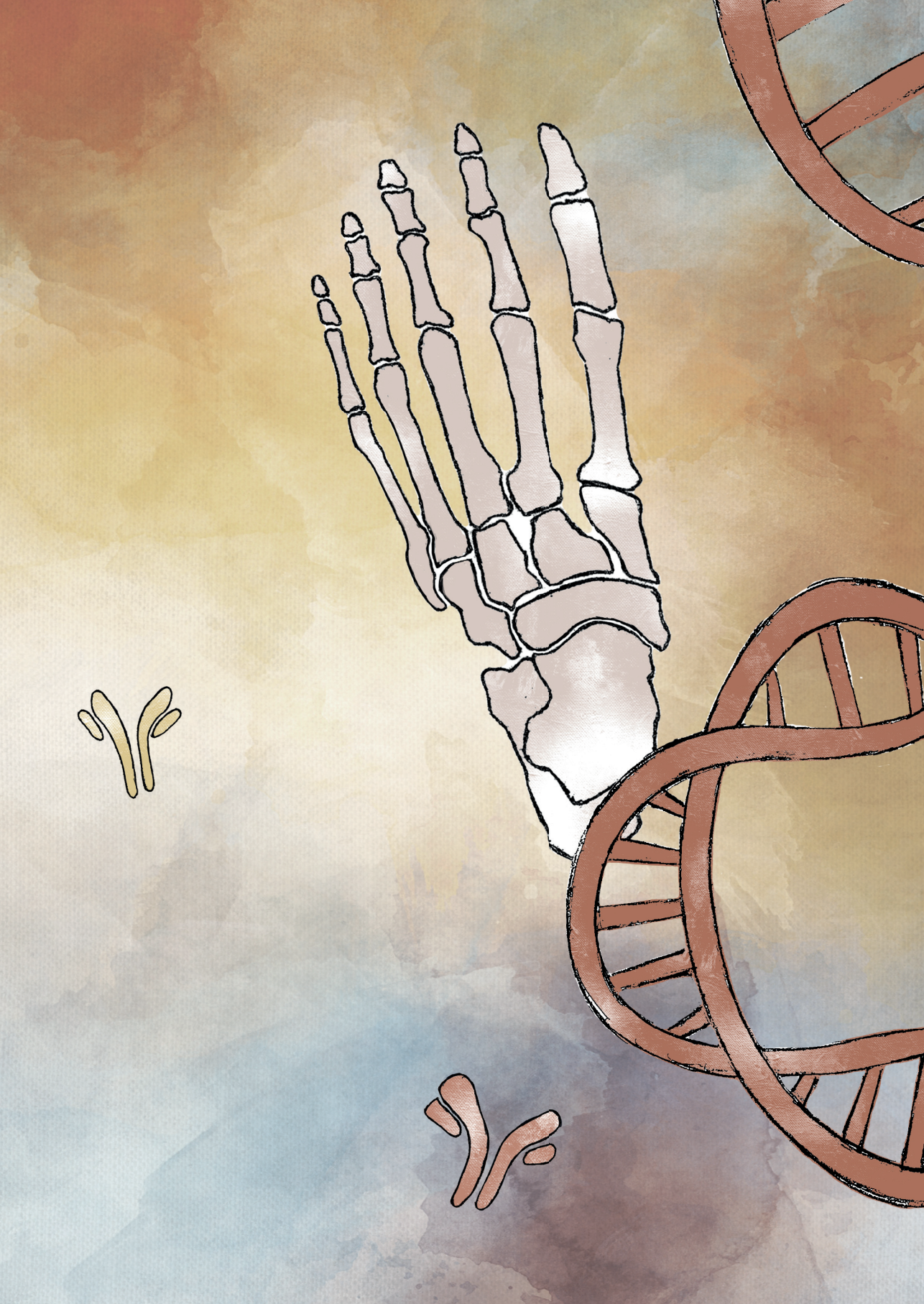
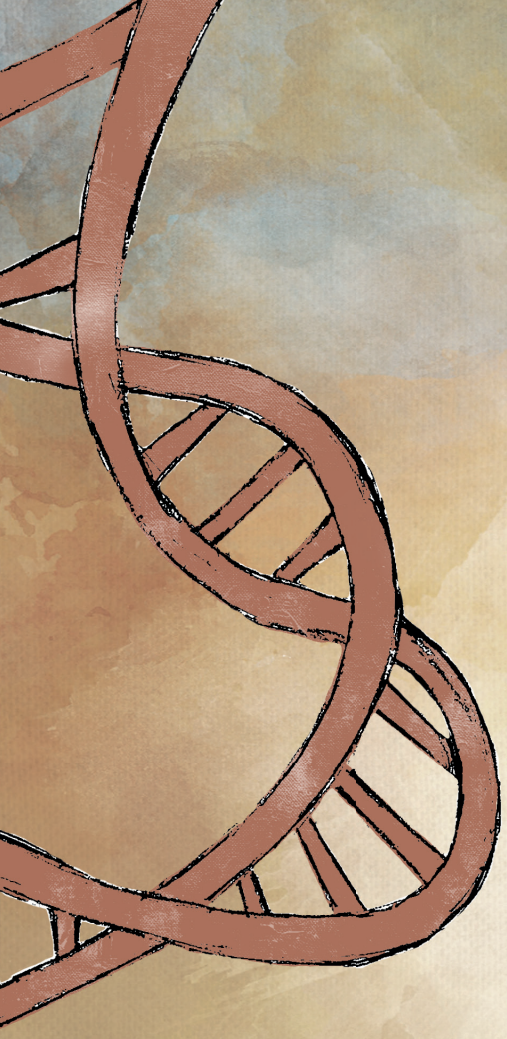
Clinically suspect arthralgia: unraveling the development of rheumatoid arthritis

Wouters, F.

