Immunogenetic and immunological aspects of rheumatoid arthritis : DERAA and anti-citrulline reactivity can make the difference

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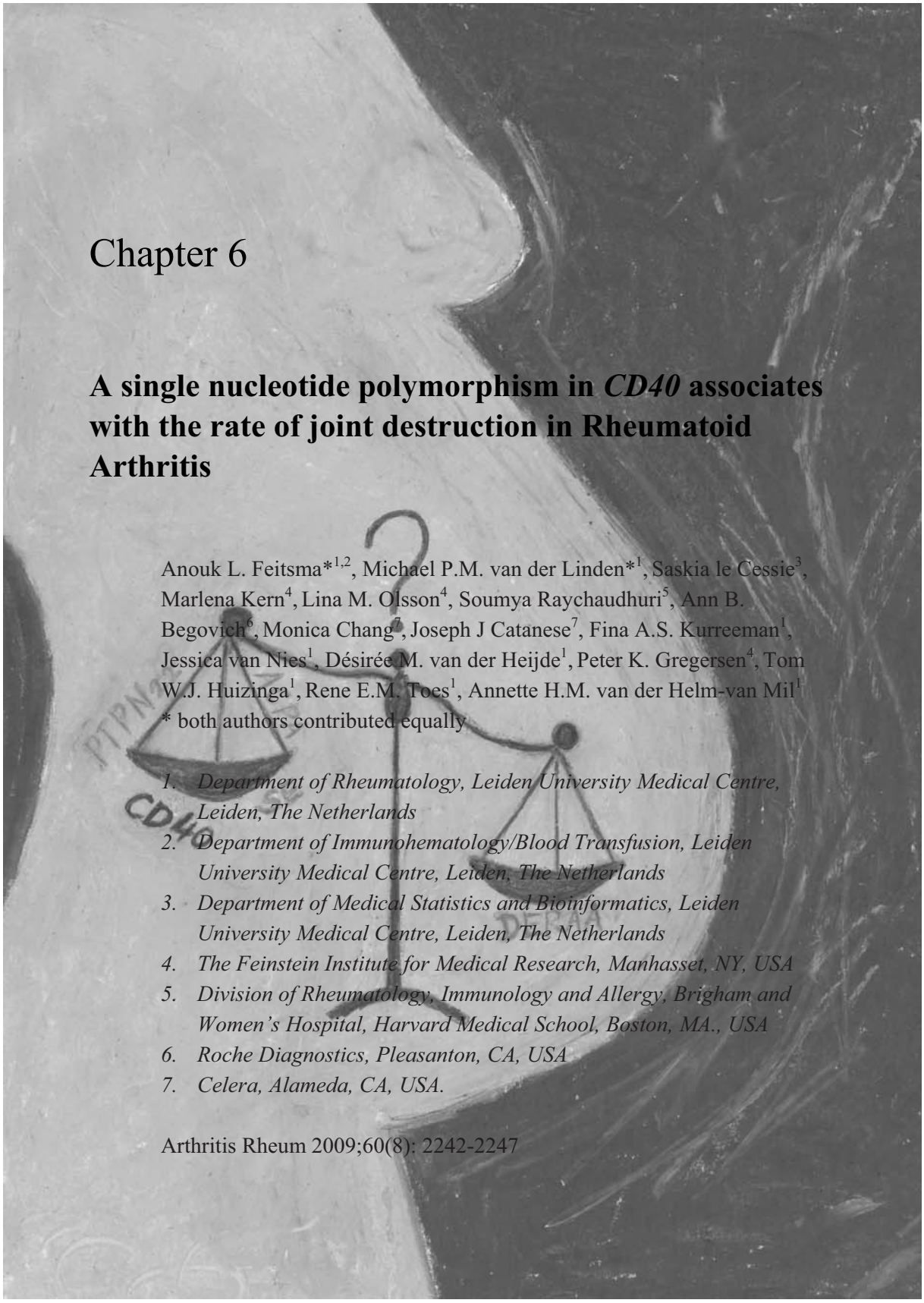

Chapter 6

A single nucleotide polymorphism in *CD40* associates with the rate of joint destruction in Rheumatoid Arthritis

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Abstract

Objective: The severity of joint destruction in Rheumatoid Arthritis (RA) is highly variable between patients and influenced by genetic factors. Genome-wide association studies (GWAs) have boosted the field of the genetics of susceptibility to RA enormously, but risk loci for severity of RA remain poorly defined. A recent meta-analysis of GWAs identified 6 genetic regions for susceptibility to autoantibody-positive RA, i.e. *CD40, KIF5A-PIP4K2C, CDK6, CCL21, PRKCQ* and *MMEL1-TNFRSF14*. We have investigated whether these newly described genetic regions associate with the rate of joint destruction.

