


ORIGINAL RESEARCH

Antibodies against citrullinated proteins of IgA isotype are associated with progression to rheumatoid arthritis in individuals at-risk

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ABSTRACT

Objective Events triggering disease outbreak in individuals at-risk for rheumatoid arthritis (RA at-risk) remain unclear, and the role of the various anticitrullinated protein antibody (ACPA) isotypes in this process is still to be established. We aimed to investigate the prevalence of IgA ACPA in RA at-risk individuals, their role in the transition from the RA at-risk status to RA and their dynamics during this transition.

Methods Cross-sectional measurement of serum IgA1 and IgA2 ACPA levels was conducted in healthy controls, RA at-risk individuals and patients with RA and compared with the frequency of RA development in at risk individuals during a follow-up of 14 months. In addition, longitudinal measurements of serum IgA1 and IgA2 ACPA levels prior to, at and after the onset of RA were performed.

Results Approximately two-thirds of RA at-risk individuals were positive for serum IgA1 and IgA2 ACPA in levels comparable to IgG ACPA positive patients with RA. IgA1, but not IgA2 ACPA positivity was associated with the transition from the RA at-risk state to RA within the following 14 months. Interestingly, during this transition process, IgA1 ACPA levels declined at RA onset and also thereafter during the early phase of RA. This decline was confirmed in a second, independent cohort.

Conclusion Both IgA1 and IgA2 ACPA are present in RA at-risk individuals, but only IgA1 ACPA are associated with the progression to RA. The observed decline in serum IgA1 ACPA levels before the onset of RA might indicate starting barrier leakiness prior to disease outbreak.

INTRODUCTION

Rheumatoid arthritis (RA) is characterised by autoimmunity against post-translationally modified proteins.¹ Especially the presence of anticitrullinated protein antibodies (ACPA) of the IgG class constitutes an important risk factor for the development of RA and IgG ACPA positive individuals are considered at-risk for the development of RA.^{2–4} However, it is still unclear how the transition from mere autoimmunity to actual disease onset occurs

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Autoantibodies against citrullinated proteins (ACPA) of the IgG class are an important risk factor for the development of rheumatoid arthritis (RA). There are only limited data if ACPA of other isotypes contribute to this risk.

WHAT THIS STUDY ADDS

⇒ We found that IgA ACPA are present in RA at-risk individuals in levels comparable to patients with established RA.
⇒ Positivity for IgA1 ACPA is associated with an increased risk for progression to RA within the following year.
⇒ IgA1, but not IgG ACPA levels decline around RA onset.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Results of this study suggest that screening for IgA ACPA positivity would help to better define the individual risk profile of at-risk individuals to develop RA.

and whether ACPA play an active role in this transition. Most knowledge on the role of ACPA in RA concerns antibodies of the IgG class.^{5,6} However, in a substantial proportion of patients with RA, ACPA are also present in the form of IgA.^{7–12}

After IgG, IgA forms the second most common immunoglobulin class in human blood.¹³ Humans possess two IgA subclasses, IgA1 and IgA2 that differ in their hinge region and their glycosylation spectrum.¹⁴ We recently found that IgA2 is a more potent activator of myeloid cells compared with IgA1.¹⁵ In addition, we found that the IgA1 to IgA2 ratio in ACPA is shifted in favour of the proinflammatory IgA2 in a cohort of patients with established RA,¹⁵ but it is unclear if IgA



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subclass composition of ACPA changes before RA onset and if it is of relevance for disease development.

IgA can occur in a monomeric form that stays in the serum as well as in a dimeric form that is secreted into mucosal tissues. In humans, monomeric serum IgA has been shown to elicit pro-inflammatory effects, such as cytokine release, formation of neutrophil extracellular traps and antibody-dependent cellular cytotoxicity via the Fc α receptor I (Fc α RI) that is mainly expressed on myeloid cells, such as neutrophils, monocytes, macrophages and some types of dendritic cells.^{16–20} It is thus possible that ACPA of the IgA class contribute to synovial inflammation and bone destruction in RA.

IgA ACPA have been associated with RA disease activity and risk of relapse in patients with RA in remission,^{11 21 22} but to date, little is known if IgA ACPA contribute to the transition from the RA at-risk state to clinical RA. A recent study reported that around half of IgG ACPA-positive non-RA individuals showed IgA ACPA positivity with IgA ACPA levels being significantly higher in individuals who in addition were defined as clinically suspect arthralgia positive.²³ These findings suggest that IgA ACPA are present before RA onset and might contribute to disease development, but longitudinal follow-up data are missing.

In order to better understand the role of IgA ACPA, we compared the serum levels of IgA ACPA from at-risk individuals with patients with established RA and healthy controls. In addition, we investigated whether IgA ACPA increase the risk of at-risk individuals for progression to RA and followed individual dynamics of IgA ACPA levels during the transition to RA.

METHODS

Patients and controls

Four groups of participants were investigated cross-sectionally: (1) healthy subjects negative for IgG ACPA and with no present or past signs of arthritis (n=32); (2) RA at-risk individuals (n=63); (3) patients with established RA according to the 2010 EULAR/ACR criteria who were IgG ACPA positive (n=30) or (iv) negative (n=29). All participants were recruited at the Department of Internal Medicine 3 of the University Clinic Erlangen. The RA at-risk cohort included people with serological evidence of IgG ACPA (anticyclic citrullinated peptide (CCP) and/or antimodified citrullinated vimentin (anti-MCV) antibodies) with or without musculoskeletal symptoms (arthralgia was present in more than 90% of at-risk individuals). Individuals with clinically apparent arthritis (at least one swollen joint with synovitis in clinical assessment in the 66 joint count, performed by an experienced rheumatologist) were excluded. RA at-risk individuals had follow-up visits every 3, 6 or 12 months depending on the severity of their symptoms.

