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Antibodies against carbamylated proteins: prevalence and associated disease characteristics in Belgian patients with rheumatoid arthritis or other rheumatic diseases

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Objectives: Anti-carbamylated protein antibodies (anti-CarP) are reported to be associated with increased disease activity and with more severe joint damage in rheumatoid arthritis (RA) patients. The present study investigated the presence of anti-CarP in various rheumatic diseases, and their specific clinical significance in RA, in Belgian rheumatology patients.

Method: We tested sera from 254 RA patients, 56 healthy controls, and 153 patients with different rheumatic conditions: juvenile idiopathic arthritis (JIA), axial spondyloarthritis, systemic sclerosis, and Sjögren's syndrome (SS). An in-house enzyme-linked immunosorbent assay was used to detect immunoglobulin G antibodies against carbamylated foetal calf serum.

Results: Anti-CarP were detected in 88 RA patients (34.6%), of whom 82% were also positive for anti-citrullinated protein antibodies (ACPAs) and 81% were also rheumatoid factor (RF) positive. Of note, 11 anti-CarP single-positive patients were detected (4.3%). The previously reported association with joint erosions was not detected. However, in ACPA- and RF-negative RA patients, the presence of anti-CarP was associated with higher disease activity and disability. Fifteen per cent of JIA patients and 30% of SS patients also tested positive for anti-CarP and their antibody levels did not differ significantly from those of anti-CarP-positive RA patients. Anti-CarP levels were, however, significantly higher in ACPA- or RF-positive patients.

Conclusion: Anti-CarP antibodies were detected in the sera of a cohort of Belgian RA patients. Moreover, they were also detected in primary SS patients and in JIA patients. In the seronegative subset of RA patients, anti-CarP antibodies showed prognostic value.

Antibodies against carbamylated proteins (anti-CarP) were first described in 2011 in the serum of rheumatoid arthritis (RA) patients and were associated with erosive radiographic progression of the disease (1). Since their original description, anti-CarP have been reported to be associated with a more severe disease phenotype and with a greater disease impact on daily life activities. The antibodies appear early in the disease history and are predictive of evolution towards arthritis in arthralgia patients (2–5).

Replication studies have so far been few, and the overlap of anti-CarP positivity with anti-citrullinated peptide antibodies (ACPAs) and rheumatoid factor (RF) has led to debate concerning their value as a novel diagnostic marker in RA. In addition, their specificity has been scrutinized because carbamylation and anti-CarP antibodies seem to occur in many inflammatory conditions (6–8).

The objective of our study was to investigate the presence and the clinical significance of anti-CarP antibodies in a cohort of Belgian patients with early and established RA, and in patients with other rheumatic conditions.

Method

Study design

In this retrospective study, we included RA patients followed in the Rheumatology Department of Erasme Hospital (Brussels, Belgium) (n = 66), and early RA patients from the CAP48 Belgian cohort (n = 188), all fulfilling the

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American College of Rheumatology/European League Against Rheumatism 2010 RA classification criteria (9). Disease controls were chosen from patients with juvenile inflammatory arthritis (JIA) (n = 80), primary Sjögren's syndrome (SS) (n = 37), axial spondyloarthritis (axSpA) (n = 25), and systemic sclerosis (SSc) (n = 11) from our rheumatology department. Sera of self-reported healthy controls (HCs) with no previous personal or family history of rheumatic disease were provided by the Department of Rheumatology biobank (n = 56).

This study was approved by the local ethics committee of Erasme Hospital, Université Libre de Bruxelles, Brussels.

Clinical assessment

Baseline and follow-up clinical and biological data (described in the Supplementary index) were obtained for all RA patients from the CAP48 and Rheumatology biobank registry. The presence of bone erosions was assessed in a dichotomic fashion on plain radiographs of the hands.

Anti-CarP ELISA

Replicating the methods of Shi et al (1), we created an in-house enzyme-linked immunosorbent assay (ELISA) using carbamylated foetal calf serum (FCS), as described in detail in the Supplementary index.

