

EXTENDED REPORT

Identification of a genetic variant for joint damage progression in autoantibody-positive rheumatoid arthritis

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ABSTRACT

Background Joint destruction is a hallmark of autoantibody-positive rheumatoid arthritis (RA), though the severity is highly variable between patients. The processes underlying these interindividual differences are incompletely understood.

Methods We performed a genome-wide association study on the radiological progression rate in 384 autoantibody-positive patients with RA. In stage-II 1557 X-rays of 301 Dutch autoantibody-positive patients with RA were studied and in stage-III 861 X-rays of 742 North American autoantibody-positive patients with RA. Sperm-Associated Antigen 16 (*SPAG16*) expression in RA synovium and fibroblast-like synoviocytes (FLS) was examined using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) and immunohistochemistry. FLS secrete metalloproteinases that degrade cartilage and bone. *SPAG16* genotypes were related to matrix metalloproteinase (MMP)-3 and MMP-1 expression by FLS in vitro and MMP-3 production ex vivo.

Results A cluster of single nucleotide polymorphisms (SNPs) at 2q34, located at *SPAG16*, associated with the radiological progression rate; rs7607479 reached genome-wide significance. A protective role of rs7607479 was replicated in European and North American patients with RA. Per minor allele, patients had a 0.78-fold (95% CI 0.67 to 0.91) progression rate over 7 years. mRNA and protein expression of *SPAG16* in RA synovium and FLS was verified. FLS carrying the minor allele secreted less MMP-3 ($p=1.60\times 10^{-2}$). Furthermore, patients with RA carrying the minor allele had lower serum levels of MMP-3 ($p=4.28\times 10^{-2}$). In a multivariate analysis on rs7607479 and MMP-3, only MMP-3 associated with progression ($p=2.77\times 10^{-4}$), suggesting that the association between *SPAG16*-rs7607479 and joint damage is mediated via an effect on MMP-3 secretion.

Conclusions Genetic and functional analyses indicate that *SPAG16* influences MMP-3 regulation and protects against joint destruction in autoantibody-positive RA. These findings could enhance risk stratification in autoantibody-positive RA.

Rheumatoid arthritis (RA) is characterised by persistent joint inflammation and joint destruction. The severity of radiographic destruction can be measured objectively and reflects the cumulative burden of inflammation. Up-to-date treatment strategies have improved the outcome of patients with RA, though the chronic and destructive character of the disease is not prevented. Anticitrullinated peptide antibody (ACPA)-positive patients with RA in particular are characterised by progressive joint destruction.¹ The severity is highly variable between ACPA-positive patients, some develop almost no joint destruction, whereas others have a severely progressive disease (see online supplementary figure 1). Current treatment is not individualised; we are neither able to identify the patients that will have the most severe disease course nor do we understand the processes underlying these interindividual differences. To improve this, risk factor studies are required.

A genome-wide association analysis (GWAs) is a hypothesis-free approach to identify common genetic risk factors. Performed GWAs in RA consisted of a case-control design, mainly yielding genetic variants involved in disease susceptibility. However, therapeutic strategies are targeted to affect pathways of disease progression. For drug discovery and to achieve personalised medicine, interrogation of the genome with disease progression is attractive. As it requires high-quality longitudinal outcome data of a considerable number of patients, such GWAs are scarce. We present the first GWAs on long-term disease progression. The notion that the severity of joint destruction in RA is influenced by genetic factors is supported by our previous work on twins and the Icelandic RA-population, estimating the heritability of joint destruction at 45–58%.^{2,3} We evaluated high-quality radiological data of cross-sectional and longitudinal studies (2812 X-rays in total) of 1427 ACPA-positive patients with RA.

METHODS

Patients

All patients with RA fulfilled the 1987-ACR criteria⁴ and were ACPA positive. Approval was given

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Basic and translational research

by the ethics committees of all cohorts. Written informed consent was obtained from all participants. First, 396 ACPA-positive patients with RA from the North American Rheumatoid Arthritis Consortium (only one of the sib-pair) with hand X-rays, information on disease onset and genome-wide single nucleotide polymorphism (SNP) data⁵ were assessed; after quality control 384 patients were studied (figure 1). These patients, diagnosed between 1953–2002, were treated conservatively; the mean follow-up was 13.9 ± 10.5 years. Stage-II included 301 ACPA-positive patients with RA included in the Leiden Early Arthritis Clinic between 1993–2006.⁶ Hands and feet X-rays were made with yearly intervals during 7 years of follow-up. Treatment regimes changed over time as described elsewhere.^{6–8} In short, patients diagnosed in 1993–1995 were initially treated with NSAIDs, patients diagnosed in 1996–1998 were mainly treated with hydroxychloroquine or sulphasalazine and patients included from 1999 onwards were treated with methotrexate. In stage-III, 742 North American and Canadian ACPA-positive patients with RA were studied. In the National Databank for Rheumatic diseases (NDB)⁹ 629 patients were included and 113 patients with RA in the Wichita cohort (Kansas, USA) in 1973–1993.¹⁰ The NDB-patients had one hand X-ray per person; the Wichita-patients had serial X-rays. Also

these patients were recruited and followed in eras when biological therapies were uncommon.

Radiological joint destruction

All X-rays were scored by trained readers at the Leiden University Medical Centre (JABvN in stage-I, MPMvdL in stage-II and DPCdR in stage-III) using the Sharp–van der Heijde method (SHS)¹¹ blinded to clinical and genetic data. Serial X-rays were scored chronologically. In every stage $\geq 10\%$ randomly selected X-rays were rescored. The intraobserver reliability (ICC) was 0.99, 0.91 and 0.98 in the three stages.

Genotyping

In stage-I GWAs genotyping was conducted with Illumina BeadChips (HumanHap 550 k).⁵ The dataset was filtered for SNPs on genotyping success rate $>98\%$, minor allele frequency >0.1 and Hardy-Weinberg equilibrium (HWE) $p < 1 \times 10^{-5}$. From chromosome 1–22, 391 733 SNPs passed quality control. Patients were excluded based on success rates $<10\%$ ($n=2$), evidence of identity-by-descent ($n=0$) or population substructures ($n=10$). Since after quality control the genomic inflation factor was 1.009 (see online supplementary figure 2), we did not further adjust for substructures. In total 384 patients were studied. In stage-II, a set of SNPs in *SPAG16* were chosen based on the strongest association in stage-1 ($p < 9.5 \times 10^{-5}$) and a pairwise $R^2 < 0.8$ were typed using Sequenom iPLEX. Doubtful calls were manually checked. Error and success rates (percentage of incorrect duplicates and of successful calls per SNP respectively) were $<1\%$ and $>97\%$. None of the SNPs was out of HWE ($p < 0.001$). In stage-III and functional studies, rs7607479 was typed using TaqMan platforms. The error rate and genotyping success rate were respectively $<1\%$ and $>98\%$.

RA synovial tissue and fibroblast-like synoviocytes

Presence of *SPAG16* protein in synovial tissue from ACPA-positive patients with RA that underwent joint replacement surgery (Hasselt University, Belgium) was studied using immunohistochemistry (see online supplementary methods). Fibroblast-like synoviocytes (FLS) were derived from synovial tissue specimens obtained from 26 patients with RA during joint replacement surgery (Department of Orthopaedic Surgery, Schulthess Clinic Zurich, Switzerland). Cells were cultured as described elsewhere¹² and used between passages 4–8 for all experiments.

To isolate DNA 5×10^4 cells were cultured in 6-well plates 24 h after splitting cells were lysed in tissue lysis buffer and DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen, Hombrechtikon, Switzerland).

Total RNA was isolated using the miRneasy Kit (Qiagen, Hombrechtikon, Switzerland) including on-column DNaseI (Qiagen) digest, reversed transcribed and used for relative quantification of mRNA expression using human glyceraldehyde-3-phosphate dehydrogenase for internal standard sample normalisation as described previously (see online supplementary table 1 for primer sequences).¹² Relative mRNA expression levels were calculated by the comparative threshold cycle method (ΔCt). The specificity of the *SPAG16* transcript was also confirmed by loading the Real-Time Quantitative Polymerase Chain Reaction-products on a 1.5% agarose gel.

Matrix metalloproteinase (MMP)-1 and MMP-3 were measured using the human total MMP-1 ELISA-kit and the human total MMP-3 ELISA-kit (BD Biosciences, Allschwil, Switzerland)

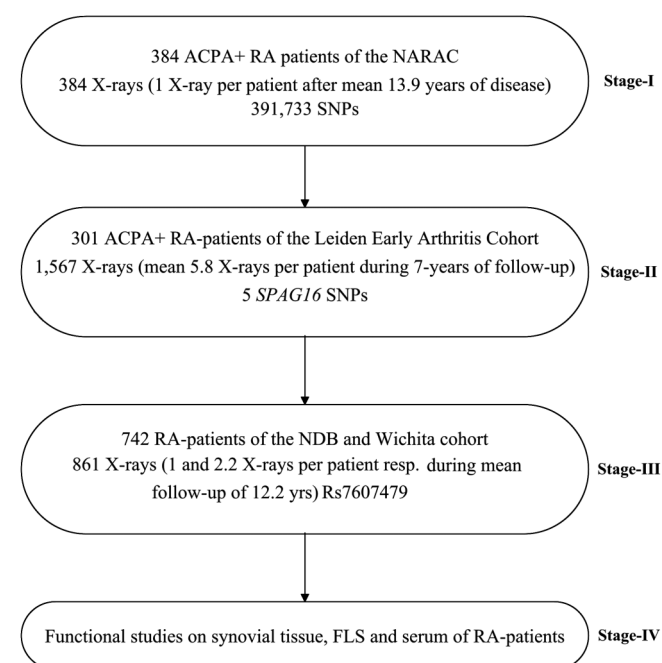


Figure 1 Study design. The mean ($\pm$ SD) ages at disease onset of the patients in the three stages were: 40.8 ± 11.9 years, 55.8 ± 14.8 years and 45.8 ± 13.3 years, respectively. The percentage of female patients was 73%, 86% and 76%, respectively. The years of diagnosis (min-max) of the patients were 1953–2002, 1993–2006 and 1963–1999, for the three stages respectively. The mean (min-max) follow-up durations were 13.9 (0.5–46) years, 5.8 (1–7) years and 12.2 (1–56) years. All patients were positive for the 1987-ACR criteria for rheumatoid arthritis (RA) and had ACPA. ACPA was measured on stored serum samples using second-generation ELISAs (anti-CCP2, Immunoscan RA Mark 2, Euro-Diagnostica, Arnhem, the Netherlands). The minor allele frequency of rs7607479 in the different cohorts were 0.36 (NARAC), 0.37 (Leiden Early Arthritis Clinic), 0.37 (NDB) and 0.35 (Wichita). ACPA, anticitrullinated peptide antibodies; FLS, fibroblast-like synoviocytes; NARAC, North-American Rheumatoid Arthritis Consortium; NDB, National Databank of rheumatic Diseases; RA, rheumatoid arthritis.

in cell culture supernatants from unstimulated cells according to the manufacturer's instructions.

Subsequently, the MMP-3 mRNA and protein expression studies were repeated in tumor necrosis factor α and interleukin (IL)-1 β stimulated FLS as described in the online supplementary methods.

Serum MMP-3 levels

Serum samples of patients with RA included in the replication cohort used in stage-II were studied. Samples were taken at mean disease duration of 4.2 ± 2.2 years (range 1–9 years) and before 2000, an era when the antirheumatic drugs were mild and tight control strategies not implemented. Standard sandwich ELISA for mature MMP-3 was performed (BD Biosciences).

