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Chapter 7

The ACPA isotype profile reflects long-term radiographic progression in rheumatoid arthritis


* These authors contributed equally.

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ABSTRACT

Objectives The presence of anti-citrullinated protein antibodies (ACPA) is a powerful predictive factor for the development and progression of rheumatoid arthritis (RA). The ACPA response has been shown to consist of various isotypes, but the consequences of differences in isotype-distribution have not been extensively investigated. In this study, we investigated the relationship between ACPA isotypes, disease progression and radiological outcome.

Methods ACPA isotypes were determined in sera of anti-CCP2-positive patients by enzyme-linked immunosorbent assay (ELISA). To investigate if the ACPA response continues to evolve during disease development, we studied the ACPA isotype profile during progression of undifferentiated arthritis (UA) to RA. The association of disease progression with ACPA isotype use was assessed using long-term radiographic follow-up data from RA patients in two independent cohorts.

Results The ACPA isotype distribution did not expand during disease progression from UA to RA, but was relatively stable over time. In both RA cohorts, the baseline ACPA isotype profile was a significant predictor of disease severity, with more isotypes indicating a higher risk of radiographic damage (odds ratio for every additional isotype: 1.4 (95% CI: 1.1-1.9) p<0.001). ACPA isotypes supplied additional prognostic information to ACPA-status alone, even after correction for other predictive factors.

Conclusions The magnitude of the ACPA isotype profile at baseline reflects the risk of future radiographic damage. These results indicate that not only the presence, but also the constitution of the ACPA response, is relevant for the disease course of RA.
INTRODUCTION

Anti-citrullinated protein antibodies (ACPA) have emerged as a very distinctive feature of rheumatoid arthritis (RA) patients. The presence of these antibodies, which is commonly assessed by reactivity against cyclic citrullinated peptides (CCP), has been shown to potently predict both the development of RA and the extent of associated joint destruction. Recent evidence indicates that well-known risk factors for RA such as the HLA-DRB1 shared epitope alleles and smoking predominantly predispose to ACPA-positive RA. These differences in clinical phenotype and underlying risk factors have led to the concept that ACPA-positive and ACPA-negative RA constitute distinct entities with different pathophysiological mechanisms of disease.

Studies using animal models to investigate the biologic processes underlying RA have shown that ACPA can exacerbate arthritis in mice. These findings suggest that anti-citrulline immunity may play a role in the pathogenesis of autoantibody-positive RA. Possibly, isotype switching may be one of the events required in order for autoantibodies to contribute to disease pathogenesis.

Isotype switching during the maturation of an immune response leads to an increased diversity of antibody structure. The generated antibody isotypes differ substantially with regard to their ability to mediate effector mechanisms. For example, IgM and IgG3 are much more potent activators of the complement system than other isotypes. Furthermore, the various isotypes can bind to specific Fc receptors on the surface of different cell-types, which in the case of IgG1 and IgG3 can lead to the activation of macrophages with concomitant cytokine production. In light of these well-known differences between antibody isotypes, a detailed investigation of the isotype distribution of an autoantibody response such as ACPA may provide important clues about the role of these antibodies in the pathogenesis of the disease.

The isotype use of the ACPA response has been studied using several tests, some of which were derived from the commercially available CCP2 assays, which were modified in order to be able to measure IgG subclass antibodies, as well as IgM and IgA ACPA. The fact that ACPA of the IgM isotype could be detected at various stages of the disease indicates that new B cells are continuously being recruited into the ACPA response. This means that the anti-citrullinated protein immune response is constantly being (re)activated during the course of ACPA-positive arthritis. It is unclear however, if during this process of continuous reactivation throughout disease progression, there is also further maturation of the ACPA response in the sense of isotype switching. In addition, the reported findings of the differences in ACPA isotype use between patients, raise the question to what extent the exact features of the ACPA response, such as isotype use, are associated with disease progression.
In this study, we examined the relationship between ACPA isotypes and disease development and progression. The use of two large, independent cohorts with long-term follow-up data enabled us to investigate the association between the ACPA isotype profile and progression of radiographic damage.

