Single Nucleotide Polymorphisms in the Toll-Like Receptor Pathway increase Susceptibility to Infections in Severely Injured Trauma Patients

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ABSTRACT

Background. Sepsis and subsequent multiple organ failure are the predominant causes of late mortality in trauma patients. Susceptibility and response to infection is, in part, heritable. Single nucleotide polymorphisms (SNPs) in Toll-Like Receptor (TLR) and Cluster of Differentiation 14 (CD14) genes of innate immunity may play a key role. The aim of this study was to assess if SNPs in *TLR/CD14* predisposed trauma patients to infection.

Methods. A prospective cohort of trauma patients (age 18-70 years, injury severity score ≥16) admitted to a Level I Trauma Center between January 2008 and April 2011 was genotyped for SNPs in *TLR2* (T-16934A and R753Q), *TLR4* (D299G and T399I), *TLR9* (T-1486C and T-1237C), and *CD14* (C-159T) using High Resolution Melting Analysis (HRMA). Association of genotype with prevalence of positive cultures (Gram-positive, Gram-negative, fungi), systemic inflammatory response syndrome (SIRS), sepsis, septic shock, and mortality was tested with chi-square and logistic regression analysis.

Results. Genotyping was performed for 219 patients, of which 51% developed a positive culture in sputum, wounds, blood, or urine. SIRS developed in 64%, sepsis in 36%, and septic shock in 17%. The *TLR2* T-16934A TA genotype increased the risk of a Gram-positive infection (Odds Ratio, OR, 2.816; 95% CI 1.249-6.348; p=0.013) and SIRS (OR 2.386; 95% CI 1.011-5.632; p=0.047). Trends were noted for TLR9 and CD14 SNPS, but did not reach statistical significance. Sepsis and septic shock were unrelated to any of the SNPs studied.

Conclusion. Aberrant functioning of the TLR/CD14 pathway of innate immunity changes the risk of infectious complications in severely injured trauma patients. Of the seven SNPs studied, the *TLR2* T-16934A increased the risk, the *TLR9* T-1486C SNPs may decrease the risk, TLR4 variation seemed unrelated to outcome. Early genotyping may prove to be helpful in the future in identifying polytraumatized patients at risk for infectious outcome. Level of Evidence II. Prognostic and epidemiological study.

INTRODUCTION

Trauma is a leading cause of morbidity and mortality, and many polytraumatized patients require treatment in an Intensive Care Unit (1). Infectious complications are common after trauma and may substantially affect morbidity and mortality. Sepsis and subsequent multiple organ failure are the predominant causes of late mortality in polytraumatized patients; sepsis may occur in 3.1-17.0% and multiple-organ failure may occur in 1.6-9.0% of trauma patients (2). Immunogenetic biomarkers have been proposed as contributing factors in the development of trauma-induced infection and organ failure (3-5).

Toll-like receptor (TLR) signalling plays an important role in the innate immune response and has been associated with the activation of inflammatory immune responses during trauma-induced infections (6, 7). TLRs recognize and bind to a wide range of pathogen-associated molecular patterns (PAMPs) on bacteria, viruses, fungi, and protozoa. This binding leads to the transcriptional activation of genes encoding for pro-inflammatory cytokines, chemokines, and co-stimulatory molecules, which subsequently control the activation of antigen-specific adaptive immune response (5, 8, 9).

To date, ten different TLRs are described in humans (TLR1-10), each binding to different PAMPs and initiating specific immunological responses (10, 11). TLR2 heterodimers bind polypeptides in the cell walls of bacteria and signal via MyD88 following initial interaction with Cluster of Differentiation 14 (CD14) (12, 13). TLR4 forms a homodimer complex with the MD-2 protein after initial binding of lipopolysaccharide (LPS) expressed by Gram-negative bacteria to CD14 (14). TLR9 is present in various cells of the immune system, such as dendritic cells and B- and T-cells and is situated intracellularly, bound to the endosome. TLR9 binds to the unmethylated cytosine guanine dinucleotide

(CpG) motifs, present in bacterial and viral DNA but also in our mitochondrial DNA. Thus this receptor recognizes pathogens as well as damaged tissue (9, 15).

CD14 is a central pattern recognition molecule in innate immunity (16). It is found in association with TLRs on the surface of monocytes, macrophages, neutrophils, and hepatocytes (17, 18), and as a soluble form in serum (19). CD14 acts as coreceptor of TLR2 and TLR4 for the detection of various microbial PAMPs (20), and initiates immune responses through TLR2 and TLR4 signalling (17, 21).

