**No associations established between single nucleotide polymorphisms in human Toll-like receptor 2 and Toll-interacting protein and Staphylococcus aureus bloodstream infections**

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**Running title:** Association of SNP in TLR2 and TOLLIP with SABSI

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**Summary**

*Staphylococcus aureus* bloodstream infections (SABSI) are associated with high morbidity and mortality. The Toll-like receptor 2 (TLR2) and Toll-interacting protein (TOLLIP) are important in recognition and regulation of human innate immunity response to *S. aureus.* Single nucleotide polymorphisms (SNPs) in the TLR2 and TOLLIP encoding genes have been associated with disease, including BSI. The aim of this study was to examine potential associations between a selection of SNPs in the genes encoding TLR2 and TOLLIP, and predisposition, severity and outcome of SABSI. All patients ≥18 years of age with at least one *S. aureus* positive blood culture collected from March 2011 through February 2014 at Akershus University Hospital, Lørenskog, Norway, were considered for inclusion. Patients attending elective orthopaedic surgery (total hip and knee replacements, lumbar surgery) served as a control group. **The** TLR2 Arg753Gln, **TLR2 Pro631His, TOLLIP** rs5743942 andrs5743867 **polymorphisms were analysed using** TaqMan SNP Genotyping Assays. A total of 209 SABSI patients and 295 controls were included. **The** TLR2 Arg753Gln and **TLR2 Pro631His** polymorphisms were infrequent with no homozygotes and <10% heterozygotes. T**he included** TLR2 and TOLLIP **polymorphisms were not associated with** susceptibility to SABSI, severity, 30-day all-cause mortality, or SABSI caused by the clonal complex 30 (CC30) genotype.

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**Introduction**

*Staphylococcus aureus* (*S. aureus*) is one of the most common causes of bloodstream infections (BSI) and is associated with high morbidity and mortality (1). *S. aureus* BSI (SABSI) has a 30-day mortality of 20-30% (2, 3). **In order to cause disease, the bacteria need to evade the first line of defence in recognition of microbial structures, the human innate immune system. The Toll-like receptor (TLR) family is the best characterized class of mammal pattern recognising receptors (PRRs) consisting of ten functional human TLR types (4)**. The TLR2 is one of t**he most important receptors in *S. aureus* recognition**. It recognizes a variety of microbial products including lipoproteins and lipoteichoic acid and possesses a protective function during *S. aureus* infection by regulating pro- and anti-inflammatory cytokine responses (5). Toll-like interacting protein (TOLLIP) is a critical regulator of innate immunity by inhibiting the TLR pathway (6), hence indirectly controlling the amount of antimicrobial peptides (7).

Single nucleotide polymorphisms (SNPs) in TLR2 and TOLLIP genes have been associated with different types of infections (8). The most frequently investigated SNP in TLR2 is the TLR2 Arg753Gln (rs5743708) which impairs TLR2 signalling and may potentially increase the susceptibility to staphylococcal infections (9). A recent meta-analysis showed a direct effect on sepsis risk and suggested that this polymorphism could be used as a relevant risk estimate for the development of sepsis (10). Another TLR2 SNP, TLR2 Pro631His (rs5743704), confers either protection or susceptibility to meningitis or tuberculosis, respectively (11, 12). A study on SNPs in TOLLIP showed that a reduced risk of sepsis was associated with a variant of rs5743867, whereas a variant of rs5743942 was associated with increased susceptibility to sepsis in a Chinese Han population (13).

In a previous study, we reported that *S. aureus* respiratory tract infection, unknown focus of infection and *S. aureus* clonal complex 30 (CC30) genotype were independent predictors of mortality in SABSI patients (3). When applying DNA microarray analysis to investigate the association of specific bacterial genes and mortality of SABSI caused by the CC30 genotype, no explanation for the increased mortality could be established (14). We therefore wanted to investigate if SNPs in TLR2 and TOLLIP may be contributing factors to increased mortality.

The aim of this study was to examine the possible association of a selection of SNPs in the genes coding for TLR2 and TOLLIP with predisposition, severity and outcome of SABSI.