For investigation if IgA ACPA associate with RA progression, a subgroup of 45 at-risk individuals from whom at least 14 months of clinical follow-up was available was

analysed. This subgroup comprised IgA ACPA positive and IgA ACPA negative individuals. RA diagnosis was based on the 2010 EULAR/ACR criteria.

Longitudinal follow-up of RA at-risk subjects was conducted in a subgroup of 14 IgA ACPA positive and 5 IgA ACPA negative at-risk individuals who developed RA and from whom sera prior to, at the time of diagnosis and/or after RA development were available from 7 individuals, sera were available only from prior to and at the time of RA diagnosis; from 8 individuals, sera were available prior to, at the time of and after onset of RA; and from 4 individuals, sera were available at the time of diagnosis (sera that were taken less than 1 month before diagnosis were considered as at diagnosis for computational reasons) and after the onset of RA. Median time of serum available prior to RA diagnosis was 5 months and median time of serum after the diagnosis was 3 months. In addition, longitudinal ACPA measurements were performed in a confirmation cohort of patients with diagnosed RA of whom sera were available prior to the diagnosis, collected and stored at the respective biobank (n=9) at the Division of Rheumatology at the Medical University of Vienna.¹⁴

Measurement of IgA ACPA

All sera were stored at -80°C . Serum levels of IgA1 and IgA2 ACPA were measured in duplicates by in-house ELISA on CCP-precoated plates (Orgentec Diagnostika #Org601) as described previously.¹¹ To avoid concurrent binding of IgG to the plates interfering with the results, sera were depleted of IgG using AcroPrep Advance 3 μm 96 well-plates from PALL (#8075) and capture beads from Thermo Scientific (Capture select FcXL Affinity Matrix, #194328005). Capture beads were prewashed with phosphate buffered saline (PBS) on the filter plate on top of an empty cell culture plate via centrifugation. Sera were prediluted 1:1 with PBS and incubated with the capture beads (1 μL beads per μL serum) on a plate shaker for 1 hour at room temperature. Following the incubation, plates were centrifuged, the same amount of PBS as of serum was added to each well and plates were incubated on a shaker for several minutes with subsequent centrifugation. This was repeated two times in total. After the final centrifugation, the sera-PBS dilutions in the collection plate were used for ELISA. Prediluted 1:4 sera were further diluted to a final dilution of 1:20 in 1% TBS/bovine serum albumin (BSA) and incubated on precoated and preblocked CCP-plates for 1 hour. For IgA1 detection, the secondary mouse anti-human IgA1-horseradish peroxidase (HRP) antibody (SouthernBiotech, 9130-05) and for IgA2—mouse anti-human IgA2-HRP (SB 9140-05) were used. After detection with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate, the absorption was measured at 450 nm with reference wavelength at 650 nm.

IgA ACPA previously isolated from the pooled serum of patients with RA were used as a standard for ACPA and total serum IgA ELISA. Initially, IgA was purified with Peptide M-Agarose (InvivoGen). Afterwards, it was

applied onto columns with MCV-Sepharose (Orgentec Diagnostika, Mainz, Germany) as described previously.^{11 24} IgA1 and IgA2 amounts in the standard were defined by ELISA using purified IgA1 and IgA2 in known concentrations.

Total IgA ACPA was calculated as the sum of IgA1 and IgA2 ACPA. Longitudinal measurements from one patient were performed on the same ELISA plate to avoid interassay variability affecting the results.

The cut-offs for IgA ACPA-positivity were set based on the standard curve and detection limits of the ELISA rather than setting the priority at clinical specificity, as is conventionally done if the test is to be used for diagnostic purposes. Comparing the healthy controls to the IgG ACPA positive patients with RA, our chosen cut-offs show the following specificity and sensitivity parameters: total IgA ACPA: cut-off 3 µg/mL, specificity 84.3%, sensitivity 86.7%; IgA1 ACPA: cut-off 2.5 µg/mL, specificity 84.3%, sensitivity 83.3%; and IgA2 ACPA: cut-off 0.6 µg/mL, specificity 84.3%, sensitivity 76.7%.

Usage of higher cut-offs (mean +3 standard deviations (SD) of the healthy population) setting the priority at clinical specificity results in the following specificity and sensitivity parameters: total IgA ACPA: cut-off 5.133 µg/mL, specificity 100%, sensitivity 43.3%; IgA1 ACPA: cut-off 4.4664 µg/mL, specificity 100%, sensitivity 46.7% and IgA2 ACPA: cut-off 1.2278 µg/mL, specificity 97.9%, sensitivity 36.7%.

Measurement of IgG ACPA

IgG ACPA levels were measured with a CCP ELISA kit (Euroimmun) according to the manufacturer's instructions, with the positivity cut-off above 5 RU/mL (relative units per ml) set by the manufacturer.