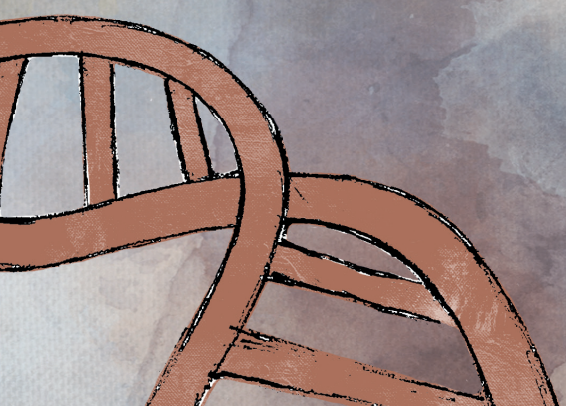


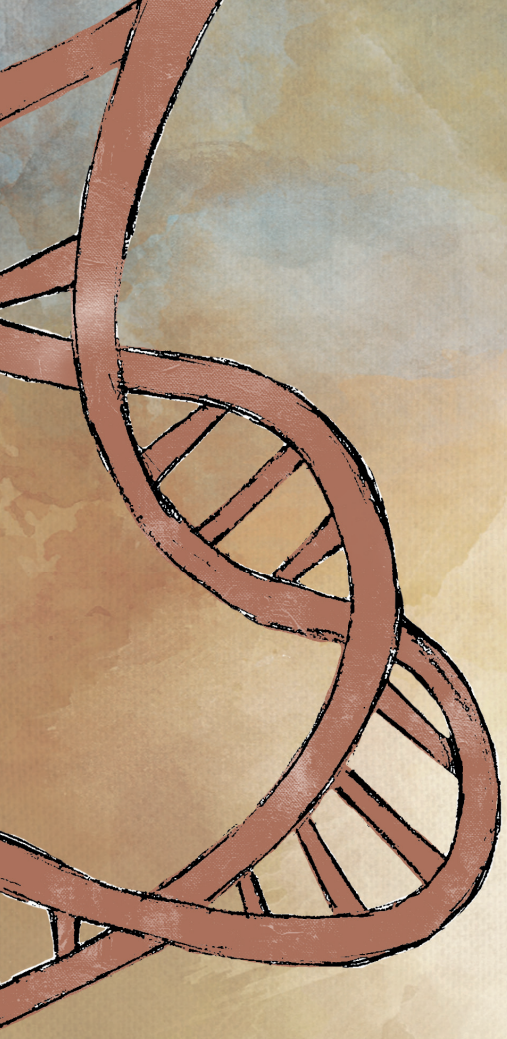


Pathogenesis of Rheumatoid Arthritis

Part

II

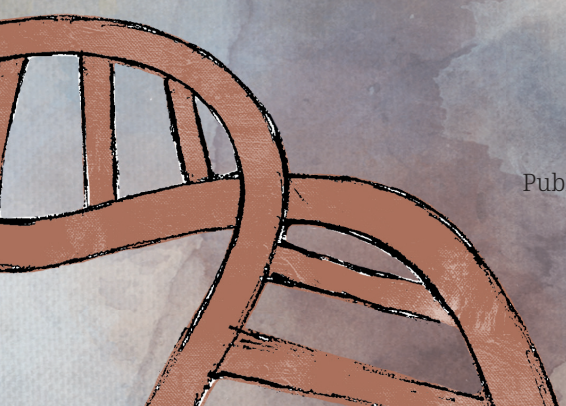




6

Do autoantibody-responses mature between presentation with arthralgia suspicious for progression to rheumatoid arthritis and development of clinically apparent inflammatory arthritis? A longitudinal serological study

Fenne Wouters
Ellis Niemantsverdriet
Nazike Salioska
Annemarie L. Dorjée
René E.M. Toes
Annette H.M. van der Helm-van Mil



Published as *Letter* in Annals of the Rheumatic Diseases
2020 Nov 3;80(4):540-542.
doi:10.1136/annrheumdis-2020-218221.

Several nested case-control studies have shown that autoantibody-response maturation in rheumatoid arthritis (RA) precedes clinical arthritis-development.[1-3] This suggests a role in disease triggering. However, nested case-control studies have, similar to case-control studies, the disadvantage that controls are selected and that prospective data from non-progressing patients in a similar pre-disease stage are absent. The phase preceding clinically apparent inflammatory arthritis (IA) can be distinguished into an asymptomatic and symptomatic (i.e. clinically suspect arthralgia, CSA) sub-phase. It is unknown whether autoantibody-response maturation occurs in the symptomatic phase. Likewise, its role in progression to clinical arthritis is undetermined; if autoantibody-response maturation relates to disease-development, maturation is expected to be more pronounced in CSA-patients that progress compared to CSA-patients that do not. To better understand the relation between autoantibody-response maturation in time and development of clinical arthritis (RA/IA), we performed a longitudinal study on autoantibody-response maturation in CSA-patients that did and did not progress.

In serum from 147 CSA-patients, we determined with in-house ELISAs the presence and levels of IgM, IgG, IgA anti-citrullinated, anti-carbamylated and anti-acetylated protein antibodies (ACPA, anti-CarP, AAPA), resulting in 9 autoantibody measurements per patient per time-point. Autoantibody-response maturation was defined as increase in number of autoantibody-reactivities or isotypes, and/or increase in autoantibody levels. CSA-patients with paired samples at first presentation at the outpatient clinic and at IA-development (n=55) or else after 2-years (n=92) were selected. Analyses were repeated with the outcome RA (the subgroup of IA-patients that fulfilled the 2010-or 1987-criteria at the time of IA-development). Detailed description of methods and baseline characteristics are shown supplementary.

In patients negative for all autoantibodies at baseline, 17% of patients that progressed to IA became positive, compared to 6% of ”non-progressors” (Figure 1A, p=0.12). In patients with ≥1 autoantibody-reactivity at baseline progressing to IA, the median number of autoantibody-reactivities was 1.0 (IQR 1.0-3.5, max. 6) at baseline and 1.0 (IQR 1.0-4.0, max. 6) at IA-development (p=0.29). In non-progressing CSA-patients with ≥1 autoantibody-reactivity at baseline, this was 1.0 (IQR 1.0-2.0, max. 4) at baseline and 1.0 (IQR 0.0-2.3, max. 5) after 2-years (p=0.07). As shown in Figure 1B; an increase in the number of autoantibody-reactivities was infrequent (16% in progressors, 18% in non-progressors (p=1.00)). Most changes in autoantibody-positivity were explained by fluctuations around the cut-off (data not shown). Levels of autoantibodies did not significantly change over time (p-values ranging 0.21-1.00) both in progressors and non-progressors (Figure 1C). Similar results were found with the outcome RA (Supplementary Figure 1), though remarkably, the number of autoantibody-

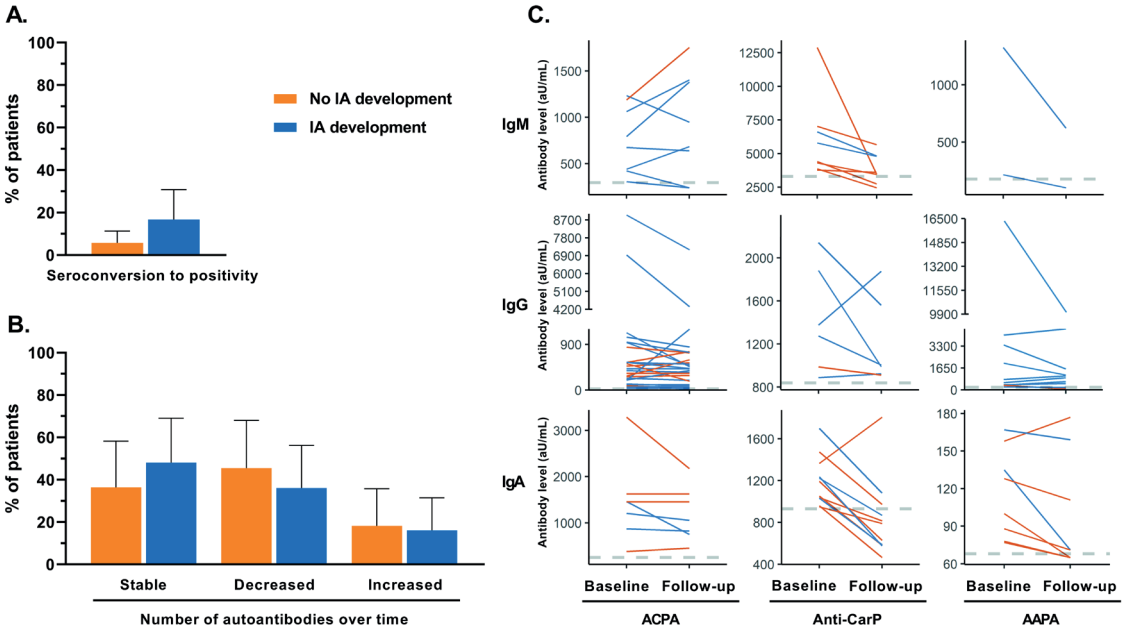
reactivities in patients not-progressing to RA significantly decreased over time (1.0 (IQR 1.0-2.0) at baseline and 1.0 (IQR 0.0-2.0) after 2-years, p=0.015). Finally, when evaluating number of autoantibody-reactivities and autoantibody-level changes within the entire study population (instead of within patients with ≥1 autoantibody-reactivity at baseline) no significant increases were found (Supplementary Figure 2).

