Anti Carbamylated Protein Antibodies (Anti-CarP) are Present in Arthralgia Patients and Predict the Development of Rheumatoid Arthritis

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ABSTRACT

Objective
Recently, we discovered a new autoantibody system in rheumatoid arthritis (RA): anti-carbamylated protein antibodies (anti–CarP). These antibodies have prognostic value in predicting joint destruction. However, it is not clear whether they are present before the diagnosis of RA and whether they have predictive value for the development of RA. Therefore we studied whether anti–CarP antibodies are present in Arthralgia patients and whether their presence associates with the development of RA.

Methods
Sera of 340 arthralgia patients without clinical signs of Arthritis and 32 healthy controls were measured for anti–CarP IgG antibodies. One hundred eleven arthralgia patients were IgM–rheumatoid factor (IgM–RF) positive/anti–cyclic citrullinated peptide 2 (aCCP2) antibody negative and 229 were aCCP2 antibody positive. Patients were followed for the development of RA (2010 criteria) during a median follow up time of 36 months. Cox regression analysis was performed to compare the risk of developing RA between Anti–CarP antibody positive and negative Arthralgia patients during follow up.

Results
Anti–CarP antibodies were present in sera of 39% patients. One hundred twenty patients developed RA after a median (IQR) of 12 (6–24) months. The presence of anti–CarP antibodies was associated with the development of RA in the whole Arthralgia cohort after correction for RF and aCCP2 antibody status (HR: 1.56; 95%CI: 1.06–2.29; p = 0.023), as well as in the aCCP2 antibody positive subgroup (OR: 2.231; 95%CI: 1.31–3.79; p = 0.003).

Conclusions
Anti–CarP antibodies were present in arthralgia patients and their presence predicted the development of RA independent of aCCP2 antibodies.
INTRODUCTION

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 (ACPA) are important diagnostic markers for RA and may also contribute to pathogenesis.\(^1\) ACPA positive RA patients have more radiological damage and a lower chance to reach disease modifying anti-rheumatic drug (DMARD) free remission than ACPA negative RA patients.\(^2-5\) In pre-RA states such as arthralgia and undifferentiated arthritis, ACPA and RF are predictive factors for future progression towards RA.\(^6,7\)

Recently, we discovered another autoantibody system present in RA patients, which we designated anti-carbamylated protein antibodies (anti-CarP).\(^8\) These antibodies target carbamylated proteins instead of citrullinated proteins. Carbamylation is a process in which lysines are converted into homocitrullines under the influence of cyanate. Homocitrulline is an amino acid which highly resembles citrulline. Cyanate can be formed in low concentrations from urea under physiological conditions or it can originate from the environment, for instance from car fumes. In inflammatory conditions it can be formed from thiocyanate catalyzed by myeloperoxidase released by for instance activated neutrophils. Whether or not anti-CarP antibodies are directly involved in the pathogenesis of RA is currently unknown.

In our previous paper, we reported that anti-CarP antibodies are present in both ACPA positive (74%) and negative (16%) RA patients.\(^8\) In RA patients, they are a prognostic factor for a higher rate of joint destruction independent of ACPA. However, at present it is unknown whether they exist in Arthralgia patients and could have predictive value in them. Therefore, we measured 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 anti-cyclic citrulline peptide 2 (aCCP2) antibodies and/or RF positive arthralgia patients.
MATERIALS AND METHODS

Study population
The inclusion procedure was as described before. In short, 340 Caucasian patients from the Amsterdam area, without arthritis but with a positive aCCP2 antibody and/or IgM–RF status and (a history of) arthralgia were included. Absence of arthritis was confirmed by physical examination of 44 joints by a trained medical doctor and a senior rheumatologist. Medical history, details of joint complaints and the number of tender joints were recorded. Patients with arthritis as revealed by chart review or baseline physical examination, negative aCCP antibody and IgM–RF status on second analysis, previous treatment with DMARD or recent glucocorticoid treatment (< 3 months) were excluded. Patients were followed semi-annually in the first year and yearly thereafter for the development of RA according to the 2010 ACR/EULAR criteria. In addition, extra visits were planned if (rheumatoid) arthritis developed. Healthy control sera were collected from Caucasian inhabitants of the Leiden area. The protocols were approved by the local ethics committee and informed consent was obtained.