Statistical analysis

In all genetic analyses an additive model (1-df. test) was used. Since SHS are non-normally distributed, all radiological data were log transformed before analyses. The residuals of the regression analysis were randomly distributed around the zero-line, indicating a good fit of the model. Obtained effect sizes were back transformed to the original scale, indicating how many fold the joint destruction increased per year in the presence of the minor allele compared with the reference common genotype. The difference in joint damage increases per follow-up year by the power of the years of follow-up.

For datasets with one X-ray per patient (North American Rheumatoid Arthritis Consortium and NDB), the estimated yearly progression rate was calculated (total SHS/disease years at time of X-ray).¹³ This method assumes a linear progression rate over time and the absence of joint damage at disease onset. Here a linear regression with $\log_e(\text{estimated progression}+1)$ as outcome variable performed. For the analyses in datasets with multiple measurements per patient (Leiden Early Arthritis Clinic and Wichita) a multivariate normal regression analysis was used to model $\log_e(\text{SHS}+1)$ over time.^{14 15} This method takes advantage of within-patient correlation between the measurements. All genetic analyses were adjusted for age and sex. In stage-I and stage-III no influence of treatment on joint destruction was observed. In stage-II an effect of treatment on the radiographic progression was observed and analyses were adjusted for the applied treatment strategy as described previously.⁸ An inverse variance weighting meta-analysis testing for random effects¹⁶ was done on the Wichita and NDB data and on stage-II and stage-III.

In stage-I, 545K SNPs were genotyped, after quality control 391.733 SNPs were analysed. For GWA-studies the family wise error rate depends on linkage disequilibrium, the SNP density, the chosen test statistic, the population and the sample size.¹⁷ Large sample sizes decrease the per test significance level, because increased sample sizes change the distribution of the test statistic at rare variants to allow smaller p values. Hoggart *et al* showed that for the European population, an additive model, 660K SNPs and a sample size of 100 cases and 100 controls the per test significance level is 2.9×10^{-7} , while for 1000 cases and 1000 controls the level decreases to 1.5×10^{-7} .¹⁷ Interpolating these results to a sample size of 400 subjects resulted in a threshold for genome-wide significance for the present study of $p \leq 2.7 \times 10^{-7}$, with $2.7 \times 10^{-7} < p < 9.5 \times 10^{-5}$ considered as highly suggestive. These cut-offs served to find variants of interest, which were proceeded to stage-II. In stage-II, stage-III and the functional analyses, p values < 0.05 were considered statistically significant.

The associations of SNPs with MMPs' expression and production were analysed using the non-parametric data for linear trend (1-df.) of Cuzick. Linear regression with $\log_e(\text{MMP-3})$ as outcome variable and SNP as test variable was used for analyses on serum levels. Similar as the Cuzick test a linear association was assumed (1-df. test). All analyses on the serum samples were corrected for sex, age, treatment and disease duration at time of serum collection. The relation between $\log_e(\text{MMP3})$ levels and progression of joint destruction in the next year ($\log_e(\text{SHS2-SHS1}+1)$) was analysed using linear regression.

PLINK V1.0.6, Stata V10.1 and SPSS V18.0 were used.

RESULTS

Genetic analyses

Evaluating joint damage progression in stage-I revealed the strongest association for a cluster of SNPs located at 2q34, in the region of Sperm-Associated AntiGen16 (*SPAG16*) (figure 2, see online supplementary table 2). Of these, rs7607479 reached the predefined level of genome-wide significance ($p = 1.59 \times 10^{-7}$). *SPAG16* is located 132 kb downstream of *IKFZ2* and there is no linkage disequilibrium (LD) ($R^2 < 0.05$) between rs7607479 and any of the variants in *IKFZ2*. Compared with the patients with the common genotype, patients with one minor rs7607479 allele had a 0.77-fold (95% CI 0.70 to 0.85) progression rate per year and patients carrying two minor alleles a 0.59-fold (95% CI 0.49 to 0.72) progression rate per year. The interindividual variance in joint destruction explained by rs7607479 was 6.6%.

In stage-II, all five tested *SPAG16* SNPs were significantly associated with the rate of joint destruction; rs7607479 had the strongest association (see online supplementary table 3). However, none remained significant when conditioned on rs7607479 (see online supplementary table 4), indicating that the five significant SNPs reflect one signal. Over the 7-years studied, patients with one rs7607479 minor allele had a 0.81-fold progression rate and patients with two minor alleles a 0.65-fold progression rate compared with patients with the common genotype (95% CIs 0.68 to 0.96 and 0.45–0.93, $p = 2.16 \times 10^{-2}$ figure 3A), indicating that 19% and 35% less joint destruction was observed.

In stage-III, again a protective effect of the rs7607479 minor allele on progression of joint destruction was observed ($p = 2.29 \times 10^{-2}$, 0.71-fold (95% CI 0.53 to 0.95) progression rate over 7 years in the presence of one minor allele, figure 3B).

Combining the results of rs7607479 on progression of joint destruction of stage-II and stage-III in a meta-analysis testing for random effects ($I^2 = 0\%$) demonstrated a 0.78-fold (95% CI 0.67 to 0.91) progression rate over 7 years per minor allele ($p = 1.65 \times 10^{-3}$, figure 3C). A meta-analysis on all three cohorts is presented in online supplementary figure 3.

SPAG16 expression in the joint

We identified an association with *SPAG16*, a region with a largely unknown function. *SPAG16* is expressed in sperm, testis,^{18–21} and in many other tissues like haematopoietic cells in the bone marrow, macrophages in the lung, glandular cells of the tractus digestivus and mammae (<http://www.proteinatlas.org>). Information on the expression in synovium was lacking. Several isoforms are described; isoforms 1 and 2 are the best validated. Considering how *SPAG16* could influence joint damage progression, we first evaluated whether it is expressed in the joint. Presence of *SPAG16* protein was observed in synovial tissue of patients with RA using immunohistochemical staining (figure 4). Fluorescent double staining showed *SPAG16*

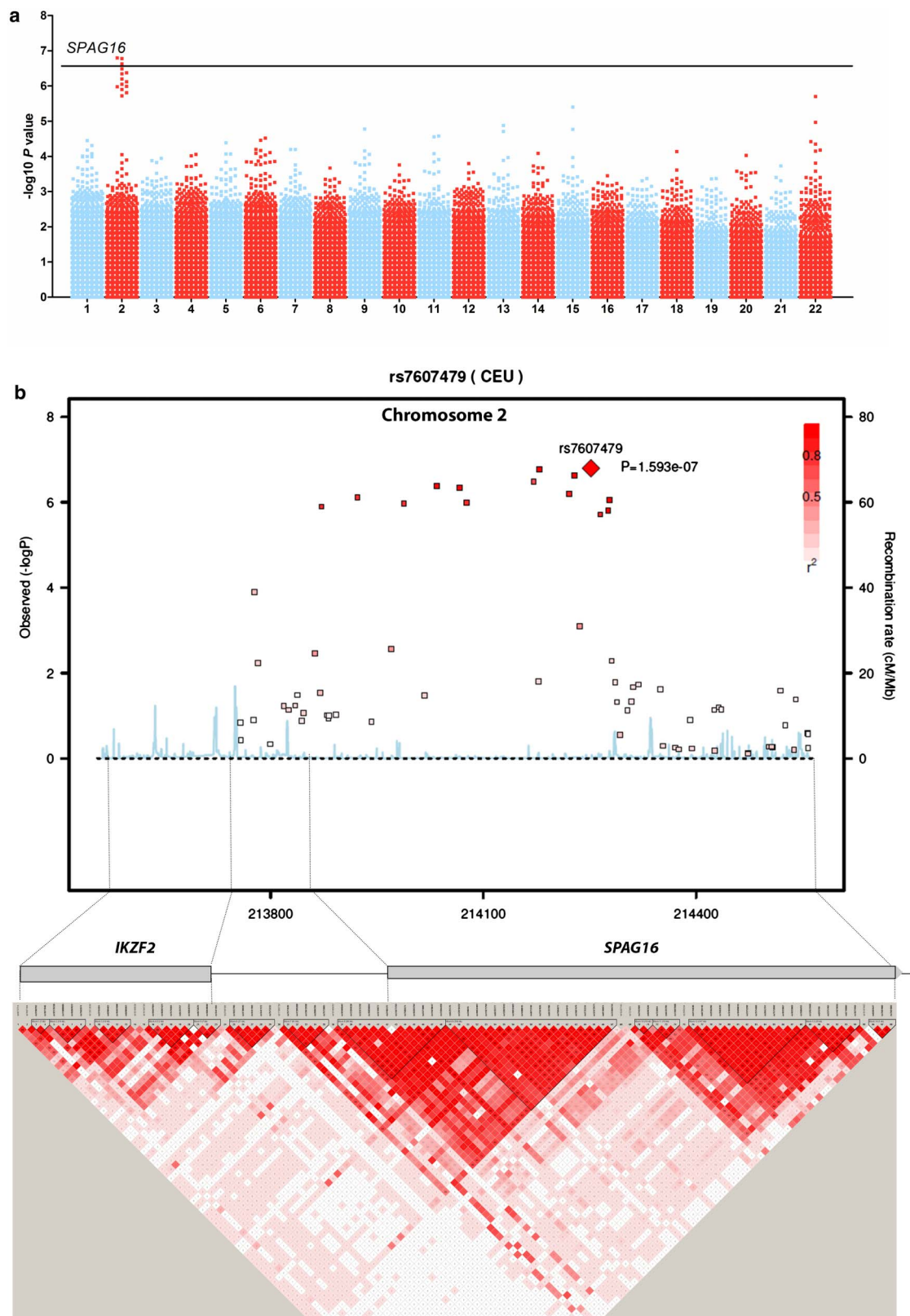


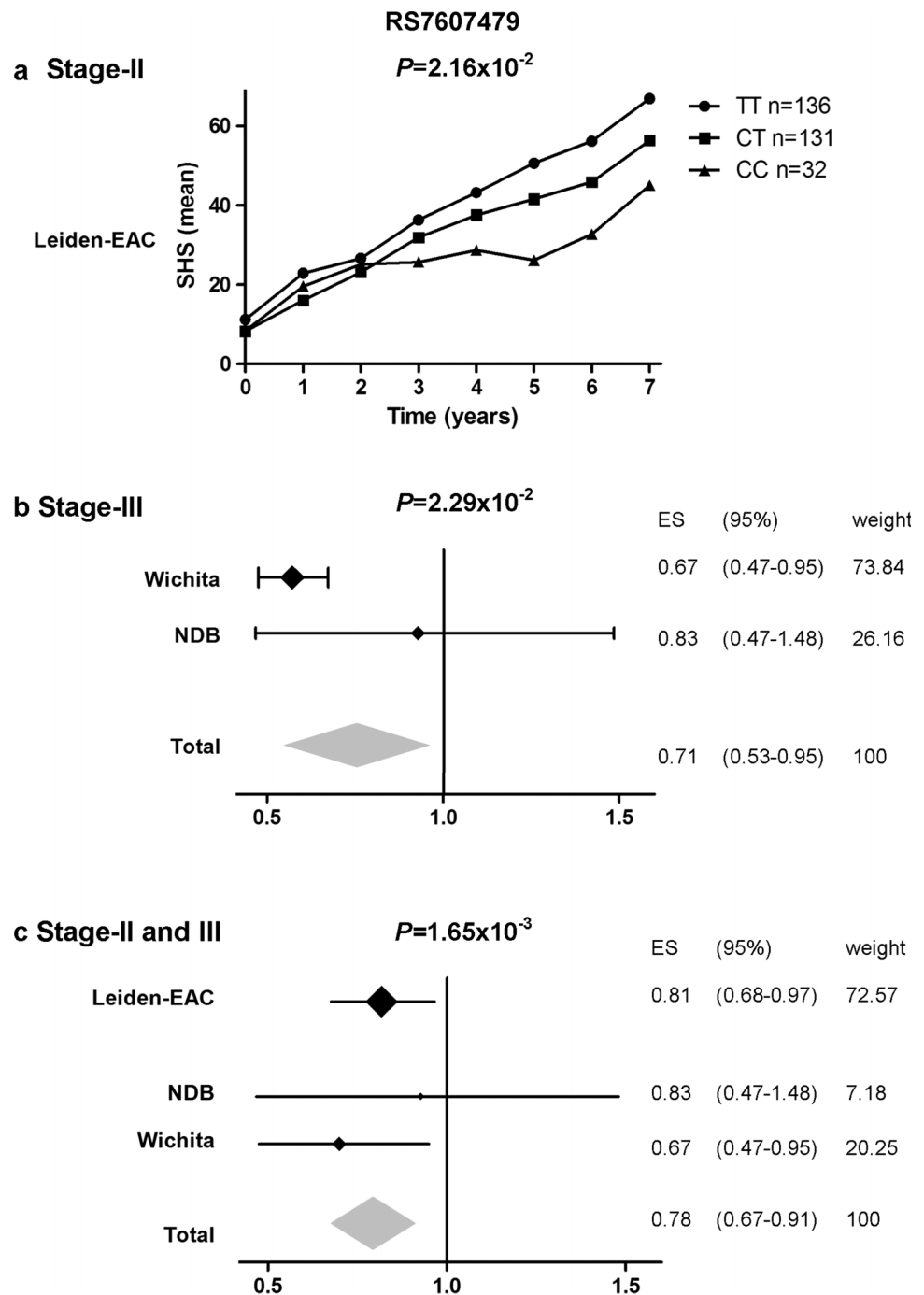
Figure 2 Results of the discovery stage. (A) Manhattan-plot showing the $-\log_{10}$ (p value) of the trend-test. p values are plotted against position on each chromosome. (B) Genomic structure (upper) and $-\log_{10}$ of SNPs in the GWAs around *SPAG16* and linkage disequilibrium map (lower) in NARAC. GWAs, genome-wide association analysis; NARAC, North-American Rheumatoid Arthritis Consortium.