To the best of our knowledge, this is the first study evaluating multiple isotypes and three anti-modified protein autoantibodies over time in CSA. Our data indicate that the presence and levels of IgM, IgG and IgA ACPA, anti-CarP and AAPA did not significantly increase over time, and that this was similar for CSA-patients that did or did not develop IA.

Autoantibody maturation in terms of cross-reactivity, affinity maturation and involvement of individual B-cell clones was not studied here, which is a limitation. We did not observe changes in isotype-usage over time, indicating that isotype switching was infrequent in both groups (Supplementary Figure 3, Supplementary Table 4). Although we cannot exclude that the results of this study would be different with a larger sample size (especially in CSA-patients autoantibody-negative at first presentation), the current data suggests that autoantibody-response maturation already occurs before presenting with CSA and that it does not increase substantially during progression to IA. Our results on characteristics of the ACPA, anti-CarP and AAPA-response expand on previous longitudinal studies showing similar ACPA- and RF-levels,[4,5] and absence of change in the ACPA antigen-recognition repertoire in ACPA-positive arthralgia.[6] The data together imply that maturation occurs predominantly in the asymptomatic phase, a finding to be confirmed in population-based studies. Moreover, in relation to a multiple-hit model for RA-development, our data suggest that autoantibody-response maturation in the CSA-phase is not related to the “final hit” as maturation was similar in CSA-patients not developing RA. These results increase the comprehension of the pathogenesis of RA.

In conclusion, autoantibody-response maturation as measured in this study occurs in the vast majority of CSA-patients before presenting with symptoms and broadening of the autoantibody-response is not specific for progression from arthralgia to clinical arthritis.

Figure 1. Changes in autoantibody-response over time: A) percentage of patients with seroconversion to positive in patients negative for all autoantibodies at baseline, B) percentage of patients that has an increasing, decreasing or stable number of positive measurements over time in patients positive for ≥1 autoantibody-reactivity at baseline, C) autoantibody levels over time in patients positive for the respective autoantibody at baseline.



All results are shown separately for CSA-patients that did and did not progress to IA. The mean time between first presentation and IA development was 5.6 months (SD 9.2). In patients that did not progress the second serum sample was obtained after 2-years.
Figure 1A autoantibody negativity at baseline was defined as negative for the nine studied measurements (n=100), Figure 1B autoantibody positive was defined as at least one (out of nine) positive measurement at baseline (n=47).
Error bars in Figure 1A and 1B represent 95% CI. Dashed grey horizontal lines in Figure 1C indicate the cut-off values for each autoantibody.
IA: clinically apparent inflammatory arthritis, ACPA: anti-citrullinated protein antibodies, anti-CarP: anti-carbamylated protein antibodies, AAPA: anti-acetylated protein antibodies.

References

1. Nielen MM, van Schaardenburg D, Reesink HW et al. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. Arthritis Rheum. 2004;50:380-6.

2. Rantapaa-Dahlqvist S, de Jong BA, Berglin E et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum. 2003;48:2741-9.

3. Gan RW, Trouw LA, Shi J 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.

4. Ten Brinck RM, van Steenbergen HW, van Delft MAM et al. The risk of individual autoantibodies, autoantibody combinations and levels for arthritis development in clinically suspect arthralgia. Rheumatology (Oxford). 2017;56:2145-53.

5. van Beers-Tas MH, Stuiver MM, de Koning M et al. Can an increase in autoantibody levels predict arthritis in arthralgia patients? Rheumatology (Oxford). 2018;57:932-4.

6. Janssen KM, Westra J, Chalan P et al. Regulatory CD4+ T-Cell Subsets and Anti-Citrullinated Protein Antibody Repertoire: Potential Biomarkers for Arthritis Development in Seropositive Arthralgia Patients? PLoS One. 2016;11:e0162101.

Supplementary File 1 – Detailed description of methods

Patients

Patients with recent-onset (<1 year) arthralgia of small joints and, according to the clinical expertise and pattern recognition of the rheumatologist a clinical suspicion for progression to RA, were included in the Leiden CSA-cohort. Autoantibody status was largely unknown at inclusion as (in line with Dutch guidelines) general practitioners in the area of Leiden are discouraged to perform autoantibody tests. Inclusion in the CSA-cohort was therefore predominantly based on history taking and physical examination. Patients were excluded if clinically apparent inflammatory arthritis was already present, or if a different explanation for the joint pain was more likely. The cohort is described in detail previously.[1] Patients were followed for at least 2 years on the development of clinically apparent inflammatory arthritis (IA) with scheduled research visits after 4, 12 and 24 months. Clinical follow-up visits took place at the scheduled visits and at additional visits (either in between or after the scheduled visits), as considered necessary by patients or rheumatologists. Serum samples were taken at baseline and when patients progressed to IA, or, when patients did not progress to IA after 2-years. Patient selection for the present study was first based on availability of paired samples and subsequently on the presence of autoantibodies at baseline. The latter was done because of limited laboratory capacity. Patients that were tested positive for RF (in house ELISA, cut-off >3.5 IU/mL) and/or ACPA (anti-CCP2, Phadia, Nieuwegein, the Netherlands, cut-off >7U/mL) during routine laboratory measurements at baseline and had paired serum samples were included (n=59, 29 progressing and 30 non-progressing patients). In addition, autoantibody-negative patients with paired samples that progressed to IA were included (n=26). Finally, from the large group of autoantibody-negative patients that did not progress to IA a random selection was made (n=62). Supplementary Table 2 suggests that selection of patients with paired samples from the total CSA-cohort did not induce substantial selection bias. Similarly, baseline characteristics of the randomly selected autoantibody-negative patients were similar to that of the patients that were not selected (Supplementary Table 3); suggesting that the selection is representative for this total group. Thus, the similarity in baseline characteristics from selected and non-selected patients implies that the selected group of patients (N=147 in total) is representative and suitable to study autoantibody characteristics over time. However the fact that not all but a selection of autoantibody-negative CSA-patients was assessed makes the current selection not suitable to determine the predictive accuracy of autoantibodies, which was also not the aim of this study.

