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Chapter 12

Genetic factors for the severity of ACPA-negative Rheumatoid Arthritis in two cohorts of early disease: a genome-wide study


Submitted
ABSTRACT

Objective
ACPA-negative and ACPA-positive Rheumatoid Arthritis (RA) are increasingly regarded as separate clinical entities. Although ACPA-negative patients have a less severe disease course at group level, considerable inter-individual differences in the amount of joint destruction occur. As no studies focusing on genetic risk factors underlying the differences in joint destruction in ACPA-negative patients have been performed thus far, we performed the present study.

Methods
A Genome-Wide Association Study was performed using Illumina Human CytoSNP-12v2 in relation to radiographic joint destruction in 276 ACPA-negative early RA-patients included in the Leiden Early Arthritis Clinic (EAC). According to the Bonferroni correction on the number of tested SNPs, the threshold for genome wide significance was \( p < 2 \times 10^{-7} \). Subsequently, the significant SNPs were evaluated for association with the progression of radiographic joint destruction in 253 ACPA-negative early RA-patients included in the BARFOT-study. As 11 uncorrelated SNPs were tested, the Bonferroni threshold for significance was 0.0045. In all patients, joint destruction was measured by Sharp-van der Heijde Score with good reproducibility.

Results
33 SNPs associated significantly to the severity of joint damage \( (p < 2 \times 10^{-7}) \) in phase-1. In phase-2, two SNPs showed a trend towards a significant association with joint damage, rs2833522 \( (p=0.0049) \) and rs17763915 \( (p=0.047) \). A combined analysis of both the Leiden and BARFOT datasets of rs2833522 showed a highly significant association with joint destruction \( (p=3.57 \times 10^{-9}) \), the presence of the minor allele associated with more severe damage.

Conclusion
Rs2833522 might be associated with the severity of joint damage in ACPA-negative RA. Larger, longitudinal, studies are needed for confirmation.
INTRODUCTION

Rheumatoid Arthritis (RA) is diagnosed by clinical characteristics and not based on pathophysiological processes. Nonetheless as recent studies have found differences in genetic susceptibility factors, histopathology and outcome of anti citrullinated peptide antibodies (ACPA)-positive and ACPA-negative RA, RA is considered to consist of two sub-entities. One subset is characterized by presence of these auto-antibodies and another subset is characterized by the absence of ACPA.\textsuperscript{1-3} On the group-level ACPA-negative RA patients have a less severe disease course with less joint damage than ACPA-positive patients. On the individual level however, disease severity is variable and severe joint destruction also occurs in auto-antibody negative patients (this is illustrated in Figure 1). The number of studies addressing risk factors for joint damage progression in the total group of RA-patients is relatively small, and joint damage progression in ACPA-negative RA has less frequently been explored as the majority of cohorts included predominantly ACPA-positive RA patients.

The heritability of joint damage in the total population of RA-patients is estimated to be 45-58%.\textsuperscript{4} Whether this is different for ACPA-positive and ACPA-negative RA is unknown. Joint destruction in RA is considered to be the net result of the influence of inflammation on bone. Hypothetically, several processes (for instance processes that determine the sensitivity of bone and cartilage to get destructed in response to inflammatory stimuli) may be similar in ACPA-positive and ACPA-negative RA, whereas other (immunological and/or inflammatory) pathways may be differently regulated in ACPA-positive and ACPA-negative RA and affect the severity of joint damage. Under the assumption that this notion is correct, risk factors for progression of joint destruction may in part be shared between these subsets of RA and in part be subset specific.

\begin{figure}[h]
\centering
\subfloat[ACPA-]{
\includegraphics[width=0.4\textwidth]{ACPA-}
}\hspace{1cm}
\subfloat[ACPA+]{
\includegraphics[width=0.4\textwidth]{ACPA+}
}
\caption{SHS scores after 5 years of follow-up in ACPA- and ACPA+ patients included in Leiden EAC Depicted are the first, second, third and fourth quartiles of patients and their SHS score after 5 years of follow-up.}
\end{figure}
We aimed to identify genetic risk factors for the severity of joint damage in ACPA-negative RA using a genome wide approach. Two cohorts of ACPA-negative early RA patients who had serial radiographs over time and were recruited in eras when treatment strategies were milder than today and when biological therapy was uncommon were evaluated.