**METHODS**

**Patient populations**

The Leiden EAC is an inception cohort of patients with recent-onset arthritis (less than 2 years of complaints) that was initiated in 1993. At standardized follow-up visits two weeks after the first presentation and yearly thereafter, diagnoses were recorded for all patients. Patients were classified as having RA when they fulfilled the 1987 revised ACR criteria.

In order to investigate the association of ACPA isotypes with radiographic damage, baseline serum samples of all ACPA-positive RA patients in the EAC (n= 171) were analyzed for ACPA isotypes. Annual radiographs of hands and feet of all RA patients were assessed for radiographic damage according to the Sharp-van der Heijde score (SHS).

To determine if the findings in the Leiden EAC cohort could be replicated in a second cohort, ACPA isotypes were measured in a Norwegian cohort which was part of the European Research on Incapacitating Disease and Social Support (EURIDISS) project. Consecutive RA patients (n=238) with a maximum disease duration of 4 years at baseline were enrolled in this study and followed longitudinally for 10 years. 125 patients had X-rays of the hands available at baseline and after 5 and 10 years of follow-up for scoring with the SHS method. There were no significant differences between the patients with X-rays available at follow-up (n=125) and the whole cohort (n=238). ACPA isotype measurements were performed on baseline serum samples. The protocols of both cohorts were approved by the local ethics committees and all participants provided written informed consent.

**Anti-CCP2 assays**

Total IgG anti-CCP2 was measured in baseline serum samples from patients with UA or RA by a second-generation ELISA (for the EAC cohort: Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands, for the EURIDISS cohort: INOVA diagnostics, San Diego, CA, USA). Samples with a value above the cut-off as specified by the manufacturer were considered positive.

**Measurement of ACPA isotypes**

ACPA IgG1, IgG2, IgG3, IgG4, IgA and IgM were determined using a sandwich ELISA technique as described previously. Briefly, assays were performed using anti-CCP2
ELISA plates (Eurodiagnostica) with isotype-specific detection antibodies. A series of successive dilutions of pooled patient sera that were positive for all isotypes was included as a standard on all plates, along with a positive control to verify comparability between plates. Sera from 8 representative healthy controls were included on each plate to determine the cut-off for positivity. Cut-off values were defined as the mean plus 2 standard deviations of the measurements of the healthy control sera.

Statistical analysis
To assess changes in ACPA isotype usage between baseline and follow-up patients who presented with undifferentiated arthritis (UA) and subsequently developed RA, McNemar’s test was used. Changes in the levels of ACPA isotypes between baseline and follow-up samples were evaluated using Wilcoxon signed ranks test. Correlation between changes in the levels of the various ACPA-isotypes were investigated using Spearman’s rank correlation coefficient, due to the non-normal distribution of the data.

To investigate the association between ACPA isotypes and radiographic progression, differences in radiographic damage between groups were assessed using Mann-Whitney U-tests and logistic regression analysis. The radiographic progression (dependent variable) was calculated as the difference in SHS between baseline and the last time point of follow-up (4 years in the EAC, 10 years in the EURIDISS). This value was subsequently dichotomized into minor and major radiographic progression based on the median values per cohort (10 in the EAC, 17 (hands only) in the EURIDISS). Patients with a change in SHS greater than or equal to the median were classified as having major progression, while the other patients were classified as having minor progression. Multivariate logistic regression analysis was performed to adjust for additional variables (age, gender, CRP, IgM rheumatoid factor and SHS at baseline). The positive likelihood ratio was calculated as the sensitivity divided by (1-specificity). Likewise, the negative likelihood ratio was calculated as (1-sensitivity) divided by the specificity. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) 16. P-values below 0.05 were considered to be statistically significant.