expression in FLS. The presence of *SPAG16* transcripts was detected by Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) and subsequent agarose gel electrophoresis on cDNA derived from FLS of RA synovium (figure 5).

SPAG16 expression in vitro in relation to genotypes

FLSs are key effector cells in the RA synovium and contribute actively to joint destruction by the release of MMPs.^{22–24} MMP-3 and MMP-1 are involved in the breakdown of

Figure 3 Results of the two replication stages and a meta-analysis on stage-II and stage-III. (A) rs7607479 and radiological progression rates in stage-II where patients with RA had serial measurements over 7 years. (B) rs7607479 and radiological progression rates in stage-III where patients with RA from the NDB had single X-rays and from the Wichita cohort serial X-rays; data of both cohorts were combined in inverse variance weighted meta-analysis. (C) The data of both replication stages were summarised in an inverse variance weighted meta-analysis testing for random effects. The I^2 was 0%. ES=effect size. For reasons of comparability all data were depicted for progression over a similar disease period (7 years). An effect size <1 indicated a protective effect. NDB, National Databank of rheumatic Diseases; RA, rheumatoid arthritis.



extracellular matrix during tissue remodelling in normal physiological processes and disease processes, such as tumour metastasis and arthritis.²⁵ Rs7607479 is located at an intronic region of *SPAG16*. *SPAG16* expression did not correlate with rs7607479 genotypes (see online supplementary figure 4). The capacity of FLS to express or secrete MMPs can be measured in vitro and we studied this in relation to rs7607479 genotypes. In unstimulated FLS a tendency towards a lower mRNA expression of MMP-3 in FLS carrying the minor alleles of rs7607479 was observed. Furthermore, MMP-3 protein levels were significantly lower in supernatants of FLS carrying the minor allele compared with that of FLS with the common genotype ($p=1.60 \times 10^{-2}$, figure 6A). Since the fold induction after cytokine stimulation was similar between genotypes, these data show that even in

inflammatory conditions rs7607479 genotypes are predictive for MMP-3 expression levels (see online supplementary figure 5). Rs7607479 was not associated with MMP-1 expression or production.

SPAG16 expression ex vivo in relation to genotypes

Given these in vitro data, we studied whether *SPAG16*-rs7607479 was associated with MMP-3 production in vivo, reflected by the serum level. Patients with the minor rs7607479 allele had lower MMP-3 serum levels. For each minor allele of rs7607479 patients displayed 0.83-fold MMP-3 levels compared with patients with the common genotype (95% CI 0.70 to 0.996, $p=4.28 \times 10^{-2}$, figure 6B). Additionally, lower MMP-3 levels were associated with less radiological progression

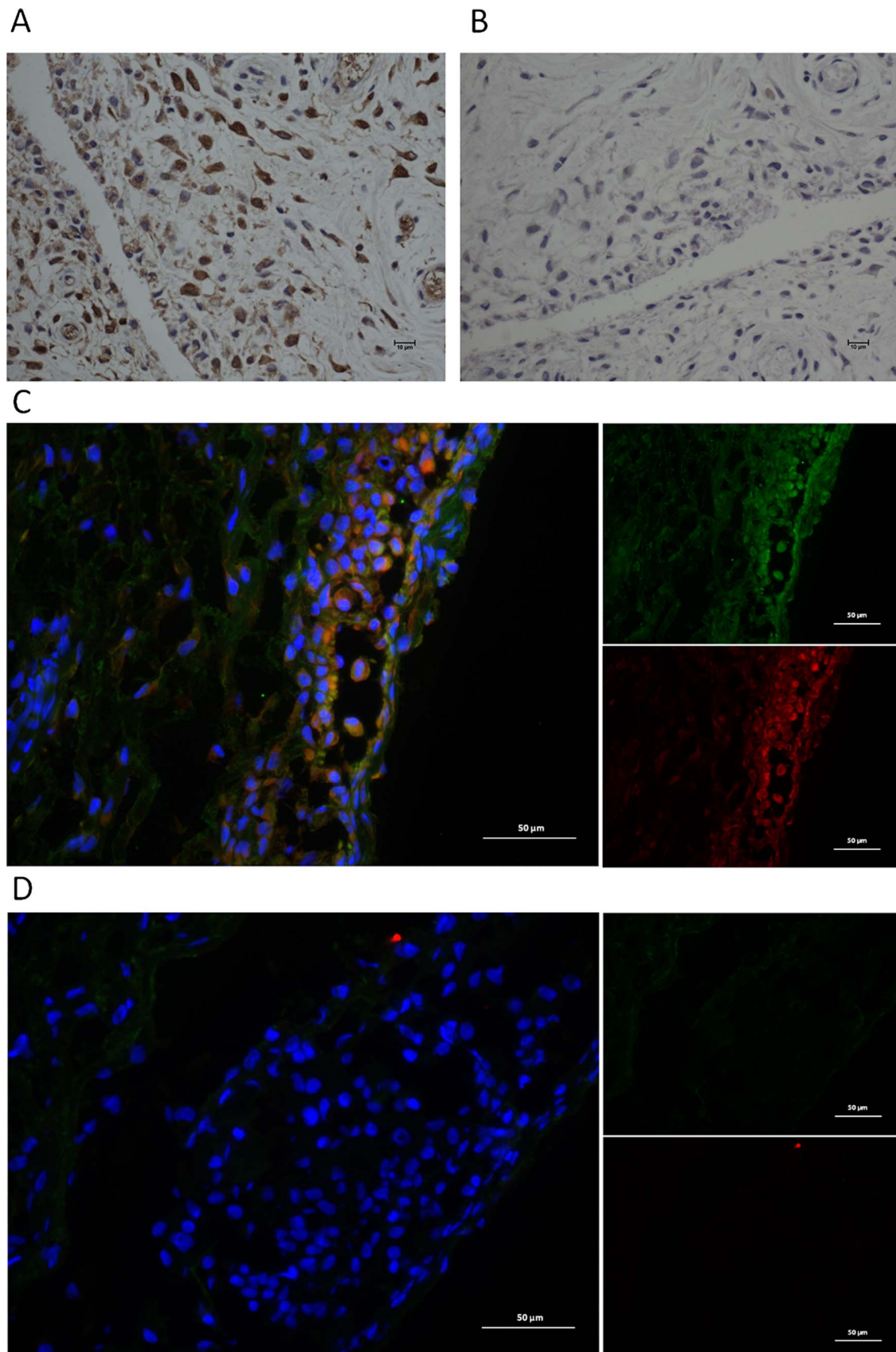


Figure 4 Immunohistochemical expression of *SPAG16* in synovial tissue and FLS. (A) Immunohistochemistry for the expression of *SPAG16* in synovial tissue of a patient with RA (B) Isotype control on consecutive synovial tissue section. Staining was performed with diaminobenzidine (brown) with haematoxylin counterstaining of nuclei (blue). Scale bars represent 10 μm . (C) Fluorescent double staining of *SPAG16* (green) and a FLS marker prolyl-4-hydroxylase- β (red) in the synovial tissue lining; cell nuclei are stained in blue. Representative photos of one of the five ACPA +RA patients tested. (D) Control staining on consecutive synovial tissue section. Scale bars represent 50 μm . ACPA, anticitrullinated peptide antibodies; FLS, fibroblast-like synoviocytes; RA, rheumatoid arthritis.

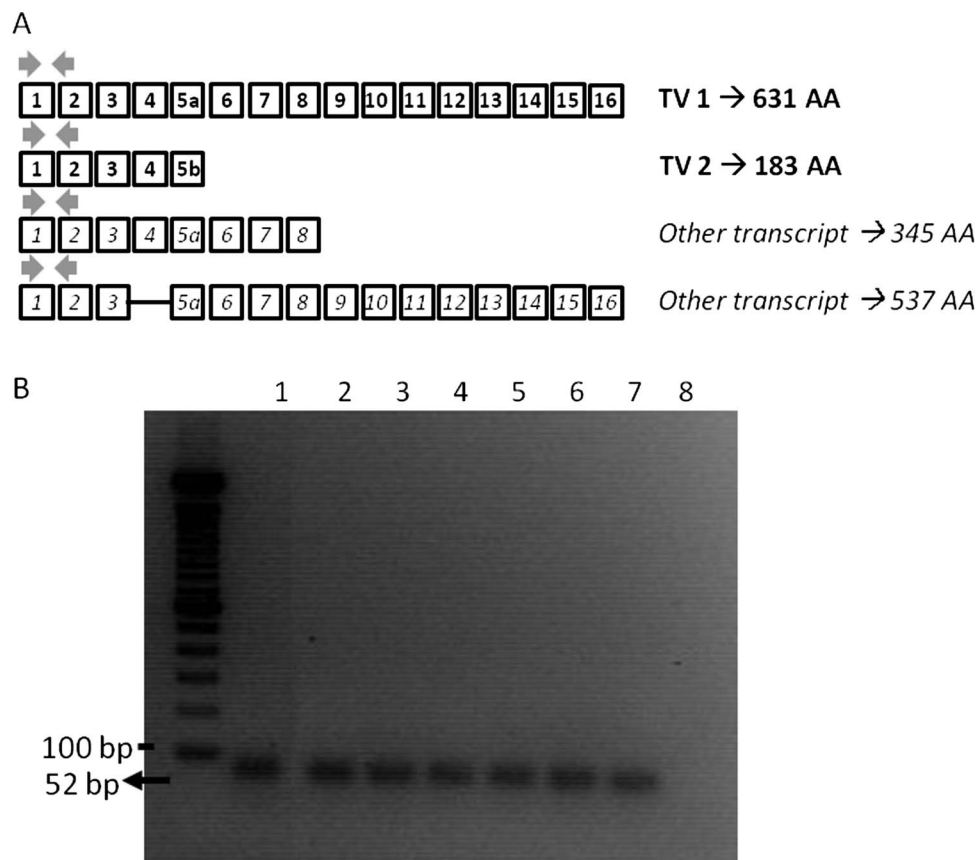


Figure 5 Expression of *SPAG16*, studied using cDNA derived from FLS of RA tissue and using quantitative Real-time PCR and agarose gel electrophoresis for visualisation of bands. (A) Location of the primers used to amplify different *SPAG16* transcripts are indicated by the arrows above the exons. Transcripts in bold have been experimentally validated. (B) PCR products were visualised on a 1.5% agarose gel; a 100 bp ladder was used and the expected amplicon length is 52 bp as indicated by the arrow. Sample 1 concerns testis cDNA as positive control, samples 2–7 are FLS of RA patients with genotypes TC, TC, TT (common genotype), CC (homozygous for the minor allele), TT and CC respectively. Sample 8 concerns the negative control. FLS, fibroblast-like synoviocytes; RA, rheumatoid arthritis.

during the next year ($p=1.76 \times 10^{-4}$, see online supplementary figure 6). When rs7607479 and MMP-3 serum levels were analysed together in one analysis, only MMP-3 serum levels were significantly associated with progression of joint destruction ($p=2.77 \times 10^{-4}$, see online supplementary table 5), suggesting that the association between *SPAG16*-rs7607479 and joint destruction is mediated by a mechanism affecting MMP-3 levels.