There is growing evidence that susceptibility and response to infectious disease is, in part, heritable; particularly Single Nucleotide Polymorphisms (SNPs) in innate immune response genes may play a key role (22-25). Two important SNPS in the *TLR2* gene (*i.e.*, T-16934A and R753Q) have been associated with Gram-positive infections, sepsis and septic shock (12, 13, 26). SNPs in the *TLR4* gene (*i.e.*, D299G and T399I) may alter the responsiveness of TLR4 to LPS, and increase the risk of Gram-negative sepsis, bacteremia, and mortality in patients admitted to the Intensive Care Unit (ICU) (4, 27, 28). SNPs in the *TLR9* gene promoter (*i.e.*, T-1237C and T-1486C), affect transcription regulation resulting in a disabled TLR9 function. A variant T-1273C genotype has been associated with increased susceptibility to viral pneumonia (29). The *CD14* C-159T SNP has been associated with sepsis and Gram-negative bacteremia (26, 30, 31).

The aim of this study was to assess the relevance of SNPs in *TLR2*, *TLR4*, *TLR9*, and *CD14* for infectious complications in trauma patients. We hypothesized that these SNPs are associated with increased prevalence of positive cultures (Gram-positive and Gram-negative bacteria and fungi), systemic inflammatory response syndrome (SIRS), sepsis, and septic shock in trauma patients.

MATERIALS AND METHODS

Patients and Data Collection

A prospective cohort of consecutive polytraumatized patients admitted to a Level I Trauma Center (Rotterdam, The Netherlands) between January 2008 and April 2011 was studied. Eligibility criteria were an Injury Severity Score (ISS) (32) of 16 or higher and age 18-80 years. Patients with a known immune disorder or those taking immunosuppressive medication were excluded. Patients with a neutrophil count of $<1x10^9$ cells/L before the onset of sepsis, infections associated with burns, or lack of commitment to full life-support measures by the primary physician were also excluded. The ISS score was calculated using the ISS update 98. After obtaining written informed consent from patient or proxy a blood sample was taken in an EDTA tube and stored at -80°C until use. The study was approved by the local medical research ethics committee and the trial was registered in the Dutch Trial Registry (NTR1625) before patient recruitment.

Cultures were taken on a standardized routine basis or at the discretion of the physician based upon clinical signs. Endpoints used were: positive cultures during hospital stay, mortality, SIRS within the first 24 hours of admission and developing sepsis or septic shock during hospital admission. SIRS, sepsis, and septic shock were defined using international criteria (33). The following data were retrieved from electronic files: age at trauma, trauma mechanism, ISS score, length of stay in the Intensive Care Unit and the hospital, all positive cultures from blood, urine, sputum, wounds, or other positive cultures during hospital stay, if patients developed SIRS within 24 hours of hospital admission and sepsis or septic shock during the hospital stay, and if applicable the cause of death.

DNA Isolation

Genomic DNA was isolated from 300 µL EDTA-treated peripheral blood using the QIAamp® DNA Blood Mini kit (QiaGen Benelux, Venlo, The Netherlands), according to the manufacturer's instructions. The purity (A260nm/A280nm index) and concentration of the isolated DNA samples were determined with the Thermo Scientific Nanodrop TM1000 spectrophotometer (Isogen Life Science, De Meern, The Netherlands). Samples were diluted to 10 ng/µL using Milli-Q and were stored at 4°C until use.

PCR Oligonucleotides

Details on the SNPs studied are given in Table 1. SNP data were retrieved from Ensembl.org, National Center for Biotechnology Information (NCBI) GenBank, and NCBI SNP Database. All oligonucleotides (see Table 2) were purchased from Eurogentec (Seraing, Belgium). For the High Resolution Melting Analysis (HRMA) 18-20 nucleotides upstream and downstream of the SNPs were used as forward and reverse primers. Oligonucleotides for direct sequencing were designed using the Oligo 6.22 software (Molecular Biology Insight, Cascade, CO, USA) and resulted in 400-555 base pair amplicons. Oligonucleotides had melting temperatures (Tm) between 65.0-66.5°C (Tm calculated using the nearest neighbour method at a salt concentration of 50mM KCl and 4mM $MgCl_2$ (303mM of Na^+ equivalent) and 300nM oligonucleotides).

