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

Running header: Anti-CarP in Belgian RA patients

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ABSTRACT:

Objectives: Anticarbamylated 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.

Methods: We tested sera from 254 RA patients, 56 healthy controls as well as 153 patients with different rheumatic conditions : Juvenile Idiopathic Arthritis (JIA), Axial Spondylarthritis (axSpA), Systemic Sclerosis (SSc) and Sjögren's Syndrome (SS). An in-house ELISA technique was used to detect IgG class antibodies against carbamylated Fetal Bovine Serum.

Results: Anti-CarP were detected in 88 RA patients (34.6%), out of which 82% were also positive for antibodies against citrullinated protein antigens (ACPA) 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 percent of JIA patients and 30% of SS patients were 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.

Introduction

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 activity. The antibodies appear early in disease history and are predictive of evolution towards arthritis in arthralgia patients (2-5).

Replication studies have been few so far, and the overlap of anti-CarP positivity with anti-citrullinated peptide antibodies (ACPA) and Rheumatoid Factor (RF) has led to debate concerning their value as a novel diagnostic marker in RA. Additionally, their specificity has been scrutinized due to the fact that 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 in a cohort of Belgian early and established RA patients and in patients with other rheumatic conditions.

Patients and Methods

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 ACR/EULAR 2010 RA classification criteria (9). Disease controls were chosen from Juvenile Inflammatory Arthritis (JIA) (n=80), primary Sjögren's Syndrome (SS) (n=37), Axial Spondylarthritis (axSpA) (n=25) and Systemic Sclerosis (SSc) (n=11) patients from our rheumatology department. Sera of self-reported healthy controls (HC) 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, ULB, 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 assay:

Replicating the methods of Shi et al (1), we created an in-house ELISA assay using carbamylated Fetal Bovine Serum, described in detail in the Supplementary index.

Statistical analysis

For statistical analysis, Graphpad Prism version 7.0 was used. Testing for normality was done using Kolmogorov-Smirnov test while variance homogeneity was assessed using Bartlett's Chi squared. Non-parametric variables were assessed using Mann-Whitney U test and Kruskal-Wallis test. For normally distributed variables Student's *t*-test was used and ANOVA was performed for comparisons between more than 2 groups.

Results

Anti-CarP antibodies status in a Belgian cohort of RA patients:

The demographic data at baseline for the RA patients are summarized in Supplementary Table 1.

The anti-CarP reactivity was significantly higher for both early RA and established RA patients, when compared to HC. 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 ACPA 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 HC. 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%) were tested positive for anti-CarP, and their anti-CarP levels were comparable to those of RA patients. (Figure 1B)

Four HC (7%) were positive for anti-CarP, along with 12 JIA patients (15%) and 5 axSpA patients (20%). By restricting the analysis to these anti-CarP positive patients, the antibody levels were not different in RA patients and in disease control patients. (Figure 1D)

Anti-CarP antibodies and presence of other autoantibodies:

ACPA were more prevalent in the anti-CarP positive RA subgroup ($p<0.0046$). RF positivity was also associated to anti-CarP positivity ($p<0.0011$). RF positive and ACPA positive patients presented significantly higher anti-CarP levels when compared to seronegative patients. Interestingly, our results showed that RF positivity was associated with higher anti-CarP levels in SS patients (Supplementary Table 2). In

contrast, the presence of antinuclear antibodies in the serum was not associated with anti-Carp positivity, nor 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, nor with other baseline characteristics was established.

Additionally, longitudinal follow-up data at 12 months were assessed: disease response to DMARDS -observed by the modification of the activity scores over time- did not differ between the groups. Disability, as measured by the 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 DAS28 and higher HAQ at baseline, as well as higher Disease Activity indexes , DAS28 and HAQ, at 12 months of follow-up. (Table 2)

Diagnostic value of anti-CarP ELISA assay.

At the positivity cut-off chosen (mean absorbance of HC + 2SD), 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% whereas the negative predictive value was at 44.0%. Positive Likelihood Ratio was 4,86 and Negative Likelihood Ratio 0,71. The Area under the Curve in the Receiver Operator 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 in the Erasme-Brussels RA cohort, a different population from the ones 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 one year of follow-up in our study may explain our results: 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 fetal calf serum 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 present 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 Fetal Calf Serum to detect anti-CarP antibodies: the heterogeneity of composition of FCS from one batch to another, as well as the structural difference between human and bovine antigens, are potential sources of bias when it comes to autoantibody detection. Additionally, missing values in clinical data **(**Table 1, annotations), as well as the fact that a subset of patients had received treatment prior to inclusion, may have influenced our ability to further demonstrate the clinical value of anti-CarP.

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.

Conclusions

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

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Disclosure Statement:

The authors declare that there is no conflict of interest.