Measurement of total serum IgA1, IgA2 and IgG

Total serum IgA1 and IgA2 levels were measured by in-house ELISA as previously described.¹¹ Briefly, ThermoFisher Nunc MaxiSorp flat-bottom plates were precoated with 2 µg/mL of goat F(ab)2 anti-human IgG (H+L) (Jackson ImmunoResearch #109-006-003) or goat F(ab)2 anti-human IgA (SouthernBiotech, #2052-01) overnight at 4°C. On the next day, plates were washed with 0.05% PBS/Tween and blocked with 1% PBS/bovine serum albumin (BSA). Sera (diluted 1:1000 000 for IgG and 1:40 000 for IgA measurement) and IgG commercial standard (Human IgG I2511 Sigma-Aldrich) or isolated IgA in known concentrations were incubated on the plates for 1 hour. After the plates were washed, they were incubated with goat anti-human IgG HRP-antibody (SouthernBiotech #2040-05) for 1 hour. After detection with TMB substrate, the absorption was measured at 450 nm with reference wavelength at 650 nm.

Statistical analysis

Data were analysed using GraphPad Prism 9 (V.9.0.2). For non-normally distributed continuous variables (tested with Shapiro-Wilk normality test), data are represented

as medians with 95% confidential intervals (CIs). For normally distributed data, mean with SD is shown. For the comparison of ACPA levels between different patient groups, Mann-Whitney test (2 groups) or Kruskal-Wallis test with Dunn's multiple comparisons correction (>2 groups) were applied for all non-normally distributed datasets. Parametric statistics (t-test for 2 groups, analysis of variance (ANOVA) or mixed effects model (modified ANOVA) with Tukey's multiple comparisons correction for >2 groups) were applied for normally distributed datasets. For progression-free survival curve analysis, log-rank (Mantel-Cox) test was used.

RESULTS

IgA ACPA levels in RA at-risk individuals are comparable to those in IgG ACPA positive patients with RA

To evaluate a potential role of IgA ACPA in RA disease onset, we first aimed to investigate to which extend IgA ACPA are present already in the RA at-risk phase and if differences between the two IgA subclasses can be seen. We therefore, assessed the serum levels of IgA, IgA1 and IgA2 ACPA in RA at-risk individuals and compared the values to healthy controls and patients with IgG ACPA-positive and IgG ACPA-negative RA. Characteristics of the study groups are summarised in table 1.

Compared with healthy controls, RA at-risk individuals displayed clearly elevated IgA ACPA levels (median 3.83–2.12 µg/mL; $p=0.0001$), with no difference to patients with established IgG ACPA-positive RA (4.47 µg/mL) (figure 1A). When using cut-offs based on the ELISA detection limits, 61.9% of RA at-risk individuals and 86.7% of IgG ACPA-positive patients with RA were positive for IgA ACPA. Also, 41.4% of ACPA-negative patients with RA displayed IgA ACPA and low concentrations were found in a small subset (15.6%) of healthy controls. However, only some of the IgG ACPA negative patients with RA and only one of the healthy controls was positive for IgA1 and IgA2 ACPA, while most of the at-risk individuals and IgG ACPA positive patients with RA were positive for both IgA subclasses (online supplemental figure 1), indicating that IgA1 and IgA2 ACPA emerge together before disease onset.

In comparison, the usage of very conservative cut-offs (mean +3 SD of the healthy population) nearly halves the IgA ACPA positive populations in the RA at-risk and the IgG ACPA positive RA groups and classifies nearly all healthy donors as IgA ACPA negative (table 1) (online supplemental figure 2A).

Similar to IgA ACPA, IgG ACPA levels did not differ between individuals at-risk and patients with RA (figure 1A).

IgA ACPA are associated with progression to RA

Since many of the RA at-risk individuals displayed IgA ACPA, we investigated whether the presence or levels of IgA ACPA could serve as a predictive factor for RA development. For this purpose, we compared IgA ACPA levels

Table 1 Characteristics of the study groups

	Healthy donors (N=32)	Individuals at-risk (N=63)	IgG ACPA-positive patient with RA (N=30)	IgG ACPA- negative patient with RA (N=29)
Age, years: mean (SD)	48 (12.7)	48.3 (12.4)	55 (11.6)	59.8 (13.9)*
Female, N (%)	20 (62.5)	41 (65.1)	18 (60.0)	17 (58.6)
DAS28-ESR: mean (SD)	–	–	2.46 (0.98)	3.56 (1.66)††
Disease duration, years: median (IQR)	–	–	5 (1–10)	2 (1–6)
Arthralgia, N (%)	–	58 (92.1)	–	–
Arthralgia duration, months: median (IQR)	–	23 (8.5–83)	–	–
IgA ACPA, µg/mL: median (IQR)	2.1 (1.6–2.7)	3.8 (2.4–7.7)	4.47 (3.4–20.7)	2.6 (1.0–4.5)‡
IgA1 ACPA, µg/mL: median (IQR)	1.8 (1.2–2.2)	2.9 (1.8–6.3)	3.5 (2.6–18.8)	1.8 (0.8–3.1)‡
IgA2 ACPA, µg/mL: median (IQR)	0.4 (0.3–0.5)	0.8 (0.4–1.7)	1.0 (0.6–1.9)	0.5 (0.3–0.9)‡
IgG ACPA, RU/mL: median (IQR)	0.9 (0.9–0.9)	109.3 (14.1–334.6)	78.1 (24.7–202.7)	0.9 (0.7–1.0)‡
IgA ACPA positive, %: low cut-off (high cut-off)	15.6 (0)	61.9 (41.3)	86.7 (43.3)	41.4 (17.2)
IgA1 ACPA positive, %: low cut-off (high cut-off)	15.6 (0)	60.3 (38.1)	83.3 (46.7)	37.9 (13.8)
IgA2 ACPA positive, %: low cut-off (high cut-off)	15.6 (3.1)	61.9 (34.9)	76.7 (36.7)	44.8 (3.4)
IgG ACPA positive, %	0	80.9	100	0

Values of antibodies are represented as median (IQR).

