

Multiplex Screening of 22 Single-Nucleotide Polymorphisms in 7 Toll-like Receptors: An Association Study in Rheumatoid Arthritis

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ABSTRACT. *Objective.* Toll-like receptors (TLR) have been implicated in the pathogenesis of arthritis. We investigated the role of functional variants of TLR in the disease phenotype and severity of rheumatoid arthritis (RA).

Methods. All patients from a longterm observational inception cohort (n = 319) were genotyped for 22 single-nucleotide polymorphisms (SNP) in TLR2, 3, 4, 5, 7, 8, and 9 using multiplex assays. Clinical characteristics including sex, age at disease onset, rheumatoid factor (RF), and shared epitope positivity and disease activity score and radiological progression were taken into account. Genotypes were analyzed for association with Disease Activity Scores (DAS28) and joint damage (Rau scores) at 3 and 6 years.

Results. After Bonferroni correction, there was a moderate association between RF positivity and TLR8-rs5741883. No other TLR variant was significantly associated with any RA clinical characteristics.

Conclusion. Using a large inception cohort and strict statistical evaluation, we could not identify an association between functional TLR variants and RA phenotype and disease severity. This suggests the functional TLR variants do not play a major role in RA phenotype and disease severity. (First Release March 1 2010; J Rheumatol 2010;37:905–10; doi:10.3899/jrheum.090775)

Key Indexing Terms:

TOLL-LIKE RECEPTOR

RHEUMATOID ARTHRITIS

RHEUMATOID FACTOR

RADIOLOGICAL JOINT DAMAGE

DISEASE ACTIVITY SCORE

Rheumatoid arthritis (RA) is an autoimmune disease affecting multiple synovial joints, leading to significant morbidity and shortened life expectancy. Despite longstanding efforts, the precise mechanisms underlying the inflammatory processes remain to be elucidated. Toll-like receptors (TLR) were discovered to be crucial receptors triggering

innate immune responses. Currently, 10 TLR subtypes have been described in humans, all thought to have their own specific ligands and cellular localization (as reviewed¹). For example TLR1, 2, 4, 5, and 6 are located on the cell surface and scavenge the environment for ligands. In contrast, TLR3, 7, 8, and 9 have an intracellular localization and recognize intracellular ligands, including ligands that are endocytosed^{2,3}. Accumulating evidence suggests a pivotal role for TLR in the recognition of endogenous ligands and, as well, linking innate and adaptive immune responses. Recently, several groups have provided evidence for a role of TLR in arthritis in experimental disease conditions and in humans. It has been demonstrated that various TLR subtypes are expressed at higher levels in synovial tissues from patients with RA compared to those from healthy controls⁴⁻⁶. Ligands for TLR3 [host-derived (RNA) and TLR4 (HSPB8)] are also abundant in the circulation as well as in the synovial joints of patients with RA^{4,5,7}. Additionally, TLR4-mediated stimulation of dendritic cells from patients with RA leads to significantly higher cytokine concentrations compared to similar cells from healthy controls, further supporting a deranged TLR response in RA. On these grounds, we hypothesized that TLR might be involved in the pathogenesis of RA. To test this, we investigated potential

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associations between TLR variants and RA phenotype and severity.

MATERIALS AND METHODS

Ascertainment of patients. Genotyping was performed in RA patients participating in an early RA inception study started in 1985. Our study includes only those patients who met the American Rheumatism Association (American College of Rheumatology) criteria for RA⁸, had a disease duration < 1 year, and had not previously been treated with disease-modifying antirheumatic drugs (DMARD) or biological therapies. The local ethics committee approved the study.

Characterization of disease activity and outcome. Patients' demographic data such as sex, age at disease onset, and the presence of rheumatoid factor (RF), HLA-DR4 and the shared epitope were included in the analysis. We used the Disease Activity Score 28 (DAS28) and the Rau score at baseline and after 3 and 6 years of followup to determine the disease course and radiological joint progression, respectively^{9,10}. The use of DMARD was analyzed using essentially the same protocols as described^{11,12}.