Methods: RA-patients enrolled in the Leiden Early Arthritis Clinic were studied (n=563). Yearly radiographs were scored using the Sharp-van der Heijde method (median follow-up 5 years, maximal follow-up 9 years). The rate of joint destruction between genotype groups was compared using a linear mixed model correcting for age, gender and treatment-strategies. 393 ACPA-positive-RA-patients included in the NARAC with radiographic data were used for replication.

Results: The TT and CC/CG genotypes of two SNPs, rs4810485 *(CD40)* and rs42041 *(CDK6)* respectively, were associated with a higher rate of joint destruction in ACPA-positive RA (p=0.003 and 0.012), of which rs4810485 was significant after Bonferroni correction for multiple testing. The association of the *CD40* minor allele with radiographic progression rate was replicated in the NARAC cohort (p=0.021).

Conclusion: A polymorphism in the *CD40* locus is associated with the rate of joint destruction in ACPA-positive RA and provides one of the first non-HLA-related genetic severity-factors that is replicated.

Introduction

Rheumatoid arthritis (RA) is characterized by inflammatory arthritis and localized destruction of bone and cartilage. The severity of joint destruction is highly variable between patients and, according to twin studies, substantially influenced by genetic factors (1). Nevertheless, the precise contribution of genetic factors still has to be determined. To date only a small number of genetic risk-factors has been identified, and apart from HLA, none of these factors have been convincingly replicated.

In contrast, the genetics of susceptibility to RA has been boosted considerably, largely due to genome-wide association studies. In addition to the HLA-DRB1 shared epitope alleles, several new susceptibility-factors, *PTPN22*, *TRAF1-C5*, *OLIG3-TNFAIP3* and *STAT4*, have been identified and were independently replicated. Intriguingly, for many of these genetic risk-factors the associations are confined to anti-citrullinated protein antibodies (ACPA)-positive RA-patients. Whether genetic factors also differently affect the severity of joint destruction in ACPA-positive and ACPA-negative RA remains unknown. Nonetheless, compelling evidence demonstrates that ACPA-positive RA-patients have a more destructive disease course compared to ACPA-negative patients.

A recent meta-analysis on two genome-wide association studies identified six new risk loci (rs4810485 (*CD40*), rs1678542 (*KIF5A-PIP4K2C*), rs42041 (*CDK6*), rs2812378 (*CCL21*), rs4750316 (*PRKCQ*) and rs3890745 (*MMEL1-TNFRSF14*)) as susceptibility factors for autoantibody-positive RA (2). The present study aimed to investigate the association between these single-nucleotide-polymorphisms (SNPs) and the rate of radiological joint destruction in RA, and ACPA+ RA in particular, using a large longitudinal cohort. A cohort of ACPA-positive RA-patients was used for replication. This study shows that a genetic variant in the *CD40* gene associates with the rate of joint destruction in ACPA-positive RA.

Patients and methods

Patients

Five hundred sixty three RA-patients, consecutively included in the Leiden Early Arthritis Cohort (EAC) between 1993 and 2006 with both DNA and radiographs available were studied. The RA-patients fulfilled the 1987 ACR-criteria. Follow-up visits were performed yearly. Treatment strategies changed in time and differed for different inclusion periods (before 1996, 1996-1998, 1999-2001, after 2001) (see ref (3) for detailed description of the EAC). Anti-CCP2 antibodies were measured using stored baseline serum samples (Immunoscan RA Mark 2; Euro-Diagnostica, The Netherlands).

Replication cohort

393 ACPA-positive RA-patients that were included in the North American Rheumatoid Arthritis Consortium (NARAC) that had hand radiographs available were studied. As the radiographs were taken at different disease durations, the estimated radiological progression per year was determined by dividing the total Sharp-van der Heijde score of the hands by the disease duration at the time of the radiograph.