Autoantibodies

In serum, we determined the presence and levels of anti-citrullinated, anti-carbamylated and anti-acetylated protein antibodies (ACPA, anti-CarP and AAPA, respectively); all three autoantibodies have been shown to be present in RA. ACPA and anti-CarP have been shown to be associated with progression and/or prediction of disease and have a specificity of 95-100% and 95%, respectively.[2-6] The specificity of AAPA IgG in patients with RA, compared to non-RA patients with persistent or resolving arthritis was 86% in a previous study.[7] Cross-reactivity between all three autoantibodies has been shown.[8] In this study, presence of ACPA, anti-CarP and AAPA was determined for three isotypes (IgM, IgG and IgA), resulting in 9 autoantibody measurements per patient per time-point. In-house ELISA was used for all measurements as described previously.[9] Briefly, plates were coated with citrullinated CCP2, carbamylated FCS and CCP1 acetylated lysine for measurements of ACPA, anti-CarP and AAPA, respectively. To determine background signal, plates were additionally coated with non-modified antigens (arginine CCP2, non-modified FCS and CCP1 norleucine, respectively). Serum samples were diluted 1:50 and incubated. After washing, plates were subsequently incubated with HRP-labeled goat-anti-human IgM (Millipore), rabbit-anti-human IgG (Dako) or goat-anti-human IgA (Novex). HRP-activity was visualized with ABTS and measurements were expressed in arbitrary units per milliliter (aU/mL). On every plate a dilution standard was included to determine the linear part of the curve; standards from all plates were used in the analyses. The fourth standard, which is expected to be in the middle (and therefore linear part) of the curve, is further diluted and additionally included as a reference sample. Serum of healthy subjects (n=199) was used to determine the cut-off of all autoantibody measurements, which was calculated as the mean plus two times the standard deviation of healthy subjects. When the background signal of non-modified antigens was >50% of the signal measured in modified proteins, the measurement was considered non-specific; non-specific measurements with values above the cut-off were considered negative. In case a sample reached the upper detection limit of the assay, the sample pair (two samples of the same individual but from different time points) was reanalyzed in a higher dilution (2 samples for ACPA IgG in 1:2000, 2 samples for ACPA IgA in 1:250, 6 samples for AAPA IgG with dilutions ranging 1:100-1:2000). Samples were measured single well and paired samples, thus two samples of the same individual but from different time points, were analyzed on the same plate. Inter-assay variation of in-house ELISAs was determined previously by reevaluation of ~10% of samples; measurements were highly correlated (Pearson's r ranges 0.88-0.99) and changes in positivity of the test were infrequent, see Supplementary Figure 4. Intra-assay variability was determined for ACPA and anti-CarP IgM, IgG and IgA by measurement of 3 samples 10 times. The mean coefficients of variation (CV, mean % (SD)) were: ACPA IgM 13.5 (15.0), IgG 8.7 (6.2), IgA 3.4 (1.2), anti-CarP IgM 5.6 (3.7), IgG

20.4 (6.8), IgA 4.2 (1.1). Of note, although not absolute at the monoclonal- or polyclonal level, cross-reactivity of ACPA towards other post translationally modified proteins have been conclusively shown in different studies,[8,10] and hence should be regarded as anti-modified protein antibody-reactivities.

Outcome

The primary outcome was development of IA, determined by physical examination of the rheumatologist (assessment of clinical joint swelling) during follow-up. DMARDs (including glucocorticoids) were not prescribed in patients with CSA. In patients that progressed to IA, the second sample was taken at IA-development. In patients that did not progress to IA serum samples were taken after 2 years (last scheduled follow-up visit with serum collection). Theoretically, IA-development could have occurred after this 2 years-visit in these patients. Reassuringly however, this did not occur during the period for which clinical follow-up data was available (median 29 months (IQR 20-46) after the scheduled 2-years visit). We also assume that patients would have visited our outpatient clinic in case of an increase in symptoms or suspected arthritis, and therefore that these data are all-encompassing, since our outpatient clinic is the only referral center in a healthcare region of approximately 400.000 inhabitants and patients (especially those participating to clinical studies) have very easy access to our outpatient clinic.

Analyses were repeated with "development of RA" as outcome, which was defined by fulfilment of the 1987 and/or 2010 classification criteria for RA at the time clinically apparent arthritis (IA) had presented.[11,12] The 1987-criteria were incorporated in this definition as autoantibody-negative patients require >10 involved joints in the 2010-criteria to be classified as RA.

Statistical analyses

Autoantibody-response maturation over time was defined as an increase in number of autoantibody-reactivities or isotypes, and/or an increase in autoantibody levels. To evaluate autoantibody-response maturation three analyses were performed, in patients that progressed to IA (n=55) and in patients that did not progress (n=92) separately. First, in patients negative for all nine measurements at baseline, we determined the frequency of conversion to seropositivity. Importantly when showing the results from the analyses of the different isotypes of ACPA, AAPA and anti-CarP, autoantibody negativity was defined as negativity for these nine isotypes at baseline (n=100). Second, in patients with at least one positive test at baseline (n=47), we studied autoantibody positivity over time by evaluating the median number of positive autoantibody-reactivities over time and the frequency that the number of positive measurements changed. Finally, we determined the change in autoantibody

levels over time, for all autoantibodies and isotypes separately. In these analyses we only included patients positive for the respective measurement at baseline, e.g. for evaluation of changes in IgG ACPA levels over time we only included patients that were positive for IgG ACPA at baseline. Frequencies and medians were reported. Statistical significance of frequencies was tested with Fisher's Exact test. The number of autoantibody-reactivities over time was tested with generalized estimating equations (GEE), taking into account that measurements over time and within one autoantibody type (ACPA, anti-CarP or AAPA) can be correlated. Changes in autoantibody levels over time were tested with Wilcoxon Signed Ranks tests with Bonferroni correction for multiple testing.

Subanalyses

Two additional analyses were performed. First, analyses were repeated with the outcome development of RA. Secondly, the number of autoantibody-reactivities and autoantibody levels over time were evaluated within the entire study population (instead of within the group of patients that were autoantibody positive at baseline).

IBM SPSS Statistics 25 was used for all analyses. P-values ≤0.05 were considered statistically significant.

References

1. van Steenbergen HW, van Nies JA, Huizinga TW et al. Characterising arthralgia in the preclinical phase of rheumatoid arthritis using MRI. Ann Rheum Dis. 2015;74:1225-32.

2. Nielen MM, van Schaardenburg D, Reesink HW et al. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. Arthritis Rheum. 2004;50:380-6.

3. Rantapaa-Dahlqvist S, de Jong BA, Berglin E et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum. 2003;48:2741-9.

4. van Steenbergen HW, Mangnus L, Reijnierse M et al. Clinical factors, anticitrullinated peptide antibodies and MRI-detected subclinical inflammation in relation to progression from clinically suspect arthralgia to arthritis. Ann Rheum Dis. 2016;75:1824-30.

5. Gan RW, Trouw LA, Shi J 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.

6. Nishimura K, Sugiyama D, Kogata Y et al. Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. Ann Intern Med. 2007;146:797-808.

7. Juarez M, Bang H, Hammar F et al. Identification of novel antiacetylated vimentin antibodies in patients with early inflammatory arthritis. Ann Rheum Dis. 2016;75:1099-107.

8. Kissel T, Reijm S, Slot LM et al. Antibodies and B cells recognising citrullinated proteins display a broad cross-reactivity towards other post-translational modifications. Ann Rheum Dis. 2020;79:472-80.

9. Shi J, van de Stadt LA, Levarht EW et al. Anti-carbamylated protein antibodies are present in arthralgia patients and predict the development of rheumatoid arthritis. Arthritis Rheum. 2013;65:911-5.

10. Sahlstrom P, Hansson M, Steen J et al. Different Hierarchies of Anti-Modified Protein Autoantibody Reactivities in Rheumatoid Arthritis. Arthritis Rheumatol. 2020.

11. Arnett FC, Edworthy SM, Bloch DA et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 1988;31:315-24.

12. Aletaha D, Neogi T, Silman AJ et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/ European League Against Rheumatism collaborative initiative. Arthritis Rheum. 2010;62:2569-81.

