Anti-carbamylated protein antibodies as a clinical response predictor in rheumatoid arthritis patients treated with abatacept

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Abstract

Objective

Carbamylation is an irreversible post-translational modification of proteins. The presence of anti-carbamylated protein antibodies (anti-CarP) has been observed in rheumatoid arthritis (RA). This study was focused to verify whether anti-CarP antibodies can be used as a predictive factor of clinical response to abatacept (CTLA4-Ig) in RA patients.

Methods

Sixty RA patients treated with abatacept were enrolled. A home-made ELISA for anti-CarP and a commercial anti-CCP3.1 kit for anti-citrullinated proteins antibodies (anti-CCP) were applied to determine serum levels every six months of therapy. Rheumatoid factor (RF) was also tested.

Results

Anti-CarP positive patients (n=18) were younger (p=0.01) and with a longer disease duration (p=0.05) when compared to anti-CarP negative patients (n=42) at baseline. Considering the entire cohort, a significant reduction of anti-CarP titre after twelve-months of treatment was shown (p<0.01). A significant reduction of Disease Activity Score (DAS) 28-C-reactive protein (CRP) in the first six months of therapy was found in the subgroup of anti-CarP positive patients in comparison with the negative ones (p=0.003). No significant results were found by dividing the cohort using the positivity to anti-CCP and/or RF.

Conclusion

Earlier onset and a longer disease duration in anti-CarP positive patients might suggest they are specific risk factors for RA in this subgroup of patients. The correlation between the anti-CarP positivity at baseline and the reduction of disease activity during the first six months of treatment with abatacept allowed us to hypothesise that anti-CarP antibodies, but not anti-CCP and/or RF, could be used as a good clinical response predictor.

Key words

anti-carbamylated protein antibodies, rheumatoid arthritis, abatacept, biomarkers

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Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease. Genetic and environmental factors are involved in its pathogenesis and autoantibodies positivity were found in the majority of patients (1). Anti-cyclic citrullinated peptide antibodies (anti-CCP), autoantibodies that specifically target proteins containing peptidyl-citrulline and rheumatoid factors (RFs), antibodies against epitopes on the Fc portion of immunoglobulin G (IgG), are the defined biomarkers of RA with diagnostic and prognostic values (2). Compared with those with seronegative RA, patients who have a persistently positive test for anti-CCPs and/or RFs are likely to have more erosion of bones and joints and more extraarticular manifestations (2). After specific post-translational modifications (carbamylation and the well-known citrullination) altered peptides bind MHC proteins enhancing specific autoimmune responses in RA (3). Carbamylation is a non-enzymatic process involving the addition of a cyanate group on self-proteins in which a lysine is converted into a homo-citrulline in the tertiary structure (4). Inflammation can enhance this process through a mechanism which involves myeloperoxidase contained in neutrophil granules. Furthermore, a recent study, demonstrated that direct or indirect exposure to tobacco smoke could have an effect by inducing carbamylation, which could trigger a pathogenic B cell response (5). It was recently demonstrated in one study that 16% of anti-CCP negative RA patients had a positivity for anti-carbamylated proteins autoantibodies (anti-CarP), showing a more severe disease course than that of seronegative RA (4). Abatacept (ABA) is a soluble fusion protein that consists of the extracellular domain of human CTLA4 linked to the modified Fc portion of human IgG1 (CTLA4-Ig) and acts as lymphocyte co-stimulation blocker. It is used as a biological disease modifying drugs in the treatment of RA (6). The post-hoc analysis of clinical trials demonstrated that in biologic-naïve patients with RA, the positivity of RF and/or anti-CCP was associated with greater efficacy of intravenous ABA than seronegative status (7). No data are available about the possible value of anti-CarP in the prediction to ABA response. Few data are available about the variations of anti-CarP titre during the course of the disease and after therapies (8). The aim of this study is to evaluate RA autoantibodies in a monocentric cohort of 60 RA patients treated with ABA and to verify whether anti-CarP antibodies can be used as a predictive factor of clinical response.