SNP genotyping

The six recently identified risk loci (2) were genotyped in the 563 RA-patients from the Leiden EAC using allele-specific kinetic PCR as previously described (4). The data were hand-curated without knowledge of clinical characteristics before statistical analysis with a 98% genotyping success rate; previous analyses suggest a genotyping accuracy of >99%. For the *MMEL1-TNFRSF14* locus, a perfect proxy of rs3890745 (reported in (2)) was used (rs6684865, r^2=1).

In the NARAC genotyping was performed using the Illumina Hapmap500 BeadChip, as described (5). Rs4810485 was not typed in the whole genome study, but a perfect proxy for this variant was genotyped (rs1569723, r^2=1). For *CDK6*, neither rs42041 nor a perfect proxy were genotyped and therefore the data on rs42041 was imputed as described (2).

Radiographs

In the EAC, radiographs of hands and feet, taken on consecutive years, were scored according to the Sharp-van der Heijde method (6). To encompass a reliable sample

size, radiographic follow-up data were restricted to a maximum of 9 years with a median of 5 years. All radiographs were scored by one experienced scorer who was blinded with respect to clinical and genetic data. 499 radiographs were rescored (149 baseline radiographs and 350 radiographs during follow-up from 60 randomly selected RA-patients). Intraclass-observer correlation coefficients (ICC) were 0.91 for all radiographs, 0.84 for baseline radiographs and 0.97 for the radiographic progression rate. In the NARAC the radiographs were scored by one reader blinded to clinical or genetic data. 25% of the radiographs were re-scored, the ICC was 0.99.

Statistical Analysis

Analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL). As radiographic data were not normally distributed, the raw data on the Sharp-van der Heijde scores are presented using medians (Figure 1-2) and were log-transformed in preparation for analysis. In the EAC, a linear model for longitudinal data was used to compare progression rates between groups. Age, gender, inclusion period (a proxy for treatment strategy) and their interactions with time were entered in the model to correct for possible confounding effects (see also the supplementary methods). As six SNPs were evaluated, a Bonferroni correction for multiple testing was applied; the p-value for significance was set at $p<0.008$. Only the SNPs that were clearly related to the progression rate in the EAC were analyzed in the replication cohort. In the NARAC, the estimated radiological progression per year was compared with the Kruskal Wallis test. No corrections were made for age, gender or treatment in this cohort.

Results

Baseline characteristics of the RA-patients are shown in Table 1. In the EAC, the minor allele frequencies were 0.242, 0.340, 0.267, 0.366, 0.204 and 0.307 for rs4810485, rs1678542, rs42041, rs2812378, rs4750316 and rs6684865 respectively and in agreement with previous results (2). The raw data on the Sharp-van der Heijde scores for the three genotypes at each SNP are depicted in Figure 1. To study the influence of the SNPs on the rate of joint destruction, a linear mixed model analysis was performed for each SNP. For rs4810485 (*CD40*) the GG and GT genotypes showed comparable radiographic scores, therefore the genotype data were combined and carriership-analysis was performed. Similarly, the CC and CG genotypes of rs42041 (*CDK6*) were pooled. In the total group of RA-patients an association was observed for rs42041 (*CDK6*) (p=0.033). For the other SNPs no significant association

Table 1. Patient characteristics at baseline

Patient Characteristics EAC	**N=563**
Age at inclusion (yrs), mean (SD)	56.0 (± 15.6)
Female, N (%)	394 (70.0)
Symptom duration at inclusion (months), mean (SD)	6.7 (± 10.5)
Swollen Joint Count, mean (SD)	5.72 (± 3.3)
Ritchie score, mean (SD)	10.3 (± 7.8)
ACPA-positive, N (%)[†]	250 (55.9)
IgM-RF-positive, N (%)[†]	322 (58.4)
HLA-DRB1 Shared Epitope +, N (%)[†]	339 (67.1)
CRP (mg/l), mean (SD)[†]	29.4 (± 34.2)
ESR (mm/h), mean (SD)[†]	39.5 (± 27.5)
HAQ, mean (SD)	1.1 (± 0.7)
Total Sharp-score, median (IQR)	5 (2-11)
Patient Characteristics NARAC	**N=393**
Age at disease onset	40.8 (± 11.9)
Female, N (%)	286 (72.8)
ACPA-positive, (%)[§]	100%
HLA-DRB1 Shared Epitope +, N (%)[§]	100%

[†] *Data on ACPA-, RF- and HLA DRB1 SE-status and CRP and ESR-levels were available in the EAC in 447, 551, 441, 520 and 544 out of 563 genotyped patients respectively.*

[§] *Data on ACPA- and HLA DRB1 SE-status was available for all of the 393 genotyped patients*.