Supplementary Table 1. Baseline characteristics of the studied CSA patients that did and did not progress to clinically apparent inflammatory arthritis (IA)

		IA during follow-up (n=55)	No IA during follow-up (n=92)	p-value
Clinical characteristics				
Female, n (%)		40 (72.7)	73 (79.3)	0.42
Age in years, mean (SD)		46.4 (12.9)	45.5 (12.8)	0.60
Symptom duration in weeks, median (IQR)		21 (8-51)	17 (10-37)	1.00
68-TJC, median (IQR)		5 (3-9)	5 (2-11)	0.82
Morning stiffness ≥60 minutes, n (%)		22 (40.0)	23 (25.0)	0.066
Difficulties making a fist, n (%)		14 (25.9)	10 (11.0)	0.036
Family history of RA, n (%)		16 (29.6)	17 (19.1)	0.16
Routine laboratory measurements				
Increased CRP (≥5 mg/L), n (%)		16 (29.1)	19 (20.7)	0.32
RF IgM positivity (≥3.5 IU/mL), n (%)		26 (47.3)	25 (27.2)	0.019
ACPA IgG positivity (≥7.0 IU/mL), n (%)		22 (40.0)	12 (13.0)	<0.001
Presence of autoantibodies with in-house ELISA, n (%)				
ACPA	IgM	8 (14.5)	1 (1.1)	0.002
	IgG	20 (36.4)	9 (9.8)	<0.001
	IgA	3 (5.5)	4 (4.3)	1.00
Anti-CarP	IgM	2 (3.6)	6 (6.5)	0.71
	IgG	5 (9.1)	1 (1.1)	0.028
	IgA	4 (7.3)	7 (7.6)	1.00
AAPA	IgM	2 (3.6)	0 (0.0)	0.14
	IgG	10 (18.2)	1 (1.1)	<0.001
	IgA	2 (3.6)	7 (7.6)	0.48

SD: standard deviation, IQR: interquartile range, TJC: tender joint count, CRP: c-reactive protein, RF: rheumatoid factor, ACPA: anti-citrullinated protein antibody, anti-CarP: anti-carbamylated protein antibodies, AAPA: anti-acetylated protein antibodies

Supplementary Table 2. Baseline characteristics of all CSA-patients included between 2012 and 2016, stratified for patients with available paired serum samples and patients with only baseline samples available

	Paired samples available	Only baseline samples available	p-value
Female, n (%)	171 (78.4)	119 (77.8)	0.90
Age in years, mean (SD)	45.3 (12.8)	40.9 (11.8)	0.001
Symptom duration in weeks, median (IQR)	17 (9-39)	17 (8-33)	0.23
68-TJC, median (IQR)	5 (2-10)	6 (2-11)	0.81
Increased CRP (≥5 mg/L), n (%)	41 (18.8)	33 (21.7)	0.51
RF positivity* (≥3.5 IU/mL), n (%)	49 (22.5)	27 (17.6)	0.30
ACPA positivity* (≥7 U/mL), n (%)	31 (14.2)	16 (10.5)	0.34

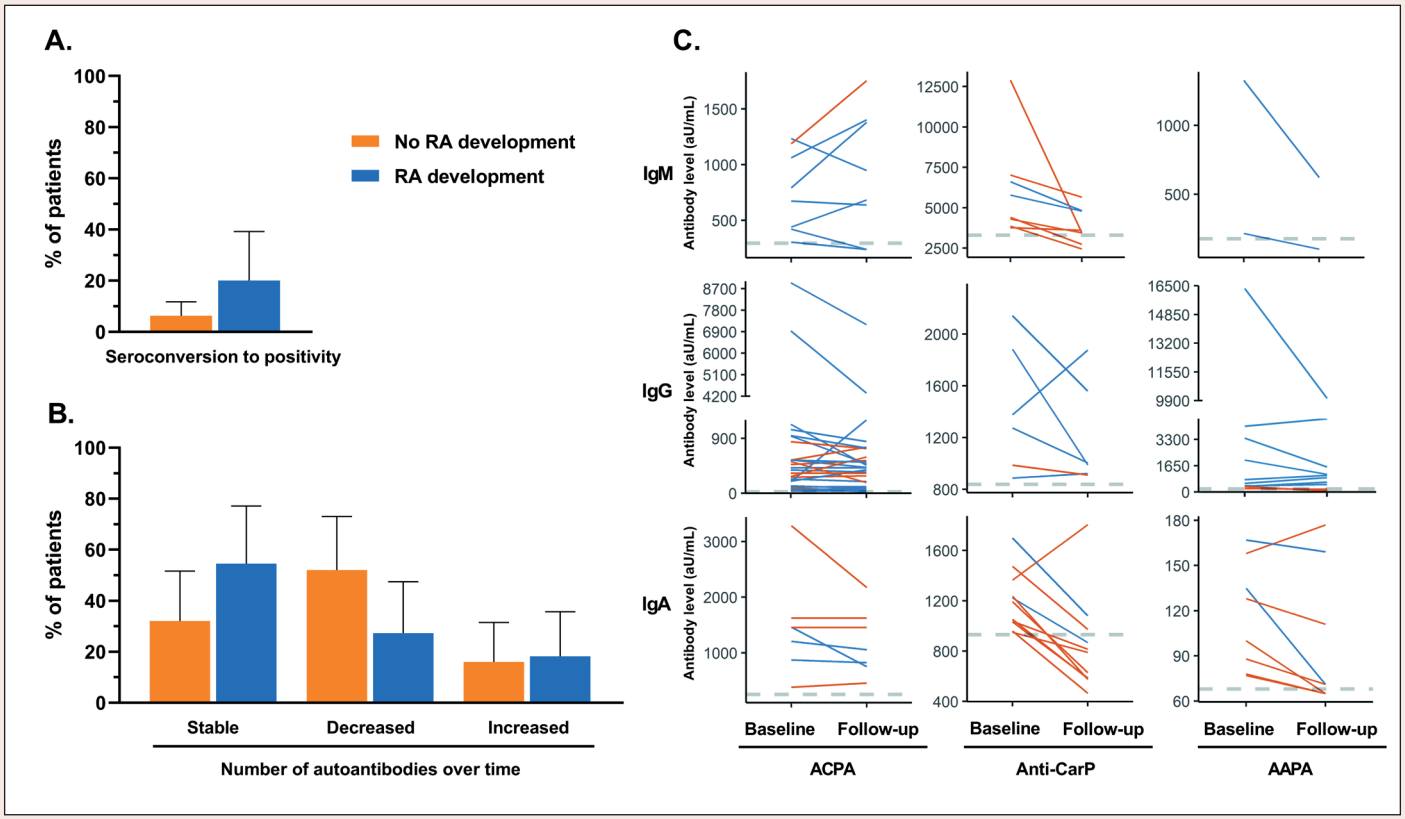
* based on routine laboratory diagnostics at baseline
CSA: clinically suspect arthralgia, ACPA: anti-citrullinated protein antibody, SD: standard deviation, IQR: interquartile range, TJC: tender joint count, RF: rheumatoid factor, CRP: c-reactive protein

Supplementary Table 3. Baseline characteristics of the autoantibody-negative CSA-patients not progressing to IA with available paired samples that were randomly selected to be included and not included in this study

	Included based on random selection (n=62)	Not included based on random selection (n=77)	p-value
Female, n (%)	49 (79.0)	62 (80.5)	0.84
Age in years, mean (SD)	44.3 (13.6)	44.7 (12.7)	0.99
Symptom duration in weeks, median (IQR)	16 (9-29)	17 (9-45)	0.47
68-TJC, median (IQR)	7 (3-13)	6 (2-10)	0.55
Increased CRP (≥5 mg/L), n (%)	14 (22.6)	9 (11.7)	0.11
RF positivity* (≥3.5 IU/mL), n (%)	0 (0.0)	0 (0.0)	NA
ACPA positivity* (≥7 U/mL), n (%)	0 (0.0)	0 (0.0)	NA

