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Anti-carbamylated protein antibodies presence in early arthritis with a poorer clinical and radiological outcome: data from the French ESPOIR cohort

Marie-Elise Truchetet[1,2*], MD/PhD, Stéphanie Dublanc[1*], MD, Thomas Barnetche[1], PhD, Olivier Vittecoq[3,] MD/PhD, Xavier Mariette[4], MD/PhD, Christophe Richez[1,2], MD/PhD, Patrick Blanco[2,5], MD/PhD, Michael Mahler[6], PhD, Cécile Contin-Bordes[2,5], PhD, and Thierry Schaeverbeke[1], MD/PhD on behalf of the FHU ACRONIM

[1]Rheumatology Department, Bordeaux University Hospital

[2]Immunoconcept, CNRS UMR 5164, Bordeaux University, Bordeaux 33076, France

[3]Rheumatology Department, Rouen University Hospital

[4]Université Paris-Sud; Hôpitaux Universitaires Paris-Sud, AP-HP ; Institut National de la Santé et de la Recherche Médicale (INSERM) U1184, Centre for Immunology of Viral Infections and Autoimmune Diseases (IMVA), Le Kremlin Bicêtre, France

[5]Immunology Department, Bordeaux University Hospital

[6]Inova Diagnostics, San Diego, US

*These authors equally contributed to this study.

Address correspondence and reprint requests to: Thierry Schaeverbeke, MD, PhD, Service de Rhumatologie, CHU de Bordeaux, Place Amélie Raba-Léon, 33076 Bordeaux, France; Phone: +33 556 795 556; Fax: +33 556 796 084; E-mail address: thierry.schaeverbeke@chu-bordeaux.fr

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ABSTRACT

Objectives: To assess the prevalence of anti-carbamylated protein (CarP) antibodies in a French cohort of early arthritis and to investigate their association with clinical features, final diagnosis, prognosis and comorbidities.

Methods: The presence of anti-CarP antibodies among patients with early arthritis belonging to the ESPOIR French cohort (n=720) was determined using ELISA. We calculated the prevalence of anti-CarP antibodies in different patient subgroups that were stratified according to their anti-citrullinated peptides antibodies (ACPA) and/or rheumatoid factor (RF) status. Diagnosis and prognosis values of the test were evaluated in this population.

Results: We observed the presence of anti-CarP in approximately one third of the patients (32.6%) and 23.6% of the RF and/or ACPA sero-negative patients. Anti-CarP positivity was associated with a more active disease status at baseline and over time with a significantly higher DAS28-ESR level at M36 (3.1±0.11 *vs.* 2.8±0.06, *p=0.03*). Anti-CarP-positive early arthritis was associated with a higher risk of developing erosions after 96 months of follow-up (55.6% among the anti-CarP+ patients *vs.* 37.3% among the anti-CarP- patients, OR=2.1 [1.2-3.6], p=0.009). This association was particularly true when anti-CarP was associated with ACPA positivity. Moreover, ACPA positivity alone-early arthritis was not associated with a higher risk of erosive evolution.

Conclusions: Anti-CarP antibodies were present among one third of patients with early arthritis and among one fourth of the RF-negative and ACPA-negative patients. They were particularly associated with a more severe radiographic fate. Anti-CarP antibody positivity may help to accurately identify those at-risk of erosive evolution in an early arthritis population.

Rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) are the two main autoantibodies associated with rheumatoid arthritis (RA) (1). ACPA and/or RF are detected in approximately 70-80% of patients with RA (2). Approximately one third of patients with established RA do not express RF or ACPA. Seronegative RA is more heterogeneous and sometimes difficult to diagnose due to the lack of specific autoantibodies (3,4).

ACPA recognize epitopes containing citrulline that arises from posttranslational modifications (5). Other posttranslational modifications of self-proteins, such as glycosylation, oxidation and carbamylation, have been described in RA (6-8).

Carbamylation results from the chemical conversion of lysine into homocitrulline in the presence of cyanate. Increases in cyanate levels, either by excess of urea, increased myeloperoxidase activity (inflammation) or through direct intake (smoking), leads to carbamylation (9). The presence and involvement of carbamylated proteins in atherogenesis and renal failure has been highlighted for years (9-11). Whereas citrullinated proteins have been identified in the joints of RA patients, the presence of carbamylated proteins has not been clearly demonstrated (9). However, indirect evidence has been strongly observed, notably through the detection of anti-carbamylated protein (CarP) antibodies (8).

The presence of anti-CarP antibodies has been described in several cohorts of RA patients, particularly in those from northern Europe (12-16). The development of autoantibodies among patients with RA depends on the genetic background, and differences have already been found for ACPA in Caucasian populations (17,18). Anti-CarP antibodies may also be present prior to onset and predict the evolution of RA, which has been shown in some studies; however, this needs to be reinforced by data from additional cohorts (19-21).

Altogether, these data suggest an interesting diagnostic and prognostic value for anti-CarP antibodies. In our work, we provide new data on anti-CarP antibodies in a French cohort of early arthritis patients (ESPOIR) (22).

We assessed the prevalence of anti-CarP antibodies, detailing different clusters of early arthritis. Herein, we describe the clinical, biological, and radiological characteristics of early arthritis in patients who tested positive for anti-CarP antibodies. We also assessed the diagnostic and prognostic predictive values of anti-CarP antibody levels for RA in early arthritis. Finally, we determined whether anti-

CarP antibodies were associated with specific treatments or comorbidities in this particular cohort.