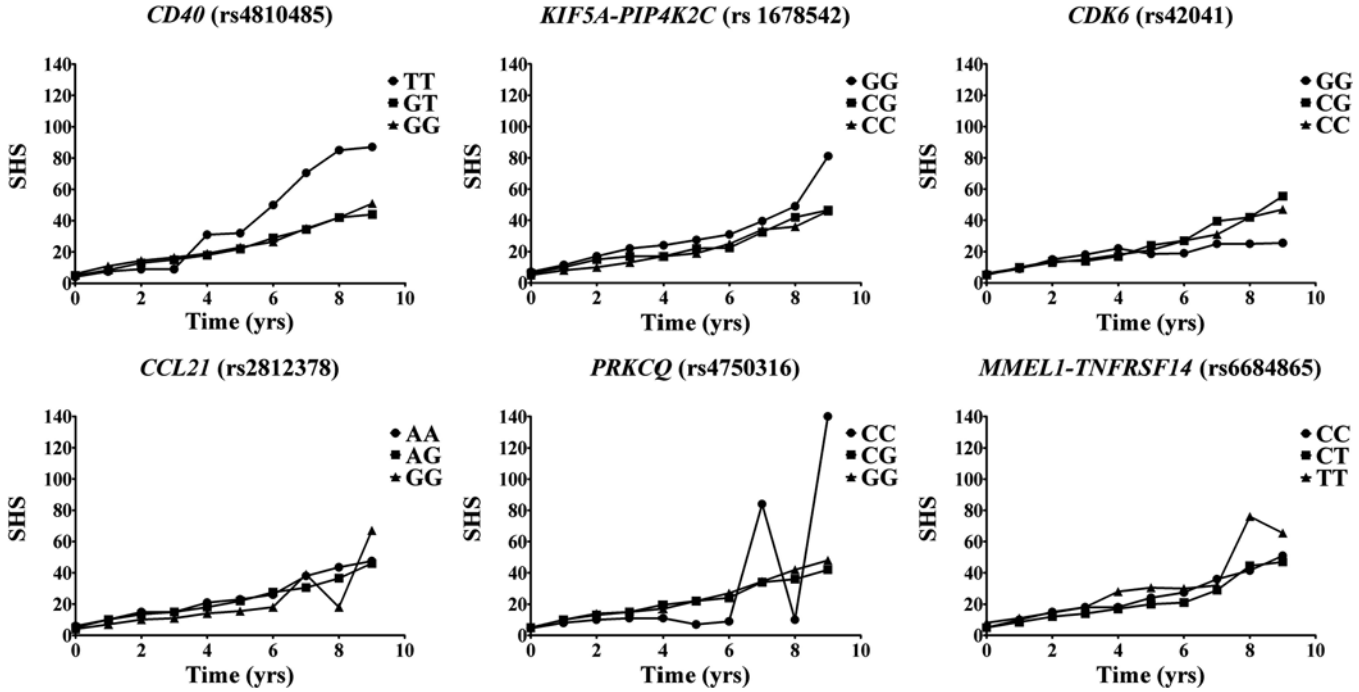
with the radiological progression over time was detected (p=0.268, 0.369, 0.679, 0.583 and 0.451 for rs4810485, rs1678542, rs2812378, rs4750316 and rs6684865 respectively). Because the genetic regions studied are thus far observed to be susceptibility-factors only for autoantibody-positive RA-patients, analyses were repeated in the ACPA-positive subgroup. Here, two polymorphisms, rs4810485 (*CD40*) and rs42041 (*CDK6*), affected the rate of joint destruction (Figure 2). For rs4810485, the G-allele was associated with a lower progression rate (GG/GT vs. TT, p=0.003). Back transforming the regression coefficient of the genotype in the model to the original scale yielded a 1.12 (95% CI 1.04-1.21) times larger increase in Sharp-score per year for carrying the risk genotype. For rs42041, the C-allele was associated with a higher rate of joint destruction (CC/CG vs. GG, p=0.012). For carriership of the C-allele a 1.09 (95% CI 1.02-1.16) larger yearly increase in Sharp-score was observed. Only rs4810485 was statistically significant after correction for multiple testing. The interaction between inclusion period and time was significant in all six analyses (p<0.001), demonstrating the effect of inclusion period on the radiological progression rate. Gender and age were not independently associated with progression.

