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Exploring seropositive rheumatoid arthritis: from immunological depths to clinical course

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Part 2

The role of mucosal immunity in the development of the AMPA response in RA

In rheumatoid arthritis patients, total IgA1 and IgA2 levels are elevated: Implications for the mucosal origin hypothesis

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Abstract

Objectives Mucosal initiated immune responses may be involved in the pathophysiology of rheumatoid arthritis (RA). The most abundant immunoglobulin at mucosal surfaces is IgA, of which two subclasses exist: IgA1 and IgA2. IgA2 is mainly present at mucosal sites and has been ascribed pro-inflammatory properties. As IgA subclasses might provide insights into mucosal involvement and pro-inflammatory mechanisms, we investigated IgA responses in sera of RA patients.

Methods In two cohorts of RA patients, the EAC and IMPROVED, total IgA1 and IgA2 were measured by ELISA. Furthermore, IgA subclass levels of rheumatoid factor (RF) and anti-citrullinated protein antibodies (anti-CCP2) were determined. The association of these IgA subclass levels with CRP and smoking was investigated.

Results Total IgA1 and IgA2 were increased in RA patients compared to healthy donors in both cohorts. This increase was more pronounced in seropositive RA versus seronegative RA. For RF and anti-CCP2, both IgA1 and IgA2 could be detected. No strong associations were found between IgA subclasses (total, RF and anti-CCP2) and CRP. In smoking RA patients, a trend towards a selective increase in total IgA2 and RF IgA1 and IgA2 was observed.

Conclusion RA patients have raised IgA1 and IgA2 levels. No shift towards IgA2 was observed, indicating that the increase in total IgA is not due to translocation of mucosal IgA into the bloodstream. However, mucosal inflammation might play are role, given the association between smoking and total IgA2 levels. Despite its pro-inflammatory properties, IgA2 does not associate strongly with pro-inflammatory markers in RA patients.

Introduction

IgA is the most abundant class of immunoglobulin at mucosal sites and it has an important function in maintaining intestinal homeostasis. Recently, mucosal immune responses have gained increasing attention for their potential role in the pathophysiology of (seropositive) rheumatoid arthritis (RA) (1). Therefore, it is worthwhile to study the IgA response in more detail in RA patients.

Several findings suggest mucosal involvement in the disease mechanisms of seropositive RA. Smoking is a well-known risk factor for the development of RA (1), implying that processes occurring at the pulmonary mucosa could play a role in disease pathogenesis. Furthermore, ACPA have been detected in sputum and saliva of seropositive RA patients, suggesting local production of autoantibodies (2, 3). The gut, another vast mucosal site, might also be important and dysbiosis of the gut microbiome has been described in RA (4). It has been hypothesized that dysbiosis of the microbiome could lead to local inflammation, loss of barrier function and possibly even bacterial translocation. Given that IgA plays an important role in mucosal immune responses, the characteristics of the IgA response might provide more insight into local inflammatory processes and thus possible mucosal origins of RA.

Humans harbor 2 IgA subclasses: IgA1 and IgA2 (5). The biggest differences between IgA1 and IgA2 are the structure of the hinge region and their distribution at different sites. In serum, over 90% of total IgA is IgA1, whereas the IgA subclasses are more balanced at mucosal sites, with exact ratios depending on the location (5). Furthermore, a recent report described a pro-inflammatory effect of IgA2 on neutrophils and macrophages, while this was not found for IgA1 (6). Thus IgA1 and IgA2 not only differ in structure and localization, but may also recruit different effector functions. Moreover, the same study reported total IgA subclass levels to be lower in RA patients compared to healthy controls, and most strikingly, that ACPA IgA in serum is more often of the IgA2 subclass (up to 80% of all ACPA IgA) compared to total IgA. A higher proportion of ACPA IgA2 was also positively correlated with disease activity score (DAS) (6) and ACPA IgA2 levels were weakly correlated with the severity of flares during DMARD tapering (7). These findings suggest that ACPA IgA may have a mucosal origin, compatible with the high amount of IgA2, and that it might translocate from the mucosa into the bloodstream, leading to an elevated ACPA IgA2 percentage in serum. Furthermore, the pro-inflammatory properties of ACPA IgA2 might contribute to disease processes in RA.

Thus, studying IgA subclasses might provide more insight into both the origin as well as the potential pro-inflammatory pathophysiological mechanisms in RA. We hypothesized that mucosal inflammation in RA patients might result in elevated IgA2 levels, which could contribute to the ongoing pro-inflammatory processes in RA. To investigate these hypotheses, we set out to examine IgA subclass levels of total and autoantibody-specific IgA in sera of RA, as well as their link with inflammation and smoking, as a proxy for mucosal inflammation.

Patients and Methods

Stored sera from 2 cohorts of early RA patients were used, the IMPROVED study and the Leiden Early Arthritis Clinic (EAC), of which details are described elsewhere (8, 9). All patients fulfilled either the 2010 (IMPROVED) or the 1987 (EAC) ACR criteria for RA and gave written informed consent. Samples were selected based on previous autoantibody measurements (8, 9). In this study, seropositivity was defined as positivity for anti-CCP2 IgG and/or RF IgM. Of the IMPROVED study, baseline samples from 125 seropositive RA patients were included, most of whom previously tested positive for anti-CCP2 IgA and RF IgA, as well as baseline sera of 68 seropositive RA patients who were negative for RF IgA and anti-CCP2 IgA, and 56 RA patients who were seronegative for both anti-CCP2 IgG and RF IgM. To investigate the generalizability of the findings, samples of a second cohort, the EAC, were used. This included sera of 95 seropositive, mostly anti-CCP2 IgA positive, and 64 seronegative RA patients collected at the one-year visit. Sera of 60 healthy donors, not matched for age or sex, were taken along as control.

Total, RF and anti-CCP2 IgA subclasses were measured with in-house enzyme-linked immunosorbent assays (ELISA) (Supplementary data S1 for details). To test whether RF IgA could influence the readout of the anti-CCP2 IgA subclass ELISA's by binding to anti-CCP2 IgG, sera of anti-CCP2 IgG-positive/IgA-negative, RF IgA-negative patients were mixed 1:1 with sera of anti-CCP2 IgG-negative/IgA-negative, RF IgA-positive patients or with control sera before addition to the plate. Moreover, part of the samples were tested side-by-side before and after IgG depletion on anti-CCP2 IgA2 ELISA (Supplementary data S1). As RF was measured in arbitrary units and not in exact amounts, no percentage RF IgA2 of total RF could be calculated. Instead the ratio RF IgA1 / RF IgA2 was used.

Mann-Whitney U tests were used to compare IgA-levels between groups. Multivariate linear regression was performed to analyze total IgA subclass levels in RA patients versus healthy controls corrected for known confounders age and gender (10), using the IgA subclass levels (^{10}log transformed due to skewness of the data) as dependent

variable. To assess the relationship between IgA subclass levels and C-reactive protein (CRP), DAS, health assessment questionnaire score (HAQ) and BMI, spearman's rank correlation coefficient were calculated, due to the presence of outliers. Patients with missing CRP values (IMPROVED n=5, EAC n=20) or smoking status (IMPROVED n=1) were excluded from analyses involving CRP and smoking respectively. For smoking analysis a multivariate linear regression model including the possible confounders age, gender and CRP was used, with the log-transformed IgA subclass levels as dependent variable. For analysis regarding CRP, similar models were performed for each IgA subclass separately (included as independent variable), together with age, gender and smoking as confounders, using the log-transformed CRP levels as dependent variable. The associations between RF IgA subclass levels and smoking or CRP were analyzed within patients who tested positive for both RF IgA1 and IgA2. Analyses regarding anti-CCP2 IgA1 levels were performed within the anti-CCP2 IgA1-positive group only. The Holm-Bonferroni method was applied to correct for multiple testing.