**PATIENTS AND METHODS**

Patients

A two-staged study was performed. In both phases RA was defined according to the 1987-American College of Rheumatology (ACR) criteria for RA. In all patients, written informed consent had been obtained and the medical ethical committee’s of the participating centers had approved the study.

In the first phase, 276 early ACPA-negative RA-patients who were included in the Leiden-EAC between 1993 and 2006 were studied. Anti-CCP2 antibodies were measured in stored sera that were collected at the first visit using enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands). Samples with a value below 25 units/ml were considered negative according to the manufacturer’s instructions. Radiographs of the hands and feet were taken at baseline and yearly thereafter during 7 years of follow-up. (in total 1,266 sets of hands and feet radiographs). Radiographs were chronologically scored by one reader using the Sharp-van der Heijde Score (SHS) with good ICC (Intra-class observer Correlation Coefficient). Treatment strategies were different in three treatment-periods (1993-1995, initial treatment with non-steroid anti-inflammatory drugs (NSAIDs), 1996-1998, initial treatment with chloroquine or sulfasalazine and 1999-2006, prompt treated with methotrexate or sulfasalazine) as described in more detail previously.

The second phase concerned 253 ACPA-negative RA-patients included in the Better Anti-Rheumatic Farmaco Therapy (BARFOT)-cohort, a Swedish multicentre observational study of patients with early (disease duration ≤1 year) RA. Clinical, laboratory and radiological assessments were performed at inclusion and after 1, 2 and 5 years of follow-up. Hands and feet radiographs (total number 842) were scored according to the SHS score by 2 readers with good ICCs. Average scores of the readers have been used. During follow-up 50 patients participated in a 2-year randomized study on low dose prednisolone as an addition to Disease Modifying Anti-Rheumatic Drug (DMARD)-therapy.

Baseline characteristics of the ACPA-negative study populations are shown in Table 1.
In phase 1, genotyping was done using Illumina Human CytoSNP-12v2. Results of 244,655 SNPs were obtained. Of these all call rates were >97% and no SNPs were excluded due to a low call rate. 31 SNPs were excluded from the analyses because they were not in Hardy-Weinberg equilibrium (threshold $10^{-4}$), leaving 244,624 SNPs to be analyzed for an association with the progression of joint destruction. DNA on 276 patients was used for genotyping; 2 patients were excluded because of failed genotyping, 1 patient due to high homozygosity and 11 patients were excluded as relatives (based on Identity By State (IBS) analysis). Thus, in total 244,624 SNPs were studied in 262 ACPA-negative patients.

In phase 2, SNPs that showed a significant association in the first phase and with MAF>0.1 ($n=18$) were genotyped using Sequenom iPLEX. Apart for rs4926674, for which genotyping failed, success rates were all >98.5% and error rates 0% (based on the samples typed in duplo). No patients were excluded from the analyses.

**Statistical methods**

In phase 1, associations with the SNPs with MAF >0.05 and radiographic joint destruction were studied based on a linear mixed effects model for the longitudinal log-transformed SHS data. Such a model can accommodate the within patient correlations induced by the repeated radiological measurements and allows studying effects on disease progression. To model the evolution of joint destruction in time we have used natural cubic splines with 3 knots located at the sample quantiles of the visiting times. This method was used to model the non-linear progression of joint damage and the correlation between the X-ray scores adequately. Adjustments have been made for gender, age and inclusion period (as a proxy for differences in treatment) as described previously and SNP under an additive genetic model. It has been shown that in this cohort, time is an adequate proxy for different treatment regimes. For the random effects component, we have assumed random-intercepts and random-slopes modeled again using the splines effects. The effect of each
SNP on the disease progression has been tested using the likelihood ratio test, which is assumed to follow under the null hypothesis the chi-squared distribution with 4 degrees of freedom. Thereby we can investigate whether the patients' profiles for patients carrying different genotypes were different. The normality assumption for the linear mixed effects model on the log-transformed SHS scores is validated using the normal quantile-quantile plot of the marginal residuals, which does not show any serious deviation from the normal distribution (Figure 2). Since analyses were done on 244,624 SNPs, the threshold for significance was set at \( p < 2 \times 10^{-7} \).

