

Anti-carbamylated protein antibodies: are they useful for the diagnosis of rheumatoid arthritis?

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Abstract

Objective

ACR/EULAR-2010 classification criteria for rheumatoid arthritis (RA) rely heavily on the presence of anti-citrullinated peptide antibody (ACPA). The role of anti-carbamylated protein antibodies (anti-CarP) in this context is uncertain. We aimed to investigate the value of anti-CarP for RA classification in patients with early inflammatory arthritis.

Methods

Patients (n=402) were recruited from an early arthritis clinic and followed for 24 months. Healthy controls (n=95) were included. An anti-CarP ELISA was performed (uA/mL). Statistical analysis used regression and AUC analysis.

Results

The criteria for RA were met by 195/402 patients at inclusion; 28 developed RA during follow-up and 179 had other diagnosis (non-RA). 97/195 (49%) RA patients were anti-CarP+ (median 250 uA/mL [IQR 25–762]). In the group that progressed to RA, 7/28 (25%) were positive (82 uA/mL [13–235]) compared to non-RA (p=0.001) with 13/179 (7%) positive (26 uA/mL [5–80]). Being anti-CarP+ alone was observed in 17 patients of whom 7 (41%) were RA. Levels/positivity were not associated with other parameters. Anti-CarP+ had an odds ratio (OR) 6.5 for predicting RA (OR=17.1 for ACPA+ and OR=2.5 for RF+). In ACPA- patients, anti-CarP+ was also predictive of RA (OR=2.39). Being ACPA+/anti-CarP+/RF+ had a high predictive value for RA (OR=29.9 sensitivity/specificity (sen/spe) 33%/99%, positive/negative predictive values (ppv/npv) 97%/54%), however, being ACPA+/anti-CarP+ was superior (OR=36.1 sen/spe=41%/99%, ppv/npv=98%/57%) while being ACPA+/RF+ was inferior (OR=11.9, sen/spe=54%/95%, ppv/npv=94%/62%).

Conclusion

For RA classification, anti-CarP+ was less sensitive than ACPA, but more specific than RF. Anti-CarP+ may prove useful, classifying early arthritis patients, notably ACPA- patients.

Key words

rheumatoid arthritis, diagnosis, autoantibodies, anti-CarP level

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Introduction

Autoantibodies have long been associated with human diseases, particularly in autoimmune diseases (AIDs). The current ACR/EULAR 2010 classification criteria for rheumatoid arthritis (RA) relies heavily on the presence of autoantibodies. Indeed, even though the exact pathogenesis of RA remains unclear, autoimmune processes are known to play a role as evidenced by linkage with Major histocompatibility complex (1), and autoantibody production (2). So far only anti-citrullinated peptide antibody (ACPA) or rheumatoid factor (RF) are accounted for however, other auto-reactivities have been described (3) notably anti-carbamylated protein (anti-CarP) antibodies (4).

In RA, autoantibodies have long been associated with the disease. Rheumatoid factor (RF) was identified in the 30s, reacting against the Fc portion of other antibodies. Post-translational modifications (PTM) are mostly enzymatic modifications of amino acids in protein sequences (4-7). Citrullination, glycosylation, oxidation/glycation, methylation, acetylation and ubiquitination are all types of physiological modifications. PTM were shown to be immunogenic and produced antibodies to the modified proteins (8). Carbamylation is a non-enzymatic, irreversible PMT. It is a natural physiological phenomenon, however excessive carbamylation may occur when proteins are exposed to too high concentrations of isocyanate (9) which is usually deleterious (10). The quantification of antibodies against carbamylated proteins (anti-CarP) is relatively novel (11-13). The presence of anti-CarP was first demonstrated in RA (14). The exact nature of the auto-antigen(s) recognised by anti-CarP remains elusive, but fibrinogen, alpha-1 anti-trypsin, enolase and vimentin are potential targets described in RA (15-18).

Here we used a large cohort of early inflammatory arthritis (IA) patients to establish the value of anti-CarP for the diagnosis of RA.