Results

Total IgA subclasses in RA

In seropositive RA IMPROVED patients, both total IgA1 (tIgA1, $p<0.001$) and total IgA2 (tIgA2, $p<0.001$) levels were strikingly elevated compared to healthy donors (Figure 1A-B). Similar results were found using linear regression including age and gender as potential confounders (Supplementary table S1). Both IgA subclasses were raised to the same extent, since the percentage of IgA2 (%tIgA2, $p=0.18$) did not differ between seropositive patients and healthy donors (Figure 1C). In seronegative IMPROVED patients total IgA1, total IgA2 and the %tIgA2 were not raised compared to healthy controls (Figure 1A-B, Supplementary table S1). To investigate if the raise in total IgA levels in seropositive RA was due to the presence of IgA autoantibodies, seropositive RA patients who were negative for RF IgA and anti-CCP2 IgA were tested. In those patients, total IgA1 ($p<0.001$) and IgA2 levels (tIgA2 $p<0.001$) were also elevated (Supplementary figure S1). This indicates that the elevation in total IgA in seropositive RA is not solely caused by the presence of IgA autoantibodies.

To investigate the generalizability of these findings, we also performed these measurements on sera of patients included in the EAC. While IMRPOVED-sera were collected at baseline, these EAC-sera were collected at the patients' one-year visit. A complete overview of difference between the cohorts can be found in Table 1. Also in the EAC, total IgA1 and IgA2 levels were raised in seropositive RA patients compared to healthy donors, although total IgA2 was just not significant after multiple testing correction (Figure 1D-E) (tIgA1

p<0.001, tIgA2 p=0.02). Increased total IgA1 levels were now also observed in seronegative RA patients compared to healthy donors (tIgA1 p<0.001, tIgA2 p=0.06). Linear regression with correction for age and gender yielded similar results (Supplementary table S1). IgA subclass levels did not differ between seropositive and seronegative EAC patients (Figure 1D-E) (tIgA1 p=0.13, tIgA2 p=0.70). Thus, both total IgA1 and IgA2 levels are raised in seropositive RA compared to healthy controls and might also be higher in seronegative RA patients with longer disease duration.

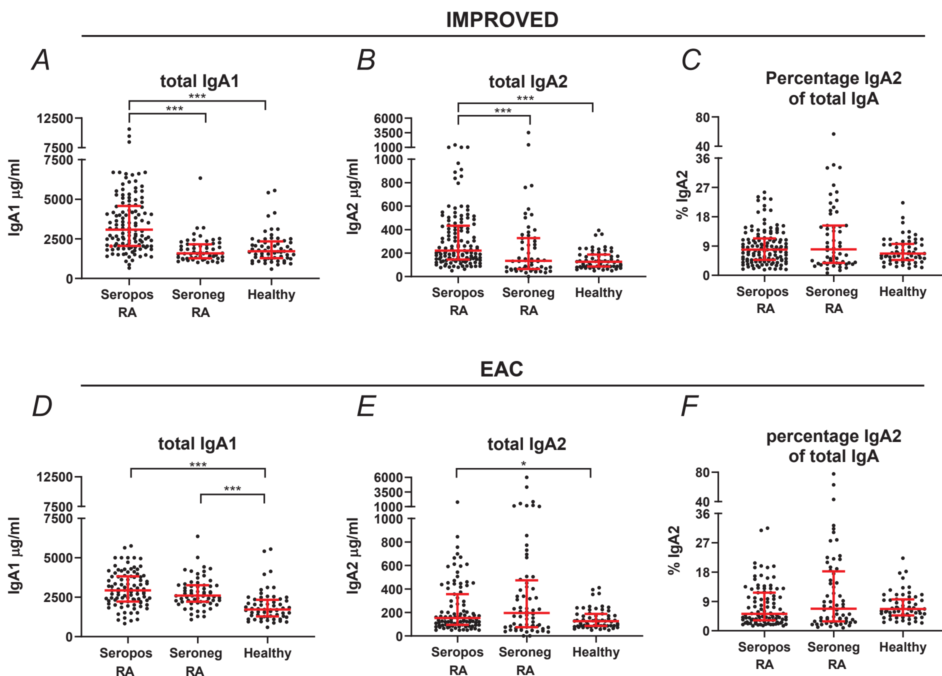


Figure 1: Total IgA1, total IgA2 and percentage IgA2 of total IgA in seropositive and seronegative RA patients compared to healthy controls. Mann–Whitney U tests to compare IgA levels between seropositive or seronegative RA patients and healthy donors. Red bars: median and interquartile range. *p<0.05, **p<0.01, ***p<0.001, no brackets shown when p>0.05. Not all samples were measured in the same experiment.

RF and anti-CCP2 IgA1 and IgA2

Next, we established assays to measure RF and anti-CCP2 IgA subclasses. For RF, both IgA subclasses could be readily detected in the seropositive IMPROVED RA patients with 77% positivity for RF IgA1 and 70% for RF IgA2 (Figure 2A-B). Replication in the EAC showed 85% positivity for RF IgA1 and 60% RF IgA2 in seropositive RA (Figure 2C-D).

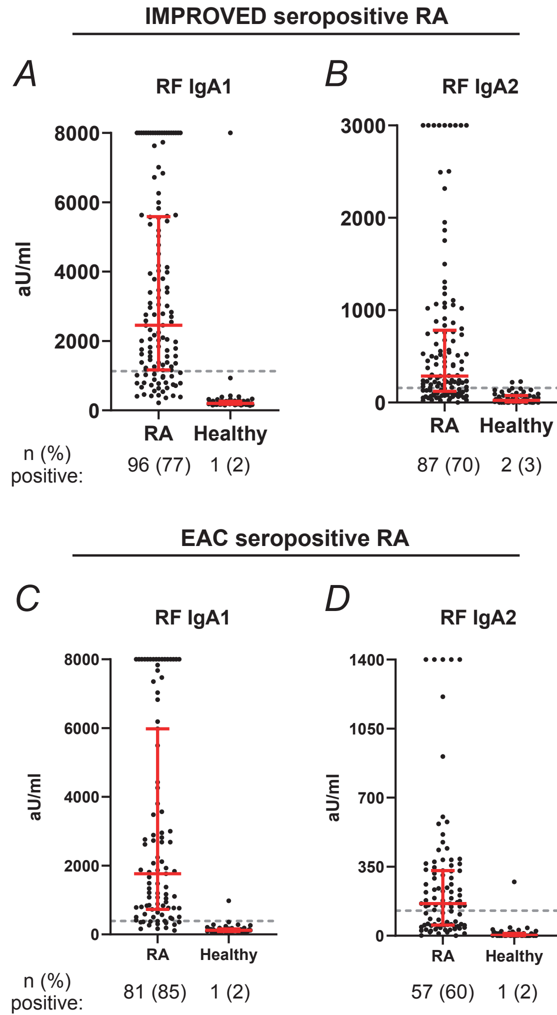


Figure 2: RF IgA subclasses in seropositive RA patients. A RF IgA1 levels and B RF IgA2 levels in IMPROVED seropositive RA patients, C RF IgA1 levels and D RF IgA2 levels in EAC seropositive RA patients. Gray dashed line represents cut-off. Red bars: median and interquartile range. Arbitrary units cannot be directly compared between subclasses.