RESULTS

The ACPA isotype distribution does not expand during disease progression
Previously, we have shown that in patients with longstanding RA, the ACPA isotype profile at baseline did not markedly differ from the ACPA isotype use after 7 years of follow-up on the population level 14. In order to extend these findings to an earlier stage of the disease, and to investigate if the ACPA response continues to evolve during disease progression, we studied the ACPA isotype profile in baseline and follow-up serum samples of 67 ACPA-
positive patients from the Leiden EAC. All patients had undifferentiated arthritis (UA) at baseline, meaning they did not fulfill the ACR criteria for RA and their clinical presentation was not compatible with any other well-defined rheumatologic disease. For the present study 579 UA patients were analyzed of whom 142 were ACPA-positive as measured by the CCP2 ELISA. Of all anti-CCP2-positive UA patients who had developed RA after one year, serum samples collected at baseline and after one year of follow-up were examined (n=67 samples available). Measurements of isotypes were restricted to patients who were positive for ACPA, since the occurrence of ACPA isotypes has been shown to be confined to ACPA-positive patients 13, 14.

As shown in Figure 1, ACPA isotype levels varied considerably during disease progression, with some patients showing an increase and others showing a decrease in levels. Most of the changes in the levels of the different isotypes were significantly correlated with each other. Especially changes in IgG3- and IgA-ACPA-levels were accompanied by similar changes in levels of the other isotypes (correlation coefficient: 0.23-0.66).

![Figure 1: ACPA isotype levels at baseline and after 1 year of follow-up of 67 UA-RA patients. All patients had UA at baseline and had developed RA at the time of follow-up. Dots depict the levels of individual patients in arbitrary units per milliliter (AU/ml) at baseline and follow-up. The number of arbitrary units/ml for one isotype is not comparable to the number of arbitrary units/ml for another isotype. Differences in the levels of ACPA isotypes between baseline and follow-up samples were assessed using Wilcoxon signed ranks test.

Table 1. ACPA isotype use in patients who experienced disease progression from undifferentiated arthritis (UA) to RA

<table>
<thead>
<tr>
<th>ACPA Isotype</th>
<th>BL (UA) n=67</th>
<th>FU (RA) n=67</th>
<th>lost</th>
<th>gained</th>
<th>p-value McNemar</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>67 (100%)</td>
<td>66 (99%)</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>IgG2</td>
<td>51 (76%)</td>
<td>44 (66%)</td>
<td>12</td>
<td>5</td>
<td>0.14</td>
</tr>
<tr>
<td>IgG3</td>
<td>34 (51%)</td>
<td>35 (52%)</td>
<td>7</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>IgG4</td>
<td>64 (96%)</td>
<td>65 (97%)</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IgA</td>
<td>45 (67%)</td>
<td>41 (61%)</td>
<td>9</td>
<td>5</td>
<td>0.42</td>
</tr>
<tr>
<td>IgM</td>
<td>39 (58%)</td>
<td>40 (60%)</td>
<td>7</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
</table>

The isotype profile at baseline (BL (UA)) compared to the isotype profile after 1 year of follow-up (FU (RA)).
Both the paired-samples analysis and the comparison of the median levels at baseline and follow-up revealed that there was no significant difference between baseline and follow-up levels for any of the isotypes. Despite the fluctuation in levels, most patients remained either positive or negative for a certain isotype at both baseline and follow-up. Thus, the number of patients which was positive for a certain ACPA isotype did not change (Table 1). Although some patients lost or acquired certain ACPA isotypes, there was no overall trend towards an increase in ACPA isotype use. The median number of ACPA isotypes present at baseline and follow-up was also the same: 5.

On the basis of these results, we can conclude that despite a worsening of the clinical phenotype, the ACPA isotype use does not expand during disease progression from UA to RA.

**Figure 2: Effect of ACPA isotypes on radiographic damage in EAC and EURIDISS.**

Depicted are the median Sharp-van der Heijde radiographic scores (SHS) over time.

Panels A and B present the results of the EAC and contain information on 251 RA patients of whom 113 were ACPA-positive with ACPA isotype data.

