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 reviewed[1]). 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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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 RA[8], 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-κB signaling and cell activation. Perhaps genetic, posttranscriptional, and/or posttranslational 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 RS1898830	Promoter (-15607)	Major	A	LUA-28	CTACAAACAAACAAACATTATCAATAGTAAAATAAATCCAGAGAAATCA	Forward	GAAAAATGAATGAGCAAGCAAA	270
		Minor	G	LUA-70	ATACCAATAATCCAATTCATATCATAGTAAAATAAATCCAGAGAAATCG	Reverse	ATGGCCTCCTGCTTATGTCA	
TLR2 RS5743704	Pro631His	Major	C	LUA-18	TCAAAATCTCAAATACTCAAATCACAGGCCAAAAGGAAGCA	Forward	GTTTCCATGGCCTGTGGTAT	493
		Minor	A	LUA-41	TTACTACACAATATACTCATCAATAGGCCAAAAGGAAGCC	Reverse	CAAAATCCTTCCCGCTGAG	
TLR2 RS5743708	Arg753Gln	Major	G	LUA-30	TTACCTTTATACCTTTCTTTTTACGTCTTGGTGTTCATTATCTTCT	Forward	GTTTCCATGGCCTGTGGTAT	493
		Minor	A	LUA-88	TTACTTCACTTTCTATTTACAATCGTCTTGGTGTTCATTATCTTCC	Reverse	CAAAATCCTTCCCGCTGAG	
TLR3 RS3775291	Leu412Phe	Major	G	LUA-12	TACACTTTCTTTCTTTCTTTCTTTAGATTTTATTCTTGGTTAGGTTGAG	Forward	TGGCTAAAATGTTTGGAGCAC	290
		Minor	A	LUA-56	CAATTTACTCATATACATCACTTTAGATTTTATTCTTGGTTAGGTTGAA	Reverse	CCTGTGAGTTCTTGCCCAAT	
TLR4 RS7873784	3'UTR	Major	G	LUA-72	TCATTTACCTTTAATCCAATAATCCAGCTGTATAGCAGAGTTCG	Forward	GGAGGAAGGGAGAAATGAGG	208
		Minor	C	LUA-07	CAATTCATTTACCAATTTACCAATCAGCTGTATAGCAGAGTTCC	Reverse	CACCTCCAAAAGCTTCCTTG	
TLR4 RS4986790	Asp299Gly	Major	A	LUA-24	TCAATTACCTTTTCAATACAATACATACTTAGACTACTACCTCGATGA	Forward	TGCAATTTGACCATTGAAGAA	463
		Minor	G	LUA-25	CTTTTCAATTACTTCAAATCTTCACTTAGACTACTACCTCGATGG	Reverse	TCAAATTGGAATGCTGGAAA	
TLR4 RS4986791	Thr399Ile	Major	C	LUA-16	AATCAATCTTCATTCAAATCATCATCAAAGTGATTTTGGGACAAC	Forward	TGCAATTTGACCATTGAAGAA	463
		Minor	T	LUA-57	CAATATCATCATCTTTATCATTACCTCAAAGTGATTTTGGGACAAT	Reverse	TCAAATTGGAATGCTGGAAA	
TLR5 RS764535	Thr82Ile	Major	G	LUA-26	TTACTCAAAATCTACACTTTTTCACTTGTCAATAGTCAAGGGGA	Forward	TCCCAAATGAAGGATGAAGG	353
		Minor	A	LUA-23	TTCAATCATTCAAATCTCAACTTTTTGTCAATAGTCAAGGGGG	Reverse	GCTCCTGCTGAGCTTCAACT	
TLR5 RS5744168	Arg392STOP	Major	C	LUA-20	CTTTTACAATACTTCAATACAATCAATTACAGACCTTGGATCTCC	Forward	CGGACTTGACAACCTCCAAG	1134
		Minor	T	LUA-67	TCATTTACTCAACAATTACAAATCAAAAATTACAGACCTTGGATCTCT	Reverse	AAAGCATTCTGCACCCATGT	
TLR5 RS2072493	Asn592Ser	Major	A	LUA-21	AATCCTTTCTTTAATCTCAAATCAAATGTGAACTTAGCACTTTTATCAA	Forward	CGGACTTGACAACCTCCAAG	1134
		Minor	G	LUA-22	AATCCTTTTTACTCAATTCAATCAAATGTGAACTTAGCACTTTTATCAG	Reverse	AAAGCATTCTGCACCCATGT	
TLR5 RS5744174	Phe616Leu	Major	T	LUA-49	TCATCAATCTTTCAATTTACTTACGTGTACCCTGACTCGC	Forward	CGGACTTGACAACCTCCAAG	1134