Statistical analysis

For statistical analysis, GraphPad Prism version 7.0 was used. Testing for normality was carried out using the Kolmogorov–Smirnov test, while homogeneity of variance was assessed using Bartlett's chi-squared test. Non-parametric variables were assessed using the Mann–Whitney U-test and Kruskal–Wallis test. The Student's *t*-test was used for normally distributed variables and analysis of variance (ANOVA) was used for comparisons between more than two groups.

Results

Anti-CarP antibody status in a Belgian cohort of RA patients

The demographic data at baseline for the RA patients are summarized in Supplementary table S1.

The anti-CarP reactivity was significantly higher for both early RA and established RA patients, compared to HCs. Anti-CarP positivity and anti-CarP levels did not differ between the established RA patients and the early RA patients (Figure 1A).

In the pooled group of RA patients, anti-CarP positivity was observed in 88 patients (34.6%), compared to 69.6% positivity for ACPAs and 66.9% for RF. Anti-CarP antibodies were found in 11 patients who were seronegative for both RF and ACPA (Figure 1E).

We then compared the pooled RA patients' anti-CarP levels to the anti-CarP levels of 153 rheumatic disease control sera: excluding scleroderma, all of the disease groups' sera exhibited a higher reactivity towards CarP compared to HCs. However, the anti-CarP levels were significantly lower compared to the corresponding values of RA sera, with the notable exception of SS: a total of 11 SS patients (30%) tested positive for anti-CarP, and their anti-CarP levels were comparable to those of RA patients (Figure 1B).

Four HCs (7%) were positive for anti-CarP, along with 12 JIA patients (15%) and five axSpA patients (20%). By restricting the analysis to these anti-CarP-positive patients, the antibody levels were not different between RA patients and disease control patients (Figure 1D).

Anti-CarP antibodies and presence of other autoantibodies

ACPAs were more prevalent in the anti-CarP-positive RA subgroup than in anti-CarP-negative RA ($p < 0.0046$). RF positivity was also associated with anti-CarP positivity ($p < 0.0011$). RF-positive and ACPA-positive patients presented significantly higher anti-CarP levels than seronegative patients. Our results showed that RF positivity was associated with higher anti-CarP levels in SS patients (Supplementary table S2). In contrast, the presence of anti-nuclear antibodies in the serum was not associated with anti-CarP positivity or with higher anti-CarP levels.

Anti-CarP and clinical characteristics

We subdivided the early RA patients into anti-CarP-positive and anti-CarP-negative subgroups, and performed statistical analysis to examine possible associations of the anti-CarP antibody status with clinical parameters, baseline characteristics, and outcome (Table 1).

No association with the presence of erosions, inflammatory markers, or other baseline characteristics was established.

In addition, longitudinal follow-up data at 12 months were assessed: disease response to disease-modifying anti-rheumatic drugs (DMARDs), as observed by the modification of the activity scores over time, did not differ between the groups. Disability, as measured by the Health Assessment Questionnaire (HAQ) score, was also not significantly different between the two groups at 12 months of follow-up (Table 1).

In the ACPA- and RF-negative early RA population, anti-CarP-positive patients exhibited higher Disease Activity Score in 28 joints (DAS28) and higher HAQ at baseline, as well as higher disease activity indices, DAS28, and HAQ, at 12 months of follow-up (Table 2).

