BRIEF REPORT

Anti–Carbamylated Protein Antibodies Are Present in Arthralgia Patients and Predict the Development of Rheumatoid Arthritis

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Objective. **Recently, we discovered a new auto-antibody system in rheumatoid arthritis (RA): anti–carbamylated protein (anti-CarP) antibodies. These antibodies have value in predicting joint destruction; however, it is not clear whether they are present before the diagnosis of RA and whether they have value as predictors of RA development. Therefore, we studied whether anti-CarP antibodies are present in patients with arthralgia and whether their presence is associated with the development of RA.**

Methods. **Sera from 340 arthralgia patients who did not have clinical signs of arthritis but who were positive for IgM rheumatoid factor (IgM-RF) and/or anti–cyclic citrullinated peptide 2 (anti–CCP-2) and 32 healthy controls were tested for anti-CarP IgG antibodies. Of the patients with arthralgia, 111 were IgM-RF positive/anti–CCP-2 antibody negative and 229 were anti–CCP-2 antibody positive. Patients were observed for the development of RA (based on the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria) during a median followup period of 36 months. Cox proportional hazards regression analysis was performed to compare the risk of developing RA between arthralgia patients who were positive for anti-CarP antibodies and those who were negative for anti-CarP antibodies during followup.**

Results. **Anti-CarP antibodies were present in the sera of 39% of the patients. One hundred twenty patients developed RA, after a median of 12 months (interquartile range [IQR] 6–24). The presence of anti-CarP antibodies was associated with the development of RA in the entire arthralgia cohort after correction for RF and anti–CCP-2 antibody status (hazard ratio 1.56 [95% confidence interval 1.06–2.29], $P = 0.023$), as well as in the anti–CCP-2 antibody–positive subgroup (odds ratio 2.231 [95% confidence interval 1.31–3.79], $P = 0.003$).**

Conclusion. **Anti-CarP antibodies are present in patients with arthralgia, and their presence predicts the development of RA independent of anti–CCP-2 antibodies.**

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Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder. The disease process often causes the destruction of joints, which can lead to considerable disability. Autoantibodies such as rheumatoid factor (RF) and anti–citrullinated protein antibodies (ACPAs) are important diagnostic markers for RA and may also contribute to pathogenesis (1). ACPA-positive patients with RA have more radiologic damage and a lower chance of attaining disease remission without disease-modifying antirheumatic drugs (DMARDs) than ACPA-negative patients with RA (2–5). In pre-RA states such as arthralgia and undifferentiated arthritis, ACPA and RF are predictive factors for future progression to RA (6,7).

Recently, we discovered another autoantibody system present in RA patients, which we designated as anti–carbamylated protein antibodies (anti-CarP) (8).

These antibodies target carbamylated proteins rather than citrullinated proteins. Carbamylation is a process in which lysines are converted into homocitrullines under the influence of cyanate. Homocitrulline is an amino acid that highly resembles citrulline. Cyanate can be formed in low concentrations from urea under physiologic conditions or it can originate from the environment, e.g., from car fumes. In inflammatory conditions, it can be formed from thiocyanate that has been catalyzed by myeloperoxidase released by, e.g., activated neutrophils. Whether anti-CarP antibodies are directly involved in the pathogenesis of RA is currently unknown.

In a previous article, we reported that anti-CarP antibodies are present in both ACPA-positive patients (74%) and ACPA-negative patients (16%) with RA (8). In RA patients, they are a prognostic factor for a higher likelihood of joint destruction independent of ACPA status. However, at present it is unknown whether they exist in patients who have arthralgia but do not meet criteria for RA and whether they could have predictive value in those patients. Therefore, we tested for the presence of anti-CarP antibodies and studied the association between anti-CarP antibody status and levels and the risk of developing RA in a cohort of patients with arthralgia who were positive for ACPA (determined based on levels of anti–cyclic citrullinated peptide 2 [anti–CCP-2] and/or RF).