Table 1: Characteristics of the different patients subsets

	IMPROVED seropositive* RA	IMPROVED seronegative RA	IMPROVED seropositive, RF IgA-/CCP2 IgA- RA	EAC seropositive* RA	EAC seronegative RA	Healthy donors
Number	125	56	68	95	64	60
Time sampling	Baseline	Baseline	Baseline	1 -year visit	1 -year visit	NA
Age, yearsm, Mean ± SD	53.3 ± 12.4	54.3 ± 15.1	47.1 ± 14.7	59.1 ± 15.3	59.1 ± 16.8	44.7 ± 14.4
Female, n (%)	80 (64)	39 (70)	48 (71)	64 (67)[#]	41 (64)[#]	35 (58)
Ever smoking, n (%)	76 (61)	23 (42)	18 (27)	50 (53)[#]	22 (35)[#]	Unknown
CRP, mg/L Mean ± SD	24.5 ± 34.1 (n=120)	22.2 ± 32.3 (n=55)	25.3 ± 33.0 (n=64)	26.3 ± 37.7 (n=75)	11.0 ± 21.8 (n=55)	NA
DAS, Mean ± SD	3.29 ± 0.91	3.70 ± 0.85	3.20 ± 0.83	Unknown	Unknown	NA
RF IgM positivity, n (%)	112 (92)	0 (0)	56 (85)	75 (79)[#]	0 (0)[#]	NA
RF IgA positivity, n (%)	106 (85)	NA	0 (0)	Unknown	NA	NA
Anti-CCP2 IgG positivity, n (%)	118 (95)	0 (0)	38 (57)	95 (100)[#]	0 (0)[#]	NA
Anti-CCP2 IgA positivity, n (%)	102 (82)	NA	0 (0)	63 (67)[#]	NA	NA
Total IgA1, µg/ml Median (IQR)	3082 (2057-4579)	1581 (1273-2155)	2913 (1968-3912)	2936 (2229-3828)	2605 (2215-3259)	1720 (1280-2338)
Total IgA2, µg/ml Median (IQR)	221 (145-433)	134 (65-328)	197 (139-400)	153 (95-356)	196 (74-473)	127 (86-188)
% IgA2 total IgA Median (IQR)	7.9 (4.7-11.4)	8.0 (3.8-15.3)	6.0 (4.5-9.2)	5.2 (3.2-11.7)	6.7 (2.9-18.3)	6.7 (4.8-9.6)

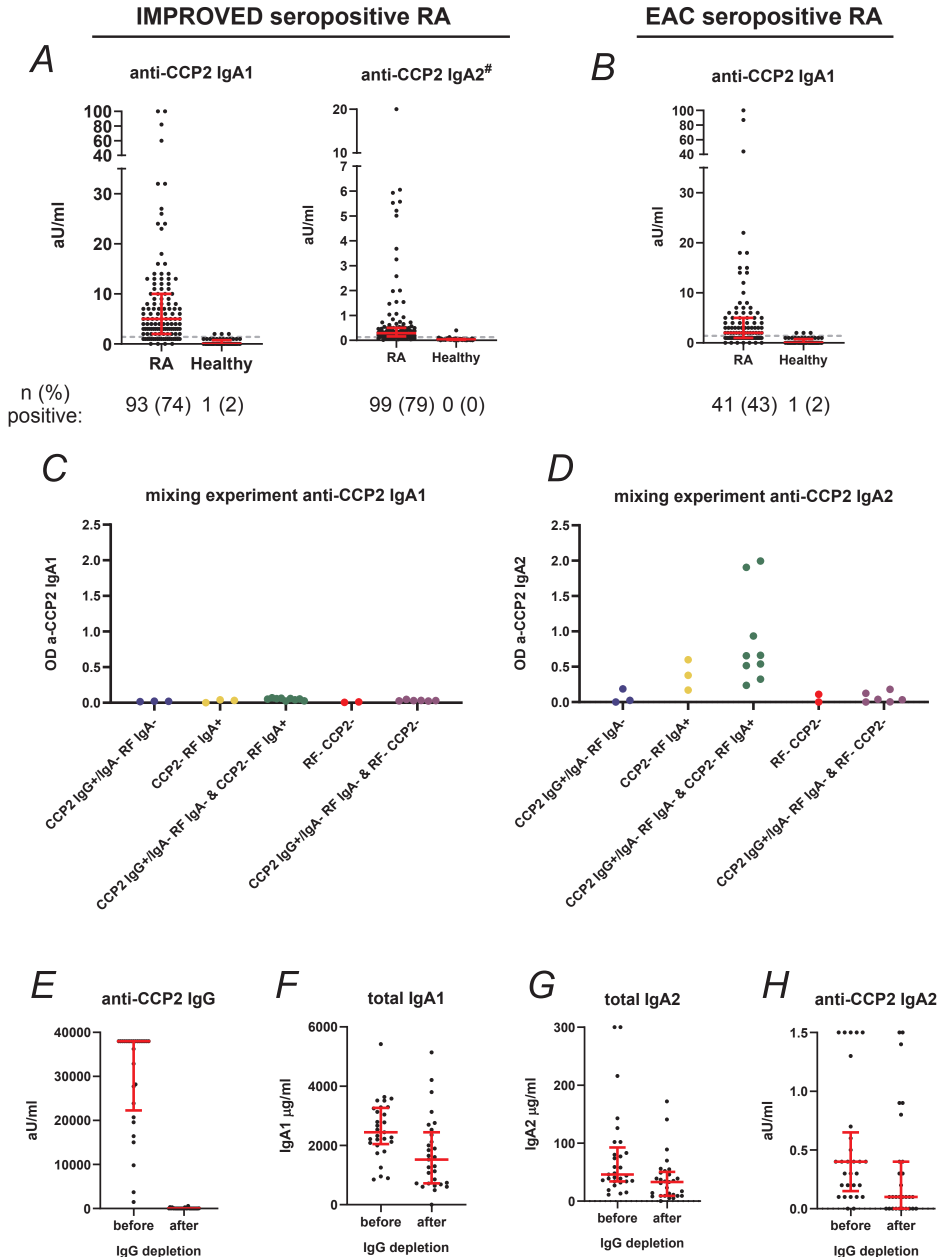
*Enriched for ACPA IgA positivity [#]Information collected at baseline

Anti-CCP2 IgA1 was present in 74% of seropositive patients in the IMPROVED (Figure 3A). In the EAC 43% of seropositive RA patients tested positive for anti-CCP2 IgA1 (Figure 3B), which is partly due to the lower amount of anti-CCP2 IgA-positive patients within the seropositive group in the EAC compared to IMPROVED. Where anti-CCP2 IgA1 could be readily detected, the detection of anti-CCP2 IgA2 proved challenging. The ELISA protocol needed to be modified extensively to obtain decent signals for anti-CCP2 IgA2 (Supplementary data S1). However, with these adaptations, including the use of less diluted serum and incubation of serum overnight, we eventually managed to obtain sufficient readouts (Figure 3A). During quality controls of this new protocol, mixing experiments showed no effect of RF on the outcome of the anti-CCP2 IgA1 ELISA (Figure 3C). However using the anti-CCP2 IgA2 protocol, RF IgA2 could bind to anti-CCP2 IgG and give a false positive anti-CCP2 IgA2 signal (Figure 3D). Therefore, we concluded that anti-CCP2 IgA2 could not be reliably detected in sera containing RF IgA2. To further assess the impact of this RF IgA interference on anti-CCP2 IgA2, IgG depletion was performed in a subset of patients and anti-CCP2 IgA2 measurements were compared before and after IgG depletion. As expected anti-CCP2 IgG was undetectable after IgG depletion (Figure 3E), whereas only a slight non-specific IgA loss was observed after the procedure (Figure 3F-G). Anti-CCP2 IgA2 could still be detected after IgG depletion in part of the seropositive RA samples (Figure 3H). Moreover, anti-CCP2 IgA2 could also be detected in some RF-negative patients, indicating that not the entire signal could be attributed to RF. In conclusion, anti-CCP2 IgA2 can be present in part of seropositive RA patients, but technical difficulties posed by RF IgA interference prohibited precise determination of anti-CCP2 IgA2 levels. Therefore, no further analyses using anti-CCP2 IgA2 levels were performed.