**Materials and Methods**

**Study design and setting**

This prospective cohort study was performed at Akershus University Hospital (Ahus), Lørenskog, Norway. The hospital is a 650-bed public secondary referral hospital with a catchment area of ~500.000 inhabitants, or almost 10% of the Norwegian population, and is the largest acute-care hospital in Norway.

**Patients**

All patients ≥ 18 years of age with a *S. aureus* positive blood culture collected from March 2011 through February 2014 were eligible for inclusion. We excluded patients that were non-residents in Ahus’ catchment area, had co-infection with other pathogens, contamination (defined as no clinical signs or symptoms of SABSI and receiving no antibiotic treatment despite one *S. aureus* positive blood culture). Immunosuppressed patients, defined as receiving immunosuppression therapy or steroids during the last 3 months, were also excluded due to possible infection susceptibility bias. Only the first episode of SABSI per patient was included.

**Controls**

Patients ≥ 18 years of age undergoing elective orthopaedic surgery (total hip and knee replacements, lumbar surgery) at Ahus were included as a control group. These patients were included in two periods during March 2011 through June 2014. Inclusion criteria were that the patients were approved for elective orthopaedic surgery and did not develop a *S. aureus* surgical site infection within 1 year after surgery.

**Demographics and clinical data**

We collected clinical information including sex, age, 30-day all-cause mortality (defined as death within 30 days after positive blood culture) and severity of infection categorized as sepsis, severe sepsis, or septic shock according to the criteria specified by (15). In statistical tests, severe sepsis and septic shock were combined. Physical status indicated by the American Society of Anesthesiologists (ASA) score was obtained for the controls.

**SNP genotyping**

EDTA blood samples from all patients and controls were centrifuged at 3000g for 10 minutes. Buffy coat was separated and stored at -20ºC. DNA was extracted from buffy coat using QIAsymphony DSP DNA Mini Kit on the QIAsymphony platform (Qiagen) according to manufacturer’s recommendations. DNA concentration was adjusted to ~10 ng/μL.

The SNPs were selected based on previously published association with increased susceptibility to infection and sepsis. See discussion for further details. The following SNP and assays were included: 1) TLR2 rs5743708 (Arg753Gln), C\_27860663\_10; 2) TLR2 rs5743704 (Pro631His), C\_25607736\_10; 3) TOLLIP rs5743867 (intron), C\_1582169\_10 and 4) TOLLIP rs5743942 (intron), C\_1582183\_10. All assays were TaqMan SNP Genotyping Assays (Applied Biosystems) performed according to manufacturer’s protocol. The assays were run on an Applied Biosystems 7900HT Fast Real-Time PCR system using 5uL reaction volume and 384-well reaction plate. Each extracted DNA sample was run in parallels and a randomly selected 10% of the samples were re-run as technical controls. SNP genotypes were designated using Taqman Genotyper version 1.2 software (Life Technologies).

**Statistical analyses**

The study was powered to detect a difference of 10% between patients (SNP frequency 20%) and controls (SNP frequency 10%) with a power of 80% and p=0.05, two-sided test. A total of 179 patients and 269 controls were needed with ratio 2:3 between the groups. The sample size in this study was therefore sufficient with 209 SABSI patients and 295 controls.

Descriptive statistics were compared using the Mann-Whitney U test or Fisher’s exact test. All statistical tests were done using the IBM SPSS Statistics software version 21 (SPSS Inc., Chicago, USA) and STATA version 14.1 (StataCorp, College Station, Texas, USA). The significance level was set to p<0.05. The distribution of alleles between the different groups was compared using Fisher’s exact test and if statistically significant adjusted for age and gender using logistic regression and presented as odds ratios (OR, 95% confidence interval). The allele frequencies were calculated using the Hardy-Weinberg equation and all tested populations (SABSI patients in total and divided into degrees of severity, 30-day all-cause mortality or SABSI caused by the CC30 genotype, and controls) were in equilibrium.

**Ethics**

The study was approved by the Regional Committee for Medical and Health Research Ethics (2009/2149). Informed consent was obtained from all individual participants included in the study.