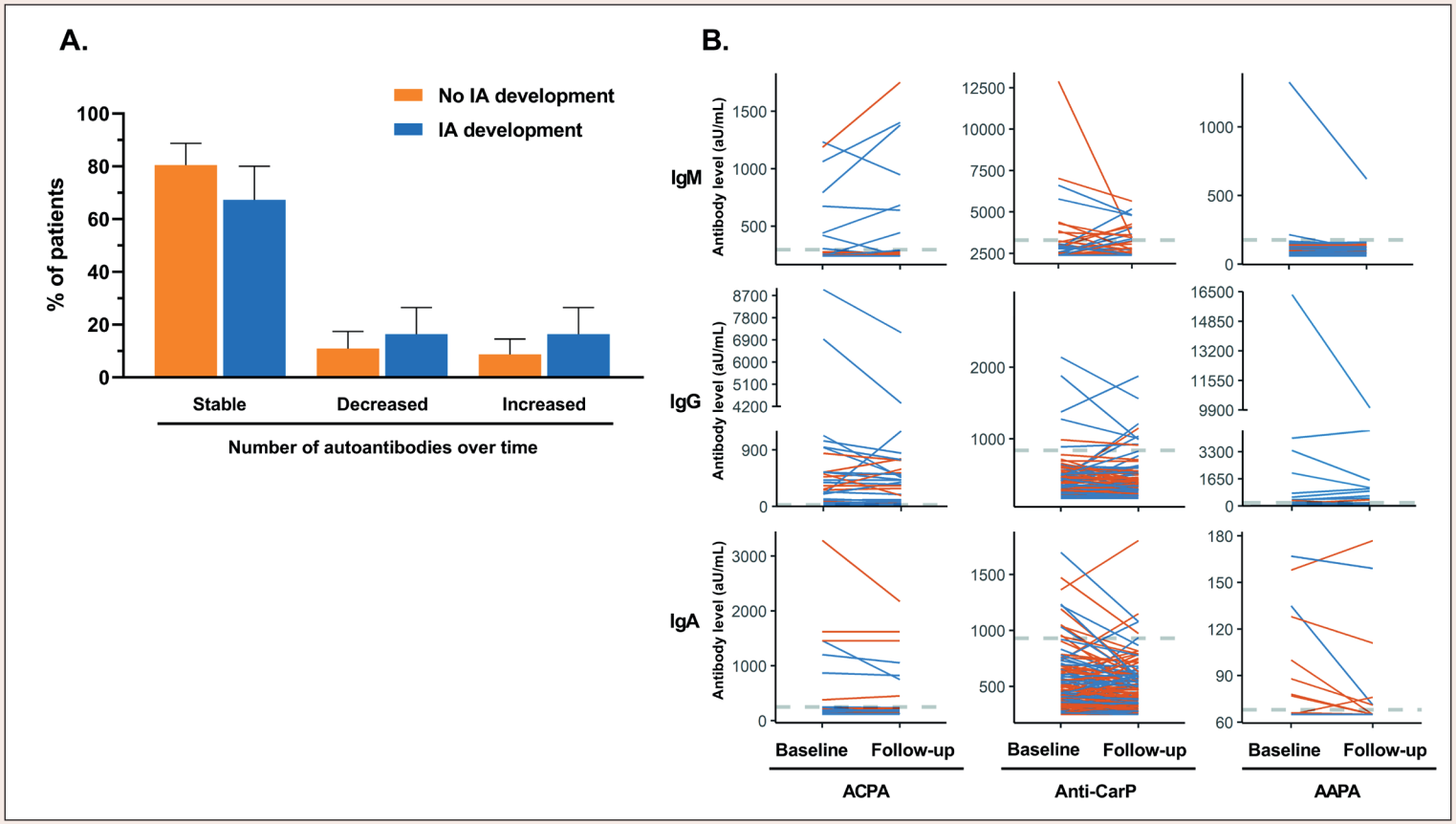
* based on routine laboratory diagnostics at baseline
The 62 RF and ACPA negative patients that did not progress and the 26 RF and ACPA negative patients that did progress to IA were selected for this study. Notably, for patient selection autoantibody negativity was defined as RF and ACPA negativity at baseline using routine diagnostics. When showing the results from the analyses of the different isotypes of ACPA, AAPA and anti-CarP in the manuscript, autoantibody-negativity was defined as negativity for the nine measured isotypes at baseline.
CSA: clinically suspect arthralgia, ACPA: anti-citrullinated protein antibody, SD: standard deviation, IQR: interquartile range, TJC: tender joint count, RF: rheumatoid factor, CRP: c-reactive protein

Supplementary Figure 1. Changes in autoantibody-response over time in patients that did and did not progress to RA: A) percentage of patients with seroconversion to positive in patients negative for all autoantibodies at baseline, B) percentage of patients that has an increasing, decreasing or stable number of positive measurements over time in patients positive for ≥1 autoantibody-reactivity at baseline, C) autoantibody levels over time in patients positive for the respective autoantibody at baseline.



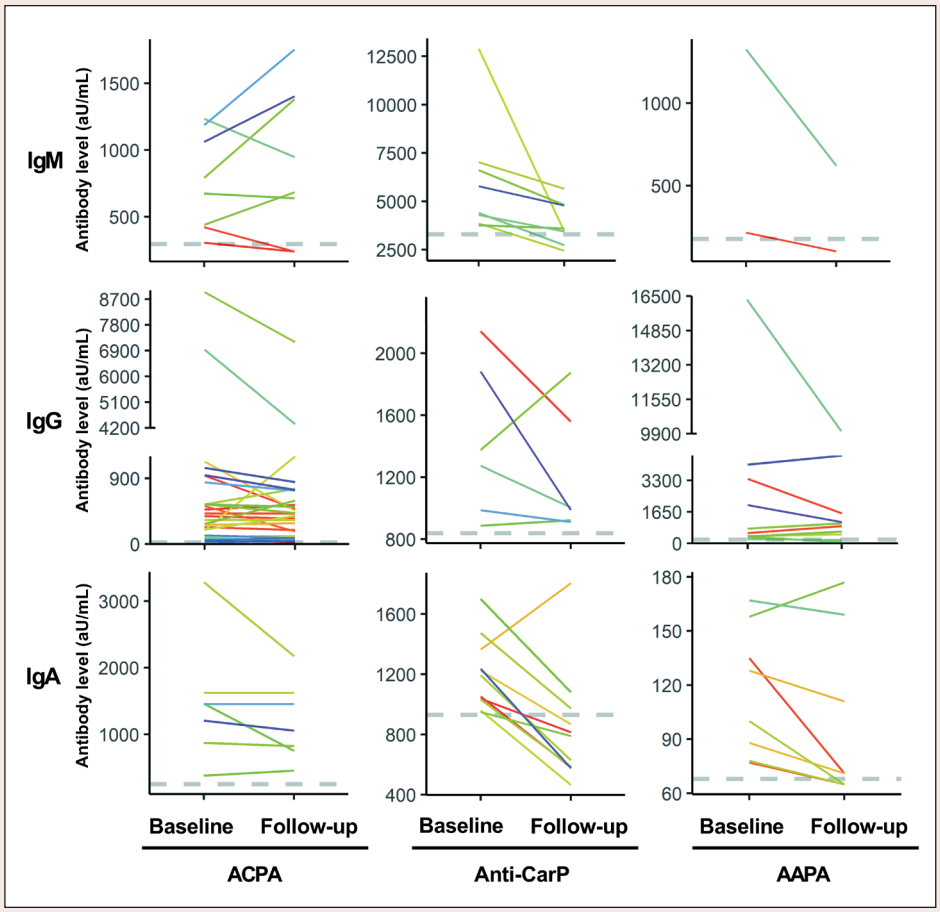
*RA defined as fulfilment of the 1987 and/or 2010 criteria at the time of clinically apparent inflammatory arthritis development. Of 55 patients with IA 42 (76%) fulfilled criteria for RA. Two patients of the non-RA group developed other diagnoses (1 inflammatory osteoarthritis, 1 psoriatic arthritis), and the remaining 11 patients had UA/clinical diagnosis of possible RA (though they did not fulfil classification criteria); nine patients received DMARD-therapy.
Figure A autoantibody negativity at baseline was defined as negative for the nine studied measurements (n=100), Figure B autoantibody positive was defined as at least one (out of nine) positive measurement at baseline (n=47).
Error bars in Figure A and B represent 95% CI. Dashed grey horizontal lines in Figure C indicate the cut-off values for each autoantibody.
No significant differences in Figure A and B were found. In patients with ≥1 autoantibody-reactivity at baseline progressing to RA, the median number of autoantibody-reactivities was 2.0 (IQR 1.0-4.0, max. 6) at baseline and 2.0 (IQR 1.0-4.0, max. 6) at RA-development (p=0.77). In CSA-patients with ≥1 autoantibody-reactivity at baseline not progressing to RA, this was 1.0 (IQR 1.0-2.0, max. 4) at baseline and 1.0 (IQR 0.0-2.0, max. 5) after 2-years (p=0.015). Levels of autoantibodies did not significantly change over time (p-values ranging 0.19-1.00).
ACPA: anti-citrullinated protein antibodies, anti-CarP: anti-carbamylated protein antibodies, AAPA: anti-acetylated protein antibodies