IgA subclasses and inflammation

To investigate whether total and antigen-specific IgA subclass levels in seropositive RA are associated with inflammation, correlations with CRP were examined. In the IMPROVED, no association was observed for either total or antigen-specific IgA1 or IgA2 and CRP in univariate analysis (Figure 4A). Also no correlations were seen for %tIgA2 and RF IgA subclass ratio (supplementary figure S2A). To correct for confounders, analyses were adjusted for age, gender and smoking. After this correction, a small difference was seen for RF IgA2 (p=0.007), but this did not remain significant after multiple testing correction (Supplementary table S2). In seropositive EAC patients, spearman correlation yielded significant results for CRP and total IgA1 (r_s=0.352, p=0.002) and RF IgA2 (r_s=0.385, p=0.007) (Figure 4B, supplementary figure S2B). However, no clear pattern was visible in the scatterplots and the strength of the correlation was limited. Similarly, in multivariate analyses, small, but significant associations were found between CRP and total IgA1 and RF IgA2 (supplementary table S2). In the IMPROVED cohort, similar analyses with DAS, HAQ, BMI and the presence of erosions were performed. No significant associations with

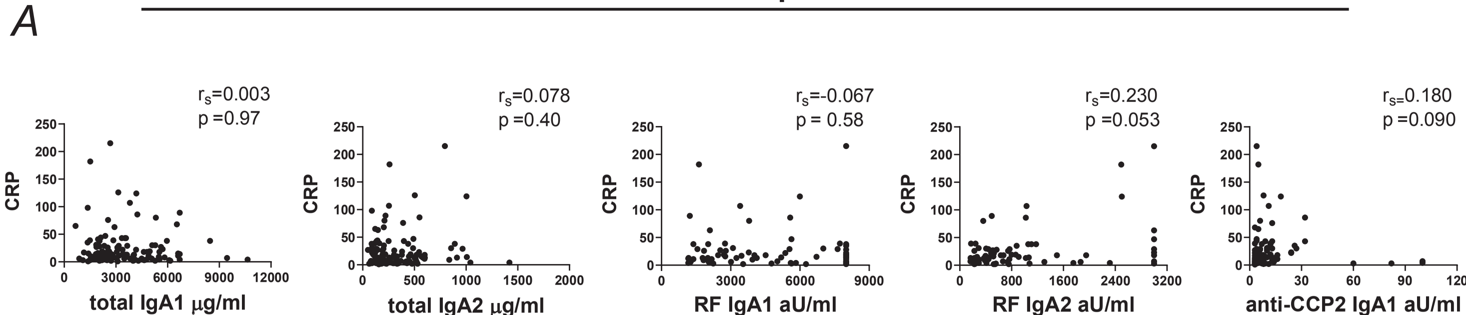
total or antigen-specific IgA subclasses was observed for these parameters, after correction for multiple testing (Supplementary figure S3). In conclusion, although there were some correlations between CRP and IgA antibodies of both subclasses, especially regarding RF IgA2-levels, these associations were not very strong in both cohorts.



◂ **Figure 3: Anti-CCP2 IgA subclasses in seropositive RA.** A Anti-CCP2 IgA1 and IgA2 subclass measurements in seropositive IMPROVED RA patients and healthy controls. Number and percentage of patients above the cut-off is indicated. Gray dashed line represents cut-off. Red bars: median and interquartile range. #Results might be influenced by RF IgA2 binding B Anti-CCP2 IgA1 in seropositive EAC RA patients C-D Mixing experiment for anti-CCP2 IgA1 and IgA2 ELISA to investigate whether RF IgA binding could influence the read-out. For anti-CCP2 IgA1 no RF interference is observed, for the anti-CCP2 IgA2 ELISA results could be influence by RF IgA2 binding, as the combination of anti-CCP2 IgG+ IgA- RF IgA- serum with anti-CCP2- RF IgA+ serum can give high OD values E-H Measurements before and after IgG depletion in a selection of IMRPOVED seropositive RA patients. After IgG depletion the anti-CCP2 IgA2 signal remains clearly visible in part of the samples, while all anti-CCP2 IgG is depleted. The procedure led to some aspecific loss of total IgA1 and total IgA2.

IgA subclasses and smoking

As IgA is the dominant antibody at mucosal surfaces, mucosal inflammation, for example caused by long-term smoking, might lead to a more prominent IgA response and higher IgA serum levels. To investigate this hypothesis, levels of total, RF and anti-CCP2 IgA subclasses were compared between smoking and non-smoking RA patients. In seropositive IMPROVED patients, smokers had significantly increased serum levels of total IgA2 (p=0.004), as well as higher RF IgA1 levels (p=0.004) (Figure 5A). Percentage IgA2 of total IgA (p=0.02) and RF IgA2 (p=0.04) were not significant after multiple testing correction. However, the ratio RF IgA1 / RF IgA2 was similar in smokers versus non-smokers (Supplementary figure S4A), indicating that both RF subclasses are elevated in smokers, even though this was not significant for RF IgA2-levels. Interestingly, levels of total IgA1 and anti-CCP2 IgA1 were not elevated in smokers. Similar results were found in multivariate analyses corrected for age, gender and CRP (Supplementary table S3). In the EAC seropositive samples, RF IgA1 levels appeared to be higher in smokers, but this was not statistically significant (p=0.36) (Figure 5B). Smoking was also not associated with other total or antigen-specific subclass levels (Figure 5B, supplementary figure S4B and table S3). Similar to the pattern observed in seropositive RA, in seronegative IMPROVED patients total IgA2 levels were also selectively increased in smokers (tIgA2 p=0.004, %tIgA2 p=0.002) (Supplementary figure S4C, table S3), while no difference was observed between smoking versus non-smoking seronegative EAC patients after 1 year of treatment (Supplementary figure S4D, table S3). In conclusion, smoking, a proxy for mucosal inflammation, might lead to a selective increase in serum total IgA2 levels and RF IgA levels at disease onset, but does not seem to affect serum total IgA1 and anti-CCP2 IgA1 in RA patients.



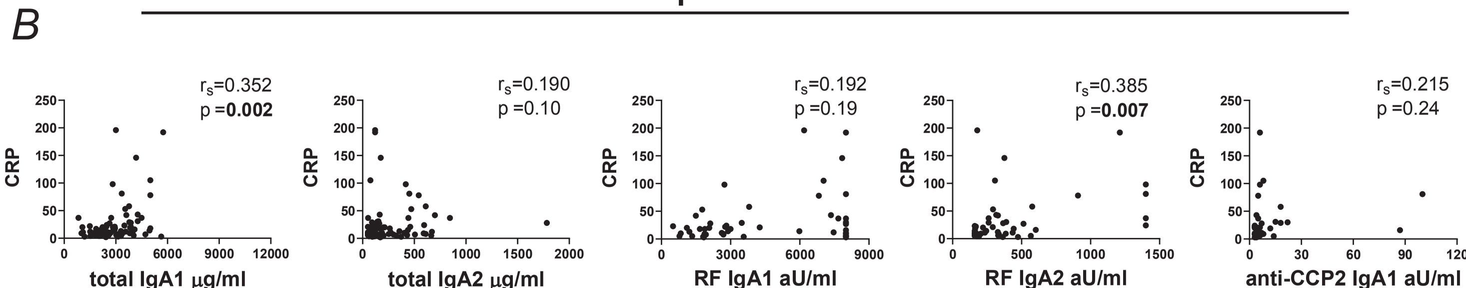


Figure 4: IgA subclass levels and CRP levels in seropositive RA. Correlation between IgA subclass levels and CRP levels in A IMPROVED seropositive RA patients and in B EAC seropositive patients, calculated using Spearman's rank correlation coefficient (r_s). In RF IgA subclass analyses, only patients positive for both RF IgA1 and RF IgA2 are included. For anti-CCP2 IgA1 analysis only anti-CCP2 IgA1 positive patients are included. Of note, RF and anti-CCP2 IgA subclass levels were not titrated.