**Results**

Of the 249 eligible SABSI patients, DNA was available from 209 patients. The control group consisted of 295 patients. Clinical data are presented in Table 1. Age was higher in the SABSI group compared to the control group (70 years versus 66 years, *p*=0.007) and there were more men among the SABSI patients (62% versus 46%, *p*<0.001). The majority of the controls had ASA score 2 (75%), 13% had ASA score 1 and 12% had ASA score 3. 30-day all-cause mortality in patients with SABSI was 15%.

Distributions and comparisons of SNPs in SABSI versus controls are presented in Table 2. Neither SABSI patients nor controls were homozygous for TLR2 Pro631His and TLR2 Arg753Gln. No statistically significant differences, suggesting predisposition, could be observed when comparing the distribution of alleles in SABSI patients versus the elective patients apart from TOLLIP rs5743867. However, this difference was only significant prior to adjustment for age and gender. The OR for having SABSI versus controls adjusted for age and gender for TOLLIP rs5743867 was 1.39 (95% CI: 0.88-2.21, *p*-value 0.158), for heterozygote (A/G), and 7.23 (95% CI: 0.82-63.65, *p*-value 0.075) for homozygote (G/G) compared with wildtype (A/A, reference group).

Distributions and comparisons of the SNP in SABSI stratified by severity and 30-day all-cause mortality are presented in Table 3. Of the 209 SABSI patients, 169 (81%) patients had sepsis, whereas the remaining 40 (19%) patients had severe sepsis or septic shock. A total of 32 (15%) patients died within 30 days. No significant associations between the selected SNPs and severity of sepsis or 30-day all-cause mortality were identified.

Of the 209 SABSI, 30 (14%) were infected with a strain belonging to the CC30 genotype. The distribution of alleles among CC30 versus non-CC30 genotype was not statistically significant (Table 4).

**Discussion**

 In t**his study, we did not detect any** associations between frequencies of the selected SNPs in TLR2 and TOLLIP and predisposition, severity and outcome of SABSI or association with the CC30 genotype. The selected polymorphisms will be discussed separately.

A number of polymorphisms have been reported in TLR2 with Arg753Gln being the most frequently discussed(10). Despite a small sample size, it was suggested to increase susceptibility to septic shock caused by Gram positive microbes (9). It has been associated with an increased risk of infective endocarditis (16) and shorter time-to-onset of severe sepsis or septic shock (17). A recent meta-analysis supported a direct effect of the TLR2 Arg753Gln polymorphism on sepsis risk. However, the sample sizes for the included studies ranged from 15-187 cases and 16-409 controls (10). Even with our sample size, we could not identify any associations between the TLR2 Arg753Gln polymorphism and susceptibility to SABSI, severity of sepsis, 30-day all-cause mortality or SABSI caused by the CC30 genotype. This is in accordance with Moore and co-workers (18), who did not identify any association between severe disease or mortality attributable to *S. aureus*. Even though the polymorphism prevents TLR2 from responding to lipoteichoic acid (19), one functional allele is enough to compensate for this polymorphism as the response to infection is the same in heterozygote as wild-type subjects (20). No homozygotes for the TLR2 Arg753Gln polymorphism were identified in our study.

**For the TLR2 Pro631His, no homozygotes were identified and only 4% of the SABSI patients and 6% of the controls were heterozygotes.** This polymorphism involves a proline to histidine substitution and is associated with a **~30%** reduction of **NF-κB activation compared to the ancestral form (21, 22). It affects the extent of the cellular response upon binding the ligand resulting in a different cytokine profile during infection that might modify severity and outcome of disease (12). TLR2 Pro631His is associated with increased susceptibility to complicated skin and skin structure infections (23) and tuberculosis (15). A protective role in meningococcal disease has been suggested (11).**