Selection of SNP and description of assay. SNP selected for the assays were primarily functional SNP, and selection was based upon information available at the dbSNP (US National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/SNP/>), SNPper (Children's Hospital Informatics Program, Boston, MA, USA; <http://snpper.chip.org/bio/>), and IIPGA (<http://www.innateimmunity.net/>) databases (Table 1). Thirteen SNP located in the human TLR2, 4, 5, and 9 genes, and 9 SNP located in the human TLR3, 7, and 8 genes were assessed in 2 multiplexed bead-based assays using a Luminex 100IS flow cytometer (Luminex Corp., Austin, TX, USA). The tests were based on described procedures, with some modifications (a detailed protocol is available from the authors)¹³⁻¹⁵.

Statistical analysis. Frequencies of the TLR genotypes were tested for Hardy-Weinberg equilibrium using the standard goodness-of-fit test. Similarity of genotype and allele distribution between patients and controls was tested with chi-square tests for 3 × 2 contingency tables. Differences in the disease characteristics between patients were analyzed using Student's t test or Mann-Whitney U test. For the TLR located on the X-chromosome, genotype and allele frequencies were recalculated after stratification for sex. Correction for multiple testing was performed using the Bonferroni correction. P values ≤ 0.002 were considered statistically significant.

Power calculations were performed using Quanto. Based on a sample size of 177 and 319 individuals the power to detect a locus that explained 5% variation of the continuous trait using a Bonferroni corrected p value of 0.002 was 47% and 83%, respectively.

RESULTS

In total, DAS28 was present for all patients at baseline and after 3 and 6 years. Radiographs were present from 272 patients at baseline, 240 at 3 years, and 177 at 6 years of followup. Demographic variables including age, sex, presence of RF, and age at disease onset were documented for these patients. The SNP in TLR5-rs5744176 (Asp694Gly), TLR7-rs3853839 (Ala448Val), and TLR8-rs5744088 (3'-UTR) were not polymorphic in our RA population.

For the extracellular TLR subtypes (TLR2, 4, and 5) no association was observed between the genotypes and age at onset, sex, or presence of RF (data not shown). Similarly, no association was found between the genotypes of the diverse TLR subtypes and the disease activity and radiological joint damage at baseline (data not shown) and after 3 and 6 years of followup (Table 2).

With respect to TLR3, 7, 8, and 9 located intracellularly,

no significant associations were observed between the genotypes and sex or age at disease onset. RA patients carrying the TLR8-rs5741883 C allele were significantly more positive for RF, which was clearly correlated with a gene-dose effect (heterozygous for the C allele 76%; p = 0.02, homozygous for the C allele 88%; p = 0.001) compared to patients homozygous for the T allele (67% RF-positive). We found an association between TLR3-rs3775291 and joint score after 6 years (Table 3). In addition, we found an association between TLR8 and DAS28 after 3 years (rs3764879) and 6 years (rs3764879 and rs3764880) (Table 3). After correction for multiple testing and sex (for TLR8, as this gene is located on the X-chromosome), the significant associations between TLR SNP and disease phenotype or severity were lost.

DISCUSSION

Much research has focused on the role of TLR in autoimmune diseases, including RA, systemic lupus erythematosus, multiple sclerosis, and inflammatory bowel diseases. A substantial body of evidence points to a role for TLR in RA. One way to investigate the role of TLR themselves is to study genetic variants (e.g., SNP) in the TLR genes that might lead to an altered ligand binding capacity and/or expression leading to an altered TLR-mediated response that might subsequently translate into variations in disease activity and/or severity.

Using a well documented prospective cohort of 319 patients with RA, we were unable to show any significant effect of TLR SNP on RA disease variability and/or severity. Although the total group could be considered large enough, the genotypic distribution of many genes led to very small subgroups, strongly affecting the power of the study and increasing the risk of rejecting clinical associations. Our investigation underscores the need for multicenter studies to evaluate the potential influence of genetic variants on the outcome and behavior of complex diseases such as RA.

