IgA anti-citrullinated protein antibodies (IgA ACPA) are associated with flares during DMARD tapering in rheumatoid arthritis

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Keywords: rheumatoid arthritis, ACPA, Autoimmunity, Remission, Immunoglobulin, IgA.

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Key messages:
- IgA ACPA are associated with a higher flare risk during DMARD tapering
- IgA2 ACPA levels declined in patients in ongoing remission but not in those who flared
- IgA2 ACPA are associated with the severity of flares during DMARD tapering

Abstract.

Objectives. A substantial proportion of rheumatoid arthritis (RA) patients flares upon withdrawal of disease modifying anti-rheumatic drugs (DMARDs), thus the definition of prognostic markers is crucial. Anti-citrullinated protein antibody (ACPA)-positivity has been identified as a risk factor for flare. However, only the role of IgG is established in this context, while the role of IgA ACPA is poorly defined. We thus aimed to investigate the role of IgA ACPA in flare of RA.

Methods. Serum levels of IgA1 and IgA2 ACPA at baseline and after 12 months were measured in 108 patients from the randomized controlled RETRO study. RA patients in stable remission for at least 6 months at study recruitment were assigned to either one of the DMARD tapering arms or to continuation of DMARDS.

Results. In patients remaining in remission but not in the ones who flared, IgA2 ACPA levels and proportion of IgA2 in ACPA (ACPA IgA2%) significantly declined (median of 17.5%; p<0.0001). This seemed to be independent of the treatment choice, as there was no difference in IgA2 ACPA dynamics between the study arms. ACPA IgA2% was associated with disease activity (DAS28) at flare (r=0.36; p=0.046). IgA and IgG ACPA showed a tendency towards independent contribution to the risk of flare with the highest risk if a patient had both antibody classes.

Conclusion. In this study, IgA ACPA was identified as a risk factor for flare in combination with IgG ACPA. IgA2 ACPA levels were associated with flare severity and declined in patients in stable remission.

Introduction

Rheumatoid arthritis (RA) is a chronic progressive inflammatory disease with an autoimmune pathogenesis. With the constant development in treatment options and progress in the understanding of disease mechanisms, DMARD-free remission has become an ambitious goal in the management of RA. To achieve this, it is important to find risk factors for flare during DMARD withdrawal in RA patients in stable remission [1,2]. Currently, the rates of achieving DMARD-free remission among RA patients are in the range of 5 to 24%. Sustained remission rates (longer than 12 months) in different studies are estimated under 20% [2]. Therefore, although DMARD tapering is suggested in the recommendations
the European Alliance of Associations for Rheumatology (EULAR) as an opportunity for patients in stable remission, there is still need for much deeper understanding of target patient populations and of factors increasing relapse risks.

About 60-70% of RA patients have autoantibodies against citrullinated peptides (anti-citrullinated protein antibodies, ACPA) [3–5]. Of note, ACPA-positivity has been shown to act as a negative prognostic factor for keeping remission during DMARD tapering [6,7]. Despite substantial research about the role of ACPA on disease activity, still little is known about the functions, origins and clinical significance of distinct ACPA subclasses. IgA and IgM ACPA were shown to be present in a large proportion of IgG ACPA-positive RA patients [8,9]. In our previous study we noticed that IgA ACPA contain a higher percentage of IgA2 and an association between IgA2 ACPA and RA disease activity score (DAS)-28 was observed [10]. Moreover, IgA2 has been shown to exhibit more pro-inflammatory functions, including stimulation of neutrophils and monocytes, compared to IgA1, which is more of anti-inflammatory nature [10,11]. Considering these data, it was of interest to investigate IgA ACPA subclasses in the context of flare prediction in RA patients in sustained remission tapering DMARDs.

Methods.

RETRO study design.