DISCUSSION

RA is composed of an ACPA-positive and ACPA-negative subset, which have different genetic susceptibility factors.^{1–26} Although ACPA-positive RA is more severe, the severity varies between patients and the mechanisms responsible are unknown. Elucidation of these mechanisms allows developing targeted interventions in patients most at risk for a severe destructive disease course. We identified a common variant in *SPAG16* of which the minor allele is predictive for less severe joint damage progression and associated with less secretion of the matrix degrading enzyme MMP-3, that is known^{25–27} and also here observed to be associated with radiological progression.

While we describe an association at this locus with RA severity, we do not at this point know the exact causal SNP. We mined 1000Genomes pilot1 CEU population to identify proxies that could be of functional relevance. We extracted all SNPs in strong LD ($R^2 > 0.8$) with rs7607479. Of the 159 SNPs identified, none were in the protein-coding region (see online supplementary table 6). The regulatory potential was subsequently

studied in Haploreg, a tool for exploring annotations of the non-coding genome (see online supplementary table 7). While there are potential candidates, further fine-mapping studies are needed to investigate functional causal alleles at this locus.

Despite the gene name, *SPAG16* expression is not confined to sperm and the association observed with joint damage was not confined to men and equally present in women (data not shown). The gene is located on chromosome 2 and expressed in various tissues. We found that *SPAG16* is expressed in the synovium and FLS. FLS are key effector cells because of their capacity to degrade cartilage and bone.^{24–25} In severe combined immunodeficiency mouse models and in vitro studies this is reflected by the invasiveness of RA FLS.²⁴ We have previously shown that the in vitro invasive capacity of FLS is associated with the expression of MMP-3.²⁸ Moreover we observed that the invasiveness of FLS is an individual characteristic that is associated with the severity of joint damage progression.²⁹ The present data revealed that rs7607479 genotypes were associated with MMP-3 production by FLS and the multivariate analyses suggested that the effect of *SPAG16*-rs7607479 was partly mediated via an effect on MMP-3. Altogether these data imply that *SPAG16*-rs7607479 influences the expression of the matrix degrading enzyme MMP-3, thereby influencing joint damage progression. Up to now little is known about the function of *SPAG16*. Since *SPAG16* is predicted to have a role in the basal structure of the primary cilium, we excluded malformation of the primary cilium in FLS with different genotypes. MicroRNA-4438 is the only known

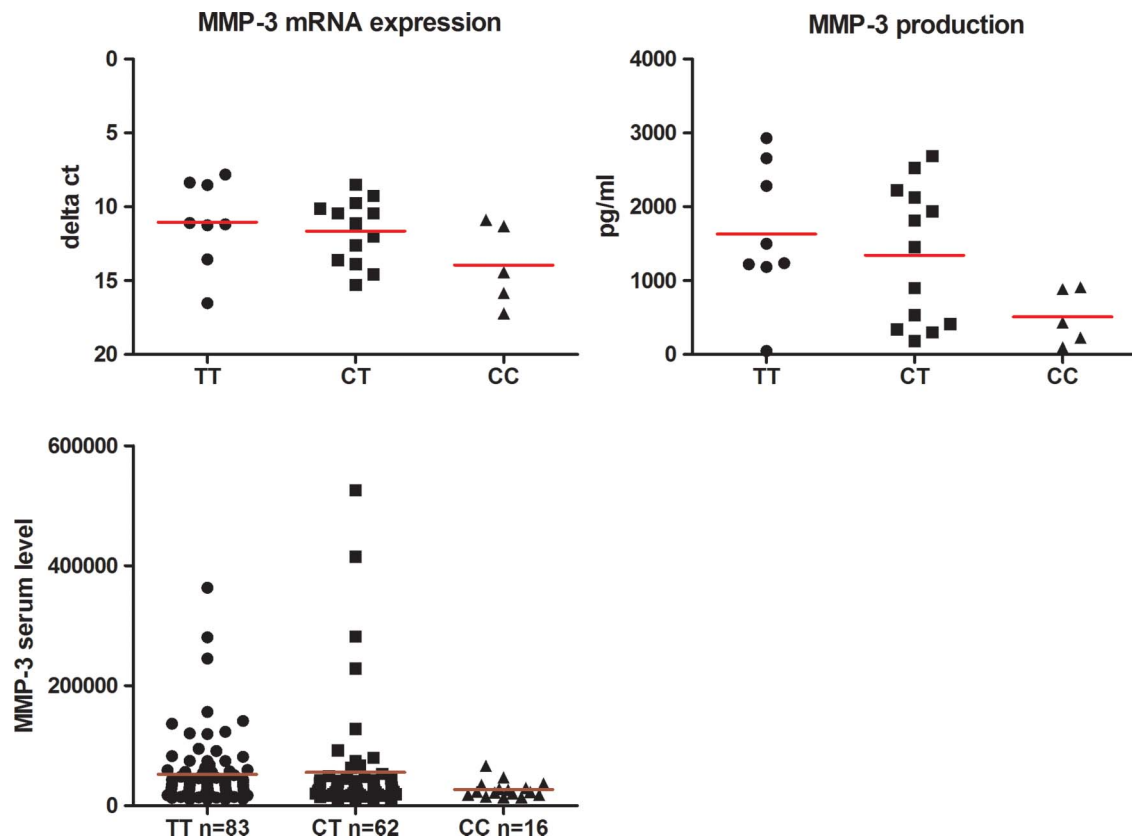


Figure 6 *rs7607479*-genotypes in relation to MMP-3 expression and production by FLS (A) and MMP-3 serum levels in patients with RA (B). (A) Presented are the mRNA expression (RT-qPCR) and protein production levels (ELISA on cell culture supernatants) of MMP-3 in relation to *SPAG16*-*rs7607479* genotypes in FLS of 26 patients with RA. In FLS carrying the minor alleles of *SPAG16*-*rs7607479* a tendency towards a lower expression of MMP-3 was observed ($p=0.08$) and a significantly lower protein production compared with FLS with the common genotype ($p=1.60 \times 10^{-2}$). (B) In patients with RA, presence of the minor allele of *rs7607479* was associated with lower serum MMP-3 levels ($p=4.28 \times 10^{-2}$). FLS, fibroblast-like synoviocytes; MMP, matrix metalloproteinase; RA, rheumatoid arthritis.

miR that is encoded within the *SPAG16* gene and is located in the same intron as *rs7607479*. However, this microRNA was not expressed in FLS (data not shown). Further studies are required to unravel the pathway with which *rs7607479* regulates MMP-3 production. Additionally, *rs7607479* may affect pathways in other cell types as well, which were not studied here.

Presence of high-quality phenotypic outcome data of populations with RA from different continents is a strength of this study. High-quality data were obtained by accurate X-ray scoring and by evaluation of cohorts with serial X-rays within patients. Having repeated measurements within patients yields more precise estimations of the progression rate than single measurements which facilitates differentiating between true effects and noise.³⁰ Another strength is studying patients treated in an era with less intense treatment strategies than today; presumably their natural disease course was relatively less affected.

This study is the first GWAs on radiological progression in ACPA-positive RA. HLA-DRB1 shared epitopes are known to associate with a severe joint damage; though these alleles actually predispose to ACPA-development, that associate with severe RA.³¹ This explains why in this ACPA-positive population the HLA-region was not associated with RA severity. Two recent candidate gene studies identified variants (among others in *IL-15*) as risk factors for radiological progression in the total population of ACPA-positive and ACPA-negative patients.^{8, 32} These variants did not appear in the top list of this study where a slightly different patient population was evaluated (we

confined to ACPA-positive RA). Furthermore, the effect size of *SPAG16*-*rs7607479* observed in stage-I (0.77-fold progression rate/year) was considerably larger than the effect size of *IL-15* observed in stage-I of the present study (1.06-fold progression rate/year, $p<0.05$) and previously published studies (1.03-fold progression rate/year; $p<0.01$).^{8, 31}

Even though our GWA contained relatively low numbers of patients, the power in the present study was largely the result of the preciseness of the outcome data. Furthermore, as mentioned above, the effect observed was considerable. This is in contrast to the results of many GWAs in complex disorders where effect sizes are generally small.

In conclusion, in three data-sets of ACPA-positive patients with RA we identified a common genetic risk factor (44% of patients with RA have one allele, 11% two alleles) that is associated with less severe joint damage progression and found a connection to a disease-relevant biological pathway, namely the production of MMP-3. These findings provide insight into the processes underlying the interindividual differences in progression of joint destruction in RA and could enhance risk stratification in ACPA-positive RA.

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Contributors RK performed the analyses, QH and RK performed the analysis of stage-I, JJH-D gave statistical advice, JABvN, DPCdR and MPMvdL scored all X-rays,

JS, GS-R, NM-O, LR-R and PKG were involved in genotyping, KK, KS, CO and LdB performed the functional experiments, PS, CO, SG and VS supervised the functional experiments, REMT and FASK provided advice, PKG, TWJH and AHMvdH-vM were involved in the design and collection of the patient cohorts, AHMvdH-vM designed and supervised the study, AHMvdH-vM and RK wrote the first draft, all authors contributed to the final manuscript.

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Competing interests A patent application has been filed on the base of the results presented in this article ('Means and methods for the determination of the joint destruction progression rate in rheumatoid arthritis patients', patent pending, US application No. 61/611.494).

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Identification of a genetic variant for joint damage progression in autoantibody-positive rheumatoid arthritis

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Identification of a genetic variant for MMP-3 regulation and joint damage progression in auto-antibody positive Rheumatoid Arthritis

SUPPLEMENTARY MATERIAL

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Supplementary Methods

Immunohistochemistry

Mouse monoclonal antibodies against SPAG16 (IgG1) were produced using the hybridoma technology¹ and affinity-purified using POROS A (Applied Biosystems, Halle, Belgium). Eluted antibodies were dialysed (Slide-A-Lyzer, Thermo Fisher Scientific, Erembodegem, Belgium) against PBS. Paraffin-embedded sections from synovial tissue obtained after knee-replacement operation of RA-patients were deparaffinized and rehydrated. After endogenous peroxidase activity was inhibited using 3% (v/v) hydrogen peroxide in methanol and nonspecific antibody binding was blocked with rabbit serum (10% (v/v) in TBS), the sections were incubated overnight at room temperature with primary mouse monoclonal antibodies against SPAG16 (5F10 and 1F1 mix, 30 µg/ml in TBS). Bound antibodies were detected with the Dako-Envision anti-mouse detection system (Dako, Heverlee, Belgium), followed by colour development with DAB substrate (Sigma-Aldrich). Counterstaining was performed with haematoxylin (Klinipath, Olen, Belgium). TBS with 0.05% (v/v) TritonX-100 was used for washing steps. An identically produced isotype monoclonal antibody (anti-human chorion gonadotrophin IgG1) was used as negative control.