Panels C and D depict the results of the EURIDISS cohort and contain information on 125 RA patients of whom 74 were ACPA-positive with ACPA isotype data.

Due to the non-normal distribution of SHS, medians were compared by Mann-Whitney U-test.

* in panels A and C indicates a significant difference with a p-value < 0.05 between ACPA-positive and ACPA-negative patients.

* in panels B and D indicates a significant difference with a p-value < 0.05 between the upper two groups: patients with less than 5 ACPA isotypes and patients with 5 or more ACPA isotypes.
ACPA isotypes at baseline predict future radiographic progression in patients with ACPA-positive RA

In view of the finding that the presence of ACPA isotypes in individual patients is relatively constant over time, we next investigated if the baseline ACPA isotype profile is associated with radiographic progression in the two independent cohorts: the Leiden EAC and in the Norwegian EURIDISS cohort. As has been described previously 1, 2, 18, ACPA-positive patients had substantially more radiographic damage than ACPA-negative patients in both cohorts (Figure 2A and 2C).

Within the ACPA-positive patients, a high number of ACPA isotypes present at baseline was associated with significantly more radiographic damage during the disease course (Figure 2B and D).

A more detailed investigation of the effect of the ACPA isotype profile on radiographic progression was performed using logistic regression analysis. As shown in Table 2 for the EAC and in Table 3 for the EURIDISS, several ACPA isotypes were significantly more prevalent in patients with major as compared to minor radiographic progression. By logistic regression analysis, these isotypes, which were more prevalent in patient with major radiographic progression, were significant predictors of radiographic progression within the ACPA-positive group of patients (Tables 2 and 3). The significant odds ratio (p ≤ 0.02) for every additional ACPA isotype which was present, reflects the effect of the number of ACPA isotypes (Tables 2 and 3).

The predictive effect of ACPA isotypes remained significant even after adjustment in multivariate logistic regression analysis for other variables known to influence radiographic progression (age, gender, disease activity, RF status and SHS at baseline) (Tables 2 and 3). The presence of five or more isotypes increased the odds of progression by 3.53 (95% CI 1.4-9.3) and 6.76 (95%CI 1.60, 28.7) in the EAC and EURIDISS cohorts, respectively.