RETRO was a multicenter, prospective, randomized controlled open trial (EudraCT 2009-015740-42). The study aimed to investigate and compare the efficacy of two tapering strategies in patients with RA in stable remission (DAS28-ESR<2.6 for a minimum of 6 months): the reduction of DMARDs by 50% (arm 2) and the reduction of DAMRDs by 50% during the first 6 months with a subsequent complete discontinuation of the drugs (arm 3) versus the continuation of treatment (arm 1) [4]. Recruited patients were receiving either conventional synthetic (cs) DMARDs and/or biological (b) DMARDs. The study duration was 12 months, during which the patients were followed up by a rheumatologist at four consequential visits, every 3 months. Flares were defined as DAS28-ESR-score exceeding 2.6 at at least one of the visits (regular visits or during flare). Additional details on the RETRO study design can be found in the main study report [4]. The study was approved by the ethic committee of the Friedrich-Alexander-University of Erlangen-Nuremberg, local ethic committees of the external centers and by the Paul Ehrlich Institute (PEI) and was conducted according to the ethical principles of the Declaration of Helsinki.

Serum samples.
By the time of the measurement initiation, 153 patients had completed the 12-month study period. Of those, serum was available from 127 patients. For further analysis, 19 patients were excluded either due to substantial differences between technical replicates; unclear flare status or an elevated DAS28 score due to elevated erythrocyte sedimentation rate (ESR) for reasons other than arthritis flare. In total, 108 patients were included in the analysis. Serum samples from the last visit were not available from 18 patients. Therefore, these patients had to be excluded in figures 3A (n=90). Figure 3B is representing only patients who flared with detectable IgA ACPA levels (>2 µg/ml; n=32). Figure 4B shows fold change in patients with serum available from the last visit and detectable IgA ACPA (>2 µg/ml; n=26 in arm 1; 26 in arm 2; 21 in arm 3).

**Total IgA and IgA ACPA concentrations measurement.**

Prior to the analysis, sera were stored at -80°C. Total serum IgA and IgA2 levels, as well as levels of ACPA of IgA and IgA2 subclasses were measured by enzyme-linked immunosorbent assay (ELISA). IgA1 levels both for total serum IgA and for ACPA were calculated by subtracting IgA2 levels from IgA levels.

The IgA ACPA pool that was used as a standard for both ACPA and total IgA ELISA were isolated from pooled serum of RA patients. First, total IgA was purified using the affinity columns with Peptide M-Agarose beads from InvivoGen followed by modified citrullinated vimentin (MCV)-Sepharose columns from Orgentec Diagnostika (Mainz, Germany) for ACPA isolation according to the previous protocol [12]. The amount of IgA1 and IgA2 in the IgA ACPA standard pool was measured by ELISA using previously isolated IgA1 and IgA2 with defined concentrations. For ACPA ELISAs, cyclic citrullinated peptide (CCP) pre-coated plates (Orgentec Diagnostika, commercial Org601 plates) were used. Diluted sera (1:10) were incubated on the plates for 1 hour, afterwards washed with 0.05% PBS/Tween and incubated with secondary antibodies for 1 hour: mouse anti-human IgA-HRP (SouthernBiotech 2050-05) or mouse anti-human IgA2-HRP (SB 9140-05). Afterwards, the plates were washed, and detection was performed with TMB substrate kit (Thermo Scientific 34021). After stopping the reaction with stop solution (Invitrogen SS04) optical density (OD) was measured at 450nm with reference wave length at 650nm. A cut-off for IgA ACPA-positivity was defined as 3 µg/ml based on the optimal sensitivity/specificity combination (72.1%/79.5%) in comparing values in RA patients from another cohort with healthy donors (unpublished data). For total IgA measurements Nunc MaxiSorp™ flat-bottom plates from ThermoFisher were coated at 4°C overnight with goat F(ab')2 anti-human IgA (unlabeled) (SB 2052-01). On the following day, the plates were blocked with 1% bovine serum albumin in PBS. Diluted samples (1:40000 for IgA; 1:20000 for IgA2) were incubated on the plates for 1 hour. Other steps were performed as described above for IgA ACPA.
Statistical analysis

Statistical analysis of the data was performed using the IBM SPSS Statistics (version 24.0.02, IBM Corporation, USA, 2016) for the descriptive statistics of demographic data and Kaplan-Meier survival curves; and GraphPad Prism 8 (version 8.3.0, USA, 2019) for group comparisons and correlations. Data for continuous variables were represented as medians with 95% confidence intervals after testing for normality of distribution. Accordingly, in further analysis nonparametric statistics was used: Mann-Whitney test, Wilcoxon test for matched-pair analysis and Spearman correlation. A two-tailed p-value of <0.05 was considered statistically significant.

Results.