For the double staining of SPAG16 in fibroblast-like synoviocytes (FLS), cryosections of synovial tissue from 5 ACPA+ RA-patients were used. Sections were fixed by incubation in acetone for 10 minutes at room temperature. After washing in PBS supplemented with 0.05% (v/v) Tween20 (PBS-T), sections were blocked for 30 minutes at room temperature with Protein Block (Dako). Tissue sections were incubated overnight at 4°C, in a humidified box, with the following primary antibodies diluted in PBS containing 0.1% (v/v) BSA: anti-prolyl-4-hydroxylase-β (mouse monoclonal, Acris Antibodies, Hiddenhausen, Germany, 1:100) and anti-SPAG16 (goat polyclonal, directed against the peptide KGVTELRTVGNVPD, Everest Biotech, Oxfordshire, United Kingdom, 1:100). The next day, the slides were washed in PBS-T and secondary antibodies (donkey anti-mouse Alexa 555 and donkey anti-goat Alexa 488, 1:250, Molecular Probes, Invitrogen) were added for 1 h at room temperature (in the dark).

Cell nuclei were fluorescently labelled using a 10 minute incubation with DAPI.

Subsequently, sections were incubated for 10 minutes in 0.1% Sudan Black (Sigma-Aldrich) in 70% ethanol to block endogenous autofluorescence. Sections were washed and cover slipped with fluorescence mounting medium (Dako). Control staining was performed by omitting the primary antibody.

Cytokine stimulation of RA FLS

For stimulation experiments 5×10^4 cells were cultured in 12-well plates. 24h after splitting, cells were stimulated with or without 10 ng/ml TNF- α or 1 ng/ml IL1 β (R&D Systems, Abingdon, UK) in DMEM/F-12 (Invitrogen, Basel, Switzerland) supplemented with 0.5% fetal calf serum (Invitrogen). Cells were harvested 24h after stimulation for isolation of RNA. Cell culture supernatants were collected and stored at -20°C for ELISA experiments.

Supplementary References

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Suppl Table 1. Primers for quantitative Real-time PCR.

gene	Sequence	dye
SPAG16	F: 5' ACACGGCGGACGCGGTGGCGGCTG 3' R: 5' GTGACCTGTTCCAGGTAGTAGGCG 3'	SYBR green
MMP-3	F: 5` GGG CTA TCA GAG GAA ATG AG 3` R: 5` CAC GGT TGG AGG GAA ACC TA 3` P: 5` AGC TGG ATA CCC AAG AGG CAT CCA CAC 3`	FAM
GAPDH	F: 5` GGG AAG CTT GTC ATC AAT GGA 3` R: 5` TCT CGC TCC TGG AAG ATG GT 3`	SYBR green

F: Forward Primer; R: Reverse Primer; P: probe.

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

Suppl Table 2. Results of the GWAs in Stage-I: SNPs with $P < 9.5 \times 10^{-5}$.

Chr	SNP	Position	Gene	Effect size	95%CI		P
2	rs7607479	214251829	SPAG16	0.77	0.70	0.85	1.59E-07
2	rs2161820	214179111	SPAG16	0.77	0.70	0.85	1.67E-07
2	rs7584873	214228332	SPAG16	0.77	0.70	0.85	2.35E-07
2	rs1400015	214171061	SPAG16	0.77	0.69	0.85	3.23E-07
2	rs6435777	214034224	SPAG16	0.77	0.70	0.85	4.19E-07
2	rs12997815	214066898	SPAG16	0.77	0.70	0.85	4.51E-07
2	rs6435780	214221392	SPAG16	0.77	0.70	0.85	6.30E-07
2	rs6435770	213922542	SPAG16	0.77	0.69	0.85	7.63E-07
2	rs6749651	214277740	SPAG16	0.78	0.71	0.86	8.87E-07
2	rs10932483	214076656	SPAG16	0.77	0.70	0.86	1.03E-06
2	rs10932481	213988295	SPAG16	0.77	0.69	0.85	1.06E-06
2	rs7580474	213871829	SPAG16	0.77	0.69	0.85	1.25E-06
2	rs12472571	214276034	SPAG16	0.78	0.71	0.87	1.56E-06
2	rs4132244	214264772	SPAG16	0.78	0.70	0.86	1.91E-06
22	rs2038326	24519026	MYO18B	0.76	0.68	0.85	1.98E-06
15	rs2120968	20483952	CYFIP1	0.77	0.68	0.86	3.95E-06
22	rs4820656	24522077	MYO18B	0.77	0.69	0.87	1.08E-05
13	rs9301836	92275312	GPC5	1.35	1.18	1.54	1.32E-05
9	rs2821204	11673901	TYRP1	1.32	1.16	1.49	1.67E-05
15	rs722410	20475538	CYFIP1	0.78	0.70	0.87	1.70E-05
13	rs9530473	75319632	GPC5	0.79	0.70	0.88	1.94E-05
11	rs7930526	25093123	LUZP2	0.81	0.73	0.89	2.64E-05
11	rs7943819	25114304	LUZP2	0.81	0.73	0.89	2.78E-05
6	rs765466	72181715	C6orf155	0.71	0.60	0.83	3.00E-05
6	rs1410664	72570991	RIMS1	1.33	1.16	1.51	3.43E-05
1	rs2284454	54138550	DIO1	0.76	0.68	0.87	3.52E-05
22	rs3859874	24539678	MYO18B	0.79	0.71	0.89	3.81E-05
5	rs4868934	164940665	MAT2B	0.80	0.71	0.89	4.06E-05
22	rs761878	48635681	ZBED4	0.74	0.64	0.85	4.42E-05
1	rs12023478	245517188	ZNF496	1.28	1.14	1.44	4.84E-05
7	rs6593122	54149426	FLJ45974	0.77	0.68	0.88	6.31E-05
7	rs2177549	54151555	FLJ45974	0.77	0.68	0.88	6.31E-05
6	rs17642884	133874977	EYA4	0.75	0.65	0.86	6.34E-05
22	rs767249	42445146	FLJ23588	0.77	0.68	0.88	6.65E-05
1	rs2272931	54128428	YIPF1/DIO1	0.77	0.68	0.88	6.67E-05
6	rs2327351	133889569	EYA4	0.79	0.71	0.89	6.83E-05

9	rs1561953	11609839	<i>PTPRD</i>	1.32	1.15	1.51	6.92E-05
22	rs138845	48571196	<i>BRD1</i>	0.76	0.66	0.87	7.14E-05
18	rs555448	64399863	<i>TXNDC10</i>	0.79	0.70	0.88	7.19E-05
6	rs2797793	37585240	<i>C6orf129</i>	0.82	1.13	1.43	7.73E-05
14	rs7156170	65484357	<i>FUT8</i>	1.23	1.11	1.36	8.08E-05
6	rs9485739	96677858	<i>FUT9</i>	0.81	0.73	0.90	8.08E-05
1	rs6690864	245557330	<i>ZNF496</i>	1.26	1.13	1.42	8.28E-05
11	rs12286647	42742731	<i>AP15</i>	1.27	1.13	1.43	8.35E-05
5	rs2913556	162589982	<i>CCNG1</i>	1.22	1.11	1.34	8.43E-05
5	rs2964350	162604595	<i>CCNG1</i>	1.22	1.11	1.34	8.43E-05
4	rs312378	18895270	<i>SLIT2</i>	0.79	0.70	0.89	8.64E-05
9	rs1335463	11452758	<i>PTPRD</i>	1.31	1.14	1.49	8.64E-05
2	rs6719342	133100325	<i>GPR39/LYPD1</i>	0.75	0.65	0.87	8.89E-05
6	rs1464074	67180065	<i>EGFL11</i>	0.82	0.74	0.90	9.03E-05
20	rs13037893	60194200	<i>SS18L1</i>	1.37	1.17	1.59	9.35E-05
4	rs312371	18880351	<i>SLIT2</i>	0.79	0.70	0.89	9.49E-05
1	rs6426239	245553359	<i>ZNF496</i>	1.26	1.12	1.42	9.83E-05
1	rs12023326	245537744	<i>ZNF496</i>	1.26	1.12	1.42	9.95E-05

The effect size is an estimate of the relative radiological progression rate per year in the presence of the minor allele compared to the homozygous major genotype. For instance patients with rs7607479-CT had 0.77-fold rate of joint destruction per year compared to patients homozygous for the major allele. The effect size over a 7-years disease period is calculated as the beta by the power of the number of follow-up years.

Five of the fourteen *SPAG16* SNPs that were not in close LD in the NARAC ($r^2 < 0.8$) were proceeded to stage-II: rs7607479, rs1400015, rs6435780, rs6435770 and rs10932481.

Suppl Table 3. Results of Stage-II.

Chr	SNP	Position	Effect size	95% CI		P
2	rs7607479	214251829	0.81	0.67	- 0.97	0.022
2	rs1400015	214171061	0.82	0.68	- 0.98	0.034
2	rs6435780	214221392	0.83	0.70	- 1.00	0.044
2	rs6435770	213922542	0.83	0.69	- 0.99	0.039
2	rs10932481	213988295	0.82	0.68	- 0.98	0.029

The effect size is an estimate of the relative radiological progression rate over 7-years of the presence of the minor allele compared to the homozygous major genotype. For instance patients with rs7607479-CT had 0.81-fold rate of joint destruction over 7-years compared to patients homozygous for the major allele.

Suppl Table 4. Results of a conditional analysis including all 5 significant *SPAG16* SNPs of Stage-II.

Chr	SNP	Position	Effect size	95% CI		P
2	rs7607479	214251829	0.82	0.50	- 1.35	0.441
2	rs1400015	214171061	1.01	0.59	- 1.72	0.979
2	rs6435780	214221392	1.01	0.67	- 1.52	0.957
2	rs6435770	213922542	1.75	0.31	- 9.74	0.525
2	rs10932481	213988295	0.55	0.10	- 2.89	0.477

Presented are the results of an analyses including all five significant SPAG-16 SNPs in stage-II. None of the SNPs remained significant, indicating the five SNPs reflect one signal.

Suppl Table 5. Analysis of both MMP-3 serum levels and rs76074749 with rate of joint destruction separately and in one regression model.

	Effect size	95% CI		<i>P</i>
Both variants were analyzed separately				
MMP-3	0.75	0.64	0.87	1.76x10 ⁻⁴
Rs7607479	0.76	0.61	0.96	0.020
Both variants were combined in one regression model				
MMP-3	0.75	0.64	0.87	2.77x10 ⁻⁴
Rs7607479	0.81	0.65	1.02	0.082

MMP serum levels were divided in quartiles as explained eFigure 8. All analyses were adjusted for sex, age, treatment and disease duration at the time of sample collection. The effect size of MMP-3 reflects the relative increase in progression rate: i.e. for each lower quartile of MMP-3 the radiologic progression rate increased 0.75-fold compared to the highest quartile. When the analysis including both rs7607479 and MMP-3 was repeated with MMP-3 levels as a continuous variable, MMP-3 levels were also associated with the rate of joint destruction ($P 7.49 \times 10^{-4}$) and the association between the SNP and joint destruction was not significant ($P 0.06$).

Suppl Table 6. All SNPs in strong LD with rs 7607479 ($R^2 > 0.8$), extracted from 1000Genomes pilot 1 CEU population.