*Older age in IgG ACPA-negative patients with RA compared with individuals at-risk ($p=0.0007$) and to healthy individuals ($p=0.0022$), ANOVA with Tukey's multiple comparisons test.

†Higher DAS28 in IgG ACPA-negative patients with RA compared with IgG ACPA-positive ($p=0.0034$), unpaired t-test with Welch's correction.

‡Higher ACPA levels in RA at-risk individuals and IgG ACPA positive RA patients compared with healthy individuals and IgG ACPA negative RA patients, Kruskal-Wallis test with Dunn's multiple comparisons correction.

ACPA, anti-citrullinated protein antibodies; ANOVA, analysis of variance; DAS28, Disease Activity Score 28; ESR, erythrocyte sedimentation rate; RA, rheumatoid arthritis; RU, relative units.

in RA at-risk individuals, from whom at least 14 months of clinical follow-up was available. Both groups (RA developed and RA non-developed) were similar in age and sex distribution. At-risk individuals who did not develop RA seem to have a tendentially longer symptoms duration, but this was not significant (table 2).

IgA1 ACPA levels were higher in individuals who developed RA than in the ones who did not (median 4.54 vs 2.05 µg/mL; $p=0.033$) (figure 1B). Furthermore, 70.8% of IgA1 ACPA-positive compared with 33.3% among IgA1 ACPA-negative RA at-risk individuals developed RA ($p=0.008$; HR=3.1 (95% CI 1.36 to 7.23)) (figure 1C), suggesting that IgA1 ACPA increase the risk of developing RA. With the conservative cut-offs, we obtained in principle the same results with 68.4% of IgA1 ACPA-positive compared with 42.3% among IgA1 ACPA-negative RA at-risk individuals developed RA ($p=0.013$; HR=2.6 (95% CI 1.11 to 6.43)) (online supplemental figure 1C).

IgA2 ACPA levels did not differ between the two groups and no difference with respect to progression to RA was observed. Similar to IgA1 ACPA, IgG ACPA levels were higher in RA at-risk individuals progressing to RA (177.6

RU/mL) than in those not progressing to RA (38.54 RU/mL) ($p=0.056$).

IgA1 and IgA2 ACPA decline at the onset of arthritis

To investigate if IgA ACPA levels further increase prior to disease development, we next investigated the dynamics of IgA ACPA during the transition to RA by longitudinal measurement of ACPA levels in sera of IgA ACPA-positive at-risk individuals who developed RA (individuals' characteristics in table 3).

Surprisingly, by the time of clinical onset of RA, IgA1 ACPA had declined in nine out of eleven individuals with an overall median reduction of 26% (range 6–185%) (figure 2). In the first 18 months after the onset of RA, IgA1 ACPA levels further significantly declined in all but one patient by an overall median of 38.5% (range 23%–70%). IgA2 ACPA did not change significantly before the disease onset, but declined directly after in 9 of 10 patients with a median reduction of 33% (range 10%–64%). One patient was IgA1 ACPA-positive but IgA2 ACPA-negative and, therefore, excluded from the IgA2 analysis. The same courses could be observed when