PATIENTS AND METHODS

Study population

The ESPOIR cohort (Etude et Suivi des Polyarthrites Indifférenciées Récentes) is a French National, multicenter, longitudinal, prospective cohort consisting of patients with early arthritis (22). Patients were recruited through general practitioners and rheumatologists who were asked to refer patients with early arthritis to one of the 14 university hospitals participating in the ESPOIR project.

Patients who were included in the study were between 18 and 70 years old; had a clinical diagnosis of certain or probable RA or a clinical diagnosis of undifferentiated arthritis potentially becoming RA; had at least 2 swollen joints for 6 weeks and less than 6 months; and had no prescriptions for disease-modifying anti-rheumatic drugs (DMARDS) or corticosteroids (except if the patient had obtained a prescription less than 2 weeks before inclusion in the study or had been administered an intra-articular injection less than 4 weeks before inclusion in the study). Patients were excluded from the study if they exhibited other inflammatory rheumatisms or connective tissue diseases already diagnosed at screening visit according to standard criteria.

All patients were examined every 6 months during the first 2 years and then every year for at least 10 years. Data about comorbidities including hypertension, dyslipidemia, MI or cerebrovascular disease were recorded as presence or absence at each follow-up visit, according to standard of care.

The protocol of the ESPOIR study was approved in July 2002 by the Montpellier ethics committee and all the patients signed an informed consent form before their inclusion.

The scientific committee of the cohort accepted our project, and we obtained patient sera during month 6 (M6) after inclusion in the study. Patient data, including demographic, clinical, biological, and radiographic data, were collected at baseline and at month 96 (M96).

Anti-CarP antibody assay

Detection of serum IgG and anti-CarP antibodies was performed using an ELISA developed by Inova Diagnostics (San Diego, CA, USA) following the manufacturer's instructions and as described previously (23). Briefly, plates were pre-coated with carbamylated fetal calf serum (FCS) before the addition of a 1:101 dilution of patient sera. Rabbit anti-human IgG coupled to horseradish peroxidase and tetramethylbenzidine substrate solution were then added. The cut-off value for positivity was determined for each plate using a Low Positive Control supplied with the kit. This internal control was measured in duplicate and the mean of both values was considered as the positivity cut-off for the corresponding plate. The absorbance was measured at 415 nm and transformed to arbitrary units per milliliter (AU/ml) using a titration curve of serum samples with increasing concentrations of anti-CarP antibody.

Data analyses

We collected the following clinical data for each patient from the ESPOIR database: gender, age, type and duration of symptoms, activity of the disease as assessed by the erythrocyte sedimentation rate (DAS28-ESR) at baseline, whether the patient

was a smoker or alcoholic, and whether the patient had suffered from hypertension (defined as a systolic or a diastolic blood pressure measurement consistently higher than respectively 139 mmHg and 89 mmHg), dyslipidemia (defined as hypercholesterolemia superior to 2.4 g/L), myocardial infarction (considering any necrosis in the setting of myocardial ischemia), or cerebrovascular disease (including strokes and transient ischemic attack) at baseline and at 8 years (M96). We collected the following biological data at baseline: erythrocyte sedimentation rate (ESR, in mm within the first hour, cut-off for positivity was >10), C reactive protein (CRP, in mg/l, cut-off for positivity was >5 mg/l), creatinine (in mmol/l). ACPA (Elisa, DiaSorin, France; Positive > 50 U/ml) and RF (IgM and IgA rheumatoid factor (Elisa, Menarini, France; Positive > 9 UI/ml) were performed for all the patients using the same technique in a central lab (Paris-Bichat). We collected the following radiological data: presence or absence of erosion joints at baseline and 8 years (M96) and the Sharp score at baseline and 3 years. We also collected patient use of DMARDS at 8 years. The final diagnosis of RA was validated if the patients fulfilled the ACR/EULAR 2010 criteria at least once over the 8-year time period.

Statistical analyses

Quantitative variables were presented as the means and standard deviations (or medians and interquartile ranges). Qualitative variables were presented as percentages. To compare positive (anti-CarP+) and negative (anti-CarP-) anti-CarP groups for the whole cohort and in each cluster, a chi-square test was performed for qualitative variables and Student's t-test was performed for quantitative variables. Non-parametric correlation analyses were performed using a Spearman correlation test. Odds ratios (ORs) with their 95% confidence intervals were also calculated. All analyses were performed using STATA 13.1 software. P-values less than 0.05 were

considered significant. The study is mainly exploratory therefore no multiple testing corrections have been included.

RESULTS

Population characteristics

Between December 2002 and March 2005, 814 subjects with early arthritis were included in the cohort. We obtained access to 720 samples of sera that were collected 6 months after patient inclusion into the study. The samples were analyzed for status.

The baseline characteristics of the 720 patients are described in Table 1. Among the 720 patients, 701 had fully available data for ACR criteria, and 91% of these early arthritis patients fulfilled the ACR 2010 criteria for RA (638/701) at least once over the 8-year period. In this cohort, which was composed mainly of women (76%), 50.1% of the patients were RF and ACPA negative at baseline, and the mean DAS28-ESR was 5.1±1.3.