Patients who used a total of 5 ACPA isotypes or more, also had significantly higher levels of total IgG anti-CCP2 (p<0.01) which is known to be associated with radiographic progression 1. Due to this strong correlation, the level of anti-CCP2 and the number of ACPA isotypes cannot be entered into the same multivariate model as explanatory variables, as this results in annulment of the effect. We therefore used a matching approach to assess the effect of ACPA isotypes independently of the effect of anti-CCP2 level. Patients with ≤4 and with ≥5 ACPA isotypes were matched for anti-CCP2 levels, resulting in 18 pairs in the EAC and 15 pairs in the EURIDISS (Figure 3A and 3C). The median SHS over time among these pairs with equivalent anti-CCP2 levels is depicted in Figure 3B and 3D. In the EAC, patients positive for 5 or more ACPA isotypes had significantly more radiographic damage at the first four timepoints, and in the EURIDISS, there was a trend towards more radiographic damage in patients using 5 or more ACPA isotypes (p=0.10 after 10 years of follow-up). These findings suggest that ACPA isotypes and the total anti-CCP2 IgG level
<table>
<thead>
<tr>
<th></th>
<th>Minor progression*</th>
<th>Major progression*</th>
<th>Positive Likelihood Ratio</th>
<th>Negative Likelihood Ratio</th>
<th>Odds Ratio</th>
<th>p-value</th>
<th>Adjusted Odds Ratio**</th>
<th>adjusted p-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACPA-positive^</td>
<td>35 (37%)</td>
<td>96 (83%)</td>
<td>2.24</td>
<td>0.27</td>
<td>8.23</td>
<td>&lt;0.01</td>
<td>3.91 (1.7-8.8)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Analysis within</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACPA-positive pts, n=</td>
<td>32</td>
<td>81</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgA ACPA-positive</td>
<td>15 (47%)</td>
<td>58 (72%)</td>
<td>1.53</td>
<td>0.53</td>
<td>2.86</td>
<td>0.02</td>
<td>3.71 (1.4-9.8)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IgM ACPA-positive</td>
<td>19 (59%)</td>
<td>54 (67%)</td>
<td>1.14</td>
<td>0.80</td>
<td>1.37</td>
<td>0.47</td>
<td>1.59 (0.6-4.1)</td>
<td>0.34</td>
</tr>
<tr>
<td>IgG1 ACPA-positive #</td>
<td>32 (100%)</td>
<td>81 (100%)</td>
<td>1.00</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IgG2 ACPA-positive</td>
<td>20 (63%)</td>
<td>69 (85%)</td>
<td>1.35</td>
<td>0.56</td>
<td>3.45</td>
<td>0.01</td>
<td>4.08 (1.5-11.3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IgG3 ACPA-positive</td>
<td>13 (41%)</td>
<td>55 (68%)</td>
<td>1.66</td>
<td>0.54</td>
<td>3.09</td>
<td>&lt;0.01</td>
<td>3.92 (1.6-9.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IgG4 ACPA-positive</td>
<td>29 (91%)</td>
<td>80 (99%)</td>
<td>1.09</td>
<td>0.11</td>
<td>8.28</td>
<td>0.07</td>
<td>16.2 (1.0-258)</td>
<td>0.05</td>
</tr>
<tr>
<td>Number of ACPA isotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(median, IQR)</td>
<td>4 (2-6)</td>
<td>5 (4-6)</td>
<td>-</td>
<td>-</td>
<td>1.45</td>
<td>&lt;0.01</td>
<td>1.61 (1.2-2.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>5 or more ACPA isotypes</td>
<td>15 (47%)</td>
<td>57 (70%)</td>
<td>1.49</td>
<td>0.57</td>
<td>2.69</td>
<td>0.02</td>
<td>3.53 (1.4-9.3)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*Patients with a change in SHS greater than or equal to the median were classified as having major progression, while the other patients were classified as having minor progression.

^ ACPA status was available for 95 patients with minor progression and 116 patients with major progression. Percentages were calculated relative to the number of patients with available measurements.

** multivariate logistic regression with adjustment for age, gender, CRP, IgM RF and SHS at baseline.

# logistic regression analysis could not be performed to investigate the effect of IgG1 ACPA, because 100% of patients in the major progression group were positive for this isotype.

Printed in bold are p-values < 0.05.
Table 3. Predictive value of ACPA isotypes for 10-year radiographic progression in the EURIDISS