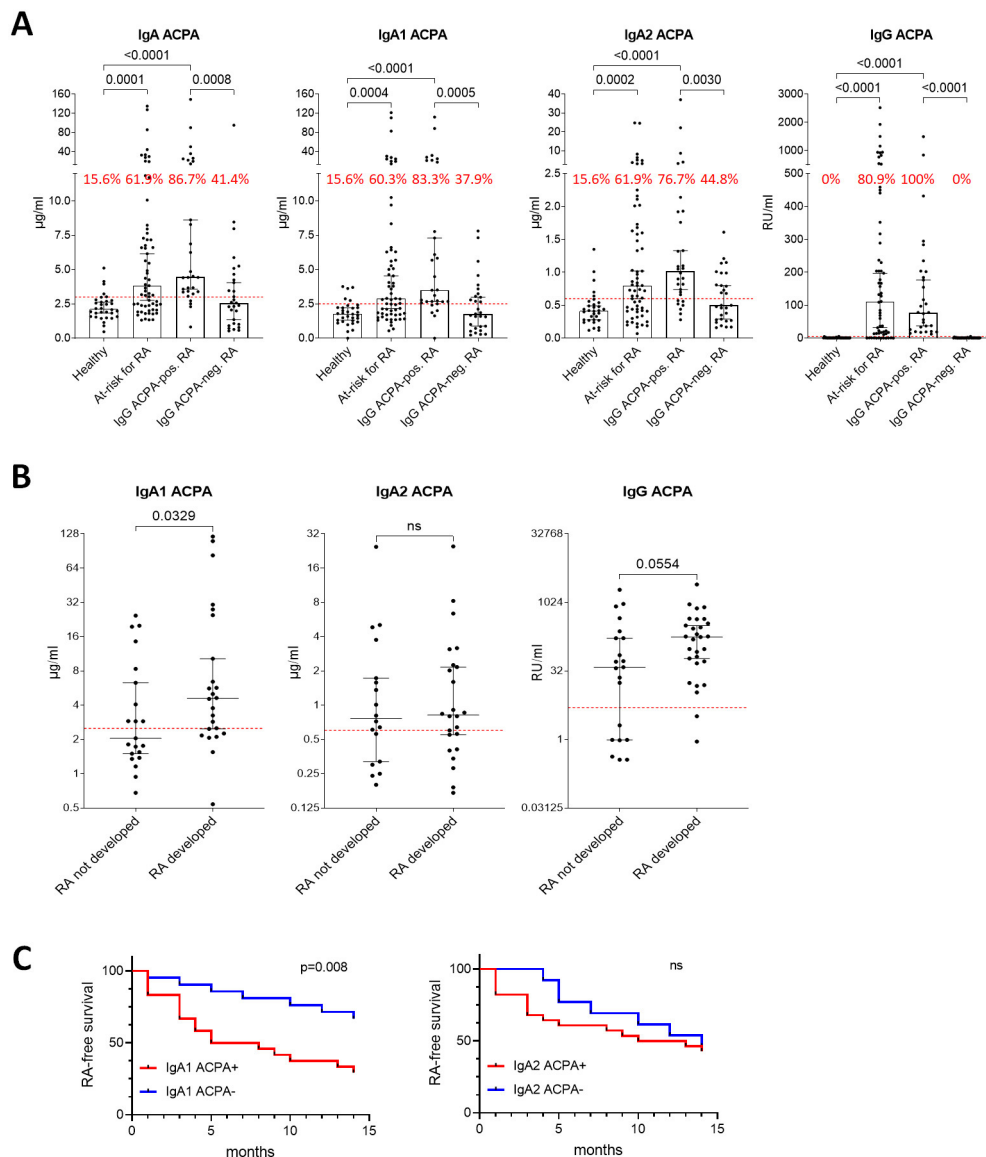


Figure 1 (A) Serum concentrations (median with 95% CI) and prevalence of IgA, IgA1, IgA2 and IgG ACPA in healthy controls (n=32), individuals at-risk for RA (n=63), IgG ACPA-positive (n=30) and IgG ACPA-negative (n=29) patients with RA. Dotted lines show the cut-offs for positivity based on the ELISA standard curves. Significances were tested with Kruskal-Wallis test with Dunn's multiple comparisons correction. (B) Serum concentrations (median with 95% CI) of IgA1, IgA2 and IgG ACPA in RA at-risk individuals who did (n=24) or did not (n=21 for IgA1 and IgG ACPA / n=18 for IgA2 ACPA) develop RA in a 14-month period. Three patients were excluded from the IgA2 ACPA analysis due to high differences between technical replicates. Significances were tested with Mann-Whitney U test. (C) Progression-free survival curves of IgA1 and IgA2 ACPA-positive (=IgA1/IgA2 ACPA+) and negative (=IgA1/IgA2 ACPA-) RA at-risk individuals with follow-up of 14 months after serum collection. Significances were tested with log-rank (Mantel-Cox) test. ACPA, anticitrullinated protein antibody; RA, rheumatoid arthritis; ns, not significant.

classifying the patients IgA ACPA positive and negative according to the higher cut-offs (online supplemental figure 3).

Total serum IgA1 and IgA2 did not change in the course of disease development (online supplemental figure 4). For IgG ACPA and total IgG, no change could be observed during the transition to RA (figure 2 and online supplemental figure 4), although there was a tendency to an IgG ACPA increase towards disease onset in IgA ACPA negative at-risk individuals (figure 2D).

As half of the at-risk individuals took non-steroidal anti-inflammatory drugs (NSAID) (table 3), we evaluated whether this could have caused the decline in IgA1 ACPA. However, we did not see a difference between individuals with and without NSAIDs (online supplemental figure 5). One of the IgA ACPA positive individuals received abatacept before disease onset which had no influence on the overall ACPA level development of the group. In addition, three of the five IgA ACPA negative individuals received abatacept. However, since all IgA ACPA negative

Table 2 Characteristics of individuals at-risk from a sub-cohort with at least 14 months of follow-up

	Developed RA (N=24)	Did not develop RA (N=21)
Age, years: mean (SD)	46 (12.6)	45.8 (13.1)
Female, N (%)	16 (66.7)	13 (61.9)
Arthralgia, N (%)	23 (95.8)	20 (95.2)
Arthralgia duration, months: median (IQR)	8.5 (5.3–32.5)	24 (10–61)
time to RA diagnosis, months: median (IQR)	4.5 (3–9.8)	–
IgA1 ACPA, µg/mL: median (IQR)	4.6 (2.3–21.1)	2.0 (1.4–7.3)*
IgA2 ACPA, µg/mL: median (IQR)	0.82 (0.4–2.2)	0.77 (0.3–2.2)
IgG ACPA, RU/mL: median (IQR)	177.6 (49.4–394.9)	38.5 (1.0–202.2)*

*Higher IgA1 and IgG ACPA levels in RA at-risk individuals who developed RA, Mann-Whitney U test.

RA, rheumatoid arthritis; RU, relative units; t, test for age, exact Fisher's test for sex and arthralgia and Mann-Whitney test for symptoms duration.

individuals showed the same tendency for IgG-ACPA levels to increase prior to disease onset, abatacept treatment is unlikely to have influenced the analysis.