Triggering TLR initiates complex cascades of downstream adapter molecules, e.g., MYD88, TRIF, and IRAK, eventually ending in nuclear factor- κ B signaling and cell activation. Perhaps genetic, posttranscriptional, and/or post-translational modifications in these adapter molecules might explain the deranged TLR response observed in RA. Such relationships would not have been detected in our study, and further research focusing on these molecules is therefore warranted.

Our results suggest potential associations between some TLR SNP and RA phenotype, such as the TLR8 SNP and RF positivity. After correction for multiple testing, however, none of these associations reached statistical significance. Multicenter studies are needed to replicate and validate these results.

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Table 1. Allele-specific primer extension sequences.

SNP	Functional Effect	Allele	FlexMAP	ASPE Primer Sequence ("Tagged")	Direction	PCR Primer Sequence	Size PCR product (bp)
TLR2	Promoter (-15507)	Major	LUA-28	CTACAAACAAACAAACATTAATCAATAGTAAATAAATCCAGAGAAATCA	Forward	GAATAATGAATGAGCAAGCAAA	270
RS1898830		Minor	LUA-70	ATACCAATATCCAAATTCATATCATAGTAAATAAATCCAGAGAAATCG	Reverse	ATGCCCTCTGCTTATGTCA	
TLR2	Pro53His	Major	LUA-18	TCMAATCTCAATACTCAATCAATCAGACGCGCAAAAGGAAGCA	Forward	GTTTCATGGCTGTGGTAT	493
RS5743704		Minor	LUA-41	TTACTACACAATATACTCATCAATAGGCAAAAGGAAGCC	Reverse	CAAAATCCTCCCGCTGAG	
TLR2	Arg753Gln	Major	LUA-30	TTACTCTTATACCTTTCTTTTACGCTTGGTGTTTATATCTTCT	Forward	GTTTCATGGCTGTGGTAT	493
TLR3	Leu412Phe	Major	LUA-88	TACACTTCTTCTTCTTCTTATAGATTATCTTGGTTAGTTGAG	Reverse	CAAAATCCTCCCGCTGAG	
RS3775291		Minor	LUA-56	CAATTTACTCATATACATCACTTTAGATTATCTTGGTTAGTTGAA	Forward	CTGTGAGTTCTTGCCCAT	290
TLR4	3'UTR	Major	LUA-72	TCATTTACCTTTAATCCAAATCAATCAAGCTGTATGACAGAGTTGG	Reverse	GGAGGAAGGAGGAATGAGG	208
RS7873784		Minor	LUA-07	CAATTCATTTACCAATTTACCAATCACTAGCTATGACAGAGTTCC	Forward	CACCTCCAAAGCTTCTCTTG	
TLR4	Asp299Gly	Major	LUA-24	TCAATTAATCTTCAATCAATCACTATGACTAGCTACTACCTCGATGA	Reverse	TCAAATTTGGAATGCTGGAA	463
RS4986790		Minor	LUA-25	CTTTTCATTTACTTCAATCTTCACTAGACTACTACCTCGATGG	Forward	TGCAATTTGACCATTAAGAA	
TLR4	Thr389Ile	Major	LUA-16	AATCAATCTTCAATCAATCATCAAGTGAATTTGGGACAAAC	Reverse	TCAAATTTGGAATGCTGGAA	463
RS4986791		Minor	LUA-57	CAATATCATCTTCTTATCACTTCACTTCACTAGACTACTACCTCGATGG	Forward	TGCAATTTGACCATTAAGAA	
TLR5	Thr82Ile	Major	LUA-26	TTACTCAAAATCTACACTTTTTTCACTTGTCAATAGTCAAGGGGA	Reverse	TCCCAATTAAGGATGAGG	353
RS764535		Minor	LUA-23	TTCAATCATCAATCTCAACTTTTTTGTCAATAGTCAAGGGGG	Forward	GCTCTGCTGAGCTTCAACT	
TLR5	Arg352STOP	Major	LUA-20	CTTTTACAATCTTCAATCAATCAATCAATCAAGCTTGGATCTCC	Reverse	CGGACTTGACACCTCCAAG	1134
RS5744168		Minor	LUA-67	TCATTTACTCAAAATTAACAAATCAAAATTAACAAATTAACAAATTAAC	Forward	AAAGCATTTCTGACCCCATGT	