The prevalence of anti-CarP antibodies in the French cohort of early arthritis (ESPOIR)

Anti-CarP antibodies were detected in the sera of 32.6% of the ESPOIR patients at M6 (235/720) and in 32.3% of the 711 patients with available data for RF and ACPA. RF was detected in 41.8% of the cohort, and ACPA was detected in 38.4% of the cohort.

Association of anti-CarP antibodies with the ACPA and/or RF status

Anti-CarP+ patients were found in every group constituted according to the RF/ACPA status, (Figure 1A and 1B). Significantly more anti-CarP+ patients were observed in the ACPA+ group than in the ACPA- group (41.8% *vs*. 26.5%, OR=2.0 [1.4-2. 8], *p*<0.0001, Figure 1C), as well as in the RF+ group than in the RF- group (42.1% *vs*. 25.4%, OR=2.1 [1.5-3.0], *p*<0.0001, Figure 1D).

Anti-CarP antibodies were present in 23.6% (n=84/356) of the RF and ACPA double negative patients. The anti-CarP single-positive patients represented 11.8% (n=84/711) of the whole early arthritis population. The prevalence of single-positive, double-positive and triple-positive patients are reported in Figure 1E. Of note, 159 patients presented with anti-nuclear antibodies (ANA) at the first visit. Anti-CarP antibodies were significantly associated with ANA (45.6% *vs*. 29.1%, OR=2.1 [1.4-3.0], *p*=0.0002, data not shown). The same association was observed between ANA and RF (OR=1.7 [1.2-2.5], *p*=0.0042, data not shown) or ANA and ACPA (OR=2.0 [1.4-2.8], *p*=0.0003, data not shown).

Altogether, anti-CarP antibodies were expressed in approximately one third of the patients with early arthritis and in one quarter of the patients with seronegative arthritis.

Characteristics of anti-CarP+ and anti-CarP- early arthritis at baseline

While anti-CarP+ and anti-CarP- patients presented similar demographic characteristics at baseline (supplementary table*)*, they exhibited differences in their clinical, biological and radiological data.

In a univariate analysis, anti-CarP positivity was associated with a more active disease state at baseline as measured by the mean DAS28-ESR value (5.0 [4.9-5.1]

vs. 5.3 [5.2-5.5], *p*=0.0004, Figure 2A). The proportion of patients in low disease activity (DAS28-ESR <3.2) was significantly lower in the anti-CarP+ patients than the anti-CarP- patients (3.4% vs. 9.7%; *p*<0.01, Fisher's exact test). Conversely, the proportion of patients in high disease activity (DAS28-ESR >5.1) was significantly higher in the anti-CarP+ patients than in the anti-CarP- patients (53.9% *vs.* 47%; *p*=0.0018, Fisher's exact test, Figure 2B). Moreover, the anti-CarP antibody levels, as measured in AU, correlated with the DAS28-ESR levels (Figure 2C). However, based on the anti-CarP antibody status, no differences were observed between patients with explosive, subacute, insidious or paroxystic disease onset.

A multivariable analysis revealed differences in the clinical characteristics based on the early arthritis antibody profiles at baseline (*p*=0.023, ANOVA). Overall, the presence of anti-CarP antibodies is linked to higher disease activity when associated with either ACPA or RF. Interestingly, this result was not observed among the patients who presented with either ACPA, RF or anti-CarP alone or the combination ACPA+RF (Figure 2D).

Biologically, CRP and ESR were more frequently abnormal (cut-offs at 5 mg/l and 20 mm, respectively) in the anti-CarP+ patients than in anti-CarP- patients (138/233 *vs.* 234/478, *p*=0.0106 and 165/232 *vs.* 303/476, *p*=0.0520*;* Figure 2E, 2F, respectively). A stratified analysis on ACPA status highlighted a slightly significant association between CARP positivity and ESR/CRP abnormality restricted to ACPA positive patients (respectively 79/114 vs. 90/160, p=0.029; 96/113 vs. 117/156, p=0.047) Nevertheless, no correlation was observed between the CRP or ESR levels and anti-CarP antibody levels.

Radiologically, both the anti-CarP+ patients and anti-CarP- patients exhibited similar values for erosive disease (33% *vs.* 34.6%, respectively) and the Sharp score at baseline (5.1 [4.1-6.1] for the anti-CarP+ patients and 5.1 [4.4-5.7] for the anti-CarP- patients, data not shown).

Predictive value of anti-CarP antibodies for RA diagnosis in an early arthritis population

The ACR/EULAR 2010 criteria were chosen to determine whether the patients with early arthritis included in the ESPOIR cohort actually developed RA.

After 8 years, 98.6% of the anti-CarP+ patients and 87% of the anti-CarP- patients, regardless of their status of ACPA or RF, fulfilled the ACR/EULAR 2010 criteria (*p=0.022*, data not shown*).* The sensitivity and specificity of the anti-CarP antibody were 34% and 95%, respectively. Clinical performance for the diagnosis of RA of the three antibodies is shown in table 2. In the same population of early arthritis, the sensitivity/specificity of ACPA and RF were 50%/91% and 52%/98%, respectively. If either anti-CarP antibodies and/or ACPA were positive, the sensitivity and specificity were 63.5% and 87.3%, respectively.