Supplementary Figure 2. Changes in autoantibody-response over time: A) percentage of patients that has an increasing, decreasing or stable number of positive measurements over time in all CSA-patients (n=147), B) autoantibody levels over time in all CSA-patients.



Error bars in Figure A represent 95% CI. Dashed grey horizontal lines in Figure B indicate the cut-off values for each autoantibody.
Note that patients with measurements below detection range at both baseline and follow-up were illustrated as a horizontal line at the lower detection limit; consequently this depicted single line actually presents data of multiple patients.
No significant differences were found in Figure A between patients progressing and not progressing to IA. In patients with ≥1 autoantibody-reactivity at baseline progressing to IA, the median number of autoantibody-reactivities was 0.0 (IQR 0.0-1.0, max. 6) at baseline and 0.0 (IQR 0.0-1.0, max. 6) at IA-development (p=0.69). In CSA-patients with ≥1 autoantibody-reactivity at baseline not progressing to IA, this was 0.0 (IQR 0.0-0.0, max. 4) at baseline and 0.0 (IQR 0.0-0.0, max. 5) after 2-years (p=0.12). A significant decrease in autoantibody levels was seen in patients progressing from CSA to IA for ACPA IgA (p<0.001) and anti-CarP IgA (p=0.036). No significant changes in levels over time were seen in the remaining autoantibodies (p-values ranging 0.18-1.00).
IA: clinically apparent inflammatory arthritis, ACPA: anti-citrullinated protein antibodies, anti-CarP: anti-carbamylated protein antibodies, AAPA: anti-acetylated protein antibodies

Supplementary Figure 3. Autoantibody levels over time in patients positive for the respective autoantibody at baseline, each colour indicates an individual patient.



Dashed grey horizontal lines indicate the cut-off values for each autoantibody.
ACPA: anti-citrullinated protein antibodies, anti-CarP: anti-carbamylated protein antibodies, AAPA: anti-acetylated protein antibodies

Supplementary Table 4. Autoantibody levels in patients positive for ≥1 autoantibody-reactivity at baseline.

	ACPA IgM		ACPA IgG		ACPA IgA		CarP IgM		CarP IgG		CarP IgA		AAPA IgM		AAPA IgG		AAPA IgA	
IA	**BL**	**FU**	**BL**	**FU**	**BL**	**FU**	**BL**	**FU**	**BL**	**FU**	**BL**	**FU**	**BL**	**FU**	**BL**	**FU**	**BL**	**FU**
-	<240	<240	<15	<15	NS	NS	<2400	<2400	246	199	***1038***	815	77	77	128	63	<65	<65
-	NS	NS	***471***	***536***	NS	NS	<2400	2835	<170	257	621	493	113	132	116	124	<65	<65
-	<240	<240	<15	<15	<120	<120	<2400	<2400	<170	<170	***1053***	583	61	64	<60	103	<65	<65
-	255	280	***522***	***176***	NS	NS	<2400	<2400	444	***1150***	729	***1151***	105	97	NS	142	***77***	<65
-	<240	<240	***43***	***56***	145	174	<2400	<2400	354	302	254	306	<60	<60	122	133	<65	<65
-	NS	***477***	***265***	***289***	NS	NS	<2400	<2400	599	494	292	429	70	71	140	165	<65	<65
-	<240	<240	<15	<15	NS	NS	2846	***4280***	340	606	***1364***	***1806***	74	101	82	109	***128***	***111***
-	<240	<240	<15	<15	NS	154	<2400	<2400	<170	239	660	611	66	79	NS	147	***88***	***71***
-	NS	<240	<15	<15	205	NS	***12892***	***3448***	249	308	***960***	466	NS	111	75	99	<65	<65
-	<240	<240	***327***	***332***	***1625***	***1621***	***3864***	2459	NS	420	618	792	90	68	NS	167	66	<65
-	NS	NS	***544***	***757***	***3287***	***2172***	***7025***	***5658***	715	391	***1193***	630	167	121	NS	NS	NS	NS
-	<240	<240	24	<15	NS	227	<2400	<2400	171	227	***1473***	***973***	67	<60	185	161	***78***	<65
-	<240	<240	<15	<15	191	156	<2400	<2400	539	361	347	364	92	75	NS	171	***100***	<65
-	<240	<240	<15	<15	NS	NS	<2400	<2400	294	217	339	448	<60	<60	166	147	***103***	NS
-	<240	<240	<15	<15	189	195	<2400	<2400	624	397	***949***	790	<60	<60	90	<60	<65	<65
-	<240	<240	<15	<15	136	<120	<2400	<2400	394	460	386	275	<60	<60	83	<60	***158***	***177***
-	<240	NS	***278***	***594***	***380***	***453***	<2400	3225	691	686	389	572	128	131	***345***	<60	<65	***76***
-	<240	<240	<15	<15	<120	<120	***3767***	***3605***	201	172	419	713	138	100	121	NS	<65	<65
-	<240	<240	<15	<15	155	154	***4399***	2732	187	173	474	392	138	139	<60	87	<65	<65