TLR5	Asn592Ser	Major	LUA-21	AATCTTTCTTAACTCAATCAATCAATCAATCAATCAATCAATCAATCAAT	Reverse	CGGACTTGACACCTCCAAG	1134
RS2072493		Minor	LUA-22	AATCTTTCTTAACTCAATCAATCAATCAATCAATCAATCAATCAATCAAT	Forward	AAAGCATTTCTGACCCCATGT	
TLR5	Phe616Ile	Major	LUA-49	TCATCAATCTTCAATTTACTTACGTTGACCTGACCTGACCTG	Reverse	CGGACTTGACACCTCCAAG	1134
RS5744174		Minor	LUA-33	TCAATTTACTTCAATTTAACTTTTGTGACCTGACCTGCTG	Forward	AAAGCATTTCTGACCCCATGT	
TLR5	Asp594Gly	Major	LUA-80	CTAATCAACAATTAATCAATCAATCAATCAATCAATCAATCAATCAATCAAT	Reverse	CGGACTTGACACCTCCAAG	1134
RS5744176		Minor	LUA-96	ATACTAACTCAACTTAATCAATCAATCAATCAATCAATCAATCAATCAATCAAT	Forward	AAAGCATTTCTGACCCCATGT	
TLR7	Exon/intron boundary	Major	LUA-65	CTTTTCATCAATCTTCAATTTACTTGTGCTGCTTGTGAATGTAACTTTT	Reverse	CGGACTTGACACCTCCAAG	1134
RS2302267		Minor	LUA-47	CITCTCAATTAATCTTCAATTTACTTGTGCTGCTTGTGAATGTAACTTTT	Forward	AAAGCATTTCTGACCCCATGT	
TLR7	Gln11Leu	Major	LUA-76	AATCTCAACAATCTCAATTAATCAATCAATCAATCAATCAATCAATCAATCAAT	Reverse	CGGACTTGACACCTCCAAG	156
RS179008		Minor	LUA-77	CAATTAATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAAT	Forward	AAAGCATTTCTGACCCCATGT	
TLR7	Ala48Val	Major	LUA-98	AATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAAT	Reverse	CGGACTTGACACCTCCAAG	192
RS5743781		Minor	LUA-50	CAATATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAAT	Forward	AAAGCATTTCTGACCCCATGT	
TLR7	3'UTR	Major	LUA-34	TCATTCATATACATACCAATTCATCAATCAATCAATCAATCAATCAATCAATCAAT	Reverse	CGGACTTGACACCTCCAAG	240
RS3853839		Minor	LUA-09	TAATCTTATATCAACATCTTCAACGAGCGGCCAAGC	Forward	ACCAATTTGCTTCCGTTGTCAT	276
TLR8	Promoter (-605)	Major	LUA-90	GTAATATCTTCAACATCTTCAACGAGCGGCCAAGC	Reverse	CTTTGACGTCGAGATAAAACA	
RS5741883		Minor	LUA-40	CTTTTACATTAATCAACATTAATCAACGAGCGGCCAAGC	Forward	ATTTTCCAGCTCAGGAATG	744
TLR8	Promoter (-129)	Major	LUA-48	AAACAACTTCAACATCTTCAACGAGCGGCCAAGC	Reverse	CTTTGACGTCGAGATAAAACA	
RS3764079		Minor	LUA-62	TCATCAATTAATCAACATTAATCAACGAGCGGCCAAGC	Forward	ATTTTCCAGCTCAGGAATG	744
TLR8	Exon (-3679)	Major	LUA-87	AACTAATCAATCAACATTAATCAACGAGCGGCCAAGC	Reverse	CTTTGACGTCGAGATAAAACA	
RS3764880		Minor	LUA-89	TATACATCAATCAACATTAATCAACGAGCGGCCAAGC	Forward	ATTTTCCAGCTCAGGAATG	744
TLR8	3'UTR	Major	LUA-32	ATTATTCATTTCAAACTTAATCAACGAGCGGCCAAGC	Reverse	CTTTGACGTCGAGATAAAACA	
RS5744088		Minor	LUA-55	TATATACATTTCAAACTTAATCAACGAGCGGCCAAGC	Forward	ATTTTCCAGCTCAGGAATG	162
TLR9	Promoter (-1486)	Major	LUA-64	TACATATCAATCAACATTAATCAACGAGCGGCCAAGC	Reverse	CTTTGACGTCGAGATAAAACA	
RS187084		Minor	LUA-82	TACATATCAATCAACATTAATCAACGAGCGGCCAAGC	Forward	ATTTTCCAGCTCAGGAATG	379
TLR9	Promoter (-1237)	Major	LUA-02	CTTTTCAATCAATCAACATTAATCAACGAGCGGCCAAGC	Reverse	CTTTGACGTCGAGATAAAACA	
RS5743836		Minor	LUA-38	TCAATCATTAATCAACATTAATCAACGAGCGGCCAAGC	Forward	ATTTTCCAGCTCAGGAATG	379