Patient characteristics

We investigated the sera of 108 patients from the RETRO study that has been described previously [4]. All patients were in stable remission at baseline; 39 patients were in study arm 1 (continued full-dose DMARD treatment), 36 patients in the tapering arm 2 (50% dose reduction) and 33 patients in arm 3 (50% dose reduction for 6 months followed by stop of DMARDs). Baseline patient characteristics are described in Table 1. The median remission duration at baseline was 11.5 months. Almost half of all patients (41.4%, N=46) were receiving biologic (b) DMARDs at baseline. None of the patients in the study received rituximab, abatacept or JAK-inhibitors. Overall, 67 patients (60%) were IgG ACPA positive at baseline.

IgA ACPA levels in IgG ACPA positive and negative patients

We first investigated all sera for the presence of IgA ACPA. A cut-off for IgA ACPA-positivity was defined as 3 µg/ml based on the optimal sensitivity/specificity combination in comparing values in RA patients with healthy donors (Supplementary Fig. 1). IgA2% in ACPA was calculated only for patients with detectable amounts of IgA ACPA. IgA ACPA levels were higher in IgG ACPA positive patients than in IgG ACPA negative (median of 4.7 versus 2.24 µg/ml, p<0.0001). Of IgG ACPA positive patients, 76.1% (n=51) had detectable IgA ACPA. Interestingly, also 34% (n=15) of IgG ACPA negative patients were IgA ACPA positive. The proportion of IgA2 in IgA ACPA was not different between the groups (Fig. 1). In addition, there was no difference in total IgA levels between IgG ACPA positive and negative patients (Supplementary Fig. 2).

Association of ACPA of IgA subclasses with flare of RA
Of 108 patients included in this study, 37 (33.3%) over all three treatment arms had a flare. IgA and IgG ACPA individually influenced flare-free survival only slightly, but patients who were both IgG and IgA ACPA positive showed a tendency (Log Rank $\chi^2$=1.748; p=0.186) to be at most risk of flare, especially in the first 6 months of the study (Fig. 2A). At the end of the study, only 60.4% of IgG and IgA ACPA-positive patients remained in remission compared to 75% of IgG and IgA ACPA-negative ones (Fig. 2B). Compared to the IgG and IgA ACPA double negative patients, the relative risk of flare for IgG and IgA double positive patients was 1.6 (95% CI 0.77-3.3) and for single IgG or IgA positive patients 1.33 (95% CI 0.51-3.49).

Regarding changes in IgA ACPA after 12 months, an average decline of 17.5% (median) from the baseline in IgA2 ACPA levels was observed in patients remaining in remission (p<0.0001), as well as a decrease in the percentage of IgA2 in IgA ACPA (IgA2% ACPA) of 13.6% (p=0.0006). This was specific for ACPA, since there was no decline in total IgA2 levels (supplementary Fig. 3). There was no clear trend in IgA2 ACPA dynamics in patients who flared, and no significant changes were registered for IgA1 ACPA for patients in flare and remission (Fig. 3A). There was a mild increase in total IgA and IgA1 levels both in patients who flared and in remission (supplementary Fig.3).

As IgA2 ACPA declined in patients remaining in remission, we aimed to investigate whether IgA2 ACPA could contribute to the severity of a flare. Indeed, baseline IgA2% in ACPA was correlated to disease activity at flare as measured by DAS28 score ($r=0.36$, p=0.046), whereas no correlation was observed between the baseline IgA1 or IgA2 ACPA levels and DAS28 (Fig. 3B).

The influence of treatment on IgA ACPA levels

To test whether the changes in IgA ACPA levels might be influenced by treatment, we first compared baseline levels– when all patients had been in remission and were still receiving DMARDs. Interestingly, patients on bDMARDs had slightly higher IgA ACPA levels than those receiving csDMARDs, but no differences in IgA2% in ACPA was observed (Fig. 4A). In addition, changes in IgA ACPA levels after the 12 months study period did not seem to depend on the management strategy, since there was no difference in the fold-change from baseline between the three study arms. Dynamics in ACPA levels were very diverse among patients and did not seem to be influenced by whether DMARDs were continued or tapered (Fig. 4B).