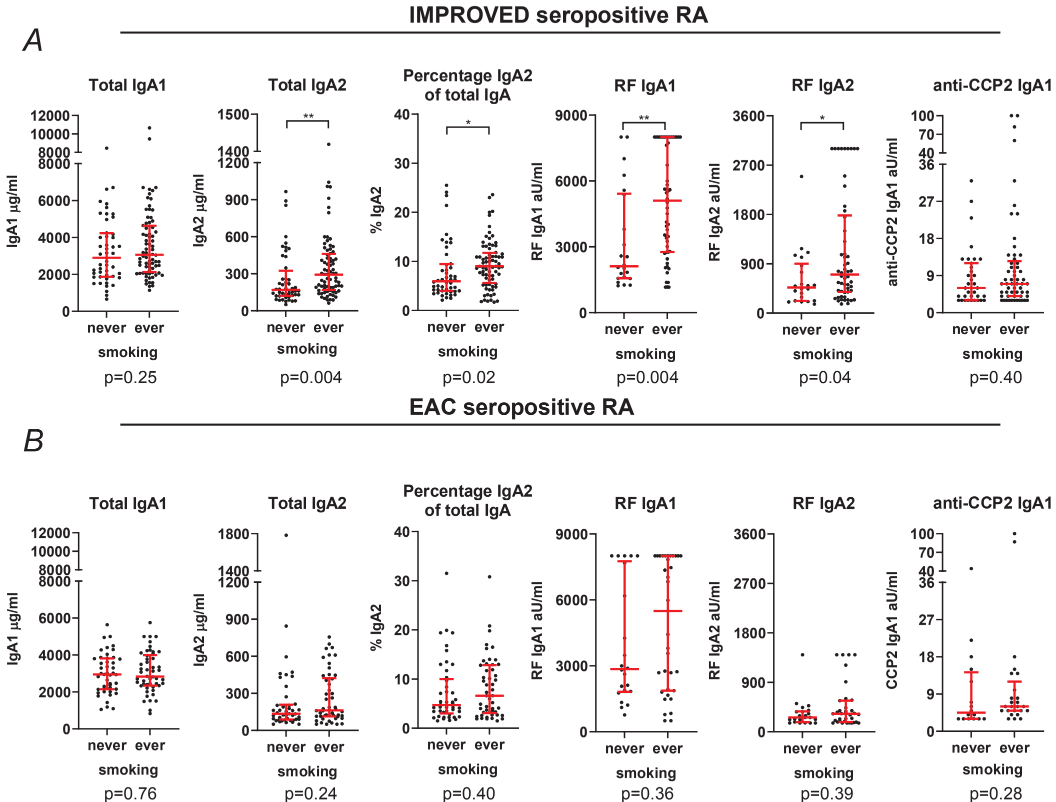


Figure 5: IgA subclass levels and smoking in seropositive RA. IgA subclass levels in ever- versus never-smoking seropositive RA patients in A IMPROVED and B EAC, analyzed using Mann–Whitney U tests. Red bars: median and interquartile range. *p<0.05, **p<0.01, ***p<0.001, #not significant after correction for multiple testing. In RF IgA subclass analyses, only patients positive for both RF IgA1 and RF IgA2 are included. For anti-CCP2 IgA1 analysis only anti-CCP2 IgA1 positive patients are included. Of note, RF and anti-CCP2 IgA subclass levels were not titrated.

Discussion

Since studying IgA subclasses can provide insight in both mucosal involvement as well as potential pro-inflammatory pathophysiological processes, we explored the IgA subclass distribution of total IgA as well as RF- and anti-CCP2-specific IgA in RA. Strikingly, total IgA1 and IgA2 levels were increased in seropositive RA patients in both the IMPROVED and EAC cohort. This was much less pronounced for seronegative patients, in whom only total IgA1 in the EAC was significant. Furthermore, both RF and anti-CCP2 IgA1 and IgA2 were detectable in a subset of seropositive RA patients. However, technical difficulties posed by RF IgA interference prohibited precise determination of anti-CCP2 IgA2 levels.

Several observations, for example the detection of RF and ACPA in sputum and saliva of seropositive RA patients, indicate that the mucosal immune system might be involved in the pathophysiology of RA (1-3, 11). Both IgA subclasses have an important function in mucosal immune responses, but the relative amount of IgA2 is increased at mucosal sites (12). A previous study suggested that also in serum of RA patients the relative amount of IgA2 is increased (6). Based on these findings, one might hypothesize that in RA patients IgA2 can translocate from (inflamed) mucosal sites, where it is highly abundant, into the circulation, leading to an elevated percentage of IgA2 in serum. However, we found that both IgA1 and IgA2 levels were elevated in seropositive RA patients. The percentage of IgA2 in serum was not increased in RA patients when compared to healthy donors. Thus, these data do not support the notion that direct translocation of mucosal IgA(2) is one of the main mechanisms leading to the elevated IgA subclass levels in RA.

Nonetheless, chronic mucosal inflammation might still be involved in the hyperproduction of IgA subclasses in RA patients. The link between mucosal immune responses and serum immunoglobulins is currently not completely understood. Research in celiac disease showed that mucosal and serum IgA are related, but produced by different plasma cells (13). Therefore, one might hypothesize that it is possible that the initial mucosal response is predominantly of the IgA2 subclass, while the related serum response is predominantly IgA1. This means the elevated IgA1 and IgA2 serum levels in RA could still be the result of increased mucosal IgA responses, most likely not by direct translocation of mucosal IgA, but potentially via the generation of specific plasma cell populations that contribute to the serum antibody pool. On the other hand, various studies have described that IgG and IgM can also be elevated in RA (14-17). This suggests the elevated IgA subclass levels could also be part of a general immunoglobulin (Ig) hyperproduction in RA patients, for example due to aspecific B

cell hyperreactivity in the context of systemic inflammation. Another possibility is that the hyperglobulinemia reflects intrinsic B cell alterations in RA patients, which could be in line with the important role that B cells play in the pathophysiology of RA (18-20).

To investigate whether chronic mucosal inflammation might play a role in the elevated IgA subclass levels in RA patients, we used smoking status as a proxy for mucosal inflammation, since smoking is known to cause chronic pulmonary inflammation (21). Intriguingly, smoking was associated with a selective increase in total IgA2 levels in serum of RA patients in the IMPROVED. Also RF IgA subclass levels tended to be increased in smoking RA patients in IMRPOVED, whereas anti-CCP2 IgA1 levels were not. This is interesting, as both RF and anti-CCP2 total IgA have been detected in sputum of RA patients, suggesting they are produced locally in the lungs (2). However, our findings suggest that smoking might have a larger influence on the production of RF IgA than on anti-CCP2 IgA. This is in line with the observations that smoking is associated with RF IgM positivity rather than the presence of ACPA IgG (22, 23). Of note, we could not replicate these associations with smoking in the EAC, where autoantibody levels were overall lower than in the IMPROVED, possibly due to the immunosuppressive treatment that most patients had received (9). In conclusion, our data suggest that chronic mucosal inflammation may be one of the mechanisms playing a role in the elevated RF IgA and total IgA2 levels in RA, although smoking status does not explain the full extent of the increase in total IgA subclasses in RA patients.

Although the presence of IgA2 in humans was described decades ago, novel findings regarding pro-inflammatory effector functions of IgA2 were recently described (6). However, in RA patients we did not observe an association between total IgA2 levels and two important markers of inflammation, CRP and DAS. On the other hand, a significant correlation between total IgA1 levels and CRP was seen in the EAC. Furthermore, significant associations between CRP and RF IgA2-levelswere found, although the effect was small and not significant after correction for multiple testing correction in the IMPROVED. Higher anti-CCP2 IgA1 levels were not associated with lower inflammation in our study, in contrast to a weak correlation between low anti-CCP2 IgA1 and high DAS described before (6). A recent study also describes that anti-CCP2 IgA2 levels decline in ongoing remission, although, based on our data regarding RF interference, this effect might be mediated by a decline in RF IgA levels (7, 9). Taken together, our findings do not appear to support an essential role for IgA2 in the ongoing pro-inflammatory processes in RA.