To find replication, the effect of *CD40* and *CDK6* on radiological progression was analysed in 393 ACPA-positive RA-patients from the NARAC. Using a perfect proxy for rs4810485, the genotype associated with severity in the EAC also revealed a higher estimated radiological progression per year in the NARAC: 3.40 Sharp-units/year (n=23) vs. 2.83 and 1.83 Sharp-units/year (n=122 and 248, p=0.021). Using imputed data for rs42041 no significant differences between the three genotypes were observed (2.76, 2.38 and 2.07 Sharp-units/year, n=32, 163 and 188 respectively, p=0.327). The total number of patients available for analysis of rs42041 was 383; genotyping data were missing in 10 cases.

Discussion

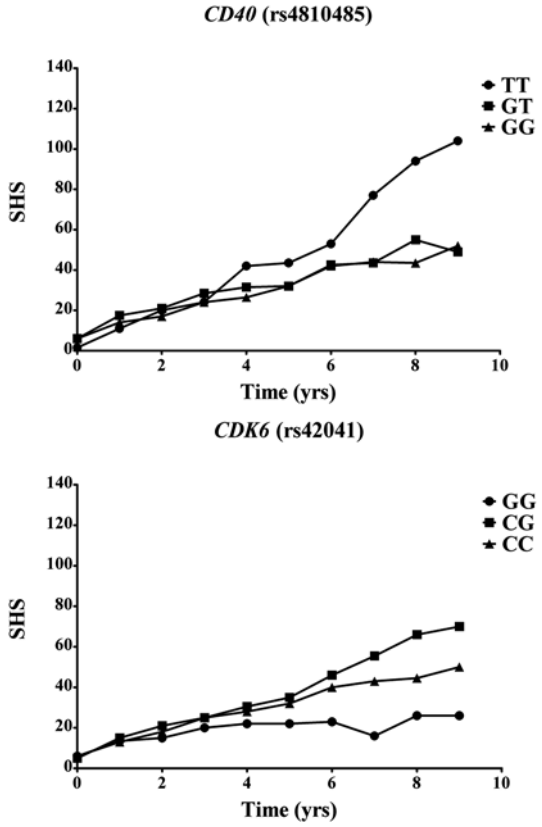
Although several clinical and serological risk factors for RA-severity are known, thus far the inter-individual variance in joint destruction is insufficiently explained and genetic factors are scarcely investigated. A better comprehension of the factors that mediate joint damage in RA may lead to the development targeted therapies or may contribute to prediction of the disease outcome in individual RA-patients. Most recently, six new loci were described to predispose to autoantibody-positive RA (2). Although susceptibility-factors do not necessarily affect disease progression, this study

Figure 1 Median Sharp-van der Heijde scores for the different SNPs per genotype in all RA-patients



Overview of the raw Sharp-van der Heijde scores, expressed as medians, of all 6 SNPs per genotype for the total patient population (n=563). The risk-alleles predisposing to RA in the study of Raychaudhuri et al (2) were the G-, C-, G-, G-, G- and T-allele for the rs4810485, rs1678542, rs42041, rs2812378, rs4750316 and rs6684865 SNPs respectively. The number of available radiographs varied per time-point and declined to 466 after 1 year of follow-up, 426, 357, 299 and 269 after 2 till 5 years of follow-up and 206, 154, 116 and 84 radiographs after 6, 7, 8 and 9 years of follow-up respectively. The number of patients in the different genotype groups were respectively: GG:280, GT:198, TT:22 (rs4810485); CC:247, CG:248, GG:67 (rs1678542); CC:305, CG:215, GG:43 (rs42041); AA:217, AG:279, GG:66 (rs2812378); CC:23, CG:183, GG:355 (rs4750316); CC:166, CT:170, TT:26 (rs6684865)*. *Due to technical difficulties genotyping was not successful in 63 and 201 of cases for rs4810485 and rs6684865 respectively. SHS: Sharp-van der Heijde score.*

Figure 2 Median Sharp-van der Heijde scores for rs4810485 and rs42041 in ACPA-positive RA



Overview of the raw Sharp-van der Heijde scores, expressed as medians, in ACPA-positive RA (n=250). The G-allele was the risk-allele predisposing to RA in the study of Raychaudhuri et al (2) for both the rs4810485 and rs42041 SNPs. The number of patients in the different genotype groups were for rs4810485*: GG: 128, GT: 88, TT: 11 and for rs42041: CC: 131, CG: 101, GG: 18. *genotype data for rs4810485 was not available in 23 cases. SHS: Sharp-van der Heijde score.

investigated whether these six SNPs are also risk-factors for a severe course of RA, measured by the rate of joint damage. The present data suggest that two SNPs, rs4810485 (*CD40*) and rs42041 (*CDK6*), influence the rate of joint destruction in ACPA-positive RA. Of these, only rs4810485 was significantly associated after correction for multiple testing and was replicated in an independent cohort of ACPA-positive RA-patients. As such, *CD40* is the first non-HLA-related genetic risk-factor for RA-severity that is independently replicated.