In phase 2, a similar model as described in phase 1 was used to study an association with joint destruction. Also here, analyses were corrected for age, gender and treatment differences (participation in the corticosteroids trial). The Bonferroni correction for the number of uncorrelated SNPs (\( R^2 = 0.8, n = 11 \)) was used; the threshold for significance was 0.0045.

The results obtained in the two cohorts for rs2833522, the strongest associating SNP, were combined into one analysis. This combined analysis was performed by combining the

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**Figure 2. Normal Quantile-quantile plot of the marginal residuals based on the fitted linear mixed effects model**

The normal Quantile-quantile plot for the marginal residuals obtained by the linear mixed effects model on the log-transformed radiographic joint destruction does not show any severe deviations from the normal distribution. This is based on the data of phase-1.
data of the two cohorts and correcting for participating in either the Swedish or the Dutch cohort in a statistical analysis similar to the one described in phase-1 and 2, so a linear mixed effects model was applied on all data. This combined analysis bears similarities with the fixed effects meta-analysis in the sense that both studies are estimating the same effect size, and the contribution of each study on the estimation of the combined effect is determined by the amount of information available by each one.

All analyses were done using the R statistical software package.\textsuperscript{10}

\section*{RESULTS}

Phase-1

In total 244,624 SNPs were studied in 262 ACPA-negative patients in relation to the radiological severity of joint destruction over 7 years of follow-up. A Q-Q plot for observed versus expected values of the likelihood ratio test statistic under a chi-squared distribution with 4 degrees of freedom is shown in Figure 2. The lambda score for genomic control was 0.994. The results are depicted in the Manhattan plot (Figure 3). 33 SNPs related significantly (p<2x10\textsuperscript{-7}) to the progression of joint destruction (Table 2).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{manhattan_plot.png}
\caption{Manhattan plot of all p-values obtained in phase-1}
\end{figure}
Table 2. SNPs that passed the significance threshold of 2x10^-7 in phase-1 and the p-value obtained in phase-2