TLR: Toll-like receptor; PCR: polymerase chain reaction; bp: basepairs.

SNP column indicates TLR gene location and RefSNP SNP identification codes (rs numbers) as applied in the public nucleic acid polymorphism databases at NCBI. Mutation indicates the location and/or effect of the polymorphism on the resulting receptor. Allele indicates major and minor alleles of the SNP. FlexMAP: FlexMAP beadset corresponding to the ASPE primer sequence. The first 24 nucleotides of all ASPE primers constitute the 'Tag' and are complementary to sequences on FlexMAP beadsets. Direction indicates the direction of PCR primers. Note that PCR primer amplicons can contain more than one SNP locus. Size is the predicted size in base pairs (bp) of the PCR product that is produced using the primers indicated. Primer sequences are in the 5' → 3' direction.

Table 2. Genotype distribution of extracellular Toll-like receptors (TLR) and association with disease severity and/or radiological joint damage.

TLR	rs Number	Genotype			p
TLR2	rs1898830	AA (n = 145)	AG (n = 139)	GG (n = 35)	
DAS28 3 yrs (n = 319)		3.9 ± 1.0	3.9 ± 1.2	4.0 ± 1.1	0.8
DAS28 6 yrs (n = 319)		3.8 ± 0.9	3.8 ± 1.2	4.0 ± 0.9	0.6
Rau Score 3 yrs (n=240)		8 (1-20)	5 (0-12)	6 (2-13)	0.2
Rau Score 6 yrs (n=177)		16 (5-29)	12 (2-21)	18 (2-26)	0.3
TLR2	rs5743704	CC (n = 297)	CA (n = 22)	AA (n = 0)	
DAS28 3 yrs		3.9 ± 1.1	4.0 ± 0.9	—	0.6
DAS28 6 yrs		3.8 ± 1.0	3.8 ± 0.9	—	0.8
Rau score 3 yrs		7 (0-17)	4 (3-9)	—	0.6
Rau score 6 yrs		15 (3-26)	5 (2-15)	—	0.06
TLR2	rs5743708	GG (n = 300)	GA (n = 19)	AA (n = 0)	
DAS28 3 yrs		3.9 ± 1.1	4.0 ± 1.1	—	0.6
DAS28 6 yrs		3.8 ± 1.0	3.8 ± 1.0	—	0.9
Rau score 3 yrs		6 (0-17)	8 (4-13)	—	0.7
Rau score 6 yrs		14 (3-26)	17 (11-24)	—	0.5
TLR4	rs7873784	GG (n = 214)	CG (n = 97)	CC (n = 8)	
DAS28 3 yrs		3.8 ± 1.1	4.0 ± 1.1	3.8 ± 0.7	0.5