<table>
<thead>
<tr>
<th></th>
<th>Minor progression*</th>
<th>Major progression*</th>
<th>Positive Likelihood Ratio</th>
<th>Negative Likelihood Ratio</th>
<th>Odds Ratio</th>
<th>p-value</th>
<th>Adjusted Odds Ratio**</th>
<th>adjusted p-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACPA-positive</td>
<td>22 (36%)</td>
<td>52 (83%)</td>
<td>2.31</td>
<td>0.27</td>
<td>8.60</td>
<td>&lt;0.01</td>
<td>4.63 (1.7-12.6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Analysis within</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACPA-positive pts, n=</td>
<td>22</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgA ACPA-positive</td>
<td>12 (55%)</td>
<td>42 (81%)</td>
<td>1.47</td>
<td>0.42</td>
<td>3.50</td>
<td>0.02</td>
<td>5.28 (1.2-22.6)</td>
<td>0.03</td>
</tr>
<tr>
<td>IgM ACPA-positive</td>
<td>5 (23%)</td>
<td>24 (46%)</td>
<td>2.00</td>
<td>0.70</td>
<td>2.91</td>
<td>0.07</td>
<td>4.32 (1.1-16.9)</td>
<td>0.04</td>
</tr>
<tr>
<td>IgG1 ACPA-positive #</td>
<td>21 (96%)</td>
<td>52 (100%)</td>
<td>1.04</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IgG2 ACPA-positive</td>
<td>7 (32%)</td>
<td>31 (60%)</td>
<td>1.88</td>
<td>0.59</td>
<td>3.16</td>
<td>0.03</td>
<td>5.81 (1.5-22.8)</td>
<td>0.01</td>
</tr>
<tr>
<td>IgG3 ACPA-positive</td>
<td>13 (59%)</td>
<td>40 (77%)</td>
<td>1.31</td>
<td>0.56</td>
<td>2.31</td>
<td>0.13</td>
<td>3.48 (0.9-13.3)</td>
<td>0.07</td>
</tr>
<tr>
<td>IgG4 ACPA-positive</td>
<td>17 (77%)</td>
<td>44 (85%)</td>
<td>1.10</td>
<td>0.65</td>
<td>1.62</td>
<td>0.45</td>
<td>1.62 (0.4-6.5)</td>
<td>0.49</td>
</tr>
<tr>
<td>Number of ACPA isotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(median, IQR)</td>
<td>3.0 (2.0-5.3)</td>
<td>5.0 (3.3-6.0)</td>
<td>-</td>
<td>-</td>
<td>1.41</td>
<td>0.02</td>
<td>1.62 (1.1-2.4)</td>
<td>0.01</td>
</tr>
<tr>
<td>5 or more ACPA isotypes</td>
<td>6 (27%)</td>
<td>29 (56%)</td>
<td>2.07</td>
<td>0.60</td>
<td>3.36</td>
<td>0.03</td>
<td>6.76 (1.6-28.7)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*Patients with a change in SHS greater than or equal to the median were classified as having major progression, while the other patients were classified as having minor progression.
** multivariate logistic regression with correction for age, gender, CRP, IgM RF and SHS at baseline.
# logistic regression analysis could not be performed to investigate the effect of IgG1 ACPA, because 100% of patients in the major progression group were positive for this isotype.
Printed in bold are p-values < 0.05.
are not interchangeable, and that determination of ACPA isotypes may supply additional information to the total ACPA IgG level.

In summary, these results demonstrate that in addition to the presence of ACPA, the constitution of the anti-citrullinated protein autoantibody response is significantly associated with the disease course of RA. Within ACPA-positive patients, an expanded ACPA isotype profile indicates a high risk for severe radiographic damage.

**DISCUSSION**

In this study, we investigated the relationship between the ACPA isotype distribution and the progression and severity of RA. A more extensive use of ACPA isotypes at baseline was associated with more radiographic damage in RA patients in both cohorts. However, disease development from UA to RA was not accompanied by an expansion of the ACPA isotype profile.
The continued presence of IgM ACPA in patients with longstanding RA is an especially noteworthy feature of the ACPA response. IgM antibodies have a half-life of approximately 1 day \(^{19}\) and since long-lived plasma cells producing IgM antibodies against T cell-dependent antigens have not been described, the presence of IgM most likely indicates that the ACPA response is continuously reactivated during the course of the disease.

In previous studies, patients who were positive for IgA ACPA were found to have higher disease activity scores (DAS), even after taking into account the higher total ACPA-levels in this group of patients \(^{13}\). We have shown here that the presence of IgA ACPA and other isotypes also affects radiographic progression. The measurement of one solitary ACPA isotype (e.g. IgA) in addition to the standard anti-CCP assay, may not have a very high diagnostic yield, in view of the relatively modest likelihood ratio statistics associated with the single isotypes. However, the risk of radiological damage increased with every additional isotype, demonstrating that the breadth of the isotype response (i.e. the total number of isotypes present) may be of particular importance.