SNP	Proxy	Distance	R2	DPrime	Arrays	Chromosome	Coordinate_HG18	GeneVariant
rs7607479	rs7607479	0	1.000	1.000	A6,I3,I5,I6,I6Q,IM,IMD,IC,I CQ,IWQ,O54,O5E,AAH	chr2	214251829	INTRONIC
rs7607479	rs6755585	2165	1.000	1.000	None	chr2	214253994	INTRONIC
rs7607479	rs9679714	5745	1.000	1.000	None	chr2	214257574	INTRONIC
rs7607479	rs11685056	6273	1.000	1.000	A6,IM,IMD,AAH	chr2	214245556	INTRONIC
rs7607479	rs2015217	7076	1.000	1.000	IM,IMD,O54,O5E,AAH	chr2	214244753	INTRONIC
rs7607479	rs1986978	9633	1.000	1.000	A6	chr2	214261462	INTRONIC
rs7607479	rs6435787	10549	1.000	1.000	None	chr2	214262378	INTRONIC
rs7607479	rs2013922	11861	1.000	1.000	AN,A5,A6	chr2	214239968	INTRONIC
rs7607479	rs6435783	17375	1.000	1.000	None	chr2	214234454	INTRONIC
rs7607479	rs6435782	17385	1.000	1.000	None	chr2	214234444	INTRONIC
rs7607479	rs7584873	23497	1.000	1.000	A6,I3,I5,I6,I6Q,IM,IMD,IC,I CQ,IWQ,O54,O5E,AAH	chr2	214228332	INTRONIC
rs7607479	rs10460417	23953	1.000	1.000	None	chr2	214227876	INTRONIC
rs7607479	rs12472571	24205	1.000	1.000	I3,I5,I6,I6Q,IM,IMD,IC,ICQ ,AxM,IWQ,O54,O5E	chr2	214276034	INTRONIC
rs7607479	rs7580818	24734	1.000	1.000	None	chr2	214227095	INTRONIC
rs7607479	rs2037179	25130	1.000	1.000	None	chr2	214226699	INTRONIC
rs7607479	rs6749651	25911	1.000	1.000	I2,I5,I6,I6Q,IM,IMD,IWQ,O 54,O5E,AAH	chr2	214277740	INTRONIC
rs7607479	rs6435791	26929	1.000	1.000	None	chr2	214278758	INTRONIC
rs7607479	rs10197286	28826	1.000	1.000	IMD	chr2	214223003	INTRONIC
rs7607479	rs11686107	30682	1.000	1.000	A6	chr2	214221147	INTRONIC
rs7607479	rs12995729	35049	1.000	1.000	None	chr2	214216780	INTRONIC
rs7607479	rs7569738	36555	1.000	1.000	None	chr2	214215274	INTRONIC
rs7607479	rs2090299	37802	1.000	1.000	None	chr2	214214027	INTRONIC
rs7607479	rs7586466	42553	1.000	1.000	None	chr2	214209276	INTRONIC
rs7607479	rs10185770	46052	1.000	1.000	IM,IMD,O54,O5E	chr2	214205777	INTRONIC
rs7607479	rs1554178	49476	1.000	1.000	I1	chr2	214202353	INTRONIC
rs7607479	rs10203782	51269	1.000	1.000	None	chr2	214200560	INTRONIC
rs7607479	rs4340451	52486	1.000	1.000	None	chr2	214199343	INTRONIC
rs7607479	rs1474003	53455	1.000	1.000	None	chr2	214198374	INTRONIC
rs7607479	rs1515913	54118	1.000	1.000	None	chr2	214197711	INTRONIC
rs7607479	rs13020325	54376	1.000	1.000	None	chr2	214197453	INTRONIC
rs7607479	rs13004439	56922	1.000	1.000	None	chr2	214194907	INTRONIC
rs7607479	rs1114446	59353	1.000	1.000	IM,IMD,O54,O5E	chr2	214192476	INTRONIC
rs7607479	rs2018907	62780	1.000	1.000	None	chr2	214189049	INTRONIC
rs7607479	rs6716478	63238	1.000	1.000	None	chr2	214188591	INTRONIC
rs7607479	rs6744795	63294	1.000	1.000	None	chr2	214188535	INTRONIC
rs7607479	rs12694307	63754	1.000	1.000	None	chr2	214188075	INTRONIC
rs7607479	rs12617938	64108	1.000	1.000	None	chr2	214187721	INTRONIC
rs7607479	rs12611569	64185	1.000	1.000	None	chr2	214187644	INTRONIC
rs7607479	rs1961052	68078	1.000	1.000	A6	chr2	214183751	INTRONIC
rs7607479	rs1304727	68197	1.000	1.000	None	chr2	214183632	INTRONIC
rs7607479	rs2161820	72718	1.000	1.000	I2,I5,I6,I6Q,IM,IMD,IWQ	chr2	214179111	INTRONIC
rs7607479	rs6754702	73327	1.000	1.000	None	chr2	214178502	INTRONIC
rs7607479	rs6712394	73362	1.000	1.000	None	chr2	214178467	INTRONIC

rs7607479	rs7577685	85284	1.000	1.000	None	chr2	214166545	INTRONIC
rs7607479	rs6715355	6621	0.965	1.000	None	chr2	214258450	INTRONIC
rs7607479	rs7602331	13356	0.965	1.000	None	chr2	214238473	INTRONIC
rs7607479	rs7591624	15989	0.965	1.000	None	chr2	214235840	INTRONIC
rs7607479	rs10804215	26615	0.965	1.000	None	N/A	N/A	INTRONIC
rs7607479	rs7596173	27353	0.965	1.000	None	chr2	214224476	INTRONIC
rs7607479	rs6749115	48441	0.965	1.000	None	chr2	214203388	INTRONIC
rs7607479	rs1554177	49983	0.965	1.000	OQ,OE,O24,O28,O54,O5E,OEE	chr2	214201846	INTRONIC
rs7607479	rs7572369	51674	0.965	1.000	IM,IMD,O54,O5E	chr2	214200155	INTRONIC
rs7607479	rs2113472	87399	0.965	1.000	None	chr2	214164430	INTRONIC
rs7607479	rs1984609	95354	0.965	1.000	None	chr2	214156475	INTRONIC
rs7607479	rs12694306	139775	0.965	1.000	None	chr2	214112054	INTRONIC
rs7607479	rs6744533	158586	0.965	1.000	None	chr2	214093243	INTRONIC
rs7607479	rs4456649	175005	0.965	1.000	None	chr2	214076824	INTRONIC
rs7607479	rs1964673	180744	0.965	1.000	I6,IM,IMD,O54,O5E	chr2	214071085	INTRONIC
rs7607479	rs12997815	184931	0.965	1.000	I3,I5,I6,I6Q,IM,IMD,IC,ICQ,IWQ	chr2	214066898	INTRONIC
rs7607479	rs7563592	189484	0.965	1.000	I1,IM,IMD,CYT,OQ,OE,O24,O28,O54,O5E,OEE	chr2	214062345	INTRONIC
rs7607479	rs7557848	195931	0.965	1.000	None	chr2	214055898	INTRONIC
rs7607479	rs6435777	217605	0.965	1.000	I2,I5,I6,I6Q,IM,IMD,IWQ	chr2	214034224	INTRONIC
rs7607479	rs12694304	208399	0.932	1.000	A6	chr2	214043430	INTRONIC
rs7607479	rs10176906	32305	0.931	1.000	AN,A6	chr2	214284134	INTRONIC
rs7607479	rs7581348	33260	0.931	1.000	AAH	chr2	214218569	INTRONIC
rs7607479	rs8179764	211287	0.931	1.000	None	chr2	214040542	INTRONIC
rs7607479	rs12694303	231601	0.931	1.000	None	chr2	214020228	INTRONIC
rs7607479	rs339821	245171	0.931	1.000	None	chr2	214006658	INTRONIC
rs7607479	rs7560793	135383	0.929	0.964	None	chr2	214116446	INTRONIC
rs7607479	rs10180751	209492	0.929	0.964	None	chr2	214042337	INTRONIC
rs7607479	rs7599738	26534	0.897	1.000	None	chr2	214225295	INTRONIC
rs7607479	rs10166400	231033	0.897	1.000	None	chr2	214020796	INTRONIC
rs7607479	rs6761365	124044	0.896	0.963	None	chr2	214127785	INTRONIC
rs7607479	rs10192180	130301	0.895	0.963	None	chr2	214121528	INTRONIC
rs7607479	rs6435779	155272	0.895	0.963	None	chr2	214096557	INTRONIC
rs7607479	rs7580222	203973	0.895	0.963	None	chr2	214047856	INTRONIC
rs7607479	rs1850130	998	0.865	1.000	IMD,O54,O5E,AAH	chr2	214252827	INTRONIC
rs7607479	rs6755913	2257	0.865	1.000	None	chr2	214254086	INTRONIC
rs7607479	rs12694310	2870	0.865	1.000	A6	chr2	214254699	INTRONIC
rs7607479	rs13026541	4007	0.865	1.000	AxM	chr2	214255836	INTRONIC
rs7607479	rs10932492	4575	0.865	1.000	None	chr2	214256404	INTRONIC
rs7607479	rs6435786	5598	0.865	1.000	IM,IMD,O54,O5E	chr2	214257427	INTRONIC
rs7607479	rs7576499	5659	0.865	1.000	None	chr2	214257488	INTRONIC
rs7607479	rs6760140	10379	0.865	1.000	None	chr2	214241450	INTRONIC
rs7607479	rs2013934	11740	0.865	1.000	None	chr2	214240089	INTRONIC
rs7607479	rs12613612	13354	0.865	1.000	None	chr2	214265183	INTRONIC
rs7607479	rs953881	16289	0.865	1.000	None	chr2	214235540	INTRONIC
rs7607479	rs12474173	16534	0.865	1.000	A6	chr2	214235295	INTRONIC
rs7607479	rs13028538	22012	0.865	1.000	None	chr2	214273841	INTRONIC
rs7607479	rs6435790	23563	0.865	1.000	None	chr2	214275392	INTRONIC
rs7607479	rs10197261	28872	0.865	1.000	None	chr2	214222957	INTRONIC