Patients and methods

Patients

Sixty people affected by RA, defined by the 2010 ACR/EULAR criteria (9), treated with ABA at the Rheumatology Unit of ASST Spedali Civili of Brescia, were enrolled in the study. Sera were collected from all patients at the first administration of ABA (T0), from 40 patients after 6 months (T6) and from 24 after 12 months (T12). Clinical data were obtained from clinical charts. The clinical status of the 60 patients after six months from the first administration of ABA evolved as follows: 2 patients were lost at follow-up; 1 patient interrupted therapy due to the occurrence of a side effect; 4 patients suspended the drug for primary inefficacy; 53 patients reached the timing T6 and only 24 samples are available for the timing T12. Fourteen patients were non-responders, accordingly with the EULAR response criteria (10). The clinical disease activity and the response to the treatment were evaluated with the Disease Activity Score (DAS) 28-C-reactive protein (CRP). Health assessment questionnaire (HAQ) was subjective state of health evaluation from patients (11). The study was approved by the local institutional ethical committee (approval number 2495) and conducted in accordance with the Declaration of Helsinki. Patients's written informed consent to publish the material was obtained.

Serum analysis

Serum samples were collected before the first administration of ABA, then after 6 months and 12 months and stored at -80°C until used. Serum laboratory analysis was performed at the end of the study in order to evaluate

antibody titres of anti-CarP IgG, anti-CCP IgG, FR and high sensitivity CRP (hs-CRP) levels.

Generation of carbamylated antigens
Fetal calf serum (FCS, Sigma Aldrich) was used to generate the carbamylated protein antigens. Briefly, 15 mg/ml of FCS was diluted in H_2O to the concentration of 4 mg/ml and then potassium cyanate was added to reach the final concentration of 1M. Then, the solution was incubated for 12h at 37°C, afterwards it was dialysed exhaustively against H_2O for 5 days, changing the H_2O every day. It was later conserved at -80°C till used.

Anti-CarP levels
A home-made IgG ELISA test was performed according to one which is already described in the literature (4), but with some minor modifications. In short, after the overnight coating with 10 μg/ml of antigen in 0.1M $Ca(CO^3)^2$ and 0.1 M $Ca(HCO^3)^2$ (pH 9.6 at 4°C), the ELISA plate was then washed and blocked with PBS-BSA 1% for 2 hours at room temperature (RT). The following incubations were done at RT for 2 hours, till the addition of substrate. The development reaction was done using alkaline phosphatase diluted in di-ethanolamine and incubated at 37°C. The absorbance was read at 405 nm with ELISA plate reader and the final reading was performed when the positive control reached the optical density (OD) of 1.500. Finally, to set up the method, we initially tested sera of 230 healthy controls and the cut-off value of 0.350 OD was established calculating the mean plus 3 times standard deviation which was similar to the 99th percentile. Later, the ROC curve analysis was also performed to evaluate the specificity and sensitivity of the method at the cut-off value. The area under curve (AUC) was 0.854±0.028; p<0.01; specificity of 0.98 and sensitivity of 0.32, with likelyhood ration of 13.6 (Fig. 1). Then, the obtained values were converted into arbitrary units per millilitre (corresponding to 340 AU/ml).

Anti-CCP levels
The analysis was performed using the QUANTA Lite® CCP3.1 IgG/IgA ELISA (Inova Diagnostics) kit, using a EUROIMMUNE automated analyzer to perform ELISA tests according to the manufacturer's instructions. The upper limit of normal (ULN; 20 U/ml) was set in accordance with the manufacturer's recommendations. Serum samples that showed high concentration (>250 U/ml) were evaluated after further dilutions (1/8 and, when necessary, 1/16) and then corrected for these additional dilution factors.

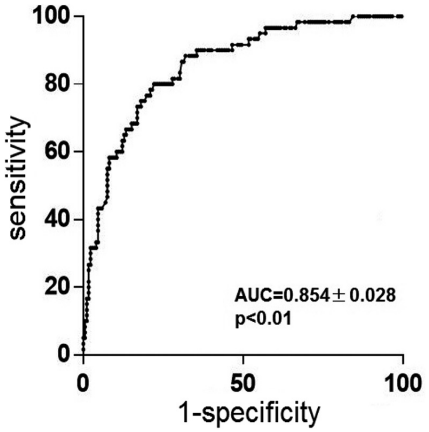

Fig. 1. Receiving-operating characteristic (ROC) analysis of anti-CarP IgG home-made ELISA.

RF and hs-CRP levels
To determine RF and hs-CRP plasma levels, peripheral blood was collected in lithium-heparin tube and concentrations were measured using the nephelometric system for RF (upper limit of normal=14 KIU/L) and the immunoassay technique for hs-CRP, used routinely in the central laboratory of our hospital. All exams were carried out in Dimension_VistaTM 1500 analyzer® (Siemens Diagnostics) according to the manifacturer's instructions. For hs-CRP: if the values were high (>10 mg/L), indicating an acute inflammatory process, the measurement was discarded or repeated. The instruments were calibrated against appropriate proprietary reference standard material and verified by using the registered quality controls.