Anti–CarP IgG antibody ELISA
Anti–CarP IgG antibodies in patients’ and controls’ sera were detected by ELISA as described before. Briefly, Nunc Maxisorp plates (Thermo Scientific) were coated with 10 mg/mL fetal calf serum (FCS) (Bodinco) and carbamylated (Ca)–FCS at 4º overnight. The plates were blocked with 1% bovine serum albumin (BSA) (Sigma) at 4º for 6 hours, followed by incubation with 1/50 diluted sera on ice overnight. Bound antibodies were detected with rabbit anti–human IgG HRP (Dako) on ice for 4 hours and subsequently visualized with 2,2’-azino–bis(3-ethylbenzothiazoline–6-sulfonate) (ABTS). Absorbance was measured at 415nm and transformed to arbitrary units per milliliter (aU/mL) using a 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 as to analyze the specific anti–CarP antibody reactivity. Sera with a level higher than 202 aU/mL were considered positive for anti–CarP antibodies. This cut–off was established by using the mean plus two times the standard deviation (SD) of the healthy controls.
Anti-CarP antibodies are associated with RA

Statistics
Statistical analysis was performed with SPSS version 17.0 software (SPSS Inc., Chicago, USA). Chi-square test, independent-samples t test, binary logistic regression and cox regression proportional hazard analysis were used to compare anti-CarP antibody positive and negative groups in the whole population and the aCCP2 antibody positive/negative population. Binary logistic regression analysis was performed to analyze the association between the Anti-CarP IgG antibody level and the risk of developing RA in Anti-CarP IgG antibody positive subgroup. P-values below 0.05 were considered statistically significant.

RESULTS

Tabel 1 Baseline characteristics of 340 Arthralgia patients from the Amsterdam Reade Arthralgia*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years, mean (±SD)</td>
<td>47 ± 11</td>
</tr>
<tr>
<td>Females, nr (%)</td>
<td>256 (75)</td>
</tr>
<tr>
<td>Duration of symptoms at baseline in months, median (IQR)</td>
<td>12 (8–36)</td>
</tr>
<tr>
<td>Number of reported painful joints, median (IQR)</td>
<td>3 (1–8)</td>
</tr>
<tr>
<td>Tender joint count, median (IQR)</td>
<td>0 (0–2)</td>
</tr>
<tr>
<td>IgM-RF positive at the baseline, nr (%)</td>
<td>209 (62)</td>
</tr>
<tr>
<td>aCCP2 positive at the baseline, nr (%)</td>
<td>229 (67)</td>
</tr>
<tr>
<td>Follow-up time in months, median (IQR)</td>
<td>36 (20–52)</td>
</tr>
</tbody>
</table>

* IQR = interquartile range; IgM-RF = IgM rheumatoid factor; aCCP2 = anti-cyclic citrullinated peptide 2.

Three hundred forty arthralgia patients were included and followed for a median (IQR) of 36 (20–52) months. Baseline characteristics are listed in table 1. One hundred twenty (35%) patients developed RA according to the 2010 ACR/EULAR criteria, after a median (IQR) of 12 (6–24) months. At the moment of RA diagnosis these patients had a median (IQR) swollen joint count of 3 (2–5). Of the remaining 220 patients, nine patients developed undifferentiated arthritis.
Figure 1.
The presence of anti-CarP antibodies in arthralgia patients and controls. 6.3% of healthy controls and 39.1% of arthralgia patients are anti-CarP IgG antibody positive.

One hundred thirty three patients (39%) were Anti-CarP antibody positive (Figure 1). Of these patients, 68 (51%) developed RA, whereas 52 (25%) of the anti-CarP antibody negative patients developed RA. Anti-CarP antibody status was significantly associated with RA development (p < 0.001). In the group of anti-CarP antibody positive arthralgia patients, the levels of Anti-CarP IgG antibodies were not associated with the risk of developing RA (p = 0.215).