One of the limitations of our study is the use of in-house ELISA's. Technical difficulties posed by RF IgA interference prohibited precise determination of anti-CCP2 IgA2 levels. To obtain decent signals in the ELISA, serum was diluted less and incubated overnight

instead of 1 hour, which might have provided RF IgA with the chance to bind anti-CCP2 IgG. As a result the anti-CCP2 IgA1/IgA2 ratio could not be calculated. This precluded our attempts to replicate the findings that the ACPA IgA response is shifted towards IgA2 (6). It is unclear whether possible interference of RF IgA2 was investigated in other studies. However, based on the fact that anti-CCP2 IgA1 was readily detectable whereas anti-CCP2 IgA2 was not, it seemed unlikely that a majority of total ACPA IgA was of the IgA2 subclass. On the contrary, both RF IgA1 and IgA2 were readily detectable, in line with previous studies (11).

Furthermore, we repeated the measurements on an independent cohort to investigate the generalizability of our findings. The results of the in-house IgA subclass ELISA's were largely reproducible between the two different cohorts. Another strength is that many anti-CCP2 IgA positive patients were included and detailed information regarding smoking status and inflammatory markers was available. Although our findings are in contrast with a previous study on IgA subclasses in RA (which found both IgA1 and IgA2 to be lower in RA patients) (6), multiple other studies have described raised total IgA levels in RA (14-16, 24-27).

In conclusion, seropositive RA patients have raised total IgA1 and IgA2 levels and can also harbor RF and ACPA IgA subclasses. Since no shift towards the IgA2 subclass was observed, the increase in total IgA levels appears not to be due to translocation of mucosal IgA over mucosal barriers into the bloodstream. However, chronic mucosal inflammation might be one of the mechanisms involved in the raise in IgA(2) levels in RA, given the association between smoking and total IgA2 levels. Despite the pro-inflammatory properties of IgA2, our data does not seem to support a large role of IgA2 in chronic inflammatory processes in RA patients.

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Supplementary information

Supplementary data S1: Methodology

ELISAs

For total IgA1 and IgA2, detection Nunc maxisorp plates (Thermo scientific; 430341) were coated with 10 μg/ml goat anti-human-IgA-Fc (Bethyl; A80-102A) and blocked with PBS/1%bovine serum/50 mM Tris. Sera were diluted 1:6000 for IgA1 and 1:2000 and 1:6000 for IgA2 in PBS/1%BSA/0.05% Tween/50 mM Tris and incubated together with a serial dilution of a commercial standard (Nordic Mubio, NOR-04) on the same plate for 1 hour at room temperature. Detection antibodies used for detecting IgA subclasses in all the different assays are described below. If samples were above the linear range of the standard, serial titrations of the sera were performed. The percentage IgA2 of total IgA was calculated by dividing total IgA2 levels by the sum of IgA1 and IgA2 levels times 100. For RF IgA1 and IgA2 ELISA, Nunc maxisorp plates were incubated overnight at room temperature with 10 μg/ml human IgG (Jackson Immunoresearch;009-000-003) and blocked for 1 hour at 37°C with PBS/1%BSA. Samples were diluted 1:25 and 1:50 for IgA1 and 1:10 for IgA2 in PBS/1%BSA/0.05% Tween and incubated for 1 hour at 37°C. A pooled serum standard was used to calculate arbitrary units. For CCP2 IgA1 and IgA2 measurements, biotinylated CCP2 (patent EP2071335) and a biotinylated control peptide containing arginine instead of citrulline (CargP2) were coated 1 μg/ml in PBS/0.1%BSA on pre-coated streptavidin plates (for IgA1 standard capacity -Microcoat 604500; for IgA2 High capacity Microcoat 604501) and incubated 1 hour at room temperature (RT) for IgA1 and overnight at 4°C for IgA2. The patent protected CCP2 and CargP2 were provided by Dr. J. W. Drijfhout (Dept. of IHB, LUMC). For IgA1, sera were diluted 1:50 in PBS/0.05% Tween/ 1% BSA and incubated for 1 hour at 37°C. For IgA2, plates were first blocked with PBS/2%BSA for 6 hours at 4°C on ice and thereafter serum was added in a 1:12.5 dilution and incubated overnight at 4°C on ice. For both anti-CCP2 IgA1 and IgA2 ELISA's the same standard of pooled isolated anti-CCP2 was used.

IgA subclasses were detected using either mouse anti-human IgA1 (Nordic MUbio, 6688) with a subsequent incubation of goat anti-mouse Ig-HRP (DAKO p0447) or with mouse anti-human IgA2-HRP (Southern biotech, 9140-05), each incubated for 1 hour at 37°C. All ELISA's are visualized with ABTS/H_2O_2. Between each incubation step the plates were washed with PBS/0.05% Tween 20.

Anti-CCP2 IgG ELISA was performed similar to the anti-CCP2 IgA1 ELISA, but with rabbit anti-human IgG-HRP (DAKO, P0214) for detection. For the anti-CCP2 IgA ELISA's, blanks were subtracted for both the citrullinated and the unmodified arginine control peptide separately. A signal was considered specific if the OD on the citrullinated

peptide was at least 0.1 higher compared to the arginine control and above the cut-off. Cut-offs for RF and anti-CCP2 were calculated using the mean plus 2 times standard deviation (SD) of healthy donors. RF and anti-CCP2 IgA subclasses levels above or below the linear part of the standard were not titrated.

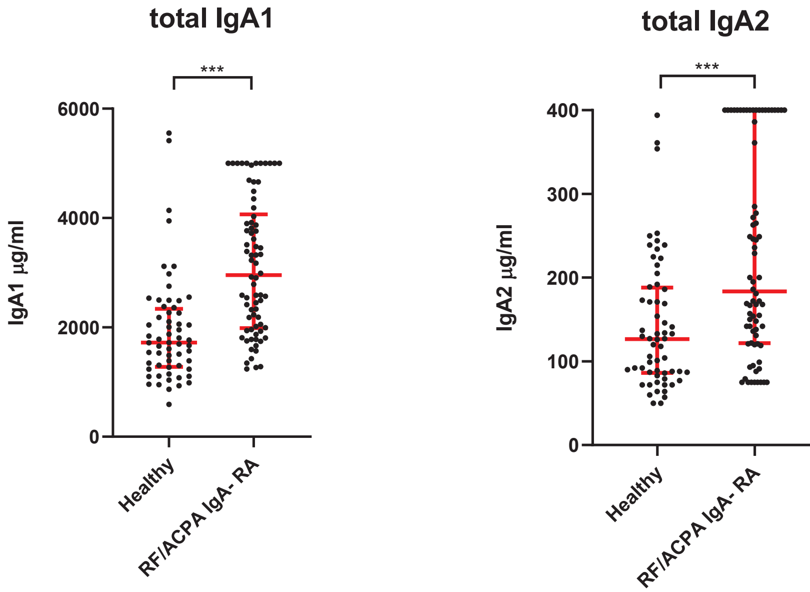
IgG depletion

To deplete IgG from the serum samples, protein G Agarose beads (Thermofisher 20397) were diluted 1:3 in PBS and pipetted 168 µl/well in a 10 µm filter plate (Orochem; OF1100). After washing, 70 µl 1:5 diluted serum was added to each well and the plate was incubated on a shaker at 900 rpm for 1 hour. Flow-through was collected after centrifugation for 1min at 350g. After regeneration of the beads with 100ul/well 0.1M glycine pH 3 and washing, the cycle was repeated using the flow-through. The flow-through of the second cycle was analysed on ELISA in a final dilution similar to the dilution of the non-depleted serum.