In our non-RA population the frequency of anti-CarP was 12% (3/25). If we added anti-CarP to the auto-Ab item of the 2010 ACR criteria, the percentage of patients fulfilling the criteria would increase from 91% to 91.4%.

The patients also suffered from other diagnoses besides RA. Seventeen patients were diagnosed with spondyloarthritis, one patient of which was anti-CarP+, ACPA- and RF-. Eight patients were diagnosed with systemic lupus erythematous, 4 of which were anti-CarP+ (but only one was single positive). Eight patients were diagnosed with primary Sjögren's syndrome of which 7 were anti-CarP+ (all were

also positive for RF). The presence of overlapping syndromes was not specified, but the ACR criteria were fulfilled at least once during the 8-year follow-up for all patients.

Overall, anti-CarP antibodies showed a high positive predictive value of developing established RA in a population of early arthritis, particularly in combination of ACPA and RF.

The prognostic value of anti-CarP antibodies in an early arthritis population

We assessed the evolution of early arthritis according to the antibody status of the patients. Patients in every group exhibited decreased DAS28-ESR over time. However, the timeline was different among the groups. Anti-CarP+ patients had a significantly higher DAS28-ESR level at M36 (3.1±0.11 *vs.* 2.8±0.06, *p=0.03*) before finally reaching the DAS28-ESR level of anti-CarP- patients at M96 (Figure 3A). In a multivariate analysis, at M36, the patients presenting with anti-CarP antibodies associated with RF exhibited more active disease states than the patients who were ACPA single positive and tended to be more active than the patients who were all negative (3.3±0.22 *vs.* 2.6±0.2, *p=0.03* and 3.3±0.22 *vs.* 2.8±0.09, *p=0.06,* respectively, ANOVA and Kruskal Wallis post-test, Figure 3B). At M96, the anti-CarP/ACPA/RF triple-positive patients exhibited more active disease states than the anti-CarP/ACPA/RF triple negative patients (2.9±0.1 *vs.* 2.4±0.2, *p=0.01*, ANOVA and Kruskal Wallis post-test, Figure 3C).

The radiological status was available for 258 patients at M96. Anti-CarP positivity was associated with the presence of erosions at M96 (55.6% in the anti-CarP+ patients *vs.* 37.3% in the anti-CarP- patients, OR=2.1 [1.2-3. 6], *p=0.009*, chi-square test, Figure 3D*)*. This difference was not due to an erosive status at M0 as shown in Figure 3E but appeared quite rapidly since the Sharp score of the patients with anti-

CarP antibodies was higher at M36 compared to the patients without anti-CarP antibodies (16.4±1.4 *vs.* 12.9±0.7, *p=0.0101,* respectively, unpaired t-test). Moreover, the patients with erosions at M96 had higher levels of anti-CarP antibodies at the beginning of their disease, suggesting the predictive value of the level of anti-CarP for the development of RA (221.5±16.2 *vs.* 166.2±11.3, *p=0.0043,* respectively, unpaired t-test, Figure 3F). Interestingly, antibody statuses were differentially associated with an erosive evolution (Figure 3G). Triple-positive patients had a higher Sharp score at M36 than the triple negative ones (18.49±2.1 *vs.* 10.1±0.7, *p<0.0001*, ANOVA and Kruskal Wallis post-test, Figure 3G). CarP+/ACPA+/RF- and CarP-/ACPA+/RF+ were also associated with a higher Sharp score at M36 than CarP-, ACPA-, RF- but not the other antibody combinations (23±6.8 and 16.6±1.3, respectively, *vs.* 10.1±0.7, *p<0.0001*, ANOVA and Kruskal Wallis post-test, Figure 3G).

Taken together, these results revealed that anti-CarP antibodies are linked to a more active disease over time and erosive polyarthritis.

Association of anti-CarP antibodies with comorbidities in early arthritis

Because the carbamylation process is related to smoking, we assessed the association of smoking with the anti-CarP antibody status. Tobacco had no influence on anti-CarP positivity. The results showed that 48% of the anti-CarP+ patients and 47% of the anti-CarP- patients were smokers (active or former, data not shown). We obtained the same result when considering only the active smokers. No associations were observed between anti-CarP positivity and cardiovascular history (stroke, cardiac infarction) or risk factors (hypertension, diabetes, hypercholesterolemia, overweight) at M0. Finally, we found no association between the presence of the

anti-CarP antibody and the occurrence of a cardiac infarction at the 8-year follow-up. However, there was a trend for a more frequent occurrence of stroke in the anti-CarP+ patients, but it was not significant (4 events in 144 anti-CarP+ patients and 2 in 332 anti-CarP- patients, *p=0.07*, Fisher's exact test).

Because carbamylation is associated with the concentration of urea, we also assessed renal function. The patients had the same level of serum creatinine at M0, regardless of their anti-CarP antibody status (data not shown). Moreover, no correlation was observed between the levels of anti-CarP antibody and serum creatinine.

Altogether, these results exclude some confounding factors that could increase carbamylation and lead to non-specific anti-CarP antibody production.