Among 111 aCCP2 antibody negative patients, 17 patients (15%) were anti-CarP antibody positive, while among 229 aCCP2 antibody positive patients, 116 patients (51%) were positive (p < 0.001). Because of this association, we next wished to study whether anti-CarP antibody positivity is also an independent predictor for RA development in the aCCP2 antibody positive and negative subgroups. In the aCCP2 antibody positive subgroup, 68 (58%) anti-CarP antibody positive patients developed RA while only 44 (40%) anti-CarP antibody negative patients 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 aCCP2 antibody negative subgroup (OR: 1.12; 95% CI: 0.22-5.63; p = 0.891). Anti-CarP antibody positive patients also display higher anti-CCP antibody levels as compared to anti-CarP antibody negative patients (p < 0.001). Similarly, after correction for anti-CCP antibody level, anti-CarP antibody positivity still increased the risk for developing RA in anti-CCP antibody positive Arthralgia patients (p = 0.032). Unlike aCCP2 antibodies, the presence of anti-CarP antibodies was not correlated with IgM-RF (p = 0.391).

Taking into account the differences in follow-up time, Cox regression analysis revealed a statistically significant association between anti-CarP antibody status and the risk of RA development. This indicates that anti-CarP antibody positive
patients not only developed RA more often, but also within a shorter time frame, with a hazard ratio (HR) of 2.53 (95%CI: 1.76–3.63; p < 0.001). This association remained significant after correction for aCCP2 antibodies and IgM–RF status (HR: 1.56; 95%CI: 1.06–2.29; p = 0.023) (Figure 2) or the levels of aCCP2 antibodies and IgM–RF status (p < 0.001).

Figure 2.
The presence of anti–CarP antibodies in arthralgia patients is associated with future development of RA. Anti–CarP IgG antibodies are associated with a higher risk of developing RA after correcting for aCCP2 antibody and RF status (HR: 1.56; 95%CI: 1.06–2.29; p = 0.023)
DISCUSSION

Although arthralgia patients often have a benign disease course, a certain subset of these patients may progress to RA. Identifying this subset at an early stage is attractive, because intervention at this stage might prevent the development of RA. As an established biomarker, the presence of ACPA increases the risk of converting to arthritis in arthralgia patients, but still only 27% of all ACPA positive arthralgia patients progress to arthritis after 1 year of follow-up. We now studied whether anti-CarP antibodies are present in arthralgia patients and whether they are an additional risk factor for RA in these patients. We found that anti-CarP antibodies were present in arthralgia patients and that they were associated with a higher risk of developing RA independent of ACPA and IgM–RF status. Within the aCCP2 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 whether anti-CarP antibodies can predict RA in aCCP2 antibody and RF double negative arthralgia patients. Another limitation is the 3 years median follow-up time, which is relatively short and may impact on the percentage of patients developing RA. However, we observed that with increasing follow-up time the percentage of arthralgia converting to RA decreases. Therefore we feel 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 ultrasound pathology was very low in this population and moreover, that ultrasound was not superior to physical examination in the prediction of RA.

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 arthralgia patients. Additionally, the presence of anti-CarP antibodies in persons at risk for RA provides a rationale for further studies on their potential pathogenic properties. Although the presence of anti-CarP antibodies associates with the risk to develop RA in ACPA-positive arthralgia patients, we previously did not obtain evidence that their presence associates with radiological progression in ACPA positive RA patients, which was only found in ACPA negative RA patients. The reasons for these findings are not yet known and require further replications, but they do resemble the observations with respect to ACPA fine specificity. The ACPA recognition profile does not correlate with radiological progression in ACPA positive RA, but the
Anti-CarP antibodies are associated with RA development in patients suffering from arthralgia or undifferentiated arthritis.\textsuperscript{13,14} Apparently, in the first stage of disease, the number of epitopes recognized and isotypes used by ACPA\textsuperscript{5} as well as the number of auto-antibodies present are determining factors for disease progression, but matter less 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 as they are largely non-cross-reactive towards defined (homo)citrullinated antigens.\textsuperscript{15} Indeed the effect of anti–CarP antibodies shown in arthralgia patients as described above is present after correcting the effect of anti–CCP2 antibodies as could be expected for two independent auto-antibody systems.

Together, our data reveal that anti–CarP antibodies are present before RA becomes clinically apparent as they can be found in patients suffering from arthralgia without signs of arthritis. Furthermore, their presence in this population is associated with the development of RA.

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Chapter 6

REFERENCES