DAS28 6 yrs		3.8 ± 1.0	3.8 ± 1.0	3.7 ± 0.7	1.0
Rau score 3 yrs		6 (0-16)	8 (0-13)	6 (2-19)	0.5
Rau score 6 yrs		15 (1-26)	14 (4-25)	11 (2-32)	1.0
TLR4	rs4986790	CC (n = 287)	CT (n = 31)	TT (n = 1)	
DAS28 3 yrs		3.9 ± 1.1	4.1 ± 1.1	2.8	0.3
DAS28 6 yrs		3.8 ± 1.0	4.1 ± 1.1	—	0.2
Rau score 3 yrs		6 (0-15)	12 (3-25)	7	0.2
Rau score 6 yrs		13 (2-25)	18 (14-31)	—	0.3
TLR4	rs4986791	AA (n = 296)	AG (n = 31)	GG (n = 1)	
DAS28 3 yrs		3.9 ± 1.1	4.1 ± 1.2	2.8	0.3
DAS28 6 yrs		3.8 ± 1.0	4.1 ± 1.2	—	0.6
Rau score 3 yrs		6 (0-16)	12 (3-24)	7	0.9
Rau score 6 yrs		13 (2-26)	18 (12-28)	—	0.2
TLR5	rs764535	GG (n = 308)	AG (n = 11)	AA (n = 0)	
DAS28 3 yrs		3.9 ± 1.1	4.6 ± 1.0	—	0.05
DAS28 6 yrs		3.8 ± 1.0	4.5 ± 0.7	—	0.07
Rau score 3 yrs		7 (1-16)	5 (0-15)	—	0.7
Rau score 6 yrs		14 (3-26)	25 (17-34)	—	0.2
TLR5	rs5744168	CC (n = 269)	CT (n = 48)	TT (n = 2)	
DAS28 3 yrs		3.9 ± 1.0	3.9 ± 1.4	4.4 ± 1.0	0.8
DAS28 6 yrs		3.9 ± 1.0	3.6 ± 1.2	4.2 ± 1.6	0.7
Rau score 3 yrs		7 (1-17)	4 (0-12)	3 (3-3)	0.3
Rau score 6 yrs		15 (3-26)	11 (1-21)	—	0.6
TLR5	rs2072493	AA (n = 262)	AG (n = 53)	GG (n = 4)	
DAS28 3 yrs		3.9 ± 1.1	3.8 ± 1.0	3.5 ± 1.2	0.6
DAS28 6 yrs		3.9 (1.0)	3.8 ± 1.0	2.9 ± 1.4	0.4
Rau score 3 yrs		6 (0-16)	9 (2-14)	22 (0-41)	0.5
Rau score 6 yrs		13 (2-26)	16 (10-23)	22 (14-34)	0.7
TLR5	rs5744174	CC (n = 68)	TC (n = 158)	TT (n = 90)	
DAS28 3 yrs		3.9 ± 1.0	4.0 ± 1.1	3.8 ± 1.0	0.4
DAS28 6 yrs		3.9 ± 1.0	3.9 ± 1.0	3.8 ± 0.9	0.5
Rau score 3 yrs		5 (0-18)	5 (0-18)	6 (0-18)	0.7
Rau score 6 yrs		11 (1-29)	16 (2-25)	14 (5-26)	0.5

For DAS28 the results are presented as mean (SD) and for the Rau score, median (range). n: number of patients in each genotype group.

Table 3. Genotype distribution of intracellular Toll-like receptors (TLR) and association with disease severity and/or radiological joint damage.

