antibodies not presented to save space. RA: rheumatoid arthritis; RF: rheumatoid factor; Neph: nephelometry; IgM: immunoglobulin M; anti-CCP2: anticyclic citrullinated peptide antibodies version 2; anti-CarP: anti-carbamylated protein; FCS: fetal calf serum; Fib: fibrinogen; +/n: n positive over the case/control group size; SN: sensitivity; SP: specificity; NA: OR, 95% CI, and p cannot be calculated because no controls met positivity criteria.

supported by other studies that have found anti-CarP in 8–16% of ACPA-negative patients with RA[8,9]. Additionally, Shi, *et al* reported anti-CarP-FCS in 27% of patients with RA prior to diagnosis[12], which is similar to our study's sensitivity for future RA of 26%.

As stated, we used the same process for determining cutoff levels for anti-CCP2 and RF as was used for anti-CarP. This was done to allow for fairer comparisons of diagnostic accuracy across antibodies. Additionally, results based on the ≥ 2 SD cutoffs for RF and anti-CCP2 antibodies were qualitatively similar to results based on clinical test-based cutoffs. One issue regarding defining cutoffs could be our use of healthy controls because the reactivity of each autoantibody system could be lower, thereby resulting in a higher specificity than if other autoimmune disease groups were used. This issue of reactivity in healthy controls should be considered in future studies.

Several features of the antibodies tested herein are of interest in the pathophysiology of RA development. There was a nonsignificant trend for anti-CCP2 to appear prior to anti-CarP-FCS and anti-CarP-Fib, and in some cases, both anti-CCP2 and anti-CarP appeared prior to RF. Given the relatively close temporal relationship between initial positivity of the anti-CCP2 and anti-CarP, it is possible that the immune processes driving the break in tolerance of these

Table 5. Appearance of first antibody in seropositive RA cases where both antibodies of interest were present in prediagnosis serum. P value for Wilcoxon signed-rank test.

Comparing Antibodies	No. Cases, +/n	Mean Yrs	p
Comparing anti-CCP2 to anti-CarP-FCS		Mean yrs of anti-CCP2 preceding anti-CarP-FCS	
Anti-CCP2 preceded anti-CarP-FCS	4/16 cases		0.13
Anti-CarP-FCS preceded anti-CCP2	0/16 cases	0.76	
First appearance in same sample	12/16 cases		
Comparing anti-CCP2 to anti-CarP-Fib		Mean yrs of anti-CCP2 preceding anti-CarP-Fib	
Anti-CCP2 preceded anti-CarP-Fib	4/12 cases		0.13
Anti-CarP-Fib preceded anti-CCP2	0/12 cases	1.01	
First appearance in same sample	8/12 cases		
Comparing RF-Neph to anti-CarP-FCS		Mean yrs of anti-CarP-FCS preceding RF-Neph	
RF-Neph preceded anti-CarP-FCS	2/16 cases		0.81
Anti-CarP-FCS preceded RF-Neph	3/16 cases	0.08	
First appearance in same sample	11/16 cases		
Comparing RF-Neph to anti-CarP-Fib		Mean yrs of anti-CarP-Fib preceding RF-Neph	
RF-Neph preceded anti-CarP-Fib	2/7 cases		1.00
Anti-CarP-Fib preceded RF-Neph	1/7 cases	0.01	
First appearance in same sample	4/7 cases		

RA: rheumatoid arthritis; anti-CCP2: anticyclic citrullinated peptide antibodies version 2; anti-CarP: anti-carbamylated protein; FCS: fetal calf serum; Fib: fibrinogen; RF: rheumatoid factor; Neph: nephelometry; +/n: n positive over the case/control group size.