Statistical analysis
Data are expressed as the mean (±SD) or median [IQR]. The comparison between unpaired quantitative variables among different groups was performed by Students' *t*-test and Mann-Whitney U-test as appropriated. Paired data were analysed using Wilcoxon matched pairs test. The correlation amongst different groups was evaluated with the linear simple regression. The association between nominal variables was assessed with chi square test with Yates's correction or Fisher's exact test. A p<0.05 was considered statistically significant.

Results

Clinical and demographical features of patients at baseline
Sixty RA patients (age (years)=57±12.1, follow-up period (months)=129.7±105, DAS28-CRP at baseline=4.59±0.99, HAQ at baseline=0.80 [0.12–1.19]) were enrolled. Dividing patients according with gender, no significant differences were found among demographical, clinical and serological features, except for HAQ at baseline which was significantly increased in female (1±0.78 *vs*. 0.43±0.44), p<0.05). At baseline, almost all patients had concomitant therapies; 56 (93%) patients took disease-modifying anti-rheumatic drugs (DMARDs) and 53 (88%) were assuming prednisone.

Prevalence of autoantibodies in RA patients at baseline
As expected, at baseline, most of the patients were positive for the classical disease bio-markers. In fact, out of 60 patients, 51 (85%) were positive for anti-CCP and 35 (58.3%) for RF. Nevertheless, 18 (30%) were positive for anti-CarP antibodies and the 25% of seronegative patients (negative for RF and/or anti-CCP) were anti-CarP positive. As anti-CarP antibodies were the aim of our study, further analyses were done by sub-grouping the patients in anti-CarP positive or negative groups. Interestingly, it revealed significant differences among the following characteristics at T0; age: anti-CarP positive subjects were younger at the beginning of the therapy than the anti-CarP negative patients (p<0.01); disease duration: the anti-CarP positive patients have on average a longer course of the disease (p<0.05); hs-CRP: anti-CarP positive patients have higher CRP levels at baseline (p<0.05) (Table I).

Table I. Baseline demographic, serological and clinical features of 60 RA patients divided according to anti-CarP autoantibodies positivity.

Feature at baseline	Anti-CarP positive n=18	Anti-CarP negative n=42	*p*-value
Gender, M/F	4/14	7/35	NS
Age (years), mean±SD	48 ± 14.10	57.40 ± 12.10	$p<0.01$
Weight (kg), mean±SD	64.90 ± 13.20	63.40 ± 16.20	NS
BMI (kg/m^2), mean±SD	24.39 ± 5.10	23.68 ± 5.13	NS
Disease duration (months), mean±SD	157.67 ± 123	117.70 ± 96	$p<0.05$
DAS28-CRP, mean±SD	4.85 ± 1.16	4.48 ± 0.92	NS
RF positivity, n (%)	13 (72%)	22 (52%)	NS
Anti-CCP positivity, n (%)	16 (89%)	35 (83%)	NS
hs-CRP (mg/L), median [IQR]	10.4 [6.13–23.80]	4.87 [1.68–9.90]	$p<0.05$
ESR (mm/h), median [IQR]	30.5 [18–36.50]	17 [11–31]	NS
DMARDs, n (%)	17 (95%)	39 (93%)	NS
Steroid, n (%)	16 (89%)	37 (88%)	NS
ABA as first line bDMARDs, n (%)	10 (55%)	23 (55%)	NS
HAQ test, median [IQR]	0.80 [0.12–1.19]	0.70 [0.37–1.38]	NS
VAS pain, median [IQR]	80 [61–90]	60 [50–70]	NS

BMI: body mass index; DAS-28: Disease Activity Score; CRP: C-reactive protein; RF: rheumatoid factor; Anti-CCP: Anti-cyclic citrullinated peptide antibodies; hs: high sensitivity; ESR: erythrocyte sedimentation rate; DMARDs: disease-modifying anti-rheumatic drugs; bDMARDs: biological disease-modifying anti-rheumatic drugs; HAQ: Health Assessment Questionnaire; VAS: visual analogue scale; NS: not significant.

Table II. Mean values of antibody titre and hs-CRP at T0, T6 and T12 from the beginning of therapy with ABA.