		Minor	C	LUA-33	TCAATTACTTCACTTTAATCCTTTTGTGTACCCTGACTCGT	Reverse	AAAGCATTCTGCACCCATGT	
TLR5 RS5744176	Asp694Gly	Major	A	LUA-80	CTAACTAACAATAATCTAACTAACCAGAACCTGATATGTACAAATATGA	Forward	CGGACTTGACAACCTCCAAG	1134
		Minor	G	LUA-96	ATACTAACTCAACTAACTTTAAACCAGAACCTGATATGTACAAATATGG	Reverse	AAAGCATTCTGCACCCATGT	
TLR7 RS2302267	Exon/intron boundary	Major	T	LUA-65	CTTTTCATCAATAATCTTACCTTTGTGCTGTCTTTGAAATGTAAACTTT	Forward	CGCATTTTAAAGCAATGATCC	156
		Minor	G	LUA-47	CTTCTCATTAACTTACTTCATAATTGCTGTCTTTGAAATGTAAACTTG	Reverse	TGGTTGAAGAGAGCAGAGCA	
TLR7 RS179008	Gln11Leu	Major	A	LUA-76	AATCTAACAAACTCATCTAAATACGTGGACACTGAAGAGACA	Forward	AGGCAGCAAATGGGAATTTT	192
		Minor	T	LUA-77	CAATTAACTACATACAATACATACGTGGACACTGAAGAGACT	Reverse	GAGTGACATCACAGGGCAGA	
TLR7 RS5743781	Ala448Val	Major	C	LUA-98	AATCATACTCAACTAATCATTCAACATAACTTTCTACAGAAGTTCTGG	Forward	TGAAGTTCTTGATCTTGGCACT	240
		Minor	T	LUA-50	CAATATACCAATATCATCATTTACTCATAACTTTCTACAGAAGTTCTGA	Reverse	TTTTTGAATCTGCAACTCCTTG	
TLR7 RS3853839	3'UTR	Major	C	LUA-34	TCATTCATATACATACCAATTCATAAGCAGGCCCAAGG	Forward	ACCAATTGCTTCCGTGTCAT	276
		Minor	G	LUA-09	TAATCTTCTATATCAACATCTTACAAGCAGGCCCAAGC	Reverse	CTTTGCAGTGCAGATAAAAACA	
TLR8 RS5741883	Promoter (-605)	Major	C	LUA-90	CTAAATACTTCACAATTCATCTAAAACACTCATTGAGCTTATACTACAC	Forward	ATTTTCCAGCCTCACGAATG	744
		Minor	T	LUA-40	CTTTCTACATTATTCACAACATTAAACACTCATTGAGCTTATACTACAT	Reverse	TCTGGGTCAGAAACCCCATA	
TLR8 RS3764879	Promoter (-129)	Major	C	LUA-48	AAACAAACTTCACATCTCAATAATACTTCTGTAAAACACACGCTAC	Forward	ATTTTCCAGCCTCACGAATG	744
		Minor	G	LUA-62	TCAATCATAATCTCATAATCCAATACTTCTGTAAAACACACGCTAG	Reverse	TCTGGGTCAGAAACCCCATA	
TLR8 RS3764880	Exon (-3679)	Major	A	LUA-87	AAACTAACATCAATACTTACATCAATGAAAAATTAGAACAACAGAAACA	Forward	ATTTTCCAGCCTCACGAATG	744
		Minor	G	LUA-89	TATACTATCAACTCAACAACATATATGAAAAATTAGAACAACAGAAACG	Reverse	TCTGGGTCAGAAACCCCATA	
TLR8 RS5744088	3'UTR	Major	G	LUA-32	ATTATTCACTTCAAACTAATCTACGGATTCAATTCCTCCTGG	Forward	GTGTCTCAGAGGCTGCAATG	162
		Minor	C	LUA-55	TATATACACTTCTCAATAACTAACGGATTCAATTCCTCCTGC	Reverse	GATGAAGCAAGCTGCCTTGT	
TLR9 RS187084	Promoter (-1486)	Major	T	LUA-64	CTACATATTCAAATTACTACTTACAGATAAAAGATCACTGCCCTT	Forward	CCTGCTTGCAGTTGACTGTG	379
		Minor	C	LUA-82	TACATACACTAATAACATACTCATAGATAAAAGATCACTGCCCTC	Reverse	GTGCTGGGCACTGTACTGG	
TLR9 RS5743836	Promoter (-1237)	Major	T	LUA-02	CTTTATCAATACATACTACAATCAGAGACTTGGGGGAGTTTT	Forward	CCTGCTTGCAGTTGACTGTG	379
		Minor	C	LUA-38	TCAATCATTACACTTTTCAACAATAGACTTGGGGGAGTTTC	Reverse	GTGCTGGGCACTGTACTGG	

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 6yrs		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 6yrs		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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