Previous reports have revealed that ACPA can be detected years before disease manifestation \(^{20-22}\) and that they tend to persist in the vast majority of patients in whom they have developed \(^{23}\). The fact that most patients are ACPA-positive for a certain period of time before they develop disease, indicates that not all ACPA are pathogenic. A potential underlying mechanism could be that the anti-citrullinated protein immune response may need to fully evolve and develop certain characteristics which then enable ACPA to cause tissue damage. Possibly, isotype switching and a resulting larger number of antibody isotypes, may be one of the features required for autoantibodies to contribute to disease pathogenesis.

The current study does not allow us to draw any conclusions about the events taking place before disease onset, when isotype switching could perhaps be involved in disease development. We therefore decided to study isotype switching in a very early phase after the onset of arthritis: during disease progression from UA to RA. Although UA-RA patients have been shown to have more ACPA isotypes at baseline than UA-nonRA patients\(^{15}\), the size of the ACPA isotype profile did not increase during the first year of follow-up, despite the development of RA in these patients during that period. Previous studies examining the ACPA response at baseline and follow-up in RA patients without overt disease progression, have reported that anti-CCP2-titers and levels of ACPA isotypes can even decrease with time \(^{14, 23}\). A possible explanation for these observations could be the effect of immunosuppressive treatment. The current first-line treatment for patients with RA and patients with severe, progressive UA \(^{24}\) consists of disease-modifying antirheumatic drugs (DMARDs), which all have more or less immunosuppressive functions. This makes it difficult to discern if the previously reported decrease in ACPA isotype levels is due to the natural course of the immune response or to the influence of medication.
Another possible reason why disease progression from UA to RA is not accompanied by a further expansion of the ACPA isotype use, could be that the clinical phenotype may lag behind the features of the autoantibody response. Analogous to observations in systemic lupus erythematosus (SLE), where a rise in anti-dsDNA antibodies is followed by a disease flare \(^{25, 26}\), the extended isotype distribution of UA-RA patients at baseline may be reflected by the disease course throughout the first year of the disease. The elucidation of the precise temporal relationship between changes in ACPA characteristics and disease phenotype will require further comprehensive follow-up studies.

The presence of 5 or more ACPA isotypes was significantly associated with higher levels of total IgG anti-CCP2 (p<0.01) which is known to be associated with radiographic progression \(^1\). Nonetheless, when patients were matched for anti-CCP2-levels, the presence of 5 or more ACPA isotypes was still associated with more radiographic progression, indicating that the presence of ACPA isotypes supplies additional information compared to the total anti-CCP2 IgG level. However, the number of matched patient pairs in which the independent effect of ACPA isotypes could be determined was limited, and more data will be required to validate these observations.

These findings are particularly important for our comprehension of the development of the ACPA response. As the anti-citrullinated protein immune response evolves and matures, it is conceivable that all features of the ACPA response, including the antibody level and the isotype use, will expand. Despite the fact that both total ACPA IgG level and the breadth of the ACPA isotype profile may, in this respect, be reflections of the same underlying immunological process, they can independently affect disease outcome. The association of radiographic outcome with these markers of ACPA maturity supports the theory that within ACPA-positive patients, the evolution of the ACPA response may be of pathophysiological importance.

A possible pathway by which differences in the ACPA isotype distribution may influence disease severity is through selective activation of the complement system. ACPA can activate the complement system in vitro and it is conceivable that a more diverse isotype profile would lead to an increase in complement activation. Furthermore, the differential effects of ACPA isotypes on disease severity may also be due to the recruitment of other effector mechanisms such as binding to Fc receptors. Ligation of Fc receptors by in vitro generated fibrinogen-ACPA immune complexes has been shown to result in cell activation and cytokine secretion \(^{27}\), both of which are of prime importance for tissue damage in RA.

The hypothesis that the nature and characteristics of the ACPA response may determine disease development and progression can certainly not be proven by the current study which was designed to investigate associations. Nonetheless, the present findings are consistent with this hypothesis and add to a growing body of evidence for the role of ACPA in the pathogenesis of the disease.
REFERENCES