PATIENTS AND METHODS

Study population. The inclusion procedure was as previously described (6). Briefly, 340 Caucasian patients from the Amsterdam area, who did not have arthritis but who were positive for anti–CCP-2 antibody and/or IgM-RF and had a history of arthralgia, were included. Absence of arthritis was confirmed by physical examination of 44 joints by a trained physician and a senior rheumatologist (DvS) (9). Medical history, details of joint symptoms, and the number of tender joints were recorded (10). Patients who had arthritis as determined by chart review or baseline physical examination, were negative for anti-CCP antibodies and IgM-RF on second analysis, had previously been treated with DMARDs, or had recently been treated with glucocorticoids (within the last 3 months) were excluded. Patients were followed up semiannually in the first year and yearly thereafter for the development of RA according to the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria (1). Additional visits were scheduled if RA developed. Healthy control sera were collected from 32 Caucasian residents of the Leiden area. Protocols were approved by the local ethics committee, and informed consent was obtained from all subjects.

Anti-CarP IgG antibody enzyme-linked immunosorbent assay (ELISA). Anti-CarP IgG antibodies in the sera from patients and controls were detected by ELISA as previously described (8). Briefly, Nunc MaxiSorp plates (Thermo Scientific) were coated with 10 μg/ml fetal calf serum (FCS; Bodinco) and carbamylated (Ca)–FCS at 4°C overnight. The plates were blocked with 1% bovine serum albumin (Sigma) at 4°C for 6 hours, followed by incubation overnight with 1:50 diluted sera on ice. Bound antibodies were detected by incubation for 4 hours with horseradish peroxidase–conjugated rabbit anti-human IgG (Dako) on ice and subsequently visualized with ABTS. Absorbance was measured at 415 nm and transformed to arbitrary units (AU) per milliliter using the titration curve of a serum pool from 3 anti-CarP antibody–positive samples. The background signal of FCS was subtracted from the signal of Ca-FCS to analyze the specific anti-CarP antibody reactivity. Sera with a level of >202 AU/ml were considered positive for anti-CarP antibodies. This cutoff was equivalent to 2 SD above the mean in the healthy controls.

Statistical analysis. Statistical analysis was performed using SPSS version 17.0 software. Chi-square test, t-test for independent samples, binary logistic regression, and Cox proportional hazards regression analysis were used to compare anti-CarP antibody–positive and anti-CarP antibody–negative groups in the whole population and in the anti–CCP-2 antibody–positive and –negative populations. Binary logistic regression analysis was performed to analyze the association between the anti-CarP IgG antibody level and the risk of developing RA in the anti-CarP IgG antibody–positive subgroup. Hazard ratios (HRs), odds ratios (ORs), and their 95% confidence intervals (95% CIs) were calculated. P values less than 0.05 were considered significant.

RESULTS

The 340 patients with arthralgia included in this study were followed up for a median of 36 months (interquartile range [IQR] 20–52). Baseline characteristics are listed in Table 1. After a median of 12 months (IQR 6–24), 120 patients (35%) developed RA according to the 2010 ACR/EULAR criteria. At the time of diagnosis of RA, these patients had a median swollen joint count of 3 (IQR 2–5). Of the remaining 220 patients, 9 patients developed undifferentiated arthritis.

One hundred thirty-three patients (39%) were

Table 1. Baseline characteristics of the 340 patients with arthralgia*

Age, mean ± SD years	49 ± 11
Sex, no. (%) female	256 (75)
Duration of symptoms, median (IQR) months	12 (8–36)
No. of reported painful joints, median (IQR)	3 (1–8)
Tender joint count, median (IQR)	0 (0–2)
IgM-RF positive, no (%)	209 (62)
Anti–CCP-2 positive, no (%)	229 (67)
Followup time, median (IQR) months	36 (20–52)

* IQR = interquartile range; IgM-RF = IgM rheumatoid factor; anti–CCP-2 = anti–cyclic citrullinated peptide 2.
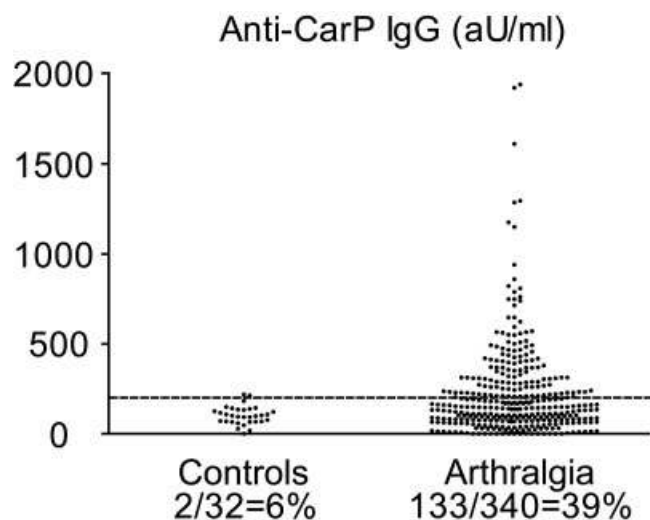
Figure 1. Presence of anti–carbamylated protein (anti-CarP) antibodies in patients with arthralgia and in controls. Anti-CarP IgG antibody positivity was found in 6% of healthy controls and 39% of patients with arthralgia. Each data point represents a single subject. The horizontal dashed line shows the cutoff for positivity.