Material and methods

Patients

402 patients were selected from an ethically approved early arthritis register

(IACON study, REC09/H1307/98, including healthy control (HC)). Diagnoses were documented at each 3 monthly visits over 24 months. EULAR 2010 criteria were used to classify patients. 95 HC were included to establish the local distribution of anti-CarP levels. All participants provided written informed consent.

AntiCarP ELISA and ACPA/RF levels

An anti-CarP antibody ELISA was performed using carbamylated FCS as target antigen as previously described (4). Results were expressed as aU/mL. Results for ACPA and RF levels were obtained from hospital records.

Statistical analysis

Continuous variables were not normally distributed and therefore data are described using median and interquartile range (Table S1). Mann-Whitney U-test and Fisher's exact test were used to compare the continuous and categorical variables between RA and non-RA patients, respectively (Table S1). A binary logistic regression was used to account for all variables. A first model included all individually significant variables from Table S1 (unadjusted OR not shown) and then only significant ones were included in the adjusted model (Table S1). ROC analyses were then performed to establish the individual predictive value of anti-CarP levels. Thresholds for the dichotomisation of data as high and low risk were set at ~80% specificity. Sensitivity/specificity (sen/spe) as well as odds ratio (OR) and positive/negative predictive values (ppv/npv) were calculated. Analyses were conducted using SPSS 24.1. The level of significance for *p*-values was set at 0.05.

Results

Anti-CarP: healthy range

We first established anti-CarP levels in a reference group of 95 HC.

We determined the top value of the 95% CI of the distribution (Fig. 1A) which allowed to define a negative/positive cut-off for the test, established at >250 aU/mL. A cut-off for positivity at Median+2 SD (200 aU/mL) proved less specificity for RA.