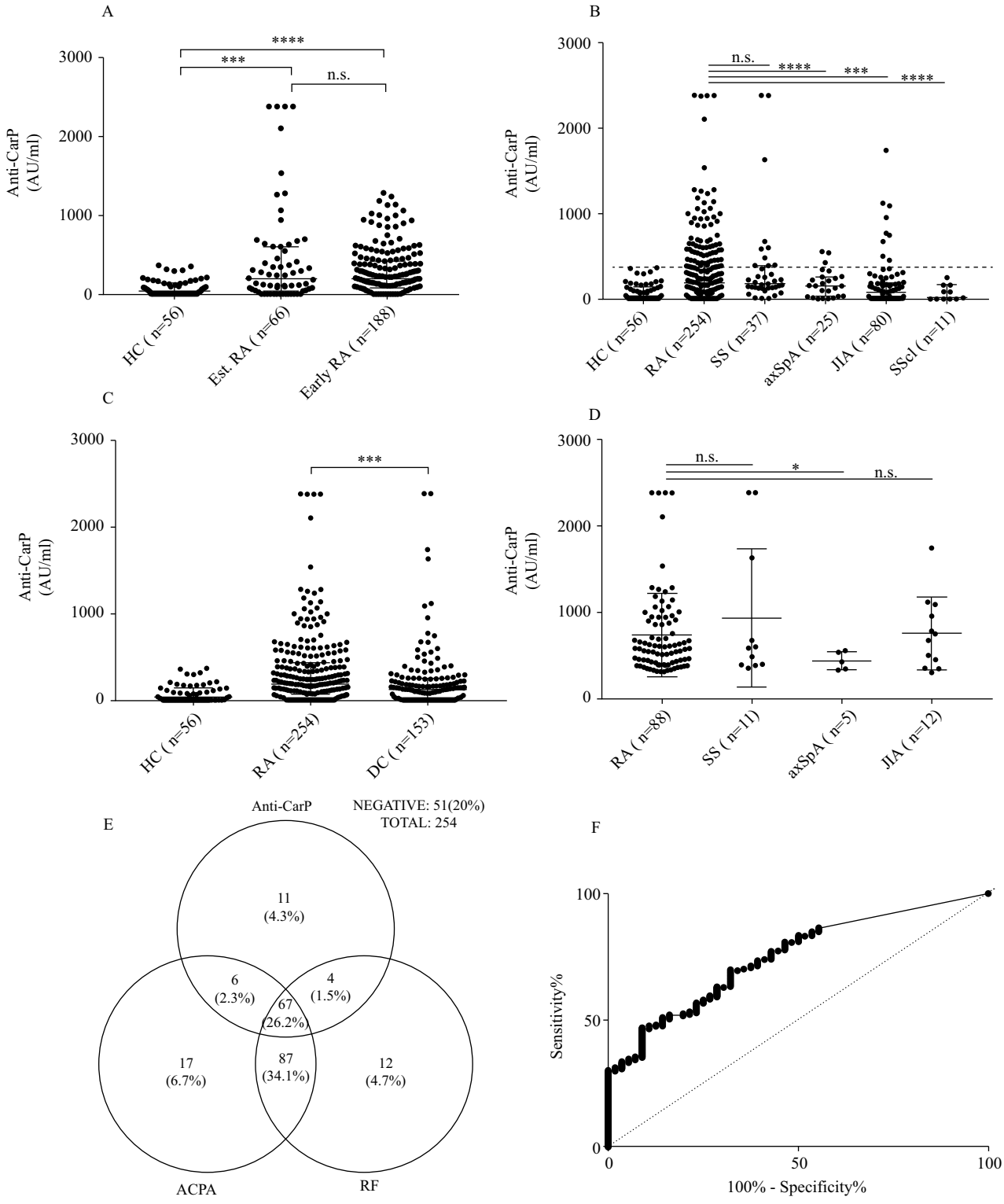

Figure 1. (A) Anti-carbamylated protein (anti-CarP) antibody levels in sera of established rheumatoid arthritis (Est. RA) patients, early RA patients, and healthy controls (HC). (B) Distribution of anti-CarP antibodies in sera of patients with RA (n = 254), Sjögren's syndrome (SS, n = 37), axial spondyloarthritis (axSpA, n = 25), juvenile arthritis (JIA, n = 80), and systemic sclerosis (SScl, n = 11). The horizontal line signifies the cut-off value (300 AU/mL). (C) Anti-CarP antibody level comparison between the tested RA population and the pooled disease controls (DC). (D) Anti-CarP antibody level comparison between anti-CarP-positive patients with various rheumatic conditions. (E) Venn diagram showing the distribution of positivity of RA sera towards rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPAs), and anti-CarP antibodies. (F) Receiver operator characteristics curve for anti-CarP antibodies in RA patients; area under the curve = 0.686. *p < 0.05, ***p < 0.005, ****p < 0.0005; n.s., not significant.

Table 1. Comparison of baseline and follow-up clinical characteristics between anti-carbamylated protein antibody (anti-CarP)-positive and anti-CarP-negative early rheumatoid arthritis (RA) patients.

