

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 controls^{4,6}. 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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Supported by the Lundbeck Foundation and the Danish Biotechnology Program. Dr. Radstake is a VIDI Laureate of the Dutch Organization of Research (NWO).

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Accepted for publication December 3, 2009.

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.

ACKNOWLEDGMENT

Pia Grothe Meinke is thanked for excellent technical assistance.

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	TCMAATCTCAATACTCAATCAATCAGAGGCCAAAGGAAGCA	Forward	GTTTCCATGGCCTGGTAT	493
RS5743704		Minor	LUA-41	TTACTACACAATATACTCATCAATAGGCCAAAGGAAGGCC	Reverse	CAAAATCCTCCCGCTGAG	
TLR2	Arg753Gln	Major	LUA-30	TTACTCTTATACCTTTCTTTTACGCTTGGTGTTCATATCTCT	Forward	GTTTCCATGGCCTGGTAT	493
TLR3	Leu412Phe	Major	LUA-88	TACACTTCTTCTTCTTCTTATAGATTTATCTTGGTAGTTGAG	Reverse	CAAAATCCTCCCGCTGAG	
RS3775291		Minor	LUA-56	CAATTTACTCATATACATCACTTTAGATTTATCTTGGTAGTTGAA	Forward	CTGTGAGTTCTTGCCCAT	290
TLR4	3'UTR	Major	LUA-72	TCATTTACCTTTAATCCAAATCAATCAAGCTGTATGACAGAGTTGG	Reverse	GGAGGAAGGAGGAATGAGG	208
RS7873784		Minor	LUA-07	CAATTCATTTACCAATTTACCAATCAATCACTAGCTATGACAGAGTTCC	Forward	CACCTCCAAAGCTTCTCTTG	
RS4986790	Asp299Gly	Major	LUA-24	TCAATTCATTTCAATCAATCAATCACTAGCTATGACAGAGTTGGA	Reverse	TGCAATTTGGAATGCTGGAAA	463
TLR4	Thr389Ile	Major	LUA-25	CTTTTCATTTCAATCTTCAATCTTCACTAGCTATGACAGAGTTGG	Forward	TGCAATTTGACCATTTGAAGAA	463
RS4986791		Minor	LUA-16	AATCAATCTTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	TGCAATTTGACCATTTGAAGAA	
TLR5	Thr82Ile	Major	LUA-57	CAATATCATCTTCTTATCAATCAATCACTAGCTATGACAGAGTTGG	Forward	TGCAATTTGACCATTTGAAGAA	463
RS764535		Minor	LUA-26	TTACTCAAAATCTACACTTTTTTCACTTGTCAATAGTCAAGGGGA	Reverse	TCCCAATGAAGGATGAGG	353
TLR5	Arg352STOP	Major	LUA-23	TTCATCAATCAATCACTTCACTTTTCACTTGTCAATAGTCAAGGGGA	Forward	GCTCTGCTGAGCTTCAACT	
RS5744168		Minor	LUA-20	CTTTTCAATCACTTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	CGGACTTGACACCTCCCAAG	1134
TLR5	Asn592Ser	Major	LUA-67	TCATTTACTCAACAAATCAAAATCAAAATTTAGACCTTTGATCTCT	Forward	AAAGCATTTCTGACCCCATGT	
RS2072493		Minor	LUA-21	AATCTTTCTTAACTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	CGGACTTGACACCTCCCAAG	1134
TLR5	Phe616Leu	Major	LUA-49	TCAATCACTTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Forward	AAAGCATTTCTGACCCCATGT	1134
RS5744174		Minor	LUA-33	TCAATCACTTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	AAAGCATTTCTGACCCCATGT	
TLR5	Asp594Gly	Major	LUA-80	CTAATCAACAAATCTCAATCAATCACTAGCTATGACAGAGTTGG	Forward	CGGACTTGACACCTCCCAAG	1134
RS5744176		Minor	LUA-96	ATACTAATCACTCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	AAAGCATTTCTGACCCCATGT	
TLR7	Exon/intron boundary	Major	LUA-65	CTTTTCATCAATCACTTCAATCAATCACTAGCTATGACAGAGTTGG	Forward	CGGACTTGACACCTCCCAAG	156
RS2302267		Minor	LUA-47	CITCTCAATCACTTCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	CGGACTTGACACCTCCCAAG	
TLR7	Gln11Leu	Major	LUA-76	AATCTCAACAAATCTCAATCAATCACTAGCTATGACAGAGTTGG	Forward	CGGACTTGACACCTCCCAAG	192
RS179008		Minor	LUA-77	CAATTAATCACTCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	CGGACTTGACACCTCCCAAG	
TLR7	Ala48Val	Major	LUA-98	AATCACTCACTCAATCAATCACTAGCTATGACAGAGTTGG	Forward	CGGACTTGACACCTCCCAAG	240
RS5743781		Minor	LUA-50	CAATATCAATCACTCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	CGGACTTGACACCTCCCAAG	
TLR7	3'UTR	Major	LUA-34	TCAATCACTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Forward	CGGACTTGACACCTCCCAAG	276
RS3853839		Minor	LUA-09	TAACTCTATATCAATCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	CGGACTTGACACCTCCCAAG	
TLR8	Promoter (-605)	Major	LUA-90	GTAAATCACTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Forward	CGGACTTGACACCTCCCAAG	744
RS5741883		Minor	LUA-40	CTTTTCACTTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	CGGACTTGACACCTCCCAAG	
TLR8	Promoter (-129)	Major	LUA-48	AAACAACTTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Forward	CGGACTTGACACCTCCCAAG	744
RS3764079		Minor	LUA-62	TCAATCACTTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	CGGACTTGACACCTCCCAAG	
TLR8	Exon (-3679)	Major	LUA-87	AACTAATCACTTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Forward	CGGACTTGACACCTCCCAAG	744
RS3764880		Minor	LUA-89	TATACATCACTTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	CGGACTTGACACCTCCCAAG	
TLR8	3'UTR	Major	LUA-32	ATTATTCATTTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Forward	CGGACTTGACACCTCCCAAG	162
RS5744088		Minor	LUA-55	TATATACATTTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	CGGACTTGACACCTCCCAAG	
TLR9	Promoter (-1486)	Major	LUA-84	TACATCACTTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Forward	CGGACTTGACACCTCCCAAG	379
RS187084		Minor	LUA-62	CTTTTCAATCACTTCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	CGGACTTGACACCTCCCAAG	
TLR9	Promoter (-1237)	Major	LUA-02	CTTTTCAATCACTTCAATCAATCACTAGCTATGACAGAGTTGG	Forward	CGGACTTGACACCTCCCAAG	379
RS5743836		Minor	LUA-38	TCAATCACTTCAATCAATCAATCACTAGCTATGACAGAGTTGG	Reverse	CGGACTTGACACCTCCCAAG	

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.

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