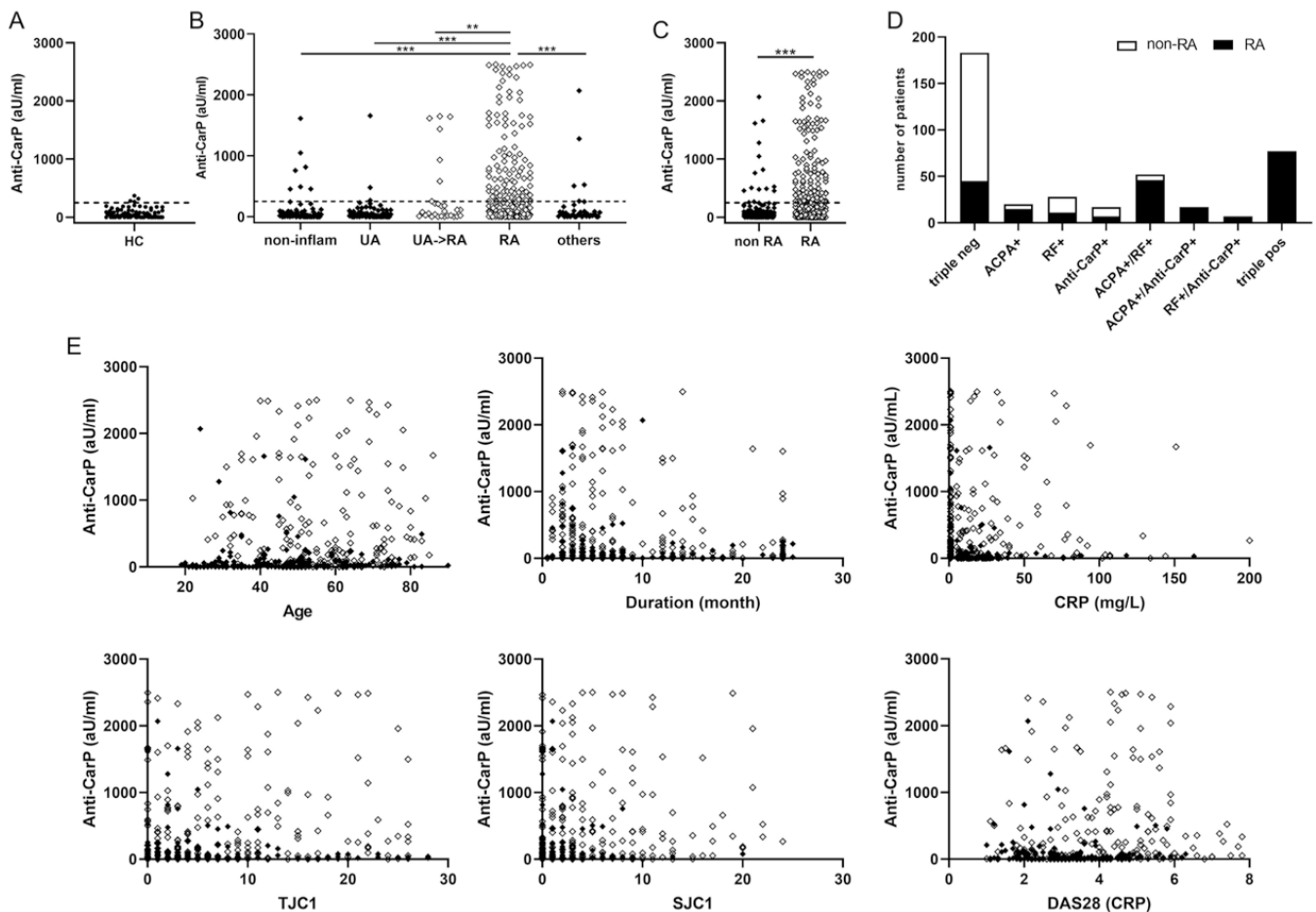


Fig. 1. Distribution of antiCarP antibodies levels.

A: HC distribution: a cut-off was set at 250 U/mL, at the top of the 95% of the anti-CarP levels distribution.

B: Serum levels of anti-CarP antibody (aU/mL) in patients from an EAC (n=402): RA n=195; UA who progressed to RA over time (UA->RA) n=28; non inflammatory n=38; persistent UA n=94, and other diagnoses n=47 (gout, psoriatic arthritis, connective tissue diseases, inflammatory OA).

C: This panel directly compare all non-RA patients n=179 to RA patients n=223

D: Number of patients with no, single, double or triple positivity for autoantibodies (n=402).

E: Anti-CarP levels were not related to clinical parameters.

EAC cohort

At inclusion 195/402 patient were classified as RA; 28 developed RA over time while 56 remained undifferentiated arthritis (UA); 83 had non persistent symptoms and 40 other inflammatory joint diseases and were grouped as non-RA (n=179). Characteristics are described in Table I. This cohort is representative of other similar early arthritis cohorts.

ELISA results

ELISA was performed on 402 samples (Fig. 1B). Anti-CarP levels were higher in RA (n=105/223, 47% positive, median 801 aU/mL, [IQR 420-1627], $p<0.0001$) compared to all other groups individually, including those who progressed to RA (25% positive) and to all non-RA (Fig. 1D) (n=15/179, 8% positive, 524 uA/mL [IQR 456-1280]).

Those who were anti-CarP+ had an odd ratio for RA of 6.48.

Seven of the 17 patients (41%) who were anti-CarP+ only were classified as RA. Similarly, 15/20 (75%) ACPA+ only were RA as well as 10/28 (35%) RF+ only patients.

234 patients were ACPA-negative. In this group, anti-CarP positivity was less prevalent (n=26/234, 11%) but was still associated with RA (n=13/69, 19% positive vs. n=13/165, 8% positive, $p=0.016$, OR=2.39). However, levels were not different in RA (475 uA/mL [IQR 305-884]) compared to non-RA (505 uA/mL [IQR 454-1162], $p=0.810$).

Anti-CarP levels association with disease parameters

We then addressed whether levels of anti-CarP were related to any demo-

graphic or clinical parameters. Gender, age, symptom duration, joint counts, CRP or smoking were not associated with anti-CarP positivity or levels (Fig. 1E). Positivity for ACPA and RF was closely associated with anti-CarP presence ($p<0.0001$). Levels in anti-CarP+ patients were significantly higher in ACPA+ patients ($p=0.009$). In contrast, there was no difference for RF positive/negative patients ($p=0.222$).