Genotyping

SNPs were detected using HRMA (34). Polymerase chain reactions (PCRs) were performed in a total volume of 10 µL, containing 20ng genomic DNA, 5pmol of both gene-specific oligonucleotides, 2pmol dNTPs (Promega, Madison, WI, USA), 1µL LC-green (Bioké,

Leiden, The Netherlands), 0.5U of Taq DNA polymerase (Roche Diagnostics, Almere, The Netherlands), and 1µl 10x PCR buffer containing 20mM $MgCl_2$. Two pmoles of calibrator oligonucleotides were added for calibration of melting curves. The PCR was run on a Biometra Thermocycler (Biometra GmbH, Göttingen, Germany). The thermocycling program included denaturation at 95°C for 10 min, followed by 55 cycles of 20 sec at 95°C, 30 sec at 60°C and 40 sec at 72°C, and a final extension step of 5 min at 72°C. HRMA was performed using a LightScanner® (HR-96, Idaho Technology, Salt Lake City, UT, USA). Melting was done from 55°C to 98°C at 0.1°C/sec. Melting curves were analyzed with the LightScanner® Software using Call-IT 1.5.

All variant genotypes found were confirmed by direct sequencing. PCR were performed in a total volume of 25 µL, containing 50 ng genomic DNA, 4mM $MgCl_2$, 3pmol of both oligonucleotides, 2pmol dNTPs, 1.0U Taq DNA polymerase, and 1µl 10x PCR buffer without $MgCl_2$. The thermocycling program included denaturation at 95°C for 5 min, followed by 35 cycles of 30 sec at 95°C, 1 min at 60°C, and 1 min at 72°C, and a final step of 7 min at 72°C. Amplicon purification and sequencing was performed by BaseClear (Leiden, The Netherlands). Results were analyzed using the SeqMan® analysis software.

Data Analysis

Allele frequencies for each SNP were determined by gene counting. The genotype distribution of each SNP was tested for departure from the Hardy-Weinberg equilibrium by means of $\chi 2$ analysis. Data were analyzed using the Statistical Package for the Social Sciences, version 16.0 (SPSS, Chicago, IL, USA). Categorical variables were analyzed using a $\chi 2$ test or a Fischer's exact test in order to determine whether the SNPs predisposed for a higher risk of any of the endpoints. A Mann-Whitney U-test was used for statistical analysis of numeric variables. Binary logistic regression models were developed in order to model the

relation between different covariates and the occurrence of an infectious complication. Herein, age, gender, injury severity score, and trauma mechanism (*i.e.*, blunt or penetrating injury) were added as covariates. A p-value of <0.05 was considered to be significant.

RESULTS

Patient demographics and genotype frequencies

From January 2008 to April 2011, 219 patients were included. Of all patients 177 (77.6%) were male, the median age was 44 years (P_{25}-P_{75} 27-56) and the median ISS score was 25 (P_{25}-P_{75} 18-29). A total of 13 patients (5.9%) sustained penetrating trauma and 159 patients (72.6%) were admitted to the ICU with a median stay of 3 days (P_{25}-P_{75} 0-14). Trauma mechanisms included traffic accidents (48.9%), fall from height (32.4%), crush injury to the chest (4.1%), head injuries with axes or screwdrivers (2.7%), gunshot injuries (2.3%), horseback injuries, street fighting, stab wounds (1.8%), and miscellaneous injuries like ice skating, helicopter crash, fireworks or collapsing buildings.

HRMA results showed that *TLR2* T-16934A rare alleles were abundantly present; 58 (26.5%) patients had a homozygous AA genotype, 111 (50.7%) had a heterozygous TA genotype, and 50 (22.8%) carried a wild-type TT genotype. For the *TLR2* R753Q SNP, 15 (6.8%) patients carried the heterozygote RQ and 204 (93.2%) a RR genotype. For the *TLR4* D299G SNP 190 (86.8%) had a DD, and 28 (12.8%) a DG genotype. The *TLR4* T399I SNP cosegregated with D299G in 217 (99.1%) patients; 191 (87.2%) had a TT, 26 (11.9%) a TI, and 2 (0.9%) an II genotype. For the *TLR9* T-1486C SNP, 80 (36.5%) had a TT, 100 (45.7%) a TC, and 39 (17.8%) a CC genotype. For the *TLR9* T-1237C SNP, 160 (73.1%) had a TT, 52 (23.7%) a TC, and 7 (3.2%) a CC genotype. For the *CD14* C-159T SNP, 59 (26.9%) had a CC, 105 (47.9%) a CT, and 55 (25.2%) a TT genotype. The genotype distribution of all SNPs was in agreement with the Hardy-Weinberg equilibrium ($\chi 2$=0.01-1.149; P=0.275-0.997).