Gene	rs Number	Genotype (no. of patients)			p
TLR3	rs3775291	CC (n = 158)	CT (n = 140)	TT (n = 31)	
DAS28 3 yrs		3.9 ± 1.1	3.9 ± 1.0	4.2 ± 1.2	0.4
DAS28 6 yrs		3.8 ± 1.1	3.9 ± 1.0	3.9 ± 1.3	0.8
Rau score 3 yrs (n = 240)		8 (1-19)	7 (1-14)	4 (0-8)	0.2
Rau score 6 yrs (n = 177)		18 (4-32)	12 (2-21)	7 (1-16)	0.04
TLR7	rs2302267	GG (n = 7)	TG (n = 17)	TT (n = 305)	
DAS28 3 yrs		3.9 ± 1.2	4.0 ± 1.2	3.9 ± 1.1	0.7
DAS28 6 yrs		3.5 ± 1.0	4.4 ± 1.0	3.8 ± 1.1	0.1
Rau score 3 yrs		6 (2-14)	8 (3-19)	6 (1-16)	0.8
Rau score 6 yrs		3 (0-16)	22 (11-28)	14 (3-26)	0.3
TLR7	rs179008	AA (n = 219)	TA (n = 72)	TT (n = 38)	
DAS28 3 yrs		3.8 ± 1.1	4.1 ± 1.2	4.1 ± 1.1	0.2
DAS28 6 yrs		3.8 ± 1.0	4.0 ± 1.1	3.87 ± 1.0	0.4
Rau score 3 yrs		6 (0-15)	6 (1-14)	12 (2-23)	0.2
Rau score 6 yrs		16 (4-26)	12 (2-24)	12 (3-32)	0.6
TLR7	rs3853839	CC (n = 258)	GC (n = 47)	GG (n = 24)	
DAS28 3 yrs		3.9 ± 1.1	3.9 ± 1.1	3.6 ± 1.4	0.5
DAS28 6 yrs		3.9 ± 1.0	3.7 ± 1.1	3.7 ± 1.3	0.5
Rau score 3 yrs		7 (1-17)	7 (3-16)	0 (0-14)	0.09
Rau score 6 yrs		14 (3-25)	17 (2-26)	15 (1-37)	0.9
TLR8	rs5741883	CC (n = 217)	CT (n = 70)	TT (n = 42)	
DAS28 3 yrs		3.9 ± 1.1	4.0 ± 1.2	3.5 ± 1.1	0.1
DAS28 6 yrs		3.9 ± 1.0	3.8 ± 1.1	3.4 ± 0.9	0.1
Rau score 3 yrs		7 (1-17)	6 (0-19)	1 (0-3)	0.8
Rau score 6 yrs		13 (3-25)	20 (4-32)	12 (5-22)	0.5
TLR8	rs3764879	AA (n = 225)	AG (n = 63)	GG (n = 41)	
DAS28 3 yrs		3.8 ± 1.1	4.2 ± 1.2	4.0 ± 1.1	0.04
DAS28 6 yrs		3.7 ± 1.1	4.1 ± 1.0	4.0 ± 0.9	0.03
Rau score 3 yrs		6 (1-14)	10 (0-21)	8 (1-18)	0.5
Rau score 6 yrs		12 (3-26)	20 (1-25)	15 (2-20)	0.8
TLR8	rs3764880	AA (n = 223)	AG (n = 62)	GG (n = 44)	
DAS28 3 yrs		3.8 ± 1.1	4.2 ± 1.0	4.0 ± 1.1	0.04
DAS28 6 yrs		3.7 ± 1.0	4.1 ± 1.0	4.0 ± 0.9	0.08
Rau score 3 yrs		6 (1-14)	9 (0-20)	8 (1-18)	0.5
Rau score 6 yrs		13 (3-26)	19 (1-25)	15 (2-21)	0.8
TLR9	rs187084	AA (n = 95)	AG (n = 178)	GG (n = 56)	
DAS28 3 yrs		3.9 ± 1.2	3.8 ± 1.2	4.1 ± 0.	0.5
DAS28 6 yrs		3.8 ± 1.1	3.8 ± 1.1	4.0 ± 0.7	0.7
Rau score 3 yrs		6 (1-13)	5 (0-15)	13 (4-21)	0.2
Rau score 6 yrs		14 (3-26)	12 (1-25)	21 (5-29)	0.5
TLR9	rs5743836	AA (n = 235)	AG (n = 82)	GG (n = 12)	
DAS28 3 yrs		3.9 ± 1.1	4.0 ± 1.0	5.0 ± 1.1	0.8
DAS28 6 yrs		3.8 (1.1)	4.0 ± 1.1	3.8 ± 1.0	0.6
Rau score 3 yrs		6 (1-16)	8 (1-23)	4 (3-8)	0.5
Rau score 6 yrs		14 (3-25)	15 (3-37)	8 (3-14)	0.4

For DAS28 results are presented as mean (SD) and for the Rau score, median (range). n: number of patients in each genotype group.