Prediction of RA classification

In this cohort, those with anti-CarP+ had an odds ratio OR=6.48 (Table II) for predicting RA, with a sensitivity $sen=91.5\%$, positive predictive value $ppv=89\%$. A ROC analysis determined that no negative/low versus high cut-off was able to predict RA better than the positive/negative status.

Table I. Cohort characteristics.

| | RA (n=223) | Non-RA (n=179) | individual <i>p</i> -value | adjusted OR (95% CI) | model <i>p</i> -value |
|--------------------|---------------|-------------------|-------------------------------|-------------------------|--------------------------|
| Age (years)* | 55 (22-86) | 46 (19-90) | <0.0001 | 0.972 (0.954-0.991) | 0.003 |
| Gender (F/M) | 120/59 | 146/77 | 0.412 | not included | |
| Duration (months)* | 9 (1-24) | 15 (1-24) | 0.067 | not included | |
| ACPA (±) | 165/13 | 154/69 | <0.0001 | 17.10 (7.932-36.889) | <0.0001 |
| RF (±) | 138/84 | 26/151 | <0.0001 | 2.51 (1.258-5.040) | 0.010 |
| Anti-CarP (±) | 103/120 | 13/162 | <0.0001 | 6.48 (3.770-11.141) | <0.0001 |
| TJC* | 8 (0-26) | 5 (0-28) | <0.0001 | 0.934 (0.892-0.978) | 0.004 |
| SJC* | 4.8 (0-24) | 1.6 (0-20) | <0.0001 | 0.880 (0.803-0.965) | 0.007 |
| CRP* | 21 (<5-241) | 10.5 (<5-163) | <0.0001 | not included | |
| DAS28* | 4.08 (1-7.80) | 3.17 (1-6.30) | <0.0001 | not included | |

*Median (range).

Table II. RA prediction.

| | ACPA alone | anti-CarPA alone | RF alone |
|-------------|------------|------------------|----------|
| Sensitivity | 69% | 47% | 62% |
| Specificity | 93% | 93% | 79% |
| OR | 9.46 | 6.48 | 4.23 |
| PPV | 92.5% | 89% | 87% |
| NPV | 70.5% | 59% | 58% |

| | ACPA+RF | ACPA+anti-CarP | Anti+CarP-RF |
|-------------|---------|----------------|--------------|
| Sensitivity | 54% | 41% | 36% |
| Specificity | 95% | 99% | 98% |
| OR | 11.9 | 36.1 | 21.5 |
| PPV | 94% | 98% | 96.5% |
| NPV | 62% | 57% | 55% |

| | ACPA + anti-CarP + RF |
|-------------|-----------------------|
| Sensitivity | 33% |
| Specificity | 99% |
| OR | 29.9 |
| PPV | 97% |
| NPV | 54% |

Those with ACPA+/RF+/anti-CarP+ had a high predictive value for RA (OR=29.9, Table II). A combination of ACPA+/anti-CarP+ was superior at predicting RA (OR=36.1) than ACPA+ alone (OR=9.46) or ACPA+/RF+ (OR=11.9).

A binary regression analysis (RA/non-RA, Table I, last column, model) confirmed that anti-CarP were the second best predictor of RA (OR=3.331) from all variables considered, ACPA remaining the best (OR=17.10) with an overall 83% accuracy.

Discussion

Our data confirmed the association of anti-CarP+ with RA in a cohort separate from the original work (4, 14). Anti-CarP positive levels were significantly higher in RA but were not asso-

ciated with any other parameters, suggesting independent added value.