As our cohort was very small, we tested whether we can confirm our results in a second cohort comprising patients with RA from the Medical University of Vienna with serum available prior to the diagnosis (2–9 months) (characteristics in [table 3](#)). In this cohort, nine patients were positive for IgA1 ACPA. In concordance with the data from our cohort, IgA1 ACPA declined in eight out of nine patients before RA onset ([figure 3A](#)). Interestingly, in contrast to the data from our cohort, in the Vienna cohort, IgG ACPA also tendentially declined before diagnosis in five out of six IgA1 and IgG ACPA-positive patients ([figure 3B](#)). Total serum IgA1 and IgG did not change significantly (online supplemental figure 6).

DISCUSSION

In this study, we show that (1) RA at-risk individuals display both IgA1 and IgA2 ACPA at comparable levels to patients with established disease, (2) IgA1 ACPA positivity in RA at-risk individuals is associated with progression to RA and (3) IgA1 ACPA levels decrease around RA development.

About two-thirds of RA at-risk individuals possessed IgA ACPA in our study. In patients with established RA, the prevalence of IgA ACPA was somewhat higher, but IgA ACPA levels were not significantly increased. These data indicate that in the majority of at-risk individuals the autoimmune response is already established, at least by the time first symptoms, such as arthralgia appear.

In general, we found a higher prevalence of IgA ACPA positivity in patients with RA than previously reported^{8 25 26}

Table 3 Characteristics of the individuals at-risk with follow-up

	IgA ACPA-positive (N=14)	IgA ACPA-negative (N=5)	Confirmation cohort from Vienna (N=9)
Age, years: mean (SD)	45.7 (15.8)	40.6 (11)	54.6 (16.5)
Female, N (%)	8 (57)	3 (60)	5 (55.6)
Arthralgia, N (%)	14 (100)	5 (100)	–
Arthralgia duration, months: median (IQR)	17.5 (5.8–32.5)	8 (2.8–22)	–
Medication before diagnosis:			
Abatacept, N (%)	1 (7.1)	3 (60)	–
NSAID, N (%)	7 (50)	1 (20)	–
Other painkillers, N (%)	3 (21.4)	0 (0)	–
IgA1 ACPA at baseline, µg/mL: median (IQR)	10.3 (4.9–43.6)	–	8.3 (4.9–66.8)
IgA2 ACPA at baseline, µg/mL: median (IQR)	1.9 (0.9–5.2)	–	–
IgG ACPA at baseline, RU/mL: median (IQR)	439.3 (57.4–856.5)	51 (21.9–115.1)	258.1 (29–867.7)
First serum prior to diagnosis, months: median (IQR)	5 (3–17)	14 (10–20)	3 (3–6)
last serum after diagnosis, months: median (IQR)	3 (3–13)	13 (13–13)	–
ACPA, anticitrullinated protein antibodies; NSAID, non-steroidal anti-inflammatory drugs; RA, rheumatoid arthritis; RU, relative units.			