	T0 n=60	T6 n=40	T12 n=24
Anti-CarP, mean ± SD (OD)	0.33 ± 0.26	0.27 ± 0.21	0.25 ± 0.26
Anti-CCP, mean ± SD (AU/ml)	783.10 ± 1062	610.10 ± 1208	366 ± 978.50
RF, mean ± SD (UI/ml)	95.82 ± 141.90	65.91 ± 101	84.04 ± 142
hs-CRP, mean ± SD (mg/L)	9.14 ± 9.51	4.49 ± 5.26	3.68 ± 3.95

Anti-CarP: anti-carbamylated proteins autoantibodies; Anti-CCP: Anti-cyclic citrullinated peptide antibodies; RF: rheumatoid factor; hs-CRP: high sensitivity C-reactive protein.

Analysis of variations of concomitant therapies over time

The 93% of patients assumed a concomitant DMARD (87% of them took methotrexate and the left 13% leflunomide) at beginning of ABA therapy and the 88% took prednisone. The dosage of leflunomide remained stable, while the dosage of methotrexate changed slightly over time (T0 *vs*. T6 *vs*. T12, 11±7 *vs*. 9±7 *vs*. 10 ±7 mg/week). These variations were not statistically significant (T0 *vs*. T6, $p=0.12$; T0 *vs* T12, $p=0.12$; T6 *vs*. T12, $p>0.99$). Prednisone dosage decreased significantly over time (T0 *vs*. T6 *vs*. T12, 33±36 *vs*. 17±14 *vs*. 15±15 mg/week; T0 *vs*. T6, $p<0.01$; T0 *vs*. T12, $p<0.01$; T6 *vs*. T12, $p=0.22$).

Levels of autoantibodies during follow-up

We evaluated autoantibodies titres in sera for every time points during follow-up. The results showed a significant reduction in the anti-CarP titre (Fig. 2A) at the T6 and T12 timepoints compared to T0 ($p<0.001$). Similarly, hs-CRP levels were decreased in patients at T6 ($p<0.001$) and T12 ($p=0.01$) in comparison with baseline (Fig. 2D). By contrast, no significant changes were observed for anti-CCP autoantibodies (T0 *vs*. T6, $p=0.49$; T0 *vs*. T12, $p=0.47$; T6 *vs*. T12, $p=0.41$) and RF (T0 *vs* T6, $p=0.44$; T0 *vs* T12, $p=0.34$, T6 *vs*. T12, $p=0.13$) (Fig. 2B-C). All values (mean±SD) are shown in Table II.

DAS28-CRP variations during follow-up

Subsequently, the distribution of the DAS28-CRP clinical index value was evaluated at different timepoints. During treatment with ABA, DAS28-CRP decreased significantly after 6 and 12 months from the start of the therapy ($p<0.01$). Moreover, it was further decreased between 6 and 12 months ($p=0.03$) (Fig. 3A).

Association of autoantibodies and δ-DAS28 or the EULAR response criteria

For further analysis, we used the variation of DAS28-CRP values between timepoints T0 and T6 (δ-DAS28). Firstly, we sub-grouped our cohort according with positivity and negativity for different autoantibodies (anti-CarP, anti-CCP, RF). Then, we calculated the δ-DAS28 in each patient of these groups. The values of δ-DAS28 were significantly higher in the anti-CarP positive patients ($p<0.01$) (Fig. 3B), whereas, other subgroups did not show any significant differences; anti-CCP ($p>0.1$) and RF ($p=0.86$). This allowed us to make an association between the δ-DAS28 value and the levels anti-CarP autoantibodies in each patient. The linear correlation between the two showed that higher values of anti-CarP were directly correlated to the higher δ-DAS28 (Spearman r=0.433, 95% CI=0.176–0.634; $p<0.01$). No associations were found between EULAR clinical response and autoantibodies positivity (RF, anti-CCP or anti-CarP).

Discussion

Anti-carbamylated protein antibodies (anti-CarP) were recently evaluated in patients with autoimmune arthritis. Currently, only RFs and anti-CCPs are included in the 2010 classification criteria for RA. In fact, a diagnostic classification did not improve by adding anti-CarP testing as RFs and anti-CCPs are already good predictor for disease (12, 13). Nevertheless, it was calculated that the anti-CarP positivity can be found in about 30% of seronegative RA patients (4). The predictive value of these antibodies was demonstrated in patients with arthralgia (14, 15) as well as in different cohort of RA patients with early disease and, in particular, in association with more active forms of disease (16). Detectable levels of anti-CarP antibodies were also found in healthy first-degree relatives of RA patients which represented a good model to study the