Effect of genotype on infectious complications

Table 3 shows an overview of infectious complications encountered separated by genotype. Overall, Gram-positive bacteria were detected in 79 patients, Gram-negative bacteria in 84 and fungi in 46 patients. Approximately half of the patients (51.1%) developed a positive culture; this was mostly in sputum (N=70), but positive cultures were also found in wounds (N=36), blood (N=32), and urine (N=32). Overall, 139 patients developed SIRS within 24 hours, of which 79 developed sepsis and 37 septic shock. Thirteen (5.9%) patients died.

Table 4 shows the results of the multivariable logistic regression models. Sustaining a Gram-positive infection was statistically significantly increased in patients with a *TLR2* T-16934A TA genotype (Odds Ratio, OR, 2.816; 95%CI 1.249-6.348; p=0.013). Forty-one percent of TA patients cultured a Gram-positive bacterium versus 24.0% of wildtype patients (Table 3). A *TLR9* T-1486C rare allele, on the other hand, seemed to result in decreased Gram-positive rates (31.0% in TC and 30.8% in CC versus 45.0% in wildtypes), however this did not reach statistical significance in the multivariable analyses (p=0.066 for TC and p=0.079 for CC).

None of the SNPs affected the risk of Gram-negative cultures or fungi. Although the rate of positive fungal cultures seemed reduced in patients with a *TLR9* T-1486C TC genotype (14.0% versus 27.5% in wildtypes) and increased in patients with a *CD14* C-159T CT genotype (23.8% versus 13.6% in wildtypes), both were not statistically associated with outcome in multivariable analyses (p=0.060 and 0.072, respectively).

As for the location of the positive cultures, the *TLR2* T-16934A AA genotype was significantly associated with fewer positive urine cultures (OR 0.230; 95%CI 0.060-0.886; p=0.033). Blood cultures occurred in 17.1% of *TLR2* T-16934A TA patients (versus 8.0% in TT) and in 23.1% of *TLR9* T-1237C TC patients (versus 12.5% in TT), but both were not

statistically significant in multivariable analysis (p=0.065 and p=0.076, respectively). The occurrence of sputum and wound cultures was unrelated to any of the SNPs studied.

The occurrence of SIRS in the first 24 hours of hospital admission was statistically significantly increased in patients with a *TLR2* T-16934A TA genotype (OR 2.386; 95%CI 1.011-5.632; p=0.047), but unrelated to any of the other SNPs. Sepsis and septic shock were unrelated to *TLR2*, *TLR4*, *TLR9*, and *CD14* SNPs.

DISCUSSION

In this study we showed that severely injured trauma patients with an inadequately functioning TLR pathway due to SNPs in *TLR2* and, to a lesser extent, in *TLR9* are at increased risk of infectious complications.

The genotype distributions of all polymorphisms studied in our trauma patient cohort were in agreement with published data (4, 13, 26, 28-30, 35-41). The genotype distributions of all SNPs were in agreement with the Hardy-Weinberg equilibrium model.

Patients with a *TLR2* T-16934A TA genotype were more prone to Gram-positive infections, and presence of homozygous AA alleles predisposed patients to SIRS. Sutherland *et al.* showed that this SNP is a risk factor for sepsis and Gram-positive bacteremia in critically ill patients in the ICU (26). We were unable to confirm their findings related to sepsis; 38.7% of our trauma patients with a TA genotype developed sepsis versus 36.0% of patients with a TT genotype. We found a 2-fold higher rate of positive blood culture in the TA group compared with the TT group (p=0.065). More patients are needed to reach statistical significance. Our finding that the T-16934A AA genotype was associated with fewer positive urine cultures (N=4) has not been shown before. Only Gram-negative bacteria were found.

The *TLR2* R753Q SNP has been associated with increased prevalence of Gram-positive infections, staphylococcal infections, sepsis, and septic shock (12, 13, 28, 42), but others reported that this SNP did not affect the risk of bacteremia in Caucasian patients admitted to the ICU (4). With only 6.8% having an RQ genotype and complete absence of a QQ genotype, our study was inadequately powered to find any associations with outcome in trauma patients. If this SNP were relevant, it would only be so in a small subset of patients.

TLR4 is important in the defense against bacteria, particularly Gram-negative bacteria. Since both the D299G and T399I SNP are associated with decreased TLR4 function it is

remarkable that we did not find any association of these SNPs with infection susceptibility. However, both SNPs were previously shown to be unrelated to outcome of Gram-negative sepsis (43) and to sepsis in trauma patients with SIRS in the ICU (13). Several other studies, however, did in fact imply that *TLR4* polymorphisms were related to increased susceptibility to infections and sepsis in burn patients (44) or in patients admitted to an ICU (4, 28, 42, 45).