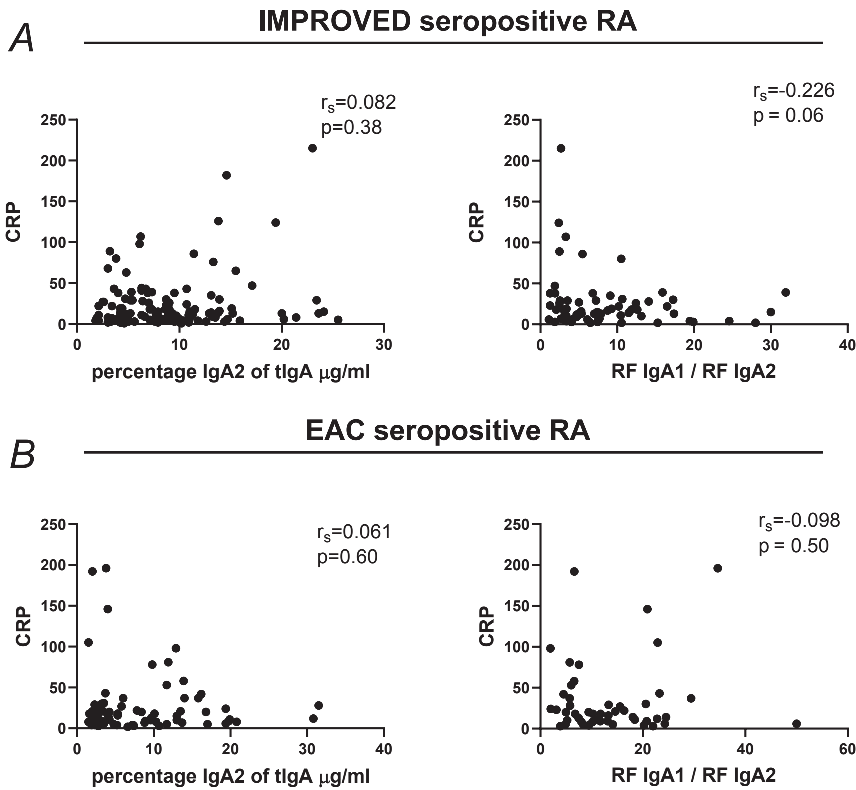
Supplementary table S1: Total IgA subclass levels in seropositive and seronegative RA patients.

	Total IgA1 levels		Total IgA2 levels		Percentage IgA2 of total IgA	
	B (95% CI)	p-value	B (95% CI)	p-value	B (95% CI)	p-value
IMPROVED seropositive RA	0.21 (0.15-0.29)	**p<0.001**	0.26 (0.17-0.35)	**p<0.001**	0.04 (-0.04-0.12)	p=0.36
IMPROVED seronegative RA	-0.04 (-0.11-0.03)	p=0.22	0.08 (-0.05-0.22)	p=0.22	0.10 (-0.03-0.23)	p=0.12
EAC seropositive RA	0.17 (0.11-0.24)	**p<0.001**	0.14 (0.03-0.25)	**p=0.01#**	-0.03 (-0.14-0.07)	p=0.53
EAC seronegative RA	0.15 (0.08-0.22)	**p<0.001**	0.22 (0.06-0.38)	**p=0.007#**	0.04 (-0.10-0.18)	p=0.59

The difference in total IgA subclass levels between RA patients and healthy controls, analysed using linear regression for each cohort separately. Due to skewness of the data ^{10}log(total IgA1), ^{10}log(total IgA2) or ^{10}log(percentage total IgA2) were used as dependent variable and age and gender were included as possible confounders. In each row the coefficients and accompanying p-value represent the effect of being an RA patient (compared to a healthy donor (reference category)) on the IgA subclass levels (dependent variable). #not significant after correction for multiple testing.



Supplementary figure S1: Total IgA subclass levels in seropositive RA patients in IMPROVED. Total IgA1 and IgA2 levels in seropositive (RF IgM+ and/or anti-CCP2 IgG+) IMPROVED RA patients, who are negative for RF IgA and anti-CCP2 IgA. Mann–Whitney U tests were used to compare IgA levels between RA patients and healthy donors (total IgA1 $p<0.001$, total IgA2 $p<0.001$). Red bars: median and interquartile range. Not all ELISA measurements were performed on the same day.

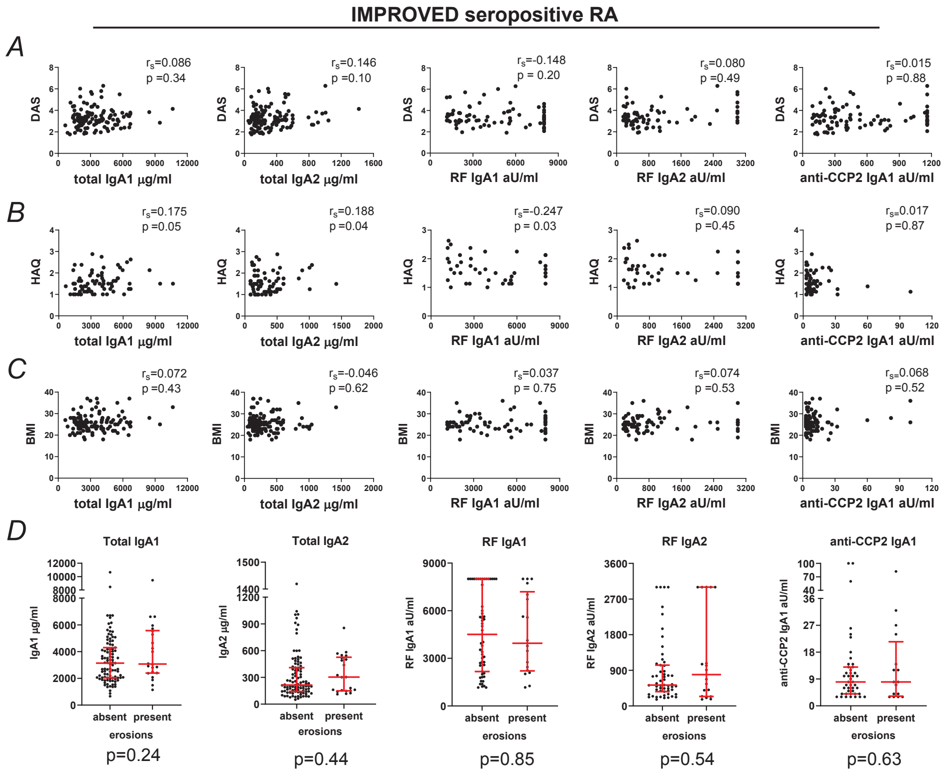


Supplementary figure S2: Correlation between CRP and total/RF IgA1:IgA2 ratio. Correlation between CRP and percentage IgA2 of total IgA and RF IgA1 / RF IgA2 ratio in A IMPROVED seropositive RA patients and in B EAC seropositive RA patients. Correlations are calculated using spearman's rank correlation coefficient (r_s). In RF IgA subclass analyses, only patients positive for both RF IgA1 and RF IgA2 were included. Of note, RF IgA subclass levels were not titrated.