Discussion.
Several risk factors for flare after DMARD tapering have been suggested, the presence of ACPA and rheumatoid factor being among them [4,13,14]. Although a couple of studies have been published in the last years on potential novel autoantibodies in RA[5,15–18], ACPA still remain the most widely used autoantibody in the clinical setting and are included in the 2010 ACR/EULAR diagnostic criteria of RA. However, in every days clinical setting, only IgG ACPA are being measured. In the present work, we show that IgA ACPA contribute to flare risk when tapering DMARDS in RA patients in stable remission.

According to the current concept, the development of RA is characterized by the expansion of the antibody subtype repertoire, affinity maturation and epitope spreading [8,9,19]. Indeed, most IgG-positive RA patients are also IgA ACPA positive, when the disease is manifesting clinically. However, we observed an appreciable proportion of patients in this study (27%) who only displayed ACPA of either IgA or IgG class alone. Interestingly, those two antibody classes seemed to make only a limited prognostic contribution separately, but if a patient had both classes, the risk of flare was higher, especially in the first 6 months after initiation of tapering. This effect was not statistically significant, putatively due to the limited sample size. Nevertheless, this trend seems intriguing in the context of the search for prognostic factors of flare.

Interestingly, IgA2 ACPA levels and its percentage decrease in patients keeping the remission state. In contrast, there was no clear trend in IgA2 ACPA dynamics in patients with flare. This effect seemed to be independent of the treatment strategy since there was no difference between the three study arms. Moreover, in patients who flared, the baseline ACPA IgA2% correlated with the intensity of the flare. IgA2 ACPA might drive the recurrence of inflammation in the context of DMARD tapering. It has been previously shown that IgA complexes from plasma and synovial fluid of RA patients are potent inducers of neutrophil extracellular traps and that IgA rheumatoid factor is enriched in the synovial fluid [20]. Recently, we demonstrated that IgA1 and IgA2 have different effector functions, which are influenced by their glycosylation profiles. IgA2 is a more powerful inductor of neutrophil-extracellular trap formation and cytokine production by neutrophils and macrophages [10]. IgA1 contains more sialic acid residues than IgA2, which is at least partially accountable for its less potent inflammatory capacity in vitro [10]. In the same study, IgA2% ACPA positively correlated with DAS28 in a cohort of RA patients, whereas IgA1 was correlating negatively. Furthermore, IgA ACPA consistently show a higher percentage of IgA2 than total serum IgA [10] , which could be confirmed in the present study. Altogether, these findings indicate a pro-inflammatory role of IgA2 ACPA in RA.

Decline in IgA2 ACPA levels in patients without flare and the association of IgA2 ACPA with DAS28 in flare could be related to the immune activation status of the individual patient. Hence, an increase in
naïve B cells 1-2 weeks before a flare has been shown, as well as activation of innate immune profiles and the appearance of a newly identified cell population with characteristic features of synovial fibroblasts [21]. Authors hypothesized that these ‘new’ fibroblast-like cells are driven by the activated B cells and both of them migrate towards the synovium where they enhance the inflammatory processes, though this hypothesis needs further validation. In view of the evidence that ACPA-positive RA patients harbor IgG as well as IgA ACPA memory B cells and plasmablasts [22,23] and that ACPA-producing antibody-secreting cells are enriched in the synovial fluid of RA patients [24], it is plausible that continuous autoantigen exposure and subsequent naïve B cells activation and differentiation towards antigen-secreting cells could play a role in disease activity. IgA2-producing cells can be generated either directly from naïve B cells or can appear as the result of the class-switch from IgG1, IgG2 or IgA1-germinal center B cells [25]. Hence, it can be hypothesized that in the events leading to flare, activation of naïve B cells and their class switch towards other subclasses, IgA2 in particular, might enhance inflammation.

Another observation from the current work was that patients receiving bDMARDs had higher levels of IgA1 ACPA at baseline than patients on csDMARDs. Since it is unlikely that bDMARDs increase ACPA levels or that csDMARDs selectively reduce them, this observation might reflect the underlying suppressed disease activity state in patients receiving bDMARDs, as this group of patients reflects a more difficult-to-treat state.

One limitation of the study is the relatively small sample size. Nevertheless, it suggests that the role of IgA ACPA should not be overseen in the prognosis of the patients to keep remission when DMARDs are tapered or withdrawn.