rs7607479	rs6435780	30437	0.865	1.000	AS,A5,A6,I1,I2,I5,I6,I6Q,I M,IMD,OQ,IWQ,OE,O24, O28,O54,O5E,OEE,AAH	chr2	214221392	INTRONIC
rs7607479	rs13008301	33754	0.865	1.000	None	chr2	214218075	INTRONIC
rs7607479	rs10932491	35740	0.865	1.000	None	chr2	214216089	INTRONIC
rs7607479	rs57936781	39887	0.865	1.000	None	chr2	214211942	N/A
rs7607479	rs13000730	41952	0.865	1.000	None	chr2	214209877	INTRONIC
rs7607479	rs10932490	49792	0.865	1.000	None	chr2	214202037	INTRONIC
rs7607479	rs6747293	57545	0.865	1.000	None	chr2	214194284	INTRONIC
rs7607479	rs13033980	59596	0.865	1.000	None	chr2	214192233	INTRONIC
rs7607479	rs11683006	60056	0.865	1.000	AN,A5,A6,AxM	chr2	214191773	INTRONIC
rs7607479	rs13029290	65467	0.865	1.000	None	chr2	214186362	INTRONIC
rs7607479	rs918839	71969	0.865	1.000	A6	chr2	214179860	INTRONIC
rs7607479	rs7569487	210493	0.865	1.000	None	chr2	214041336	INTRONIC
rs7607479	rs6751454	31223	0.861	0.962	None	chr2	214283052	INTRONIC
rs7607479	rs7561615	171935	0.861	0.962	None	chr2	214079894	INTRONIC
rs7607479	rs4132244	12943	0.833	1.000	AX,I3,I5,I6,I6Q,IM,IMD,IC, ICQ,OQ,IWQ,OE,O24,O2 8,O54,O5E,OEE,AAH	chr2	214264772	INTRONIC
rs7607479	rs6435784	17333	0.833	1.000	A6	chr2	214234496	INTRONIC
rs7607479	rs7562444	26600	0.833	1.000	None	chr2	214278429	INTRONIC
rs7607479	rs7425324	38458	0.833	1.000	None	chr2	214213371	INTRONIC
rs7607479	rs1606936	44413	0.833	1.000	None	chr2	214207416	INTRONIC
rs7607479	rs2171626	344049	0.833	1.000	None	chr2	213907780	INTRONIC
rs7607479	rs62195175	87822	0.828	0.960	None	chr2	214164007	N/A
rs7607479	rs7560229	94941	0.828	0.960	AN,A5,A6	chr2	214156888	INTRONIC
rs7607479	rs13012341	94966	0.828	0.960	None	chr2	214156863	INTRONIC
rs7607479	rs7421588	95110	0.828	0.960	None	chr2	214156719	INTRONIC
rs7607479	rs1984610	95325	0.828	0.960	None	chr2	214156504	INTRONIC
rs7607479	rs12464278	97627	0.828	0.960	None	chr2	214154202	INTRONIC
rs7607479	rs12996833	112579	0.828	0.960	None	chr2	214139250	INTRONIC
rs7607479	rs2113473	149079	0.828	0.960	IM,IMD,CYT,OQ,OE,O24, O28,O54,O5E,OEE	chr2	214102750	INTRONIC
rs7607479	rs6755408	150729	0.828	0.960	AS,A5,A6,IM,IMD,O54,O5 E	chr2	214101100	INTRONIC
rs7607479	rs10932486	158672	0.828	0.960	AS,A5,A6	chr2	214093157	INTRONIC
rs7607479	rs10932485	159027	0.828	0.960	IMD,O54,O5E	chr2	214092802	INTRONIC
rs7607479	rs12988703	164484	0.828	0.960	IM,IMD	chr2	214087345	INTRONIC
rs7607479	rs10932483	175173	0.828	0.960	I2,I5,I6,I6Q,IM,IMD,CM,IW Q	chr2	214076656	INTRONIC
rs7607479	rs6650781	182112	0.828	0.960	None	chr2	214069717	INTRONIC
rs7607479	rs12993900	188517	0.828	0.960	None	chr2	214063312	INTRONIC
rs7607479	rs12987615	189363	0.828	0.960	None	chr2	214062466	INTRONIC
rs7607479	rs13005828	190721	0.828	0.960	None	chr2	214061108	INTRONIC
rs7607479	rs10498006	198501	0.828	0.960	AH,AN,A5,A6	chr2	214053328	INTRONIC
rs7607479	rs12617636	216578	0.828	0.960	None	chr2	214035251	INTRONIC
rs7607479	rs10165804	32340	0.827	0.960	AN,A5,A6	chr2	214284169	INTRONIC
rs7607479	rs12620874	45606	0.827	0.960	None	chr2	214206223	INTRONIC
rs7607479	rs1850131	62807	0.827	0.960	None	chr2	214189022	INTRONIC
rs7607479	rs13024335	13608	0.802	1.000	AN,A5,A6,OQ,OE,O24,O2 8,O54,O5E,OEE	chr2	214238221	INTRONIC
rs7607479	rs13001102	13631	0.802	1.000	None	chr2	214238198	INTRONIC
rs7607479	rs13020757	17484	0.802	1.000	A6,I6,IM,IMD,OQ,OE,O24, O28,O54,O5E,OEE	chr2	214269313	INTRONIC

rs7607479	rs13002044	18632	0.802	1.000	None	chr2	214270461	INTRONIC
rs7607479	rs12988010	219255	0.802	1.000	None	chr2	214032574	INTRONIC
rs7607479	rs1114610	248214	0.802	1.000	IMD,O54,O5E	chr2	214003615	INTRONIC
rs7607479	rs6435775	249604	0.802	1.000	None	chr2	214002225	INTRONIC
rs7607479	rs13021887	251964	0.802	1.000	None	chr2	213999865	INTRONIC
rs7607479	rs12473341	263277	0.802	1.000	AS,A6	chr2	213988552	INTRONIC
rs7607479	rs10932481	263534	0.802	1.000	AN,A5,A6,I3,I5,I6,I6Q,IM,I MD,IC,ICQ,IWQ,O54,O5E ,AAH	chr2	213988295	INTRONIC
rs7607479	rs11687463	279749	0.802	1.000	None	chr2	213972080	3PRIME_UTR
rs7607479	rs2200758	281927	0.802	1.000	None	chr2	213969902	3PRIME_UTR
rs7607479	rs7609021	307191	0.802	1.000	None	chr2	213944638	INTRONIC
rs7607479	rs13017229	315673	0.802	1.000	None	chr2	213936156	INTRONIC
rs7607479	rs11674674	328057	0.802	1.000	I6Q,IM,IMD,IC,ICQ	chr2	213923772	INTRONIC
rs7607479	rs6435770	329287	0.802	1.000	I1,I3,I5,I6,I6Q,IM,IMD,IC,I CQ,OQ,IWQ,OE,O24,O28 ,O54,O5E,OEE	chr2	213922542	INTRONIC
rs7607479	rs35041810	342150	0.802	1.000	None	chr2	213909679	INTRONIC
rs7607479	rs11676531	367149	0.802	1.000	None	chr2	213884680	INTRONIC
rs7607479	rs11692961	367303	0.802	1.000	I1,IM,IMD,O54,O5E	chr2	213884526	INTRONIC
rs7607479	rs1488971	369588	0.802	1.000	I1,IM,IMD,O54,O5E	chr2	213882241	INTRONIC
rs7607479	rs7580474	380000	0.802	1.000	I3,I5,I6,I6Q,IM,IMD,IC,ICQ ,IWQ,O54,O5E	chr2	213871829	INTRONIC
rs7607479	rs6759627	385486	0.802	1.000	None	chr2	213866343	INTRONIC
rs7607479	rs6723575	387199	0.802	1.000	CYT,OQ,OE,O24,O28,O5 4,O5E,OEE	chr2	213864630	INTRONIC

Rs7607479 is situated in SPAG16, Sperm-associated antigen 16 protein (Pf20 protein homolog).

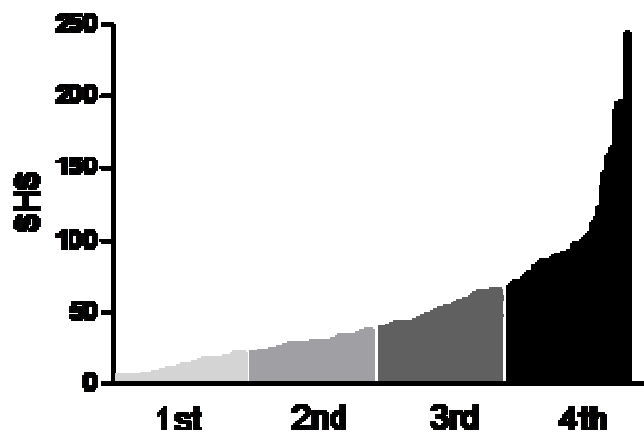
Source:Uniprot/SWISSPROT;Acc:Q8N0X2]

Suppl Table 7. Evaluation of regulatory potential of proxies of rs7607479 in Haploreg, a tool for exploring annotations of the non-coding genome.

chr	pos (hg19)	LD	variant	Ref	Alt	ASN freq	CEU freq	YRI freq	GERP cons	Promoter histone marks	Enhancer histone marks	DNase	Proteins bound	eQTL tissues	Motifs changed	RefSeq genes	dbSNP func annot
2	214458300	1	rs7577685	C	G	0.26	0.38	0.58							DMRT7	SPAG16	intronic
2	214470222	1	rs6712394	T	A	0.28	0.38	0.26								SPAG16	intronic
2	214470257	1	rs6754702	G	A	0.28	0.38	0.53								SPAG16	intronic
2	214470866	1	rs2161820	G	A	0.28	0.38	0.52								SPAG16	intronic
2	214475387	1	rs1304727	A	G	0.28	0.38	0.54								SPAG16	intronic
2	214475506	1	rs1961052	G	A	0.28	0.38	0.56							RORalpha1,RAR	SPAG16	intronic
2	214479399	1	rs12611569	G	A	0.28	0.38	0.53							4 altered motifs	SPAG16	intronic
2	214479476	1	rs12617938	A	G	0.28	0.38	0.53								SPAG16	intronic
2	214479830	1	rs12694307	A	T	0.28	0.38	0.29								SPAG16	intronic
2	214480290	1	rs6744795	A	G	0.25	0.38	0.55								SPAG16	intronic
2	214480346	1	rs6716478	C	A	0.28	0.38	0.57							5 altered motifs	SPAG16	intronic
2	214480804	1	rs2018907	A	G	0.28	0.38	0.55							6 altered motifs	SPAG16	intronic
2	214484231	1	rs1114446	G	A	0.28	0.38	0.53							Pax-5	SPAG16	intronic
2	214486662	1	rs13004439	A	C	0.28	0.38	0.6							Nkx2,Zfp105,CEBP G	SPAG16	intronic
2	214487570	1	rs11405535	T	TA	0.27	0.3	0.5								SPAG16	intronic
2	214488965	1	rs10693252	A	AATA AT	0.17	0.35	0.5								SPAG16	intronic
2	214489208	1	rs13020325	G	A	0.28	0.38	0.59				SK-N- MC				SPAG16	intronic
2	214489466	1	rs1515913	G	A	0.28	0.38	0.59							PU.1	SPAG16	intronic
2	214490129	1	rs1474003	C	T	0.28	0.38	0.53								SPAG16	intronic
2	214491098	1	rs4340451	G	A	0.28	0.38	0.56							Ik-2,AIRE,LUN-1	SPAG16	intronic
2	214492315	1	rs10203782	G	C	0.28	0.38	0.29			GM12878	4 cell types	NFKB		Smad3	SPAG16	intronic
2	214494108	1	rs1554178	A	T	0.28	0.38	0.61							Pou3f1,Pou5f1	SPAG16	intronic
2	214497532	1	rs10185770	T	C	0.28	0.38	0.56							TEF-1	SPAG16	intronic
2	214501031	1	rs7586466	G	A	0.28	0.38	0								SPAG16	intronic
2	214505782	1	rs2090299	T	A	0.28	0.38	0.56								SPAG16	intronic
2	214507029	1	rs7569738	T	C	0.28	0.38	0.54								SPAG16	intronic
2	214508535	1	rs12995729	G	C	0.27	0.38	0.54							BLIMP1,Irf,Brachy ury	SPAG16	intronic
2	214512902	1	rs11686107	G	A	0.28	0.38	0.3							Ik-2,Irf,NF-AT	SPAG16	intronic
2	214514758	1	rs10197286	C	T	0.28	0.38	0.31							Zfp105,Hoxb13	SPAG16	intronic
2	214517832	1	rs10562719	AAAG	A	0.23	0.33	0.4								SPAG16	intronic
2	214518454	1	rs2037179	A	T	0.28	0.38	0.56								SPAG16	intronic
2	214518850	1	rs7580818	G	A	0.28	0.38	0.56							5 altered motifs	SPAG16	intronic
2	214519631	1	rs10460417	T	G	0.28	0.38	0.58							DMRT2	SPAG16	intronic
2	214520087	1	rs7584873	G	A	0.3	0.38	0.56							Homez	SPAG16	intronic
2	214526199	1	rs6435782	G	T	0.28	0.38	0.55								SPAG16	intronic
2	214526209	1	rs6435783	T	C	0.28	0.38	0.58							Gm397	SPAG16	intronic
2	214529560	1	2:214237805	CAT	C	0.2	0.34	0.53								SPAG16	intronic
2	214531723	1	rs2013922	C	T	0.28	0.38	0.56								SPAG16	intronic
2	214536508	1	rs2015217	C	T	0.28	0.38	0.59							Hbp1	SPAG16	intronic
2	214537311	1	rs11685056	C	T	0.28	0.38	0.39							Foxp1	SPAG16	intronic
2	214543584	1	rs7607479	T	C	0.29	0.38	0.59								SPAG16	intronic
2	214544267	1	rs10628838	C	CAT	0.32	0.35	0.58							RP58	SPAG16	intronic
2	214545749	1	rs6755585	C	G	0.29	0.38	0.36				Caco-2				SPAG16	intronic
2	214549329	1	rs9679714	C	T	0.29	0.38	0.59							Foxq1,PLZF,Pou6f1	SPAG16	intronic
2	214553217	1	rs1986978	A	G	0.29	0.38	0.58					POU5F1		Sp100	SPAG16	intronic
2	214554133	1	rs6435787	A	G	0.29	0.38	0.58							HMG-IY	SPAG16	intronic
2	214565966	1	2:214274211	AAAAT	A	0.13	0.28	0.32								SPAG16	intronic
2	214567789	1	rs12472571	C	A	0.23	0.38	0.64							STAT	SPAG16	intronic
2	214569394	1	rs11396573	A	AC	0.27	0.34	0.44								SPAG16	intronic
2	214569495	1	rs6749651	G	A	0.28	0.38	0.59								SPAG16	intronic
2	214570513	1	rs6435791	G	T	0.23	0.38	0.47									