Treatment and anti-CarP antibodies

In general, the patients with early arthritis who were positive for anti-CarP antibodies were more frequently treated with at least one DMARD during the 8-year follow-up (81.6% *vs.* 69.5%, OR at 2.0 [1.2-3.2], *p=0.006*, Fisher's exact test, Figure 4A). Patients with anti-CarP antibodies were more frequently treated with biological agents during the 8-year follow-up (35% *vs.* 21%, OR at 2.0 [1.2-3.0], *p=0.005*, Fisher's exact test, Figure 4B). This association remains significant after adjusted analysis on RF and ACPA status (OR= 1.60 [1.02-2.53], p=0.03). Interestingly, anti-CarP antibody positivity increased the risk of receiving a biological agent as a first line of treatment (17.6% *vs.* 9.8%, OR at 2.0 [1.1-3.5], *p=0.02*, Fisher's exact test, Figure 4C). Moreover, patients who received a biological agent during the 8-year follow-up had the highest rate of anti-CarP antibodies compared to the patients who

did not received those therapeutics (211.2±14.8 *vs.* 179.5±7.4, respectively, *p=0.03*, unpaired t-test, Figure 4D).

Regardless of the presence or absence of anti-CarP antibodies, the same proportion of patients was treated with glucocorticoids (data not shown). We did not observe a correlation between the patients' sera levels of anti-CarP antibodies and the maximum or mean doses of glucocorticoids received by the patients (data not shown).

Overall, these data seem to confirm that the early arthritis patients with anti-CarP antibody positivity at baseline are those with the most severe disease course over time.

DISCUSSION

In this study, we replicated data obtained from a cohort of patients with early arthritis from northern Europe (24). Using an innovative technique, this is the first large study investigating an assay that uses CarP-FCS without the subtraction of non-CarP-FCS. Novel antibodies that may be utilized for RA diagnosis and/or prognosis should be validated among several populations and different experimental conditions to test reliability. In the French cohort ESPOIR, we observed the presence of anti-CarP antibodies in approximately one third of the patients (32.6%). Importantly, anti-CarP antibodies were present in one quarter of the seronegative (RF- and ACPA-) patients, i.e., approximately 12% of the patients in the cohort were anti-CarP+, ACPA- and RF-. The sensitivity was comparable to that observed in the Leiden early arthritis cohort (24). However, we observed a slightly higher anti-CarP specificity to diagnose RA in an early arthritis population (24). The combination of both anti-CarP

and ACPA increased the sensitivity to diagnose RA in an early arthritis population of both tests alone (63.5% *vs.* 34 or 50% respectively), which is interesting for a screening test.

Shi *et al.* (8) were the first to describe anti-CarP antibodies in RA with a prevalence of 43-45%. Since then, other studies have reported the prevalence of anti-CarP antibodies to be 30-49% (8,12,15,16,20,23). In our study, anti-CarP antibodies were associated with more active forms of RA. This finding is consistent with other studies of patients with early arthritis (25,26). This is the first study to find a statistical link between the presence of anti-CarP antibodies and disease activity as measured via DAS28-ESR. The association was stronger when considering anti-CarP positivity associated to ACPA or RF. Interestingly, this was not the case with ACPA or RF alone. Clinically relevant and in agreement with this result, the anti-CarP+ patients with early arthritis required prescription DMARDs and biologic agents more frequently than the anti-CarP- patients. These observations converge towards an association between anti-CarP antibodies at baseline and a poor prognosis of early arthritis. In the same line, anti-CarP antibodies were associated with the development of erosions and a worse clinical course. This observation is consistent with three previous studies (8,20,23). Brink *et al.* (20) reported this association at baseline, which was not the case in our study. The worst radiological course at M36 was observed among patients who were CarP/ACPA double positive. We report, for the first time, an association between the level of anti-CarP antibodies and the presence of erosions at M96 in a cohort of patients with early arthritis regardless of their antibody status at M0. Shi *et al.* (8,25) previously showed that anti-CarP antibodies could predict joint erosion in RA or in a cohort of patients with arthralgia who were positive for ACPA and/or RF. This finding strengthens the use of anti-CarP antibody

levels as a prognostic indicator and is in line with recently published data showing an additive value of anti-CarP to ACPA and RF for the determination of radiographic progression (27).

Because of the link between carbamylation and smoking or renal failure, we analyzed the association between smoking and renal failure with the concentration of anti-CarP antibodies (9). Importantly, renal function and smoking status were not associated with anti-CarP antibody levels. This lack of association suggests that anti-CarP antibodies in patients with early arthritis are not only affected by cardiovascular failure, renal failure or the carbamylation process but, in predisposed patients, are mainly affected by an inability to handle an immunological conflict.

A novel antibody that could be used to diagnose RA is of interest if it outperforms or completes the existing test, i.e., the association of RF and ACPA. In the ESPOIR cohort, 357 patients were labeled seronegative (for RF and ACPA). The diagnosis for this type of patient is left unaddressed until the appearance of specific symptoms or antibodies. With one quarter of positivity in seronegative patients, anti-CarP antibodies may be important for diagnosis. Only 2 anti-CarP single-positive patients did not fulfill the ACR criteria at the end of the 8-year follow-up. Scinocca *et al.* (28) found anti-CarP antibodies in 5% of patients with systemic lupus and in 3% of patients with psoriatic arthritis, which was significantly less than the patients with RA ($p<0.005$). However, these antibodies were also found in juvenile patients with idiopathic arthritis, and the prevalence was higher in this population than among patients with ACPA and RF (29). In our study, the prevalence of anti-CarP in causes of arthritis other than RA was similar to ACPA. In the literature, ACPA are also