Shalhub *et al.* showed that a *TLR4* D299G variant G allele caused a decreased rate of complicated sepsis in Caucasian trauma patients admitted to the ICU (45). Our multivariable analysis did not support an association between genotype and infection or outcome of sepsis; almost 50% of patients with sepsis developed septic shock, both in the DG and TI group (5 of 11 patients) and in wildtypes (32 of 68 patients).

Our data support the findings of Henckaerts *et al.* Who showed that a *TLR4* D299G variant genotype resulted in a 2-fold increase of bacteremia in ICU patients (4).

Reduced expression of TLR9 could render patients more susceptible to pathogens, yet increased TLR9 expression after stimulation by damage-associated molecular patterns (DAMPs) could trigger an exaggerated immune response (15, 46, 47). The *TLR9* T-1486C SNP has been associated with sepsis and multiple organ dysfunction (MOD) in trauma patients (37), puerperal group A streptococcal sepsis (48), and invasive fungal infections in paediatric patients receiving chemotherapy (49). We found a trend towards reduced prevalence of Gram-positive bacteria and fungi for this SNP (p=0.060-0.066), but no significant association with SIRS, sepsis, or septic shock. As opposed to our results, however, Chen *et al.* showed that patients with a T-1486C variant genotype were at increased risk of sepsis (OR 1.36) and had slightly increased MOD scores (37).

We found a trend towards increased risk of positive blood cultures the T-1237C TC genotype (p=0.076), but no significant association with SIRS, sepsis, or septic shock. In

agreement with our data, Chen *et al.* found no association between *TLR9* T-1237C variant genotype and infection in severe blunt trauma patients (37).

The *CD14* C-159T SNP reduces the promoter activity of CD14, resulting in decreased TLR2/4 downstream signalling. A CT or TT genotype has been associated with increased rates of positive bacterial culture findings and Gram-negative sepsis in critically ill patients (26, 30, 31, 41). However, in other studies this SNP was not associated with altered risk of Gram-negative infection or sepsis in critically ill patients (28, 51), and was unrelated to sepsis prevalence and severity in trauma patients (45, 52). Our data support the latter.

A limitation of our study is that we retrospectively collected data on clinical manifestations of infection like SIRS, sepsis, and septic shock. Also, cultures were initially collected at the discretion of the physician based on clinical parameters but during the study protocols changed towards routine culturing of all patients.

The human innate immune system consists of more pathways in addition to the TLR/CD14 pathway. The advantage of our study is that the growing DNA bank of severely injured trauma patients enables us to increase the panel of SNP assays or even perform a genome-wide SNP analysis.

In summary, our data showed that an aberrant functioning of the TLR/CD14 pathway due to presence of the *TLR2* T-16934A polymorphism predisposed severely injured trauma patients to increased risk of developing an infectious complication. The *TLR9* T-1486C SNPs on the other hand may reduce the risk of positive culture findings, and TLR4 variation was unrelated to infection. Additional research is needed in order to fully understand the roles these SNPs play in the complex injury and infection processes.

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Table 1. Positions of SNPs and their corresponding nucleotide and amino acid transition

Gene	SNP	dbSNP ID	Nucleotide transition (Accesion code)	Amino-Acid transition (Accesion code)	Location on gene / Domain
TLR2	T-16934A	rs4696480	6686T>A	N.A.	Promoter, 5' untranslated region
	R753Q	rs5743708	25877G>A (NG_016229.1)	Arg753Gln (NP_003255.2)	Exon 3; Toll/Interleukin-1 receptor (TIR) domain
TLR4	D299G	rs4986790	13843A>G	Asp299Gly	Exon 3 Extracellular domain
	T399I	rs4986791	14143C>T (NG_011475.1)	Thr399Ile (NP_612564.1)	Exon 3 Extracellular domain
TLR9	T-1486C	rs187084	52261031A>G	N.A.	Promoter, 5' untranslated region
	T-1237C	rs5743836	52260782A>G (NC_000003.11)	N.A.	Promoter, 5' untranslated region
CD14	C-260T	rs2569190	5371T>C (NG_023178.1)	N.A.	Promoter, 5' untranslated region

SNP, Single Nucleotide Polymorphism;

Amino acids: Arg (R), Arginine; Asp (D), Asparctic acid; Gln (Q), Glutamine; Gly (G), Glycine; Ile (I), Isoleucine; Thr (T), Threonine.