<table>
<thead>
<tr>
<th>Chrom</th>
<th>SNP</th>
<th>MAF</th>
<th>p phase-1</th>
<th>Gene</th>
<th>p phase-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>rs4926674</td>
<td>0.35</td>
<td>3.7x10^-8</td>
<td>USP24</td>
<td>na</td>
</tr>
<tr>
<td>2</td>
<td>rs7605224</td>
<td>0.15</td>
<td>8.7x10^-9</td>
<td>ITGA6</td>
<td>0.17</td>
</tr>
<tr>
<td>2</td>
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<td>4.6x10^-9</td>
<td>AF279775</td>
<td>0.26</td>
</tr>
<tr>
<td>2</td>
<td>rs11681769</td>
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<td>2.2x10^-8</td>
<td>AF279775</td>
<td>0.48</td>
</tr>
<tr>
<td>2</td>
<td>rs1316929</td>
<td>0.06</td>
<td>1.6x10^-7</td>
<td>GPC1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>rs9973786</td>
<td>0.12</td>
<td>1.5 x10^-7</td>
<td>GPC1</td>
<td>0.74</td>
</tr>
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<td>3</td>
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<td>1.0x10^-11</td>
<td>CBBL</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>rs11919628</td>
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<td>3.5 x10^-8</td>
<td>PVRL3</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>rs17763915</td>
<td>0.14</td>
<td>1.8 x10^-4</td>
<td>SLCA4A</td>
<td>0.05</td>
</tr>
<tr>
<td>4</td>
<td>rs316440</td>
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<td>1.2 x10^-7</td>
<td>ADAMTS3</td>
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</tr>
<tr>
<td>4</td>
<td>rs2726468</td>
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<td>1.9 x10^-4</td>
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<td>-</td>
</tr>
<tr>
<td>4</td>
<td>rs9307305</td>
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<td>2.6 x10^-7</td>
<td>PPA2</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>rs1388040</td>
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<td>5.0x10^-10</td>
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</tr>
<tr>
<td>5</td>
<td>rs161034</td>
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<td>PPP2R2B</td>
<td>0.49</td>
</tr>
<tr>
<td>6</td>
<td>rs17654008</td>
<td>0.05</td>
<td>3.1 x10^-4</td>
<td>AK098665</td>
<td>-</td>
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<tr>
<td>6</td>
<td>rs6931103</td>
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<td>8.7 x10^-4</td>
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<td>0.23</td>
<td>3.1 x10^-4</td>
<td>ARID1B</td>
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</tr>
<tr>
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<td>7</td>
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<td>1.6 x10^-7</td>
<td>Cullin 1</td>
<td>-</td>
</tr>
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<td>1.6 x10^-7</td>
<td>Cullin 1</td>
<td>-</td>
</tr>
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<td>8</td>
<td>rs16906415</td>
<td>0.10</td>
<td>1.6 x10^-4</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>rs1418247</td>
<td>0.44</td>
<td>3.4 x10^-4</td>
<td>GRIN3A</td>
<td>0.24</td>
</tr>
<tr>
<td>10</td>
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<td>4.6x10^-9</td>
<td>CDH23</td>
<td>0.53</td>
</tr>
<tr>
<td>12</td>
<td>rs11044895</td>
<td>0.10</td>
<td>1.4 x10^-4</td>
<td>AEBP2</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>rs2028809</td>
<td>0.20</td>
<td>1.5 x10^-9</td>
<td>C13orf31</td>
<td>0.51</td>
</tr>
<tr>
<td>14</td>
<td>rs28840384</td>
<td>0.15</td>
<td>2.0 x10^-7</td>
<td>AX746996</td>
<td>na</td>
</tr>
<tr>
<td>15</td>
<td>rs4646644</td>
<td>0.09</td>
<td>6.6 x10^-4</td>
<td>ALDH1A2</td>
<td>-</td>
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<tr>
<td>15</td>
<td>rs1834210</td>
<td>0.09</td>
<td>1.1 x10^-7</td>
<td>AK057337</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
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<td>0.10</td>
<td>7.8 x10^-4</td>
<td>AK057337</td>
<td>-</td>
</tr>
<tr>
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<td>4.4 x10^-4</td>
<td>ERCC4</td>
<td>0.45</td>
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<tr>
<td>16</td>
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<td>2.0 x10^-10</td>
<td>KIFC3</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>rs16960143</td>
<td>0.09</td>
<td>2.6 x10^-4</td>
<td>GINS3</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>rs268909</td>
<td>0.12</td>
<td>1.2 x10^-4</td>
<td>KLK5</td>
<td>0.84</td>
</tr>
<tr>
<td>21</td>
<td>rs2833522</td>
<td>0.37</td>
<td>1.7 x10^-7</td>
<td>SFRS15 – HUNK</td>
<td>0.01</td>
</tr>
</tbody>
</table>

In phase 2, data for rs4926674 and rs28840384 were not available (na) because the assay for this SNP failed. SNPs with MAF<0.1 were not tested in phase 2, therefore, no p-value is reported for these SNPs.

Phase-2

From the 33 variants identified in phase 1, only the SNPs that had a MAF of >0.1 (n=18) were studied for an association with joint destruction in ACPA-negative patients included in the BARFOT-cohort. Here, two SNPs, rs2833522 (p=0.0049) and rs17763915
(p=0.047) associated with the progression of joint destruction. However, none of these passed the Bonferroni threshold for multiple testing, though the p-value of rs2833522 was just above this threshold. The joint destruction scores of the ACPA-negative Leiden and BARFOT-patients having a minor, heterozygous or major genotype of this SNP are depicted in Figure 4A and 4C respectively. The fitted profiles by the model of both cohorts are presented as well (Figure 4B and 4D). As shown, in both cohorts presence of the minor allele was associated with more severe damage progression.