present in other types of inflammatory rheumatisms, such as psoriatic arthritis or lupus (30). ANA were found to be associated to anti-CarP antibodies but also with ACPA and this could be the reflection of a general dysimmune context. In line with other studies, we suggest that anti-CarP antibodies are highly relevant for the diagnosis and prognosis of RA (24,31). When combined with ACPA and/or RF, anti-CarP antibodies provide high ORs for the RA diagnosis, but they are more interesting when present alone or in the prognosis point of view. This is important in light of the paradigm shift in the management of RA patients. Although in the past, RA was diagnosed when patients presented with fairly established disease, it is now widely acknowledge that early diagnosis and therapeutic intervention is essential to prevent joint damage. Going forward, there is evidence that interventions might occur in the pre-clinical phase of the disease to stabilize the immune system and to ultimately prevent the disease. This, however, puts more weight on reliable biomarkers or combinations that yield very high ORs, which are important in settings with lower pre-test probabilities. Nevertheless, anti-CarP antibody ELISA is at this time for research. Comparisons with other test such as ACPA and RF with more established clinically available cut-offs may ultimately be different and reproducibility studies will have to be performed.

In conclusion, anti-CarP antibodies were present in patients with early arthritis and were associated with more severe clinical and radiographic diseases. Our results suggest the advantage of anti-CarP antibody dosages in association with ACPA and RF in the initial assessment of early arthritis.

REFERENCES

1. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, 3rd, et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum 2010;62:2569-81.
2. Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, Breedveld FC, et al. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. Arthritis Rheum 2000;43:155-63.
3. Nordberg LB, Lillegraven S, Lie E, Aga AB, Olsen IC, Hammer HB, et al. Patients with seronegative RA have more inflammatory activity compared with patients with seropositive RA in an inception cohort of DMARD-naive patients classified according to the 2010 ACR/EULAR criteria. Ann Rheum Dis 2016. doi: 10.1136/annrheumdis-2015-208873.
4. Jantti JK, Kaarela K, Lehtinen KE. Seronegative oligoarthritis: a 23-year follow-up study. Clin Rheumatol 2002;21:353-6.
5. Cantagrel A, Degboe Y. New autoantibodies associated with rheumatoid arthritis recognize posttranslationally modified self-proteins. Joint Bone Spine 2016;83:11-7.
6. Mastrangelo A, Colasanti T, Barbati C, Pecani A, Sabatinelli D, Pendolino M, et al. The role of posttranslational protein modifications in rheumatological diseases: focus on rheumatoid arthritis. J Immunol Res 2015;2015:712490.
7. Strollo R, Ponchel F, Malmstrom V, Rizzo P, Bombardieri M, Wenham CY, et al. Autoantibodies to posttranslationally modified type II collagen as potential biomarkers for rheumatoid arthritis. Arthritis Rheum 2013;65:1702-12.

8. Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, et al. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. Proc Natl Acad Sci U S A 2011;108:17372-7.
9. Shi J, van Veelen PA, Mahler M, Janssen GM, Drijfhout JW, Huizinga TW, et al. Carbamylation and antibodies against carbamylated proteins in autoimmunity and other pathologies. Autoimmun Rev 2014;13:225-30.
10. Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Horkko S, Barnard J, et al. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. Nat Med 2007;13:1176-84.
11. Pruijn GJ. Citrullination and carbamylation in the pathophysiology of rheumatoid arthritis. Front Immunol 2015;6:192.
12. Jiang X, Trouw LA, van Wesemael TJ, Shi J, Bengtsson C, Kallberg H, et al. Anti-CarP antibodies in two large cohorts of patients with rheumatoid arthritis and their relationship to genetic risk factors, cigarette smoking and other autoantibodies. Ann Rheum Dis 2014;73:1761-8.
13. Verheul MK, Shiozawa K, Levarht EW, Huizinga TW, Toes RE, Trouw LA, et al. Anti-carbamylated protein antibodies in rheumatoid arthritis patients of Asian descent. Rheumatology 2015;54:1930-2.
14. Koppejan H, Trouw LA, Sokolove J, Lahey LJ, Huizinga TJ, Smolik IA, et al. Role of anti-carbamylated protein antibodies compared to anti-citrullinated protein antibodies in indigenous north americans with rheumatoid arthritis, their first-degree relatives, and healthy controls. Arthritis Rheumatol 2016;68:2090-8.

15. Martinez G, Gomez JA, Bang H, Martinez-Gamboa L, Roggenbuck D, Burmester GR, et al. Carbamylated vimentin represents a relevant autoantigen in Latin American (Cuban) rheumatoid arthritis patients. Rheumatol Int 2016;36:781-91.

16. Li L, Deng C, Chen S, Zhang S, Wu Z, Hu C, et al. Meta-analysis: diagnostic accuracy of anti-carbamylated protein antibody for rheumatoid arthritis. PLoS One 2016. doi: 10.1371/journal.pone.0159000.

17. Laki J, Lundstrom E, Snir O, Ronnelid J, Ganji I, Catrina AI, et al. Very high levels of anti-citrullinated protein antibodies are associated with HLA-DRB1*15 non-shared epitope allele in patients with rheumatoid arthritis. Arthritis Rheum 2012;64:2078-84.