Table 2. Oligonucleotide sequence for primers and probes used in *TLR2, TLR4, TLR9,* and *CD14* genotyping

Name (Accession code)	SNP	Assay	Primer	Start Position	Sequence	Amplicon size (base pairs)
Calibrators	-	HRMA	Forward	-	5'-TTAAATTATAAAATATTTATAATATTAATTATATA TATATAAATATAATA-Amine-C6-3'	50
			Reverse	-	5'-TATTATATTTATATATATATAATTAATATTATAAA TATTTTATAATTTAA-Amine-C6-3'	
TLR2	T-16934A	HRMA	Forward	6666	5'-GATTGAAGGGCTGCATCTGG-3'	41
(NG_016229.1)			Reverse	6687	5'-AATGTAGCCAGATGACCCTC-3'	
		Sequencing	Forward	6401	5'-TTGGGGGTTTCTAAGCTAT-3'	406
			Reverse	6790	5'-TCACCAAGGGAGCAGTT-3'	
	R753Q	HRMA	Forward	25857	5'-CCAGCGCTTCTGCAAGCTGC-3'	41
			Reverse	25878	5'-TCTTGGTGTTCATTATCTTC -3'	
		Sequencing	Forward	25622	5'-CCCCCTTCAAGTTGTGT-3'	468
			Reverse	26071	5'-CCTCAAATGACGGTACATC-3'	
TLR4	D299G	HRMA	Forward	13817	5'-AGCATACTTAGACTACTACCT-3'	53
(NG_011475.1)			Reverse	13849	5'-TCAAACAATTAAATAAGTCAA-3'	
		Sequencing	Forward	13576	5'-TGTCCCTGAACCCTATGA-3'	501
			Reverse	14057	5'-ACTCAAGGCTTGGTAGATCA-3'	
	T399I	HRMA	Forward	14223	5'-TCAAAGTGATTTTGGGACAA-3'	41
			Reverse	14241	5'-GATCTAAATACTTTAGGCTG-3'	
		Sequencing	Forward	14035	5'-GTGGGAATGCTTTTTCAG-3'	552
			Reverse	14567	5'-GGAGGGAGTTCAGACACTTA-3'	
TLR9	T-1486C	HRMA	Forward	52261011	5'-AGATAAAAGATCACTGCCCT-3'	41
(NC_000003.11)			Reverse	52261032	5'-TGCTGGAATGTCAGCTTCTT-3'	
	T-1237C	HRMA	Forward	52260762	5'-TATGAGACTTGGGGGAGTTT-3'	41
			Reverse	52260783	5'-TGTGCTGTTCCCTCTGCCTG-3'	
	Both SNPs	Sequencing	Forward	52260931	5'-CTGACTGCTGGGTGTACAT-3'	508
			Reverse	52260925	5'-AAAGCCACAGTCCACAGA-3'	
CD14	C-260T	HRMA	Forward	5352	5'-AGAATCCTTCCTGTTACGG-3'	41
(NG_023178.1**)**			Reverse	5373	5'-AAGGATGTTTCAGGGAGGGG-3'	
		Sequencing	Forward	5180	5'-ATTGGGGGGTTGGATAG-3'	443
			Reverse	5604	5'-AGTCTTCCGAACCTCTGAG-3'	

HRMA, High-Resolution Melting Analysis.

Table 3: Demographic description of positive cultures and infectious complications by genotype