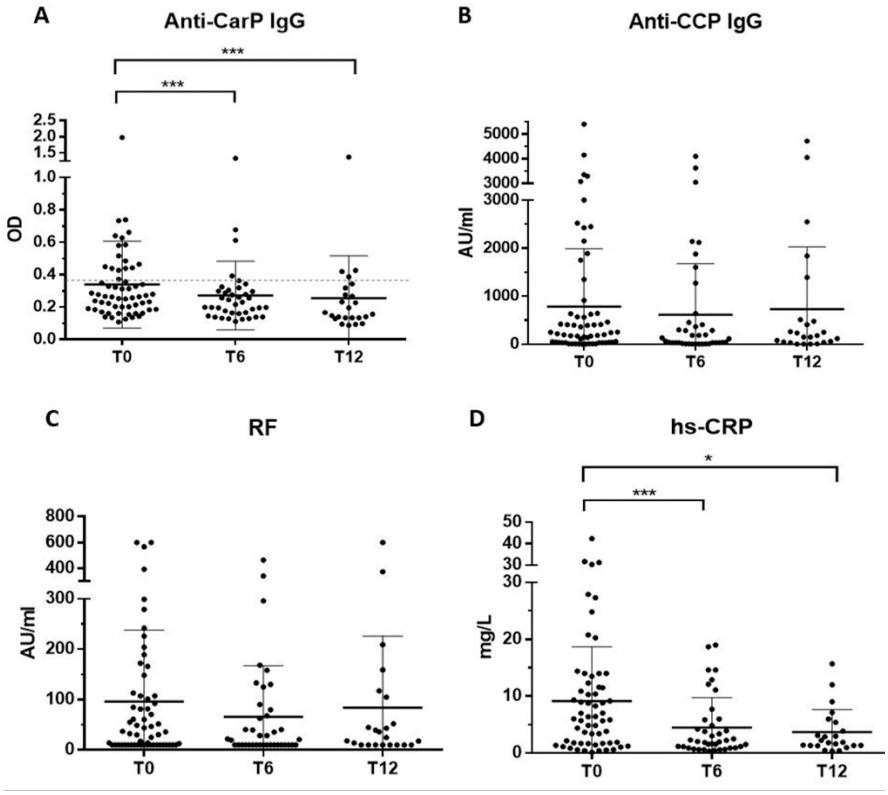

Fig. 2. Antibody titres at the beginning (T0), after 6 months (T6) and after 12 months (T12) of therapy with abatacept.
A: Anti-carbamylated proteins autoantibodies (Anti-CarP) IgG;
B: Anti-cyclic citrullinated peptide antibodies (Anti-CCP) IgG;
C: RF: rheumatoid factor IgG;
D: high sensitivity C-reactive protein (hs-CRP) levels. **p*-value <0.05; ****p*-value <0.01.

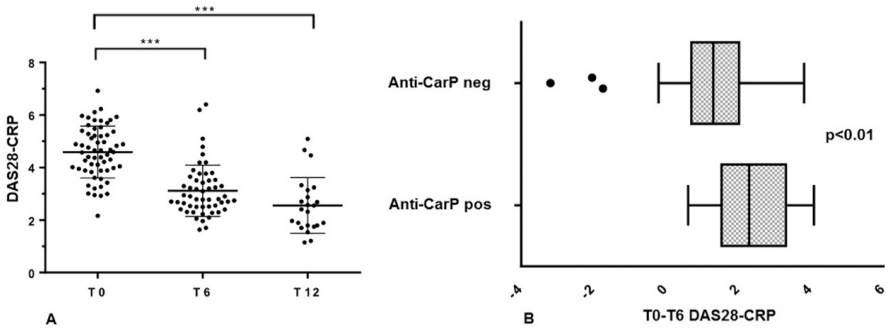

Fig. 3. Disease Activity Score (DAS) 28-C-Reactive Protein (DAS28-CRP) index values during the therapy with abatacept (**A**) and DAS28-CRP index variation after six months correlated with the initial positivity and negativity to anti-carbamylated proteins autoantibodies (Anti-CarP) (**B**); ****p*-value <0.01.

preclinical phases of autoimmune diseases (17). Furthermore, lots of studies showed a link between the presence of these antibodies and the development of erosions with a worse clinical course (4, 16, 18), suggesting also a prognostic significance.