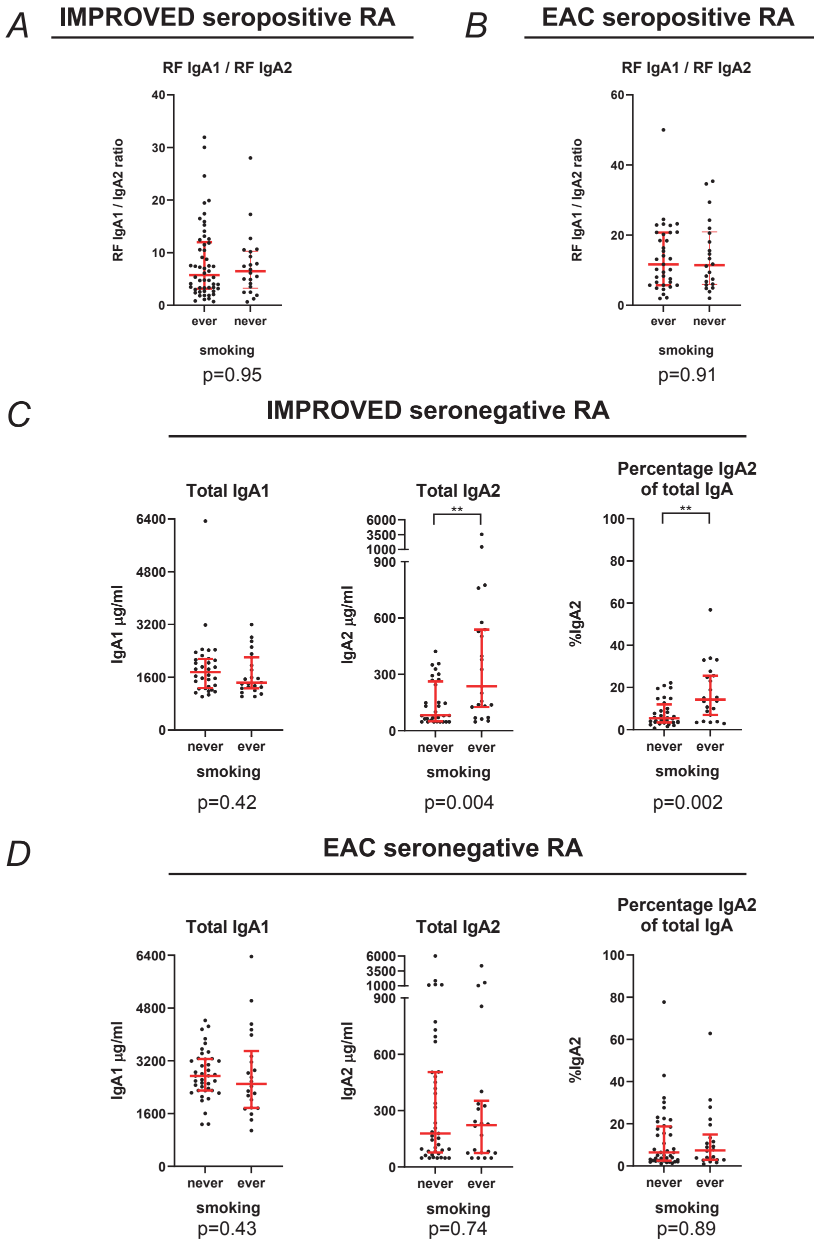
Supplementary table S2: Total and autoantibody-specific IgA subclass levels and CRP in RA.

	Dependent variable: CRP levels			
	IMRPOVED seropositive RA		EAC seropositive RA	
	B (95% CI)	p-value	B (95% CI)	p-value
total IgA1 levels	0.000 (0.000-0.000)	p=0.81	0.000 (0.000-0.000)	**p=0.002**
total IgA2 levels	0.000 (0.000-0.001)	p=0.42	0.000 (0.000-0.001)	p=0.17
percentage IgA2 of total IgA	0.009 (-0.008-0.026)	p=0.29	0.002 (-0.014-0.018)	p=0.79
RF IgA1 levels	0.000 (0.000-0.000)	p=0.61	0.000 (0.000-0.000)	p=0.052
RF IgA2 levels	0.000 (0.000-0.000)	**p=0.007**#	0.001 (0.000-0.001)	**p=0.001**
Anti-CCP2 IgA1levels	-0.002 (-0.008-0.004)	p=0.53	0.003 (-0.005-0.011)	p=0.47
	Dependent variable: CRP levels			
	IMRPOVED seronegative RA		**EAC seronegative RA**	
	B (95% CI)	**p-value**	**B (95% CI)**	**p-value**
total IgA1 levels	0.000 (0.000-0.000)	p=0.67	0.000 (0.000-0.000)	p=0.07
total IgA2 levels	0.000 (0.000-0.000)	p=0.31	0.000 (0.000-0.000)	p=0.58
percentage IgA2 of total IgA	0.008 (-0.006-0.022)	p=0.26	0.003 (-0.006-0.013)	p=0.48

Multivariate linear regression investigating the association between the different IgA subclass levels and CRP in each cohort of RA patients, using log transformed CRP levels as dependent variable. In each row the coefficients and accompanying p-value represent the effect of the specific IgA subclass level (independent variable) on CRP levels (dependent variable) per cohort. Besides IgA subclass levels, also age, gender and smoking were included as independent variables. #not significant after correction for multiple testing.



Supplementary figure S3: IgA subclass levels and disease severity in RA. Correlation between IgA subclass levels and A DAS, B HAQ (all non-significant after correction for multiple testing) and C BMI and D association between the presence of erosions at baseline and IgA subclass levels in IMPROVED seropositive patients. Correlations are calculated using spearman's rank correlation coefficient (r_s). Differences in IgA subclass levels between patients with and without erosions at baseline were tested using Mann-Whitney U tests. Patients with missing values for BMI (n=3), HAQ (n=2) and erosions (n=4) were excluded from the respective analyses. In RF IgA subclass analyses, only patients positive for both RF IgA1 and RF IgA2 were included. In graphs of anti-CCP2 IgA1 only anti-CCP2 IgA1 positive patients are included. Of note, RF and anti-CCP2 IgA subclass levels were not titrated.



Supplementary figure S4: Smoking and IgA subclass ratios in RA. A-B Ratio RF IgA1 / RF IgA2 in ever- versus never-smoking seropositive RA patients in A IMPROVED and B EAC. C-D Total IgA subclass levels in ever- versus never-smoking seronegative RA patients in C IMPROVED and D EAC, analysed using Mann–Whitney U tests. Red bars: median and interquartile range. *p<0.05, **p<0.01, ***p<0.001. In both cohort one seronegative patient had unknown smoking status and was excluded. Of note, RF IgA subclass levels were not titrated.

Supplementary table S3: Smoking and IgA subclass levels in RA patients.

	Total IgA1 levels		**Total IgA2 levels**		**Percentage IgA2 of total IgA**	
	B (95% CI)	**p-value**	**B (95% CI)**	**p-value**	**B (95% CI)**	**p-value**
Smoking IMPROVED seropositive RA	0.05 (-0.04-0.13)	p=0.27	0.16 (0.06-0.27)	**p=0.006**	0.10 (0.002-0.20)	**p=0.05#**
Smoking EAC seropositive RA	-0.03 (-0.12-0.05)	p=0.43	-0.002 (-0.18-0.17)	p=0.98	0.03 (-0.14-0.20)	p=0.72
Smoking IMPROVED seronegative RA	-0.04 (-0.13-0.05)	p=0.42	0.34 (0.11-0.58)	**p=0.006**	0.33 (0.11-0.54)	**p=0.004**
Smoking EAC seronegative RA	-0.02 (-0.11-0.08)	p=0.68	-0.16 (-0.49-0.18)	p=0.35	-0.11 (-0.40-0.18)	p=0.46
	RF IgA1 levels		**RF IgA2 levels**		**Anti-CCP2 IgA1 levels**	
	B (95% CI)	**p-value**	**B (95% CI)**	**p-value**	**B (95% CI)**	**p-value**
Smoking IMPROVED seropositive RA	0.21 (0.07-0.35)	**p=0.005**	0.23 (0.04-0.42)	**p=0.02#**	0.13 (-0.03-0.29)	p=0.12
Smoking EAC seropositive RA	0.11 (-0.09-0.32)	p=0.28	0.09 (-0.09-0.26)	p=0.34	0.11 (-0.24-0.45)	p=0.52

Multivariate linear regression investigating the association between smoking and IgA subclass levels in RA patients. The log transformed IgA subclass levels were used as the dependent variable. The coefficients and p-values represent the effect of smoking (independent variable) in each cohort on the levels of the different IgA subclasses (dependent variables). Besides smoking, also potential confounders age, gender and CRP were included as independent variables. Smoking is defined as current or former smoking. #not significant after correction for multiple testing.