A recent study (2) reported a common variant at the *CD40* locus (the minor T-allele) to be protective for the development of RA. Surprisingly, here the minor T-allele associates with a higher rate of joint destruction in two cohorts. This finding is counter-intuitive, if one assumes that genetic variants associating with susceptibility also associate with severity. Although our findings were observed in two independent cohorts, and thus replicated, a type I error cannot be ruled out. The disease associated (common) allele marks a haplotype of *CD40* that contains a polymorphism in the upstream Kozak sequence that results in increased surface expression on B cells (7). To our knowledge, the effect of this haplotype on CD40 surface expression in synovial fibroblasts has not been directly studied. However, CD40-expression is increased on synoviocytes in RA and triggering of CD40 in synovial fibroblasts is associated with production of proinflammatory cytokines and osteoclastogenesis (8;9). It is likely that the biological pathways underlying susceptibility and severity are distinct with respect to CD40 triggering. This would provide an explanation for the finding that the minor T-allele has a protective effect in susceptibility studies but associates with a more severe disease course. Clearly it is essential to perform further studies on the mechanisms by which *CD40* polymorphisms associate with erosive outcome in RA.

A second SNP tended to associate with the rate of joint damage in RA in the EAC, rs42041. Absence of replication in the NARAC indicates that the observed association with the progression rate in the EAC cannot be interpreted. Nonetheless, it will be interesting to see the results on other studies analyzing *CDK6* and RA-severity. Thus, at present, of the two SNPs that tended to show an association with the rate of joint destruction, only the genetic variant in *CD40* is statistically significant after correction for multiple testing and is replicated and is therefore identified as a severity-factor for RA.

The other four studied SNPs in the loci encoding for *KIF5A-PIP4K2C, CCL21, PRKCQ* and *MMEL1-TNFRSF14* were not observed to associate with the severity of joint destruction. Therefore, these polymorphisms appear to be genetic risk-factors that are primarily associated with RA-susceptibility. Indeed, all of these SNPs were recently replicated as true susceptible loci in RA-patients of European ancestry (10).

The prospective nature of the data of the EAC strengthens the impact of the findings because higher radiological scores for risk-genotypes were present at subsequent time points; as such the present data set is advantageous in comparison to studies that

assessed cross-sectional radiological data. The fact that a large number of patients with a long follow-up of up to 9 years were included for analysis is clearly an advantage, but also has a limitation. Inherent to the design of an inception cohort, not all patients had achieved maximum follow-up, so the number of missing data that the mixed-model had to take into account increased with longer follow-up. Small numbers of radiographs available at the latest time points are also the most likely explanation for the observed "bump" at the 8 year time point for the genotypes GG, CC and TT of the SNPs rs2812378, rs4750316 and rs6684865 respectively (Figure 1).

Evaluation of the effect of genetic factors on the rate of joint destruction during the disease course inevitably implies that other factors that affect the disease course should be taken into consideration as well. Analyses for all six SNPs revealed that inclusion period, a proxy for treatment strategy, was significantly associated with the rate of joint damage, which is in line with previous results from the EAC (11). The analyses on *CD40* and *CDK6* showed that these SNPs were associated with joint damage, independent from treatment strategy. Nevertheless, corrections for treatment strategy were made on group-level and thus were an approximation for the real effect of treatment on the rate of joint destruction for individual RA-patients.

In conclusion, a polymorphism in the *CD40* locus shows a significant association with the rate of joint destruction in ACPA-positive RA, a finding that is replicated in an independent cohort. Although further studies are needed to identify the causal variant, the data presented provide a foundation for further investigations of the role of CD40 in joint destruction in RA.