Figure 4. Progression of joint damage for the ACPA-negative RA patients carrying the homozygous major, heterozygous and homozygous minor genotype of rs2833522
Portrayed are the sample mean SHS-scores, thus the raw SHS data on the normal scale (A, Leiden EAC C, BARFOT), the mean log-transformed SHS as modeled by the linear mixed model (B, Leiden EAC, D, BARFOT) and the mean log-transformed SHS as modeled by combined analysis of both cohorts (E).
Combined analysis

Finally the data of rs2833522 of both cohorts were analyzed in a combined analysis. This showed a significant association ($p=3.57 \times 10^{-9}$); the fitted profiles are depicted in Figure 4E.

DISCUSSION

Several successful Genome-Wide Association studies (GWAs) on genetic risk factors for RA-development have been performed.\textsuperscript{11,12} The majority of these studies included ACPA-positive RA-patients. Hence the majority of identified genetic factors predispose to both ACPA-positive and negative RA or ACPA-positive RA only. A recent study using the Immunochip custom SNP array on almost 12,000 RA patients and 16,000 controls performed stratified analyses in ACPA-positive and ACPA-negative patients separately. Two variants predisposed to ACPA-negative RA with a genome-wide significant p-value: rs4143322 in the HLA region and rs71624119 in \textit{ANKRD55}.\textsuperscript{13} No GWAs has thus far been published on ACPA-negative RA and disease severity. One possible reason for a lack of genetic studies in ACPA-negative RA patients is the fear of phenotypic misclassification, as ACPA-negative RA is sometimes thought to be heterogeneous and containing patients with unrecognized diseases other than RA.\textsuperscript{14,15} A recent study attempted to address this issue and evaluated a broad range of characteristics (clinical, serological and radiological), both at first presentation and during the course of the disease, using different variable reduction techniques. Based on the characteristics studied no subgroups of patients could be discerned\textsuperscript{16} and it was concluded that, for risk-factor studies, ACPA-negative RA patients can be studied as one group. Although no phenotypic subgroups could be discerned, not all ACPA-negative RA patients are alike; some patients develop no or mild erosions, whereas others have a severe destructive disease course, which is illustrated by Figure 1. Studying genetic factors underlying these differences in joint destruction in ACPA-negative RA is important as it may lead to new insights into the pathophysiology of the progression of radiographic joint destruction in this disease subset. We therefore performed the first GWAs on the severity of joint destruction over time in ACPA-negative RA.

The heritability of the severity of joint destruction in the total RA population is estimated at 58%.\textsuperscript{14} Although the heritability in ACPA-negative RA is not known, we assumed that also here genetic factors play a role. In our two-staged study, 33 SNPs passed the threshold of genome-wide significance in phase-1 and two of these were associated with the severity of joint damage in phase-2 at 5% significance level. After the rather conservative Bonferroni correction for multiple testing in phase-2, rs2833522 showed a trend towards statistical significance ($p=0.0049$, whereas Bonferroni threshold for significance is $p=0.0045$).
In both cohorts presence of the minor allele of rs2833522 was associated with a higher rate of joint damage progression. Rs2833522 is located on chromosome 21, between \textit{SFRS15} and \textit{HUNK}. As thus far little is known about the functions of these genes, it is difficult to speculate about the potential pathophysiologic effect of this SNP. In addition, since we did not perform fine-mapping of the region, it is likely that another SNP in this locus is the actual genetic variant associated with the progression of joint damage in ACPA-negative RA.