REFERENCES

- Roelofs MF, Abdollahi-Roodsaz S, Joosten LA, van den Berg WB, Radstake T. The orchestra of Toll-Like receptors and their potential role in frequently occurring rheumatic conditions. *Arthritis Rheum* 2008;58:338-48.
- Bave U, Magnusson M, Eloranta ML, Perers A, Alm GV, Ronnblom L. Fc gamma RIIa is expressed on natural IFN-alpha-producing cells (plasmacytoid dendritic cells) and is required for the IFN-alpha production induced by apoptotic cells combined with lupus IgG. *J Immunol* 2003;171:3296-302.
- Means TK, Latz E, Hayashi F, Murali MR, Golenbock DT, Luster AD. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. *J Clin Invest* 2005;115:407-17.

4. Radstake TR, Roelofs MF, Jenniskens YM, Oppers-Walgreen B, van Riel PL, Barrera P, et al. Expression of toll-like receptors 2 and 4 in rheumatoid synovial tissue and regulation by proinflammatory cytokines interleukin-12 and interleukin-18 via interferon-gamma. *Arthritis Rheum* 2004;50:3856-65.
5. Roelofs MF, Joosten LA, Abdollahi-Roodsaz S, van Lieshout AW, Sprong T, van den Hoogen FH, et al. The expression of toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. *Arthritis Rheum* 2005;52:2313-22.
6. Ospelt C, Brentano F, Rengel Y, Stanczyk J, Kolling C, Tak PP, et al. Overexpression of toll-like receptors 3 and 4 in synovial tissue from patients with early rheumatoid arthritis: toll-like receptor expression in early and longstanding arthritis. *Arthritis Rheum* 2008;58:3684-92.
7. Brentano F, Schorr O, Gay RE, Gay S, Kyburz D. RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3. *Arthritis Rheum* 2005;52:2656-65.
8. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315-24.
9. Westhoff G, Rau R, Zink A. Radiographic joint damage in early rheumatoid arthritis is highly dependent on body mass index. *Arthritis Rheum* 2007;56:3575-82.
10. Westhoff G, Rau R, Zink A. Rheumatoid arthritis patients who smoke have a higher need for DMARDs and feel worse, but they do not have more joint damage than non-smokers of the same serological group. *Rheumatology* 2008;47:849-54.
11. Radstake TR, Blom AB, Sloetjes AW, van Gorselen EO, Pesman GJ, Engelen L, et al. Increased Fc gamma RII expression and aberrant tumour necrosis factor alpha production by mature dendritic cells from patients with active rheumatoid arthritis. *Ann Rheum Dis* 2004;63:1556-63.
12. Radstake TR, Franke B, Hanssen S, Netea MG, Welsing P, Barrera P, et al. The Toll-like receptor 4 Asp299Gly functional variant is associated with decreased rheumatoid arthritis disease susceptibility but does not influence disease severity and/or outcome. *Arthritis Rheum* 2004;50:999-1001.
13. Taylor JD, Briley D, Nguyen Q, Long K, Iannone MA, Li MS, et al. Flow cytometric platform for high-throughput single nucleotide polymorphism analysis. *Biotechniques* 2001;30:661-6, 668-9.
14. Ye F, Li MS, Taylor JD, Nguyen Q, Colton HM, Casey WM, et al. Fluorescent microsphere-based readout technology for multiplexed human single nucleotide polymorphism analysis and bacterial identification. *Hum Mutat* 2001;17:305-16.
15. Rozen S, Skaletsky H. Primer 3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000;132:365-86.