		N	Gram-Positive	Gram-Negative	Fungus	Sputum Culture	Blood Culture	Urine Culture	Wound Culture	SIRS *	Sepsis *	Septic Shock *	Mortality
Entire population		219	79 (36.1)	84 (38.4)	46 (21.0)	70 (32.0)	32 (14.6)	32 (14.6)	36 (16.4)	139 (63.5)	79 (36.1)	37 (16.9)	13 (5.9)
***TLR2* T-16934A**	**TT**	50	12 (24.0)	21 (42.0)	8 (16.0)	15 (30.0)	4 (8.0)	9 (18.0)	8 (16.0)	28 (56.0)	18 (36.0)	9 (18.0)	2 (4.0)
	TA	111	46 (41.4)	42 (37.8)	28 (25.2)	36 (32.4)	19 (17.1)	19 (17.1)	17 (15.3)	70 (63.1)	43 (38.7)	18 (16.2)	8 (7.2)
	AA	58	21 (36.2)	21 (36.2)	10 (17.2)	19 (32.8)	9 (15.5)	4 (6.9)	11 (19.0)	41 (70.7)	18 (31.0)	10 (17.2)	3 (5.2)
***TLR2* R753Q**	**RR**	204	73 (35.8)	80 (39.2)	46 (22.5)	66 (32.4)	31 (15.2)	31 (15.2)	35 (17.2)	130 (63.7)	74 (36.3)	36 (17.6)	13 (6.4)
	RQ	15	6 (40.0)	4 (26.7)	0 (0.0)	4 (26.7)	1 (6.7)	1 (6.7)	1 (6.7)	9 (60.0)	5 (33.3)	1 (6.7)	0 (0.0)
	QQ	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
***TLR4* D299G**	**DD**	190	70 (36.8)	73 (38.4)	40 (21.1)	61 (32.1)	27 (14.2)	30 (15.8)	34 (17.9)	118 62.1)	68 (35.8)	32 (16.8)	10 (5.3)
	DG	28	9 (32.1)	11 (39.3)	6 (21.4)	9 (32.1)	5 (17.9)	2 (7.1)	2 (7.1)	20 (71.4)	11 (39.3)	5 (17.9)	3 (10.7)
	GG	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)
***TLR4* T399I**	**TT**	191	70 (36.6)	74 (38.7)	41 (21.5)	62 (32.5)	27 (14.1)	30 (15.7)	35 (18.3)	119 (62.3)	69 (36.1)	33 (17.3)	10 (5.2)
	TI	26	8 (30.8)	9 (34.6)	5 (19.2)	8 (30.8)	5 (19.2)	1 (3.8)	1 (3.8)	19 (73.1)	10 (38.5)	4 (15.4)	3 (11.5)
	II	2	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (50.0)	0 (0.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)
***TLR9* T-1486C**	**TT**	80	36 (45.0)	34 (42.5)	22 (27.5)	27 (33.8)	15 (18.8)	12 (15.0)	19 (23.8)	51 (63.8)	31 (38.8)	17 (21.2)	4 (5.0)
	TC	100	31 (31.0)	34 (34.0)	14 (14.0)	32 (32.0)	10 (10.0)	10 (10.0)	12 (12.0)	62 (62.0)	33 (33.0)	14 (14.0)	7 (7.0)
	CC	39	12 (30.8)	16 (41.0)	10 (25.6)	11 (28.2)	7 (17.9)	10 (25.6)	5 (12.8)	26 (66.7)	15 (38.5)	6 (15.4)	2 (5.1)
***TLR9* T-1237C**	**TT**	160	57 (35.6)	60 (37.5)	34 (21.2)	49 (30.6)	20 (12.5)	26 (16.3)	26 (16.3)	100 (62.5)	55 (34.4)	23 (14.4)	6 (3.8)
	TC	52	20 (38.5)	21 (40.4)	9 (17.3)	19 (36.5)	12 (23.1)	5 (9.6)	7 (13.5)	34 (65.4)	21 (40.4)	12 (23.1)	7 (13.5)
	CC	7	2 (28.6)	3 (42.9)	3 (42.9)	2 (28.6)	0 (0.0)	1 (14.3)	3 (42.9)	5 (71.4)	3 (42.9)	2 (28.6)	0 (0.0)
***CD14* C-159T**	**CC**	59	20 (33.9)	22 (37.3)	8 (13.6)	20 (33.9)	11 (18.6)	8 (13.6)	7 (11.9)	38 (64.4)	22 (37.3)	10 (16.9)	3 (5.1)
	CT	105	36 (34.3)	37 (35.2)	25 (23.8)	36 (34.3)	14 (13.3)	13 (12.4)	14 (13.3)	69 (65.7)	37 (35.2)	15 (14.3)	8 (7.6)
	TT	55	23 (41.8)	25 (45.5)	13 (23.6)	14 (25.5)	7 (12.7)	11 (20.0)	15 (27.3)	32 (58.2)	20 (36.4)	12 (21.8)	2 (3.6)

* SIRS, sepsis, and septic shock were only determined for patients admitted to the Intensive Care Unit.