positive for anti-CarP antibodies (Figure 1). Of these patients, 68 (51%) developed RA, whereas 52 patients (25%) who were negative for anti-CarP antibodies developed RA. Anti-CarP antibody positivity was significantly associated with RA development ($P < 0.001$). In the group of arthralgia patients positive for anti-CarP antibodies, the levels of anti-CarP IgG antibodies were not associated with the risk of developing RA ($P = 0.215$).

Among 111 anti–CCP-2 antibody–negative patients, 17 (15%) were positive for anti-CarP antibodies, while among 229 anti–CCP-2 antibody–positive patients, 116 (51%) were positive for anti-CarP antibodies ($P < 0.001$). Given this association, we next studied whether anti-CarP antibody positivity is also an independent predictor of RA development in the anti–CCP-2 antibody–positive and anti–CCP-2 antibody–negative subgroups. In the anti–CCP-2 antibody–positive subgroup, 68 patients (58%) who were positive for anti-CarP antibodies developed RA, while only 44 patients (40%) who were negative for anti-CarP antibodies developed RA. The association between anti-CarP antibodies and RA remained significant (OR 2.231 [95% CI 1.31–3.79], $P = 0.003$), while this was not the case in the anti–CCP-2 antibody–negative subgroup (OR 1.12 [95% CI 0.22–5.63], $P = 0.891$).

Anti-CarP antibody–positive patients also displayed higher anti-CCP antibody levels as compared to anti-CarP antibody–negative patients ($P < 0.001$). Similarly, after correction for anti-CCP antibody levels, anti-CarP antibody positivity still increased the risk of developing RA in anti-CCP antibody–positive arthralgia patients ($P = 0.032$). Unlike anti–CCP-2 antibodies, the presence of anti-CarP antibodies was not correlated with IgM-RF ($P = 0.391$).

Taking into account the differences in followup time, Cox proportional hazards regression analysis revealed a statistically significant association between anti-CarP antibody status and the risk of developing RA. This indicates that anti-CarP antibody–positive patients not only were more likely to develop RA, but also were more likely to develop RA within a shorter time frame, with an HR of 2.53 (95% CI 1.76–3.63, $P < 0.001$). This association remained significant after correction for anti–CCP-2 antibody status and IgM-RF status (HR 1.56 [95% CI 1.06–2.29], $P = 0.023$) (Figure 2) or after
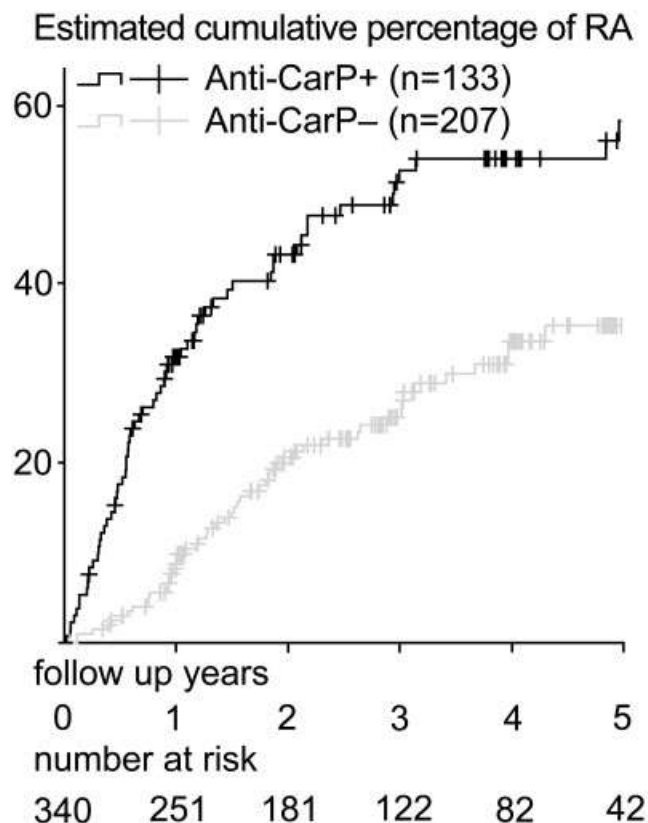
Figure 2. Association of presence of anti–carbamylated protein (anti-CarP) antibodies in arthralgia patients with future development of rheumatoid arthritis (RA). Anti-CarP IgG antibodies are associated with a higher risk of developing RA after correction for anti–cyclic citrullinated peptide 2 antibody and rheumatoid factor status (hazard ratio 1.56 [95% confidence interval 1.06–2.29], $P = 0.023$).