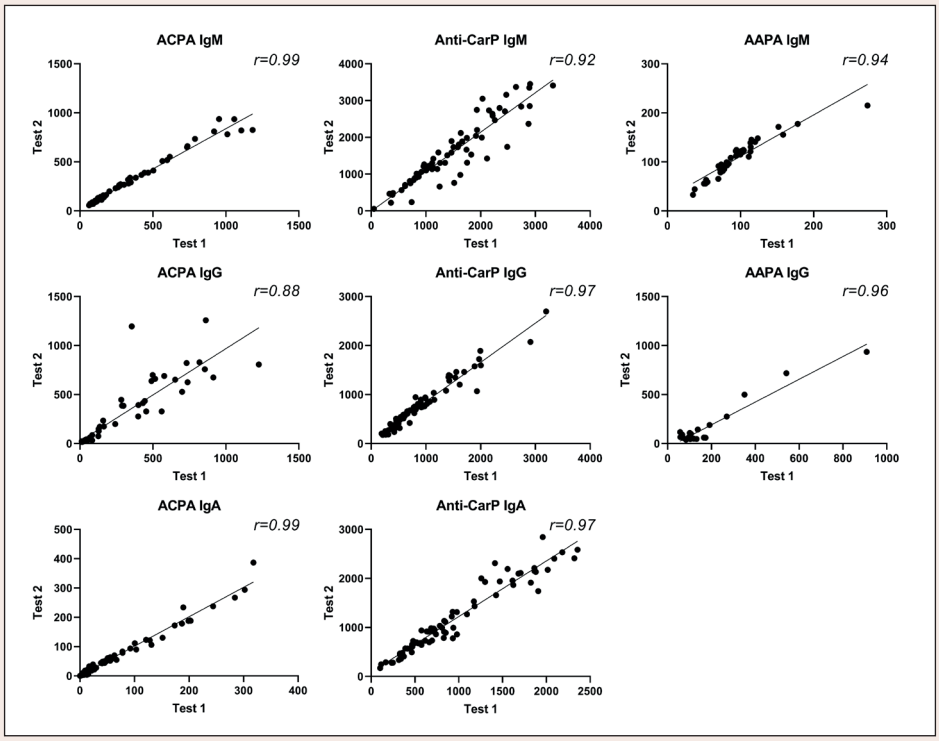
Supplementary Table 4. *Continued*

	ACPA IgM		ACPA IgG		ACPA IgA		CarP IgM		CarP IgG		CarP IgA		AAPA IgM		AAPA IgG		AAPA IgA	
IA	**BL**	**FU**	**BL**	**FU**	**BL**	**FU**	**BL**	**FU**	**BL**	**FU**	**BL**	**FU**	**BL**	**FU**	**BL**	**FU**	**BL**	**FU**
-	NS	285	<15	<15	140	132	***4300***	***3448***	<170	<170	373	266	NS	138	68	<60	<65	<65
-	<240	<240	***83***	***43***	<120	<120	2529	2587	285	447	<250	<250	115	103	<60	<60	<65	<65
-	***1188***	***1754***	***845***	***733***	***1455***	***1454***	3022	<2400	***987***	***912***	491	752	<60	65	143	***381***	<65	<65
+	<240	<240	***81***	20	243	<120	<2400	<2400	593	504	505	473	69	66	72	84	<65	<65
+	***304***	<240	***939***	***487***	NS	NS	<2400	<2400	625	590	311	324	77	73	137	NS	<65	<65
+	<240	<240	***233***	***191***	130	<120	<2400	<2400	406	609	<250	<250	97	116	103	120	<65	<65
+	***421***	<240	***385***	***350***	NS	NS	3081	<2400	***2142***	***1560***	833	565	***215***	102	***3383***	***1577***	***135***	***71***
+	<240	<240	***419***	***422***	170	134	<2400	2520	646	498	<250	258	86	81	***542***	***909***	<65	<65
+	<240	<240	***1128***	***458***	NS	NS	<2400	<2400	623	541	***1223***	868	105	96	NS	NS	<65	<65
+	<240	***443***	***191***	***1200***	NS	***1465***	<2400	3263	413	***1212***	730	615	89	91	112	NS	<65	<65
+	<240	<240	***199***	***387***	NS	NS	<2400	<2400	336	346	397	405	100	105	118	98	<65	<65
+	<240	<240	***104***	***111***	NS	228	<2400	<2400	561	599	396	318	88	69	***377***	***475***	<65	<65
+	<240	<240	20	<15	166	<120	2837	***5185***	477	***1039***	578	<250	111	120	***212***	NS	<65	<65
+	<240	<240	<15	<15	251	235	<2400	<2400	274	763	***1032***	588	<60	64	NS	NS	<65	<65
+	***791***	***1380***	***8940***	***7194***	***870***	***822***	<2400	2834	***1376***	***1876***	374	599	82	83	***774***	***1053***	<65	<65
+	256	<240	***79***	***40***	192	183	***6613***	***4821***	NS	786	567	490	97	74	NS	198	<65	<65
+	***440***	***684***	***549***	***424***	NS	NS	2574	***3669***	502	616	922	776	86	92	<60	***621***	<65	<65
+	<240	<240	<15	<15	<120	<120	<2400	<2400	<170	<170	***1699***	***1080***	150	132	88	<60	<65	<65
+	***673***	***638***	***546***	***510***	***1459***	***750***	<2400	<2400	***887***	***923***	780	553	74	67	156	178	<65	<65

+	<240	<240	<15	<15	<120	<120	<2400	<2400	237	255	<250	<250	67	64	***218***	168	<65	<65
+	***1235***	***947***	***6931***	***4327***	NS	NS	2993	<2400	***1274***	***1005***	693	679	***1327***	***621***	***16338***	***10023***	***167***	***159***
+	<240	<240	***63***	***58***	<120	<120	<2400	<2400	509	535	<250	<250	105	87	100	154	<65	<65
+	<240	<240	***120***	***82***	247	125	<2400	<2400	253	216	279	277	110	113	76	128	<65	<65
+	<240	<240	***59***	***78***	138	125	<2400	***3389***	285	297	584	604	75	82	183	156	<65	<65
+	<240	<240	***34***	***42***	<120	<120	<2400	<2400	489	826	531	507	86	104	163	NS	<65	<65
+	<240	<240	<15	<15	NS	231	<2400	<2400	<170	<170	***1236***	577	<60	65	80	83	<65	<65
+	***858***	NS	***1045***	***850***	<1204	***1055***	***5779***	***4790***	446	288	547	540	134	NS	***4132***	***4596***	<65	NS
+	***1061***	***1403***	***946***	***743***	NS	NS	2976	2414	***1882***	***989***	689	NS	NS	NS	***2011***	***1120***	<65	<65

Switches within autoantibodies were defined as a decrease in IgM levels (level at follow-up < level at baseline) accompanied by an increase in IgG levels (level at follow-up > level at baseline) within the same autoantibody. Note that even small changes in autoantibody levels, including level changes below the cut-off, could result in a 'switch'. Switches were evaluated per autoantibody. In patients progressing to IA no switches occurred in ACPA and anti-CarP, and 5 patients (20%) switched in AAPA. In patients not progressing to IA no switches occurred in ACPA and AAPA, and 1 patient (4.5%) switched in anti-CarP.
IA: clinically apparent inflammatory arthritis, ACPA: anti-citrullinated protein antibodies, anti-CarP: anti-carbamylated protein antibodies, AAPA: anti-acetylated protein antibodies, NS: non-specific measurement

Supplementary Figure 4. Inter-assay variation of in-house ELISAs



Inter-assay variation resulted in changes in positivity of the test infrequently: ACPA IgM 0%, IgG 1.3%, IgA 1.3%, anti-CarP IgM 9.2%, IgG 3.9%, IgA 7.9%, AAPA IgM 0%, IgG 4.2%, IgA 0%.
No correlation plot was created for AAPA IgA because too little samples were above the detection limit.
ACPA: anti-citrullinated protein antibodies, anti-CarP: anti-carbamylated protein antibodies, AAPA: anti-acetylated protein antibodies