In the present study, a prevalence of anti-CarP was found in 30% of our cohort of RA patients at the beginning of ABA treatment. Our results showed that the anti-CarP positive patients had a significantly higher hs-CRP values at baseline, lower mean age and a longer disease duration, compared to anti-CarP negative patients. This suggests that there is an ongoing active systemic inflammation in our anti-CarP positive patients which is in accordance with the observation that a distinct more inflammatory phenotype at disease onset of early arthritis is associated to anti-CarP antibodies, reported in different studies (16, 19). The early onset and consequent longer disease course could consolidate the hypothesis for which the presence of anti-CarP is associated to RA with atypical risk factors yet not known, compared to those classical such as anti-CCP and RF (20). The factors associated with anti-CarP antibody could support an earlier pathological onset leading to a more abrupt loss of tolerance.

We did not find any significant differences in DAS28-CRP value in the two groups at baseline, differently from other studies where an association between anti-CarP antibodies and DAS28 or HAQ levels were demonstrated (21). In our cohort, it was observed that HAQ was significantly higher while DAS28 also showed a trend to be higher in women, supporting the hypothesis of a higher disease activity compared to males. Accordingly, higher DAS28 values in female have also been reported in literature (22).

During the course of treatment with ABA, we observed a reduction of anti-CCP and RF titres, though not significant, confirming results of previous studies (23, 24). By contrast, for the first time, we recorded a significant reduction in anti-CarP antibody titres at 6 and 12 months from the start of the therapy. Moreover, both the hs-CRP and the DAS28-CRP clinical index values decreased significantly along with the reduction of anti-CarP antibodies. Taken together, these data suggest that ABA might modulates the levels of anti-CarP by acting on co-stimulatory pathway. In fact, direct and indirect effects of ABA treatment on T and B lymphocytes compartment are well demonstrated (24, 25). These findings confirm the reduction of signs of polyclonal B cell activation in RA patients treated with ABA who showed a general clinical improvement (25). Furthermore, the clinical index variation (DAS28-CRP) after 6 months of therapy, when compared to individual markers at the beginning of the therapy, showed a better clinical response in anti-CarP positive in comparison with anti-CarP negative patients, suggest-

ing a possible role of these antibodies as clinical response predictors to ABA in RA patients. Conversely, anti-CCP and RF did not show significant difference between the positive and negative subgroups when evaluated for DAS28 change. However, it must be emphasised that other studies have reported a better response to ABA treatment in anti-CCP+ patients at baseline (26) and that the positivity for anti-CCP and RF at baseline is associated with a higher retention rate (27). This difference in our results might be explained due to the small study size, which could create bias for the statistical significance. In spite of this, our study shows that anti-CarP is a stronger predictor of a clinical response in comparison with anti-CCP and/or RF. Furthermore, the fact that anti-CarP autoantibodies were the only autoantibodies detected in animal models of collagen-induced arthritis and their titres decreased after ABA therapy but not during tocilizumab (interleukin-6 receptor antagonist), suggest intrinsic differences among anti-CarP autoantibodies and the other biomarkers, and a possible more effective action of ABA at this level (28).

A recent published study suggests that any changes in autoantibody levels in RA patients are not associated with treatment response but only reflects intensity of immunosuppression (29). The analysis of the possible variations of concomitant therapies in our cohort resulted only in a significant reduction in prednisone weekly cumulative dosage, which reflects less immunosuppression, without any variations of the dosage of other concomitant DMARDs. This was due to a general clinical improvement with a subsequent reduced need of steroids. Therefore, in our study the change in intensity of immunosuppression was mainly due to the effect of ABA and is only associated with the significant variation of anti-CarP antibody titre while it does not affect the anti-CCP and RF titres over time.

Conclusion

Actually, nothing is known about the variation of anti-CarP antibody with other conventional synthetic or biologic DMARDs (bDMARDs) with different mechanism of action. The present study is a first report in this field. Nevertheless, our study has some limitations. Firstly, we assessed the variations of autoantibodies titre with only one bDMARDs with a peculiar mechanism of action. Secondly, although we have found a substantial stability of both anti-CCP and RF titres, it is possible that the number of patients enrolled in this study is insufficiently powered to demonstrate statistical significance. Furthermore, in the present study anti-CarP IgA were not analysed, even if some authors (4) demonstrated that there were patients positive for anti-CarP IgA in association with more joint destruction in comparison with seronegative ones. The strengthen of this association was lower if compared with the correlation between anti-CarP IgG seropositivity and severe disease progression (4). Strengths of this study include the first attempt to correlate variations of different biomarkers during ABA treatment in a real-life scenario, through the longitudinal analyses on the three major serological markers of RA and with disease activity measures and high-sensitive inflammation parameters.

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