18. Ajeganova S, Humphreys JH, Verheul MK, van Steenbergen HW, van Nies JA, Hafstrom I, et al. Anticitrullinated protein antibodies and rheumatoid factor are associated with increased mortality but with different causes of death in patients with rheumatoid arthritis: a longitudinal study in three European cohorts. Ann Rheum Dis 2016;75:1924-32.

19. Gan RW, Trouw LA, Shi J, Toes RE, Huizinga TW, Demoruelle MK, et al. Anti-carbamylated protein antibodies are present prior to rheumatoid arthritis and are associated with its future diagnosis. J Rheumatol 2015;42:572-9.

20. Brink M, Verheul MK, Ronnelid J, Berglin E, Holmdahl R, Toes RE, et al. Anti-carbamylated protein antibodies in the pre-symptomatic phase of rheumatoid arthritis, their relationship with multiple anti-citrulline peptide antibodies and association with radiological damage. Arthritis Res Ther 2015;17:25.

21. Stoop JN, Liu BS, Shi J, Jansen DT, Hegen M, Huizinga TW, et al. Antibodies specific for carbamylated proteins precede the onset of clinical symptoms in mice with collagen induced arthritis. PLoS One 2014;9:e102163.
22. Combe B, Benessiano J, Berenbaum F, Cantagrel A, Daures JP, Dougados M, et al. The ESPOIR cohort: a ten-year follow-up of early arthritis in France: methodology and baseline characteristics of the 813 included patients. Joint Bone Spine 2007;74:440-5.
23. Yee A, Webb T, Seaman A, Infantino M, Meacci F, Manfredi M, et al. Anti-CarP antibodies as promising marker to measure joint damage and disease activity in patients with rheumatoid arthritis. Immunol Res 2015;61:24-30.
24. Shi J, van Steenbergen HW, van Nies JA, Levarht EW, Huizinga TW, van der Helm-van Mil AH, et al. The specificity of anti-carbamylated protein antibodies for rheumatoid arthritis in a setting of early arthritis. Arthritis Res Ther 2015;17:339.
25. Shi J, van de Stadt LA, Levarht EW, Huizinga TW, Toes RE, Trouw LA, et al. Anti-carbamylated protein antibodies are present in arthralgia patients and predict the development of rheumatoid arthritis. Arthritis Rheum 2013;65:911-5.
26. Humphreys JH, Verheul MK, Barton A, MacGregor AJ, Lunt M, Toes RE, et al. Anticarbamylated protein antibodies are associated with long-term disability and increased disease activity in patients with early inflammatory arthritis: results from the Norfolk Arthritis Register. Ann Rheum Dis 2016;75:1139-44.
27. Ajeganova S, van Steenbergen HW, Verheul MK, Forslind K, Hafstrom I, Toes RE, et al. The association between anti-carbamylated protein (anti-CarP) antibodies and radiographic progression in early rheumatoid arthritis: a study

exploring replication and the added value to ACPA and rheumatoid factor. Ann Rheum Dis 2017;76:112-8.

28. Scinocca M, Bell DA, Racape M, Joseph R, Shaw G, McCormick JK, et al. Antihomocitrullinated fibrinogen antibodies are specific to rheumatoid arthritis and frequently bind citrullinated proteins/peptides. J Rheumatol 2014;41:270-9.

29. Muller PC, Anink J, Shi J, Levarht EW, Reinards TH, Otten MH, et al. Anticarbamylated protein (anti-CarP) antibodies are present in sera of juvenile idiopathic arthritis (JIA) patients. Ann Rheum Dis 2013;72:2053-5.

30. Aggarwal R, Liao K, Nair R, Ringold S, Costenbader KH. Anti-citrullinated peptide antibody assays and their role in the diagnosis of rheumatoid arthritis. Arthritis Rheum 2009;61:1472-83.

31. Lopez-Hoyos M, Alvarez-Rodriguez L, Mahler M, Torices S, Calvo-Alén JV, I., Seaman A, et al. Anti-carbamylated protein antibodies in patients with ageing associated inflammatory chronic disorders. Rheumatology 2016;55:764-6.

Tables

Table 1: Demographic characteristics of the 720 patients from the ESPOIR cohort who were included in the study.

	ESPOIR cohort (n=720)
Age, years, mean (SD)	48 (12)
Sex, female, n (%)	547 (76)
Duration of symptoms, days, median (IQR)	147 (124,25)
BMI, mean (SD) (n=718)	25 (4.6)
DAS28-ESR, mean (SD) (n=708)	5.1 (1.3)
IgM-RF positive, n (%) (n=711)	297 (41.8)
ACPA positive, n (%) (n=711)	273 (38.4)
IgM-RF negative and ACPA negative, n (%) (n=711)	356 (50.1)

SD, standard deviation; IQR, interquartile range; BMI, body mass index; IgM-RF, IgM rheumatoid factor; ACPA, anti-cyclic citrullinated peptide antibodies

Table 2: Clinical performance for the diagnosis of RA according to 2010 criteria.