correction for anti–CCP-2 antibody levels and IgM-RF status ($P < 0.001$).

DISCUSSION

Although arthralgia patients often have a benign disease course, in a certain subset of these patients the condition may progress to RA. Identifying this subset at an early stage may be beneficial because intervention at this stage might prevent the development of RA. As an established biomarker, the presence of ACPA increases the risk of developing arthritis in patients with arthralgia. Still, the condition progresses to arthritis in only 27% of all ACPA-positive patients with arthralgia after 1 year of followup (6). In the present study we investigated whether anti-CarP antibodies are present in arthralgia patients and whether they are an additional risk factor for RA in these patients. We demonstrated that anti-CarP antibodies are present in arthralgia patients and that they are associated with a higher risk of developing RA independent of ACPA and IgM-RF status. Within the anti–CCP-2 antibody–negative subgroup, we did not observe a significant association between anti-CarP antibodies and RA, possibly due to the low number of RA cases in this group.

Limited by the nature of the cohort, we were unable to address the question of whether anti-CarP antibodies can predict the development of RA in arthralgia patients who are negative for anti–CCP-2 antibodies and RF. Another limitation was the 3-year median followup time, which is relatively short and may have affected the percentage of patients developing RA. However, we observed that with increasing followup time the percentage of patients with arthralgia who develop RA decreases. Therefore, we believe that this effect will be limited. A further concern could be that these patients might have subclinical arthritis at baseline, undetected by physical examination. However, we have previously seen that the frequency of pathology as determined on ultrasound was very low in this population and moreover, that ultrasound was not superior to physical examination in the prediction of RA (11).

Our findings suggest that not only ACPA positivity, but also the presence of anti-CarP antibodies, can have clinical value in the prediction of RA in patients with arthralgia. Additionally, the presence of anti-CarP antibodies in persons at risk of developing RA provides a rationale for further studies on their potential pathogenetic properties. Although the presence of anti-CarP antibodies is associated with the risk of developing RA in ACPA-positive arthralgia patients, we previously did not obtain evidence that their presence is associated with radiologic progression in ACPA-positive patients with RA; such an association was only found in ACPA-negative RA patients (8). The reasons for these findings are not yet known and further replication is required; however, these results do resemble observations made in studies on ACPA fine specificity (12–14). The ACPA recognition profile does not correlate with radiologic progression in ACPA-positive RA (12), but the number of citrullinated epitopes recognized by ACPA is associated with RA development in patients who have arthralgia or undifferentiated arthritis (13,14). Apparently, in the first stage of disease, the number of epitopes recognized and isotypes used by ACPA (5), as well as the number of autoantibodies present, are determining factors for disease progression. They matter less, however, when a certain threshold has been passed, possibly explaining the lack of association in established RA.

Despite the similarity between the presence of anti-CarP antibodies and the broadening of ACPA fine specificities with respect to prediction of RA, anti-CarP antibodies are not a fine specificity of ACPA, since they are largely non–cross-reactive with defined (homo)citrullinated antigens (15). Indeed, the effect of anti-CarP antibodies in arthralgia patients as described herein is still present after correction for the effect of anti–CCP-2 antibodies, as would be expected for two independent autoantibody systems.

Taken together, our data reveal that anti-CarP antibodies are present before RA becomes clinically apparent, since they can be found in patients who have arthralgia without signs of arthritis. Furthermore, their presence in this population is associated with the development of RA.

ADDITIONAL DISCLOSURE

Author van de Stadt is an employee of Sanquin Research.

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