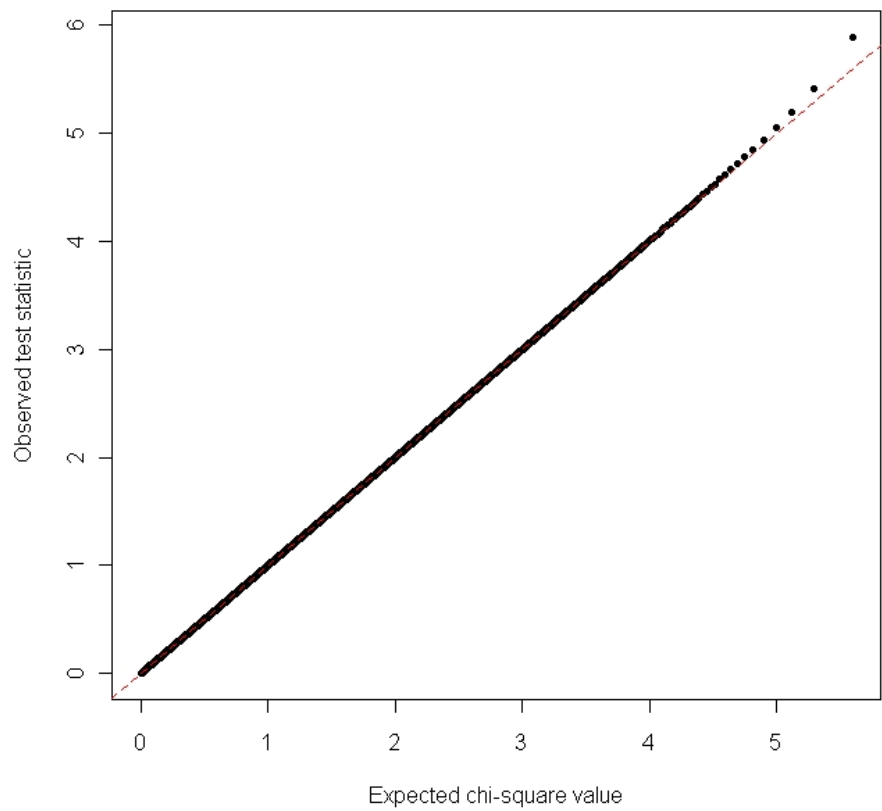
Extended documentation on haploreg is available at <http://www.broadinstitute.org/mammals/haploreg/documentation.html>.

Suppl Figure 1. The severity of joint destruction is highly variable between ACPA-positive RA-patients.

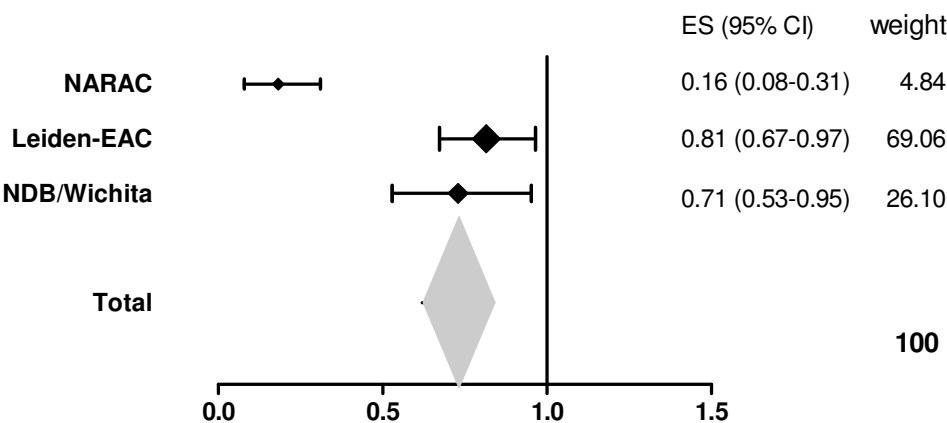


Depicted is the severity of radiological joint destruction, measured by the Sharp-van der Heijde score, after 7 years of disease in RA-patients included in the Leiden Early Arthritis Clinic. The patients were divided in quartiles based on the radiological data. These quartiles are presented on the x-axis, every bar reflects one patient and its Sharp van der Heijde score (SHS), a measure of the severity of radiological damage, is presented on the y-axis.

Suppl Figure 2. Quantile-quantile (QQ) plot for joint damage progression in stage-I; the lambda inflation factor is 1.009.

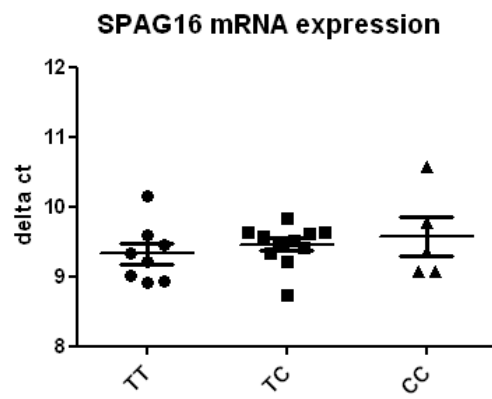


Suppl Figure 3. Meta-analysis on the three stages.



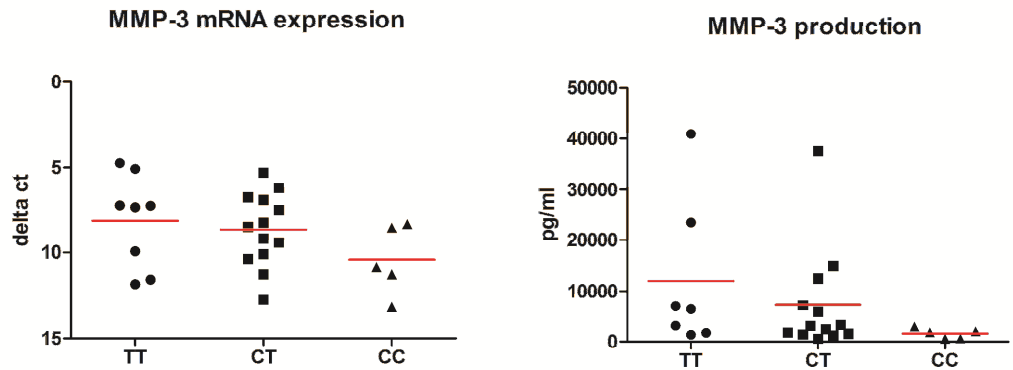
Results of an inverse variance meta analysis including all the three stages.
For reasons of comparability all data were depicted for progression over a similar disease period (7 years).
P value= 2.19×10^{-5} when evaluating fixed effects and 0.023 when evaluating random effects.

Suppl Figure 4. SPAG16 expression in relation to rs7607479 genotypes.

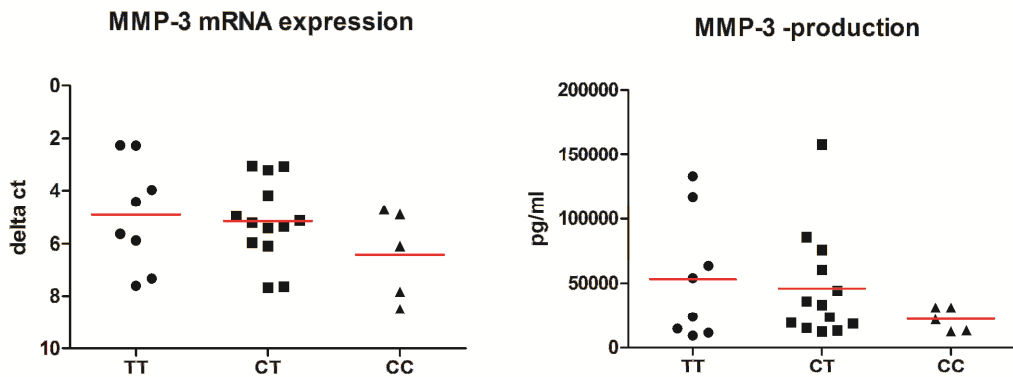


Suppl Figure 5. Expression and production of MMP-3 in stimulated FLS in relation to the different rs7607479 genotypes.

a stimulated by TNF

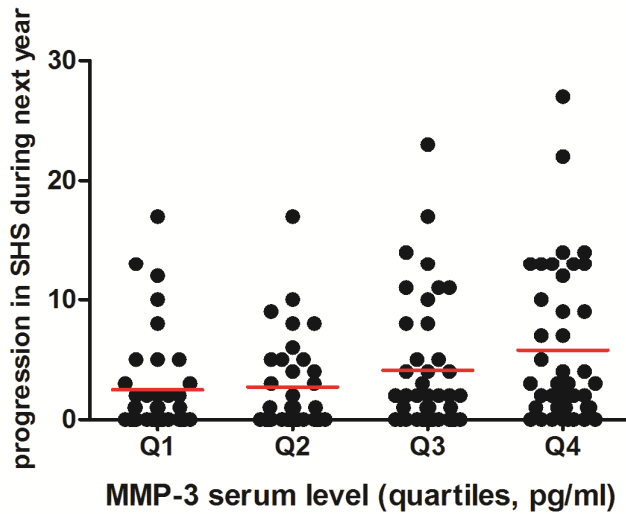


b stimulated by IL-1b



Presented are MMP-3 mRNA expression (A,C) and MMP-3 production (B,D) after TNF-alpha and IL-1beta stimulation. The p-values obtained were 0.11, 0.32, 0.17 and 0.11 respectively.

Suppl Figure 6. Relation between serum level MMP-3 and progression of joint destruction in RA-patients.



Patients with lower MMP-3 levels were observed to have less progressive joint destruction the next year of follow-up ($P=1.76 \times 10^{-4}$). SHS indicates Sharp-van der Heijde score. Horizontal lines indicate the mean. Patients were divided in four quartiles (Q) based on their serum level– each quartile included an equal number of patients. Quartile 1 relates to serum levels $<2.0 \times 10^4$ pg/ml, quartile 2 is 2.0×10^4 - 3.1×10^4 pg/ml, quartile 3 is 3.1×10^4 - 4.9×10^4 pg/ml and quartile 4 relates to levels $>4.9 \times 10^4$ pg/ml.

When the linear regression analysis was repeated with serum level as a continuous outcome (again adjusting for age, gender, treatment, and disease duration at serum collection) the association with radiological progression was significant as well ($P=3.0 \times 10^{-3}$).