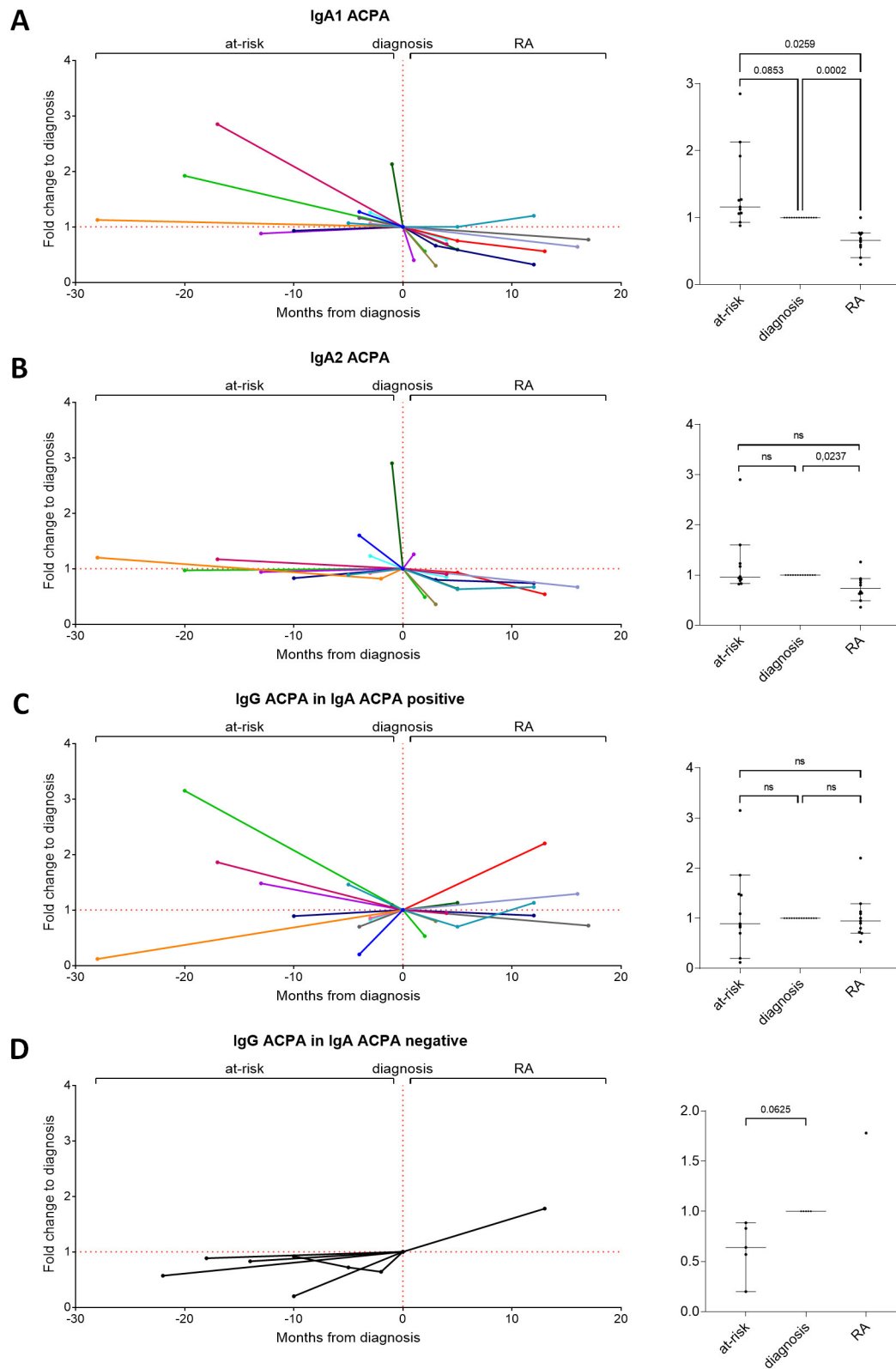


Figure 2 Dynamic changes in serum ACPA levels in RA at-risk individuals during transition from the at-risk state to RA. Only at-risk individuals positive for IgA ACPA according to the cut-off based on the ELISA standard curves were used for the analysis. Shown are fold changes of (A) IgA1 ACPA (n=14), (B) IgA2 ACPA (n=13) and IgG ACPA in (C) IgA ACPA-positive individuals (n=14) and (D) IgA ACPA-negative individuals (n=5) compared with the levels at RA onset (set as 1). Each line represents one at-risk individual (same colour represents the same individual). Significances were tested with mixed effects model (modified ANOVA) with Tukey's multiple comparisons correction. ACPA, anticitrullinated protein antibody; ANOVA, analysis of variance; ns, not significant; RA, rheumatoid arthritis.

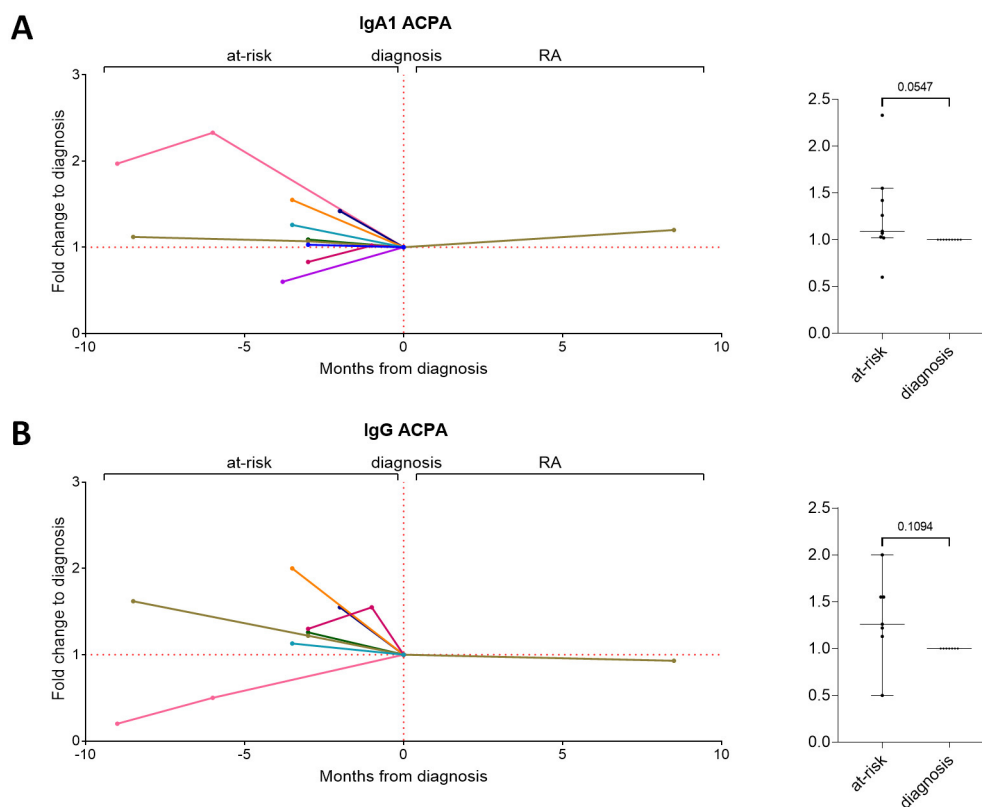


Figure 3 Dynamic changes in serum ACPA levels in individuals from the confirmatory cohort from the Medical University of Vienna during transition from the at-risk state to RA. Shown are fold changes of (A) IgA1 ACPA (n=9) and (B) IgG ACPA (n=6) compared with the levels at RA onset (set as 1). Each line represents one individual (same colour represents the same individual). Significances were tested with Wilcoxon test. ACPA, anticitrullinated protein antibody; ns, not significant; RA, rheumatoid arthritis.