	Anti-CarP$^+$ (N = 62)	Anti-CarP$^-$ (N = 126)	p
Age (years), mean ± sd	39.1 ± 9.6	36.5 ± 8.9	0.14
Gender: female	50/62 (80.7)	101/126 (80.2)	0.99
ACPA$^+$	51/62 (82.3)	78/126 (61.9)	0.0046*
RF$^+$	49/58 (84.5)†	73/126 (59.8)	0.0011*
ANA$^+$ (> 1:80)	25/55 (45.5)‡	38/107 (35.5)§	0.23
CRP (mg/dL), median ± sd	1.23 ± 1.78	1.83 ± 3.4	0.10
Erosive disease, baseline	14/57 (24.6)‖	32/117 (27.4)¶	0.85
Smoker (ever)	15/54 (27.8)††	26/110 (23.6)‡‡	0.57
RA family history	11/50 (22)§§	22/105 (17.3)‖‖‖	0.52
Corticosteroid therapy	13/62 (21)	22/123 (17.9)¶¶	0.69
DAS28, baseline	4.38 (3.44, 5.32)	4.44 (3.44, 5.47)	0.54
SDAI, baseline	20.6 (12.4, 31.2)	22.4 (13.3, 33.1)	0.63
CDAI, baseline	23.4 (12.63, 12.2)	22.1 (12.2, 30.3)	0.85
HAQ, baseline	1.15 (0.63, 1.75)	1.06 (0.5, 1.66)	0.49
DAS28, 12 months	2.36 (1.69, 3.58)	2.66 (1.70, 3.39)	0.70
SDAI, 12 months	5.95 (1.4, 13.1)	7.1 (2.3, 13.3)	0.43
CDAI, 12 months	5.8 (0.9, 13)	6.5 (2.1, 13)	0.42
HAQ, 12 months	0.375 (0.03, 1.13)	0.375 (0, 1)	0.53
ΔDAS28	−1.80 (−2.78, −0.59)	−1.68 (−3.08, −0.62)	0.86
ΔSDAI	−15 (−21.1, −3.3)	−12 (−24.8, −3)	0.80
ΔCDAI	−14.75 (−20.1, −3.4)	−11.3 (−22.5, −2.8)	0.61
ΔHAQ	−0.5 (−1.26, 0)	−0.375 (−1, 0)	0.54
Erosive disease, 12 months	17/50 (34)§§	38/103 (36.9)	0.85

Data are shown as n/N (%) or median (interquartile range), unless otherwise indicated.
†Missing four values; ‡missing seven values; §missing 19 values; ‖missing five values; ¶missing nine values; ††missing eight values; ‡‡missing 16 values; §§missing 12 values; ‖‖‖missing 21 values; ¶¶missing three values.
ACPA, anti-citrullinated peptide antibody; RF, rheumatoid factor; ANA, anti-nuclear antibody; CRP, C-reactive protein; DAS28, Disease Activity Score in 28 joints; SDAI, Simplified Disease Activity Index; CDAI, Clinical Disease Activity Index; HAQ, Health Assessment Questionnaire; IQR, interquartile range.
*Significant difference ($p < 0.05$).

Diagnostic value of anti-CarP ELISA

At the positivity cut-off chosen (mean absorbance of HC + 2 sd), the specificity of anti-CarP antibodies for RA was 92.8% and the sensitivity was 35.0%. Overall, the positive predictive value of the antibodies was estimated at 88.4% and the negative predictive value was at 44.0%. The positive likelihood ratio was 4.86 and the negative likelihood ratio 0.71. The area under the curve of the receiver

Table 2. Anti-carbamylated protein antibodies (anti-CarP) in anti-citrullinated protein antibody/rheumatoid factor-negative early rheumatoid arthritis patients.