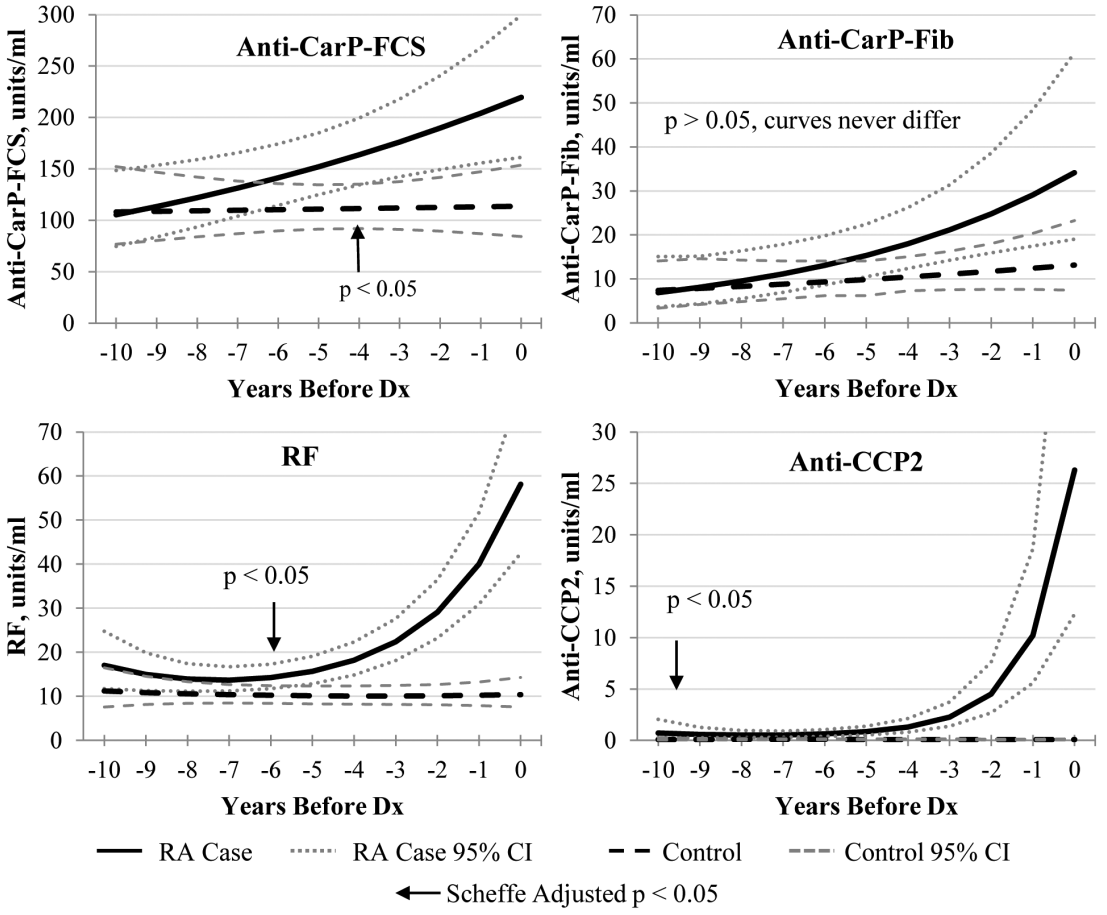

Figure 1. Trends in the mean levels of anti-CarP-FCS, anti-CarP-Fib, RF-Neph, and anti-CCP2 antibodies between RA cases and controls during the prediagnosis period. Arrows indicate where the mean levels between cases and controls are significantly different in the prediagnosis period. RF-Neph: rheumatoid factor-nephelometry; anti-CarP: anti-carbamylated protein; anti-CarP-FCS: anti-CarP fetal calf serum; anti-CarP-Fib: anti-CarP fibrinogen; anti-CCP2: anticyclic citrullinated peptide antibodies version 2; RA: rheumatoid arthritis; Dx: diagnosis.

structurally distinct autoantigens are similar in time and mechanism[15], or could suggest a degree of cross-reactivity between ACPA and anti-CarP in some of the patients at this early timepoint in the evolution of disease[16,17,18]. The higher sensitivity for disease of anti-CCP could represent a dominant autoimmune response to citrullinated antigens; alternatively, differences in the assay sensitivity between a commercially developed, optimized, and validated assay compared to a preclinical research-based method may underlie this difference.

In future studies, a larger number of RA cases would increase the ability to determine potential differences in biologic processes behind the RF, ACPA, and anti-CarP systems.

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