as we used lower cut-offs based on detectable amounts of IgA ACPA according to the standard curve of the ELISA. Our purpose was not to reach a high specificity for RA, as required for diagnostic purposes. Instead, we aimed for a high sensitivity regarding the presence of IgA ACPA. Using this detection-based cut-off, IgA ACPA were not only confined to IgG ACPA-positive patients with RA, but were also detected in a considerable number of IgG ACPA-negative patients with RA and a minority of healthy controls. However, the IgA ACPA levels of positive healthy individuals were much lower than those of most RA at-risk individuals and were in most cases only for one of the two subclasses above the cut-off. In recent years, the observed presence of ACPA at several mucosal sites, lead to the suggestion that the initial immune response against citrullinated proteins might be triggered by mucosal inflammatory processes.²⁷ Non-arthritic first degree relatives of patients with RA without serum IgG ACPA displayed IgA ACPA in the sputum which was associated with elevated myeloid cell counts and neutrophil extracellular trap levels.²¹ In addition, it has been reported that citrullinated proteins are present in the gut.²⁸ From this perspective, it is plausible that some healthy individuals possess IgA ACPA, as they might reflect immune reactions to citrullinated neoantigens in the context of chemical or infectious mucosal stress. The published observation that IgA ACPA are more prevalent

than IgG ACPA in healthy relatives of patients with RA might support such a concept.^{29 30} However, the fact that most of the IgA ACPA positive healthy individuals were weakly positive and only positive for one IgA subclass suggests that there is no directed immune response against citrullinated proteins, but that the positive events derive either from single reactive plasma cell clones or from cross reactivity.

IgA1 ACPA were associated with a higher risk of developing RA within the following 14 months. This finding is in concordance with recent studies showing that elevated levels of ACPA containing secretory component (which partially include IgA ACPA) are associated with RA development in IgG ACPA positive individuals with musculoskeletal pain³¹ and that serum IgA ACPA levels are increased in at-risk individuals with clinically suspect arthralgia.²³ However, in the former study, IgA ACPA positivity was not different between RA progressors and non-progressors, which might be related to the high cut-off used in that study. A lower cut-off as we used in our study might be beneficial in this regard due to an increased sensitivity. A clear limitation of a low cut-off is that it increases the chance of false positive signals especially in the low concentration range that may emerge from unspecific binding. We have therefore reanalysed the main data from the manuscript using quite conservative cut-offs based on the mean +3 SD of the healthy

cohort. With the higher cut-offs, nearly all of the healthy controls were IgA ACPA negative. The main outcomes of the study did not change. However, using a higher cut-off was accompanied by a substantial loss of sensitivity. Therefore, it is worth considering lower cut-offs especially when IgA ACPA values are not used for diagnostic purposes, but as additional risk factors in at-risk individuals already predefined by IgG ACPA positivity.

In addition, in contrast to other studies, we depleted the sera from IgG before IgA ACPA measurement. This procedure might further increase sensitivity and specificity as it reduces competition with IgG ACPA for binding sites and unspecific signals arising from IgA rheumatoid factor.

Interestingly, at-risk individuals who did not develop RA during the following 14 months tended to have longer symptoms duration at the time of serum analysis, although this difference was not significant. This finding is consistent with other studies describing that most IgG ACPA positive at-risk individuals with clinically suspect arthralgia who progress to RA do so within 1 year of arthralgia onset.³² However, some at-risk individuals from our study developed RA 10 years or even later after first signs of arthralgia.

Like IgG ACPA, IgA ACPA can induce proinflammatory effector functions in various cell types.³³ In addition, IgA complexes are even superior in inducing the formation of neutrophil extracellular traps²⁰ which are thought to contribute to the disease.³⁴ Surprisingly, our data did not show that serum IgA2 ACPA, which possess higher proinflammatory properties than IgA1,¹⁵ are associated with RA onset. This might be due to the fact that IgA2 ACPA levels are much lower than IgA1 ACPA levels and thus harder to measure which was also reflected by the fact that we had to remove some individuals from the IgA2 ACPA analysis due to unacceptably high differences between the technical replicates.

An unexpected and interesting finding that has not been previously described is the decline especially in IgA1 ACPA levels shortly before and after the RA onset, which has not been observed for IgG ACPA. These data at first glance contradict another study that reported an elevation of IgA ACPA levels overtime.³⁵ However, the authors used mixed models including all patients, regardless of autoantibody levels, while in this study, we included only individuals with reliably detectable amounts of IgA ACPA and evaluated the ACPA dynamics separately for each individual. In addition, the mentioned study observed a much longer time-period comprising several years. Hence, the discrepancy might also be explained by the switch from an IgA ACPA-negative to IgA ACPA-positive state over time in some individuals, as has been previously shown.^{30, 35} Here, we show that in at-risk individuals who possess IgA ACPA prior to RA onset, IgA ACPA levels decrease in a relatively short time frame around disease onset. This does not contradict the fact that in IgA ACPA-negative individuals an expansion of autoantibody

isotype repertoire is taking place, and that levels of autoantibodies increase in the long term.

The observed decline in IgA ACPA levels around RA onset could indicate their antigen engagement in the tissues due to the 'leakiness' of inner barriers, such as in the joints, lungs and gut, which are affected in RA.^{36–38} Another possibility would be the migration of ACPA-producing plasmablasts/plasma cells into the joints, as it has been proposed during flare.³⁹

A clear limitation of this study is a rather low number of patients for whom longitudinal serum samples were collected. Nonetheless, the fact that the decline in IgA ACPA during RA onset was observed in two independent cohorts strengthens this finding. Taken together, our data suggest that IgA ACPA are prevalent in RA at-risk individuals, are associated with the development of RA and decline in levels immediately before the onset of RA.

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Competing interests HB is an employee of Orgentec Diagnostika, a supplier of CCP-ELISAs for IgG ACPA measurements. All other authors declare no conflict of interest.

Patient consent for publication Not applicable.

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