	Anti-CarP$^+$ (n = 7)	Anti-CarP$^-$ (n = 40)	p
DAS28, baseline	5.2 (4.01, 6.29)	4.2 (3.1, 5.35)	0.043*
SDAI, baseline	35.8 (16.1, 57.6)	26.5 (15.2, 33.45)	0.15
CDAI, baseline	34.7 (16, 55.6)	23 (14, 29.13)	0.10
HAQ, baseline	1.75 (0.87, 2.5)	1 (0.44, 1.75)	0.038*
DAS28, 12 months	4.42 (1.82, 5.72)	2.35 (1.54, 3.44)	0.078
SDAI, 12 months	21.9 (5.89, 39.7)	6.16 (1.1, 13.75)	0.028*
CDAI, 12 months	21.8 (5.8, 39.3)	4.8 (0.75, 13)	0.027*
HAQ, 12 months	1.5 (0.625, 1.75)	0.18 (0, 1)	0.01*
ΔDAS28	−0.81 (−2.6, 0.02)	−1.94 (−3.2, −0.69)	0.15
ΔSDAI	−17.5 (−20.5, 10.3)	−11.9 (−25.2, −5.32)	0.58
ΔCDAI	−17.5 (−20.4, 10.8)	−11.5 (−24, −5.3)	0.70
ΔHAQ	−0.625 (−0.75, 0)	−0.375 (−1.09, 0)	0.85

Data are shown as median (interquartile range).
DAS28, Disease Activity Score in 28 joints; SDAI, Simplified Disease Activity Index; CDAI, Clinical Disease Activity Index; HAQ, Health Assessment Questionnaire.
*Significant difference ($p < 0.05$).

operator characteristics curve was calculated at 0.686 (Figure 1F).

Discussion

We have studied the prevalence of anti-CarP antibodies in a Belgian RA cohort and in other rheumatic diseases. We independently replicated the anti-CarP ELISA described by Shi et al (1), thereby detecting anti-CarP antibodies in the Erasme-Brussels RA cohort, a different population from those previously studied.

In the present study, anti-CarP antibodies were shown to be specific for RA compared to the general population. However, their sensitivity remains low and the significant overlap with ACPA and RF also limits their diagnostic use: only a small proportion of RA patients (4%) considered seronegative for ACPA and RF are positive for anti-CarP. This result is consistent with previous studies (10).

Contrary to what was previously shown, no significant correlations could be established with erosive disease in our study (1, 11). The high proportion of early RA patients with only 1 year of follow-up in our study may explain our results, since previous studies required several years of follow-up to demonstrate the increased risk for erosion in early RA (1, 12).

In our study, antibodies against carbamylated FCS did not demonstrate an association with clinical parameters in ACPA-positive patients. However, in the ACPA/RF-negative subgroup of patients, anti-CarP-identified patients showed higher disease activity and worse disease outcome after 12 months of treatment. This observation highlights the potential prognostic use of anti-CarP antibodies in ACPA/RF-negative patients.

Our study confirms that a subgroup of patients with SS presents anti-CarP in their serum, in line with previous studies (6, 13). While anti-CarP positivity was more frequent in RA, anti-CarP levels did not differ significantly between positive patients with RA or SS. This, along with the association of anti-CarP with RF in both conditions, may point to immune system dysregulation as the reason for the immunogenic potential of carbamylated proteins.

Our study has several limitations. As described in the literature, we used carbamylated FCS to detect anti-CarP antibodies; however, the heterogeneity of composition of FCS from one batch to another, and the structural difference between human and bovine antigens, are potential sources of bias when it comes to autoantibody detection. In addition, missing values in clinical data (see footnotes to Table 1), and the fact that a subset of patients had received treatment before inclusion, may have influenced our ability to further demonstrate the clinical value of anti-CarP antibodies.

While the diagnostic value of anti-CarP antibodies is relatively low, our study shows their prognostic role in seronegative RA. In the future, longer studies should be performed to assess the role of anti-CarP as a potential biomarker of treatment response or relapse after DMARD tapering.

Conclusion

We report the presence of anti-CarP antibodies in a Belgian cohort of patients with RA and other rheumatic conditions, such as SS and JIA. Based on our study, anti-CarP antibodies can help to identify seronegative RA patients at risk of more severe disease.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Supplementary index. CAP48 RA inclusion criteria, Disease controls inclusion criteria, Clinical assessment, ELISAs for antibody detection, RF, and ACPA assays.
Supplementary table S1. Baseline characteristics of the RA study population.
Supplementary table S2. Anti-CarP in Sjögren's syndrome patients.

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