**Data availability statement**

Data are available upon reasonable request by any qualified researchers who engage in rigorous, independent scientific research, and will be provided following review and approval of a research proposal and Statistical Analysis Plan (SAP) and execution of a Data Sharing Agreement (DSA). All data relevant to the study are included in the article.

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**Disclosure statement.**
Holger Bang is an employee of Orgentec Diagnostika, a supplier of ACPA diagnostic tools. All other authors declare no conflict of interest.

**References.**


Figure legends

Table 1. Baseline characteristics of patients from the RETRO study.
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<td>Duration of remission, months</td>
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<td>bDMARDs use, N (%)</td>
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<td>VAS patient global (0-100), mm</td>
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<tr>
<td>IgG ACPA-seropositivity, N (%)</td>
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</table>

Data presented as median with IQR or percentage and absolute numbers.
IgA, IgA1 and IgA2 anti-citrullinated protein antibody (ACPA) levels and percentage of IgA2 ACPA of total IgA ACPA (IgA2% ACPA) in serum of IgG ACPA positive (N=65/63) and IgG ACPA negative (N=43/26) rheumatoid arthritis patients. Shown are single values with median+95% confidence interval (CI). Significances were tested by Mann-Whitney test.

Fig. 1. Baseline IgA ACPA levels in IgG ACPA positive and IgG ACPA negative patients.
Fig. 2. Influence of IgG and IgA ACPA on flare rates in RA.

(A) Kaplan-Meier curves for flare-free survival based on the subgroups with no IgG and IgA anti-citrullinated protein antibodies (ACPA; IgG-IgA-; N=28), only IgG ACPA (IgG+IgA-; N=15), only IgA ACPA (IgG-IgA+; N=15) or both IgG and IgA ACPA (IgG+IgA+; N=50). (B) Percentage of patients flaring (blue) or staying in remission (orange) according to IgA and IgG ACPA-status after 12 months.

Fig. 3. Development of IgA ACPA over time.

(A) IgA, IgA1 and IgA2 anti-citrullinated protein antibody (ACPA) levels and percentage of IgA2 ACPA of total IgA ACPA (IgA2% ACPA) at baseline (V0) and 12 month (V4) in RA patients who remained in remission (n=60/50) or flared (N=30/25). Each line represents one patient. The numbers represent median change from the visit 0. Wilcoxon matched-pairs test. (B) Correlation between IgA, IgA1 and IgA2
ACPA levels as well as IgA2% ACPA and disease activity score (DAS) 28 score after flare (n=32). Spearman correlation.

Fig. 4 Cross-sectional and longitudinal IgA ACPA levels in the treatment groups.
(A) IgA, IgA1 and IgA2 anti-citrullinated protein antibody (ACPA) levels and percentage of IgA2 ACPA of total IgA ACPA (IgA2% ACPA) at baseline in rheumatoid arthritis (RA) patients treated with conventional synthetic (cs) and biological (b) disease modifying anti-rheumatic drugs (DMARDs). (B) Changes in IgA, IgA1 and IgA2 ACPA levels and IgA2% ACPA after 12 months in arm 1 (continuation of full-dose DMARDs; N=26), arm 2 (tapering of DMARDs to 50% dose; N=26) and arm 3 (tapering of DMARDs to 50% dose followed by stopping them; N=21). Individual values with median+ 95% CI. Fold-change at visit 4, normalized to baseline.
Supplementary Fig. 1. Receiver operating characteristic (ROC) curve and the curve coordinates for the setting of the IgA ACPA cut-off

The curve is based on the IgA ACPA values in healthy individuals (N=39) and diagnosed RA patients (N=61). The cut-off was chosen based on the optimal combination of sensitivity and specificity.

<table>
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Supplementary Fig. 2. Total IgA levels in seropositive and seronegative RA patients

Total serum IgA, IgA1 and IgA2 levels and percentage of IgA2 of total IgA (IgA2% total) at baseline in rheumatoid arthritis (RA) patients with (seropos) and without (seroneg) IgG ACPA. Individual values with median+ 95% CI.

Supplementary Fig. 3: Development of total IgA levels over time

Total IgA, IgA1 and IgA2 levels and percentage of IgA2 of total IgA (IgA2%) at baseline (V0) and 12 month (V4) in RA patients who remained in remission (n=60) or flared (N=30). Each line represents one patient. The numbers represent median change from the visit 0. Wilcoxon matched-pairs test.