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Table 1: Baseline and follow-up clinical characteristics comparison between Anti-CarP (+) and Anti-CarP (-) in early RA patients

	Anti-CarP (+) (n=62)	Anti-CarP (-) (n=126)	p-value
Age (years, mean)	39.1±9.6	36.5±8.9	0.14
Sex: 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
Smokers (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
DAS 28 (median, IQR) – Baseline	4.38 [3.44-5.32]	4.44 [3.44-5.47]	0.54
SDAI (median, IQR) - Baseline	20.6 [12.4-31.2]	22.4	0.63
CDAI (median, IQR) - Basline	23.4 [12.63-12.2]	22.1 [12.2-30.3]	0.85
HAQ (median, IQR) - Baseline	1.15 [0.63-1.75]	1.06 [0.5-1.66]	0.49
DAS 28 (median, IQR) - 12 months	2.36 [1.69-3.58]	2.66 [1.70-3.39]	0.70
SDAI (median, IQR) - 12 months	5.95 [1.4-13.1]	7.1 [2.3-13.3]	0.43
CDAI (median, IQR) – 12 months	5.8 [0.9-13]	6.5 [2.1-13]	0.42
HAQ (median, IQR) – 12 months	0.375 [0.03-1.13]	0.375 [0-1]	0.53
Δ DAS 28 (median, IQR)	-1.80 [-2.78 ~ -0.59]	-1.68 [-3.08 ~ -0.62]	0.86
Δ SDAI (median, IQR)	-15 [-21.1 ~ -3.3]	-12 [-24.8 ~ -3]	0.80

Δ CDAI (median, IQR)	-14.75 [-20.1 ~ -3.4]	-11.3 [-22.5 ~ -2.8]	0.61
Δ HAQ (median, IQR)	-0.5 [-1.26 ~ 0]	-0.375 [-1 ~ 0]	0.54
Erosive Disease – 12 months	17/50⁂ (34%)	38/103 (36.9%)	0.85

*missing 4 values ** missing 7 values $ missing 19 values † missing 5 values ‡ missing 9 values ※ missing 8 values ⁑ missing 16 values ⁂ missing 12 values ⁃ missing 21 values ⁑ missing 3 values

ACPA, anti-citrullinated peptide antibodies; RF, rheumatoid factor; ANA, antinuclear antibodies; CRP, C-reactive protein; RA, rheumatoid arthritis; DAS 28, Disease Activity Score in 28 joints; SDAI, Simplified Disease Activity Index; CDAI, Clinical Disease Activity Index; HAQ, Health Assessment Questionnaire; IQR, interquartile range

Table 2: Anti-CarP in ACPA-/RF- early RA patients

	Anti-CarP+ (n=7)	Anti-CarP- (n=40)	P
DAS 28 (median, IQR) – Baseline	5.2 (4.01-6.29)	4.2 (3.1-5.35)	**0.043**
SDAI (median, IQR) - Baseline	35.8 (16.1-57.6)	26.5 (15.2-33.45)	0.15
CDAI (median, IQR) - Basline	34.7 (16-55.6)	23 (14-29.13)	0.10
HAQ (median, IQR) - Baseline	1.75 (0.87-2.5)	1 (0.44-1.75)	**0.038**
DAS 28 (median, IQR) - 12 months	4.42 (1.82-5.72)	2.35 (1.54-3.44)	0.078
SDAI (median, IQR) - 12 months	21.9 (5.89-39.7)	6.16 (1.1- 13.75)	**0.028**
CDAI (median, IQR) – 12 months	21.8 (5.8-39.3)	4.8 (0.75-13)	**0.027**
HAQ (median, IQR) – 12 months	1.5 (0.625-1.75)	0.18 (0-1)	**0.01**
Δ DAS 28 (median, IQR)	-0.81 (-2.6 – 0.02)	-1.94 (-3.2 – -0.69)	0.15
Δ SDAI (median, IQR)	-17.5 (-20.5 – 10.3)	-11.9 (-25.2 – -5.32)	0.58
Δ CDAI (median, IQR)	-17.5 (-20.4 – 10.8)	-11.5 (-24 – -5.3)	0.70
Δ HAQ (median, IQR)	-0.625 (-0.75 – 0)	-0.375 (-1.09 – 0)	0.85

RA, rheumatoid arthritis; DAS 28, Disease Activity Score in 28 joints; SDAI, Simplified Disease Activity Index; CDAI, Clinical Disease Activity Index; HAQ, Health Assessment Questionnaire; IQR, interquartile range

FIGURE LEGENDS

Figure 1A.Anti-CarP antibody levels in established RA (Est. RA) patients, early RA patients and in healthy control sera. 1B. Distribution of anti-CarP antibodies in sera of RA patients (n=254), juvenile arthritis patients (JIA, n=80), axial spondylarthritis patients (axSpA, n=25), Sjögren's syndrome patients (SS, n=37) and systemic sclerosis patients (SSc, n=11). The horizontal line signifies the cut-off value (300 AU/ml). 1C: Anti-CarP antibody level comparison between the tested RA population and the pooled disease controls. 1D: Anti-CarP antibody level comparison between Anti-CarP positive patients from various rheumatic conditions. 1E: Venn diagram showing the distribution of positivity of RA sera towards rheumatoid factor (RF), antibodies against citrullinated protein antigens (ACPA) and anti-carbamylated protein antibodies (Anti-CarP). 1F: Receiver Operator Curve for anti-CarP antibodies in RA patients. AUC calculation: 0.686.

* = $p<0.05$; *** = $p<0.005$; **** = $p<0.0005$; n.s.= not significant.