Table 4: Association between genotype and infections outcome in polytraumatized patients

		Gram-Positive	Gram-Negative	Fungus	Sputum Culture	Blood Culture	Urine Culture	Wound Culture	SIRS	Sepsis	Septic Shock
		Odds Ratio	**Odds Ratio**	**Odds Ratio**	**Odds Ratio**	**Odds Ratio**	**Odds Ratio**	**Odds Ratio**	**Odds Ratio**	**Odds Ratio**	**Odds Ratio**
***TLR2* T-16934A**	**TA**	**2.816 * (1.249-6.348)**	0.908 (0.436-1.892)	1.793 (0.684-4.700)	1.246 (0.580-2.677)	3.194 (0.929-10.982)	0.864 (0.323-2.316)	0.763 (0.281-2.077)	1.514 (0.730-3.143)	1.230 (0.614-2.879)	0.913 (0.353-2.359)
	AA	2.114 (0.851-5.248)	0.817 (0.352-1.895)	1.243 (0.406-3.746)	1.290 (0.540-3.082)	2.748 (0.701-10.780)	**0.230 * (0.060-0.886)**	1.303 (0.438-3.873)	**2.386 * (1.011-5.632)**	0.996 (0.408-2.430)	1.126 (0.386-3.279)
***TLR2* R753Q**	**RQ**	0.859 (0.266-2.782)	0.356 (0.099-1.288)	N.D.	0.583 (0.167-2.033)	0.240 (0.025-2.272)	0.283 (0.032-2.521)	0.297 (0.035-2.554)	0.805 (0.260-2.497)	0.559 (0.162-1.933)	0.215 (0.024-1.923)
***TLR4* D299G[§]**	**DG**	0.640 (0.248-1.654)	0.898 (0.369-2.189)	0.946 (0.304-2.947)	0.846 (0.337-2.123)	1.170 (0.328-4.173)	0.390 (0.078-1.958)	0.322 (0.068-1.525)	1.277 (0.505-3.233)	0.946 (0.375-2.283)	0.957 (0.299-3.059)
	GG	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
***TLR9* T-1486C**	**TC**	0.532 (0.271-1.043)	0.735 (0.376-1.436)	0.447 (0.193-1.036)	0.916 (0.463-1.811)	0.489 (0.186-1.287)	0.454 (0.163-1.263)	0.511 (0.214-1.218)	1.028 (0.527-2.004)	0.913 (0.454-1.833)	0.757 (0.320-1.791)
	CC	0.456 (0.190-1.096)	0.934 (0.401-2.176)	0.791 (0.296-2.113)	0.761 (0.310-1.870)	0.976 (0.315-3.021)	1.928 (0.650-5.716)	0.397 (0.127-1.232)	1.144 (0.479-2.730)	1.022 (0.416-2.510)	0.727 (0.241-2.188)
***TLR9* T-1237C**	**TC**	0.994 (0.479-2.066)	1.170 (0.569-2.405)	0.534 (0.201-1.422)	1.108 (0.534-2.299)	2.365 (0.915-6.111)	0.501 (0.159-1.274)	0.743 (0.273-2.019)	1.185 (0.571-2.447)	1.413 (0.672-2.971)	1.905 (0.782-4.643)
	CC	0.522 (0.085-3.218)	1.107 (0.215-5.707)	2.751 (0.434-17.426)	1.004 (0.168-5.996)	N.D.	0.620 (0.058-6.663)	4.033 (0.635-25.609)	2.062 (0.334-12.717)	1.491 (0.270-7.239)	2.263 (0.353-14.490)
***CD14* C-159T**	**CT**	0.858 (0.411-1.788)	0.864 (0.420-1.779)	2.473 (0.923-6.630)	0.987 (0.479-2.034)	0.538 (0.199-1.457)	0.713 (0.249-2.037)	1.377 (0.475-3.992)	1.122 (0.543-2.322)	0.842 (0.399-1.774)	0.843 (0.323-2.202)
	TT	1.309 (0.573-2.991)	1.408 (0.627-3.164)	2.265 (0.765-6.706)	0.667 (0.281-1.584)	0.545 (0.171-1.742)	1.746 (0.563-5.415)	2.563 (0.869-7.557)	0.709 (0.312-1.613)	0.829 (0.354-2.942)	1.379 (0.495-3.842)

Multivariable logistic regression models were made for all individual outcome measure (*i.e.*, positive culture or infectious complication) as dependent variable. Age, gender, trauma mechanism, ISS, and individual SNPs were entered as covariate. Data are shown as odds ratio, with the 95% CI between brackets. For all SNPs the wildtype genotype was used as reference category. Male gender and blunt trauma were used as reference categories for gender and trauma mechanism, respectively.

[§]Since the *TLR4* D299G and T399I SNPs cosegregated in >99% of patients, only the D299G SNP was used for designing a multivariable model.

N.D., not determined due to insufficient numbers.

Statistically significant data are indicated in boldface. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$.