**The association of polymorphisms in TOLLIP,(6) with infections has not been well documented. However, Song and co-workers (13) reported that the minor C-allele in TOLLIP rs5743867 was associated with a decreased risk of sepsis in a Chinese Han population. Homozygotes for this allele showed a higher TOLLIP mRNA expression level in peripheral blood mononuclear cells (PBMCs) after exposure to lipopolysaccharide (13). A recent study showed a decreased risk of post-operative sepsis after complex open heart surgery in infants with the TOLLIP rs5743867 polymorphism (24). We identified a higher frequency of homo- and heterozygotes for the TOLLIP rs5743867 polymorphism in SABSI patients in bivariate analysis compared to controls, but that finding was not statistically significant after adjusting for age and gender.**

**There are several limitations in this study**. Both TLR2 SNPs were infrequent with no homozygotes identified and less than 10% heterozygotes. Only TOLLIP rs5743942 showed a higher homo- and heterozygotes distribution with 16% homozygotes in SABSI patients and 19% in controls. As frequencies of polymorphisms differ among ethnic groups, this may explain why we could not identify any associations with the TOLLIP rs5743867 polymorphism. Although ethnicity was not a recorded parameter in our SABSI patients and controls, we estimate roughly (based on medical records) that less than 10% are non-Caucasian, excluding population heterogeneity as a possible explanation. The heterozygote allele frequencies were higher in the sepsis studies on Han Chinese and Indonesian infants, (~44% in both groups) (13, 24), than in our SABSI and control group (21% and 17%, respectively). These studies did, however, include sepsis caused by all microbes, not restricted only to SABSI as in the present study. We included only immunocompetent patients to ensure “more true” associations removing important factors affecting the immune system and bias towards SABSI susceptibility, as the immunocompromised patient has an omnipresent risk of infection predisposing to systemic compromise like sepsis and septic shock (25).

The use of patients undergoing elective, orthopaedic surgery as a control group may be questionable. However, these patients represent the same ethnicity and come from the same geographic region as the SABSI patients and they had no ongoing infection or surgical site infections during the first year after surgery. In addition, previous publications have shown that the distribution of *S. aureus* clones causing BSI and other invasive infections, as well as colonization, in different local populations are dominated by the same clones, ensuring a uniform *S. aureus* pressure (14, 26, 27). Finally, we did not **study downstream signalling pathways, which may be important as several mediators are known to modulate signalling through the TLR cascade.**

**The presence of polymorphisms in innate immunity genes may result in major effects on risk and development of disease, and detection of polymorphisms may help predicting patients at a higher risk of developing SABSI as well as identifying new therapeutic intervention candidates. In the present study, the** TLR2 Arg753Gln, **TLR2 Pro631His, and TOLLIP** rs5743942 andrs5743867 **polymorphisms were not associated with** susceptibility to SABSI, severity, 30-day all-cause mortality, or SABSI caused by the CC30 genotype. Further larger studies on homogeneous groups are warranted especially on the infrequent TLR2 polymorphisms in SABSI patients.

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**Table 1. Demographic and clinical data on controls and patients with *S. aureus* bloodstream infection (SABSI) and stratification by severity of sepsis, N(%).**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Controls** | **SABSI total** | **Sepsis** | **Severe sepsis and toxic shock** |
| **N** | 295 | 209 | 169 | 40 |
| **Median age in years [range]** | 66 [18-88] | 70 [21-95] | 70 [21-95] | 67 [22-92] |
| **Male** | 136 (46) | 130 (62) | 107 (63) | 23 (58) |
| **30-day all-cause mortality** | - | 32 (15) | 16 (9) | 16 (40) |

**Table 2. Comparison of single nucleotide polymorphisms (SNPs) in patients with *S. aureus* bloodstream infection (SABSI) and controls, N(%).**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **SABSI** | **Controls** |  |
| **SNP** | N=209 | N=295 | ***p*-value** |
| **TLR2 Pro631His** |  |  |  |
|  C/C | 201 (96) | 276 (94) | **0.23** |
|  A/C | 8 (4) | 19 (6) |  |
|  A/A | 0 | 0 |  |
|  |  |  |  |
| **TLR2 Arg753Gln** |  |  |  |
|  G/G | 192 (92) | 275 (93) | **0.61** |
|  A/G | 17 (8) | 20 (7) |  |
|  A/A | 0 | 0 |  |
|  |  |  |  |
| **TOLLIP rs5743867** |  |  |  |
|  A/A | 160 (77) | 245 (83) |  **0.041\*** |
|  A/G | 44 (21) | 49 (17) |  |
|  G/G | 5 (2) | 1 (0) |  |
|  |  |  |  |
| **TOLLIP rs5743942** |  |  |  |
|  A/A | 65 (31) | 85 (29) | **0.70** |
|  A/G | 110 (53) | 154 (52) |  |
|  G/G | 34 (16) | 56 (19) |  |