Anti-CarP+ alone had an odds ratio OR=6.48 for predicting RA, which was better than RF+ but less than compared to ACPA+. In ACPA- patients, anti-CarP+ also had potential value (OR=2.39) allowing 70% of patients to be properly diagnosed, similar to RF (OR=2.1). A combination of ACPA+/antiCarP+ was superior (OR=36.1) to using all 3 antibodies ACPA+/RF+/anti-CarP+ (OR=29.9). A classification and regression tree (CART) analysis (see supplementary files), also suggested that ACPA/anti-CarP were able to classify correctly 5.6% more patients than ACPA alone, while the regression eliminated RF. Although RF+ is more frequently positive in RA than anti-CarP+ (62% and 47% respectively),

anti-CarP+ is more specific for RA than RF (92% vs. 79%). The combination of ACPA+/anti-CarP+ therefore proved more useful at classifying early IA patients than other antibody combinations including triple-positivity.

Recently the triple combination ACPA+/antiCarP+/RF+ was reported to be superior in identifying individuals at-risk of developing RA from the healthy population (19). Our data therefore suggest that depending on the comparator population, (*e.g.* the healthy population versus patients visiting the outpatient clinic) a combination of ACPA+/antiCarP+ autoantibodies may be sufficient for accurate prediction, when patient present with complaints (IA symptoms). Furthermore, in our study, UA patients presenting with IA symptoms that did not evolve to RA (over 2 years of follow-up) were no more positive for anti-CarP (2/94 (2%)) or ACPA+ (5/94 (5.3%)) than HC. In contrast, UA evolving to RA (UA->RA) may be considered a pre-RA stage, and this group is positive for anti-CarP in 6/28 (21.5%) of cases and ACPA+ in 11/28 (39%). This is recapitulating previous data suggesting independent value for ACPA and anti-CarP measures in pre-RA disease (20). Carbamylation (also referred to as homocitrullination) is a non-enzymatic PTM that occurs when cyanate levels are enhanced, notably during inflammation and aging (11, 21). Although homocitrullination is chemically close to citrullination, our data confirmed that anti-CarP and ACPA are generated independently in early IA (and at pre-clinical stages) (20). Anti-CarP are notably present in 14% (33/235) of ACPA- IA patients (or vice-versa 24% (64/268) of anti-CarP-patients are ACPA+) showing that these auto-antibodies do not cross-react between citrullinated or homocitrullinated antigens.

High levels of ACPA have also been suggested to be more predictive of progression to RA in at risk-individual compared to positivity (22). An analysis of whether high (above 3SD of healthy range (cut-off at 315 aU/mL) of positive levels, n=45/134) versus low/neg levels of anti-Carp antibody were better at classifying RA, showed

no particular improvement over the original dichotomisation (lower SPE 41% (-6%), same SEN 92% (-1%), lower OR 5.6 (-0.9), lower PPV 87% (-2%), higher NPV 64% (+5%)). Alternatively, an AUROC analysis suggested a cut-off at 1000 aU/ml which was highly specific (SPE 97%, OR 7.4, PPV 90%) for RA but with low sensitivity (SEN 20%) and NPV (51%). Therefore, the positive/negative status for anti-CarP altogether performed the best. In our cohort, a similar analysis showed that high/low-negative ACPA or RF levels were also less good at classifying RA (data not shown). The limitations of this work are first, that ACPA/RF positivity being included in the EULAR-2010 classification criteria may have strongly influenced the statistical analysis, biasing against accurately detecting the value of anti-CarP. Secondly, there is currently no commercial ELISA test for anti-CarP and future work needs to establish a standardised assay and replicate this data (as well as established standardised references, see also a STARD checklist in supplementary files). Therefore, these data suggest more clinical utility of using anti-CarP than RF. Furthermore, anti-CarP has some value in ACPA- patients, although it is less frequently observed there (19%) than in ACPA+ (59%). Anti-CarP positivity (41%) has also been observed in IA patient who progressed to RA over time and this work needs to be extended to even earlier stage of the inflammatory arthritis continuum. The presence of anti-CarP will not only provide diagnostic information, but also prognostic information as the presence of anti-CarP is associated with more severe joint damage, especially in the ACPA- patients (4).

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