	ACPA	*RF*	*Anti-CarP*	*RF or ACPA*	*Any two*	*All three*
Sensitivity (95% CI)	*50% (46.1 – 54)*	*52.7% (48.7 – 56.6)*	*34% (30.3 – 37.8)*	*60.5% (56.5 – 64.3)*	*49.4% (45.4 – 53.3)*	*17.6% (14.7 – 20.8)*
Specificity	*91% (80.4 – 96.4)*	*98% (91.5 – 99.9)*	*95% (86.7 - 99)*	*88.9% (78.4 - 95.4)*	*98.4% (91.5 – 99.9)*	*100% (94.3 – 100)*
Likelihood ratio +	*5.26 (2.45 – 11.3)*	*33.18 (4.7 – 232.2)*	*7.14 (2.35 – 21.7)*	*5.44 (2.7 – 11)*	*31.1 (4.4 – 217.7)*	*NE*
Likelihood ratio -	*0.55 (0.49 – 0.62)*	*0.48 (0.44 – 0.53)*	*0.69 (0.64 – 0.75)*	*0.44 (0.39 – 0.51)*	*0.51 (0.47 – 0.56)*	*0.82 (0.79 – 0.85)*
Odds ratio	*9.5*	*69*	*10.3*	*12.2*	*60.4*	*NE*

(4 – 27)	*(11.7 – 2776)*	*(3.3 – 51.9)*	*(5.4 – 32.2)*	*(10.3 – 2433)*

CI=confidence interval, NE=not evaluable

Figure legends

Figure 1: Distribution of anti-CarP antibodies as measured by ELISA in the early arthritis ESPOIR cohort (n=711). (A)(B) Anti-CarP-FCS antibody distribution according to the presence of anti-citrullinated peptide antibodies (ACPA) or rheumatoid factor (RF), respectively. Percentages (%) of patients in each subset are shown in brackets. (C)(D) Contingency histogram showing the odds of being anti-CarP+ when presenting with ACPA or RF, respectively. (E) Distribution of seropositivity RF, ACPA and anti-CarP. Each circle represents a different autoantibody, and numbers represent the percentage of subjects that were positive for the autoantibody.

FR, rheumatoid factor; ACPA, anti-cyclic citrullinated peptide antibodies; CarP, anti-carbamylated protein antibodies.

Figure 2: Baseline clinical and biological profiles of anti-CarP+ patients in the ESPOIR cohort. (A) Activity of RA at baseline as assessed by DAS28-ESR according to the anti-CarP antibody status (unpaired t-test). (B) Risk of being in a low-value DAS28-ESR or a high-value DAS-28-ESR according to the anti-CarP antibody status (Fisher's exact test). (C) Correlation between the DAS28-ESR at baseline and the level of anti-CarP antibody (Pearson test). (D) Activity of RA assessed by the DAS28-ESR at baseline according to a multiparameter antibody status (RF/ACPA/anti-CarP) (ANOVA). (E) Risk of having a high ESR (>10 mm) based on the anti-CarP antibody status (Fisher's exact test). (F) Risk of having a positive CRP (>5 mg/l) according to the anti-CarP antibody status (Fisher's exact test). A value of $p<0.05$ was considered significant. *<0.05, **<0.01, ***<0.001

ESR, erythrocyte sedimentation rate; FR, rheumatoid factor; ACPA, anti-cyclic citrullinated peptide antibodies; CarP, anti-carbamylated protein antibodies, AU, arbitrary Unit.

Figure 3: Evolution of early arthritis based on anti-CarP antibody levels among patients from the ESPOIR cohort. (A) DAS28-ESR evolution between M0 and M96 in anti-CarP+ and anti-CarP- populations (unpaired t-test). (B) Activity of RA assessed by the DAS28-ESR at M36 according to the multiparameter antibody status (RF/ACPA/anti-CarP) (ANOVA). (C) Activity of RA assessed by the DAS28-ESR at M96 according to the multiparameter antibody status (RF/ACPA/anti-CarP) (ANOVA). (D) Risk of having an erosive form of the disease at M96 according to the anti-CarP antibody status (Fisher's exact test). (E) Sharp score comparison at M0 and M36 in anti-CarP+ and anti-CarP- patients (unpaired t-test). The white and black bars show anti-CarP- and anti-CarP+, respectively. (F) Anti-CarP antibody level (AU) in erosive and non-erosive forms of arthritis at M96 (unpaired t-test). (G) Radiological evolution of the disease as assessed by the Sharp score at M36 according to the multiparameter antibody status (RF/ACPA/anti-CarP) (ANOVA). A value of $p<0.05$ was considered significant. *<0.05, **<0.01, ***<0.001

ESR, erythrocyte sedimentation rate; FR, rheumatoid factor; ACPA, anti-cyclic citrullinated peptide antibodies; CarP, anti-carbamylated protein antibodies, AU, arbitrary Unit.

Figure 4: Therapeutic intervention based on the anti-CarP status. (A) The likelihood of being treated with DMARD during the 8-year follow-up according to the anti-CarP status at baseline (Fisher's exact test). (B) Likelihood of being treated with

a biological agent during the 8-year follow-up according to the anti-CarP status at baseline (Fisher's exact test). (C) Likelihood of being treated with a biological agent as a first line therapeutic during the 8-year follow-up according to patients' anti-CarP levels at baseline (Fisher's exact test). (D) Anti-CarP antibody level (AU) in patients who received at least one biological agent during the 8-year follow-up (unpaired t-test). A value of $p<0.05$ was considered significant. *<0.05, **<0.01

ESR, erythrocyte sedimentation rate; FR, rheumatoid factor; ACPA, anti-cyclic citrullinated peptide antibodies; CarP, anti-carbamylated protein antibodies, AU, arbitrary Unit.