\*Not significant when adjusted for age and gender

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Sepsis severity** |  | **30-day all-cause mortality** |  |
|  | **Sepsis** | **Severe sepsis and toxic shock** | ***p*-value** | **Alive** | **Dead** | ***p*-value** |
| **SNP** | N=169 | N=40 |  | N=177 | N=32 |  |
|  |  |  |  |  |  |  |
| **TLR2 Arg753Gln** |  |  |  |  |  |  |
|  G/G | 155 (92) | 37 (92) | **1.00** | 161 (91) | 31 (97) | **0.48** |
|  A/G | 14 (8) | 3 (8) |  | 16 (9) | 1 (3) |  |
|  A/A | 0 | 0 |  | 0 | 0 |  |
|  |  |  |  |  |  |  |
| **TLR2 Pro631His** |  |  |  |  |  |  |
|  C/C | 162 (96) | 39 (98) | **1.00** | 170 (96) | 31 (97) | **1.00** |
|  A/C | 7 (4) | 1 (3) |  | 7 (4) | 1 (3) |  |
|  A/A | 0 | 0 |  | 0 | 0 |  |
|  |  |  |  |  |  |  |
| **TOLLIP rs5743867** |  |  |  |  |  |  |
|  A/A | 126 (75) | 34 (85) | **0.39** | 134 (76) | 26 (81) | **0.12** |
|  A/G | 38 (22) | 6 (15) |  | 40 (22) | 4 (13) |  |
|  G/G | 5 (3) | 0 |  | 3 (2) | 2 (6) |  |
|  |  |  |  |  |  |  |
| **TOLLIP rs5743942** |  |  |  |  |  |  |
|  A/A | 53 (31) | 12 (30) | **0.77** | 52 (29) | 13 (41) | **0.31** |
|  A/G | 90 (53) | 20 (50) |  | 97 (55) | 13 (41) |  |
|  G/G | 26 (16) | 8 (20) |  | 28 (16) | 6 (18) |  |

**Table 3. Comparison of single nucleotide polymorphisms (SNPs) in 209 *S. aureus* BSI patients stratified by severity and 30-day all-cause mortality, N(%).**

**Table 4. Comparison of single nucleotide polymorphisms (SNPs) and clonal complex 30 genotype in patients with *S. aureus* bloodstream infection (N=209), N(%).**

|  |  |  |  |
| --- | --- | --- | --- |
| **SNP** | **CC30**N=30 | **non-CC30**N=179 | ***p*-value** |
| **TLR2 Arg753Gln** |  |  |  |
|  G/G | 25 (83)  | 167 (93)  |  |
|  A/G | 5 (17) | 12 (7) | 0.08 |
|  A/A | 0 | 0 |  |
|  |  |  |  |
| **TLR2 Pro631His** |  |  |  |
|  C/C | 29 (97) | 172 (96) |  |
|  A/C | 1 (3) | 7(4) | 1.00 |
|  A/A | 0 | 0 |  |
|  |  |  |  |
| **TOLLIP rs5743867** |  |  |  |
|  A/A | 23 (77) | 137 (76) | 0.91 |
|  A/G | 7 (23) | 37 (21) |  |
|  G/G | 0 | 5 (3) |  |
|  |  |  |  |
| **TOLLIP rs5743942** |  |  |  |
|  A/A | 15 (50) | 50 (28) | 0.07 |
|  A/G | 12 (40) | 98 (55) |  |
|  G/G | 3 (10) | 21 (17) |  |
|  |