Supplementary Methods

Statistical Analysis

To take advantage of the prospective character of the data of the EAC, consisting of repeated measurements, and to avoid multiple testing by performing statistical tests for each time point, a linear model for longitudinal data was used, with the log transformed Sharp-score as response variable, to compare the radiological progression rates between genotype groups. Different correlation structures between the repeated measurements were explored, and based on the Akaike's information criterion, an autoregressive correlation structure with heterogeneous variances was chosen. Due to

the study design (an inception cohort) not all patients achieved a similar duration of follow-up. The model takes missing observations into account, assuming that the missing is at random. Differences in progression rates between the different genotypes were tested by considering the significance of the interaction between genotype and time with time as linear covariate. Age, gender and inclusion period (before 1996, 1996-1998, 1999-2001, after 2001) and their interactions with time were entered in the model to correct for possible confounding effects. In order to prevent overfitting of the data no corrections were applied for other variables. Inclusion period is a proxy for treatment modalities, because treatment strategies improved over time and an influence of the treatment strategy on the progression of radiographic joint damage was observed previously, as well as in the present study. The following treatment strategies were applied in the subsequent inclusion periods. Patients included between 1993 and 1995 were treated initially with analgesics and subsequently with chloroquine or sulfasalazin if they had persistent active disease (delayed treatment). From 1996 to 1998 RA-patients were promptly treated with either chloroquine or sulfasalazin (early treatment) (3). From 1998 to 2002 patients were promptly treated with either sulfasalazin or methotrexate (early treatment) and patients included in 2002 or later were promptly treated with either sulfasalazin or methotrexate combined with treatment adjustments based on the disease activity (early and disease activity based treatment).

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References

(1) van der Helm-van Mil AH, Kern M, Gregersen PK, Huizinga TW. Variation in radiologic joint destruction in rheumatoid arthritis differs between monozygotic and dizygotic twins and pairs of unrelated patients. Arthritis Rheum 2006; 54(6):2028-30.

(2) Raychaudhuri S, Remmers EF, Lee AT, Hackett R, Guiducci C, Burtt NP et al. Common variants at CD40 and other loci confer risk of rheumatoid arthritis. Nat Genet 2008; 40(10):1216-23.

(3) van Aken J, van Bilsen JH, Allaart CF, Huizinga TW, Breedveld FC. The Leiden Early Arthritis Clinic. Clin Exp Rheumatol 2003; 21(5 Suppl 31):S100-S105.

(4) Begovich AB, Carlton VE, Honigberg LA, Schrodi SJ, Chokkalingam AP, Alexander HC et al. A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. Am J Hum Genet 2004; 75(2):330-7.

(5) Plenge RM, Seielstad M, Padyukov L, Lee AT, Remmers EF, Ding B et al. TRAF1-C5 as a risk locus for rheumatoid arthritis--a genomewide study. N Engl J Med 2007; 357(12):1199-209.

(6) van der Heijde D. How to read radiographs according to the Sharp/van der Heijde method. J Rheumatol 2000; 27(1):261-3.

(7) Jacobson EM, Concepcion E, Oashi T, Tomer Y. A Graves' disease-associated Kozak sequence single-nucleotide polymorphism enhances the efficiency of CD40 gene translation: a case for translational pathophysiology. Endocrinology 2005; 146(6):2684-91.

(8) Yellin MJ, Winikoff S, Fortune SM, Baum D, Crow MK, Lederman S et al. Ligation of CD40 on fibroblasts induces CD54 (ICAM-1) and CD106 (VCAM-1) up-regulation and IL-6 production and proliferation. J Leukoc Biol 1995; 58(2):209-16.

(9) Lee HY, Jeon HS, Song EK, Han MK, Park SI, Lee SI et al. CD40 ligation of rheumatoid synovial fibroblasts regulates RANKL-mediated osteoclastogenesis: evidence of NF-kappaB-dependent, CD40-mediated bone destruction in rheumatoid arthritis. Arthritis Rheum 2006; 54(6):1747-58.

(10) Barton A, Thomson W, Ke X, Eyre S, Hinks A, Bowes J et al. Rheumatoid arthritis susceptibility loci at chromosomes 10p15, 12q13 and 22q13. Nat Genet 2008; 40(10):1156-9.

(11) Lard LR, Boers M, Verhoeven A, Vos K, Visser H, Hazes JM et al. Early and aggressive treatment of rheumatoid arthritis patients affects the association of HLA class II antigens with progression of joint damage. Arthritis Rheum 2002; 46(4):899-905.