A proportion of ACPA-negative patients was rheumatoid factor-positive (24%). A stratified analysis of rs2833522 in ACPA-negative RA-patients positive and negative for rheumatoid factor yielded significant results in both groups (p=0.015 and p=0.011 respectively), indicating that the association observed was not driven by the presence of rheumatoid factor in part of the patients.

In phase-2 a significant association was observed also for rs17763915, which is located 257821 kb from the \textit{SLC4A4} gene, encoding for a sodium bicarbonate cotransporter. To our knowledge, this gene has thus far not been related to RA or other, auto-immune or anti-inflammatory disorders. Since this association disappeared after correction for multiple testing, we cannot make a definite conclusion on the value of this SNP for joint damage severity.

A genome wide approach is focused on identifying true positive risk factors and false negative results are inherent to the study design. In our case, the risk of false negatives is of special importance. The Bonferroni correction for multiple testing applied in phase-1 is rather conservative, especially because we corrected for the total number of SNPs and not the number of uncorrelated SNPs; this choice may have introduced false negatives. Furthermore, the array that was used in phase-1 covered relatively few variants, making it likely that also for this reason not all genetic variants associating with joint damage severity in ACPA-negative RA were found. Nonetheless, the choice of the array does not affect the validity of the positive results obtained in this study.

Furthermore several issues may have affected the power of the present study to find statistically significant associations. The numbers of patients and radiographs studied were relatively small. Additionally, the severity of joint damage was relatively mild in a large number of ACPA-negative RA-patients. The differences in progression rates between genotype groups are therefore lower than those observed in total population of RA-patients or the ACPA-positive RA patients, making it more difficult to find significant associations. A limited power may be an explanation for the fact that we could not achieve independent replication of our results.
Our study also has several strengths. The two observational cohorts studied had many similarities in design and populations, they consisted both of adult, western European early RA-patients and in both cohorts treatment was much less intense than today. Although the number of ACPA-negative patients was relatively small, the patients in both cohorts were radiographed serially in time. As shown recently, the presence of repeated measurements yields more precise estimations of the radiographic progression rate and a considerable increased power. Our study did not include a third phase as we did not identify a longitudinal observational cohort with ACPA-negative RA patients including an equal or larger amount of radiographs compared to the number of radiographs studied in phases-1 and -2. Cohorts of ACPA-negative RA-patients with repeated radiological measurements available are extremely rare.

The statistical model used in this study took advantage of the within patient correlations present in repeated radiological measurements and combined all radiological data in one test. This model was chosen because, especially in ACPA-negative patients, the rate of joint destruction is not homogenous through the whole follow-up period and differences between the patients’ joint damage progression profiles are present. We decided to adopt the splines functions into the linear mixed effects model used for our analyses. This allows more precise modeling the progression of joint damage over the total follow-up period. Despite the advantage of being able to flexibly model the non-linear profiles in time, a disadvantage of the chosen model is that we cannot quantify the SNP effect over the whole follow-up period using an overall effect size. Therefore, to visualize the SNP effects on the progression of joint destruction, we presented the raw SHS data and the fitted marginal profiles in time per genotype category (see Figure 4).

In phase-1 of the study, an interesting association was found for rs7778273 and rs11979066, which are both located on the *Cullin 1* gene. Cullin 1 has been thought to influence RA by altering lymphocyte signal transduction. However, due to the chosen selection criterion for genotyping in stage-2 of a MAF >0.1, this SNP (MAF 0.06) was not proceeded to phase-2. Whether this variant comprised a false negative result remains undetermined.

Eyre et al. recently described that rs414332 in the HLA region and rs71624119 in *ANKRD55* are associated with the development of ACPA-negative RA. These variants were not in the list of variants identified in phase 1 of this study. This might indicate that the genetic variants, and hence the underlying pathophysiological processes, of developing ACPA-negative RA and progression of ACPA-negative RA are different.

In conclusion, we found that rs2833522 might be associated with the severity of joint damage in ACPA-negative RA. Larger, longitudinal studies are needed for confirmation.
and subsequent functional studies are required to elucidate the processes relevant for joint
destruction in RA that are influenced by this genetic variant.

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