Genetic
Variation in
Trauma Patients

Sequence Variations in Innate Immune
Response Genes Influence Outcome

M.W.G.A. Bronkhorst

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Genetische variatie in traumapatiënten
Genomische variatie in het aangeboren immuunsysteem beïnvloedt de uitkomst

Proefschrift

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Ter nagedachtenis aan mijn vader
J.B.M. Bronkhorst
1944 – 2010

GACCTTAAAGTACTTAGTAAGGGA

Chapter 1

Introduction and outline of this thesis

INTRODUCTION

Trauma is a major public health problem worldwide, ranking as the fourth leading cause of death. Approximately 5 million people die each year as a result from injuries worldwide (1-3). Injuries account for 9% of global mortality. For every trauma death it is estimated that there are dozens of hospitalizations, hundreds of emergency department visits and thousands of doctor's appointments (2). The total number of deaths from injuries was greater than the number of deaths from HIV/AIDS, tuberculosis, and malaria combined (3.8 million) (4, 5) (Figure 1). According to the World Health Organization (WHO), nearly one third are the result of violence (suicide, homicide and war) and nearly one quarter are the result of road traffic crashes. Motor vehicle collisions were the ninth leading cause of death in 2004 with 1.3 million deaths annually and will rise to the fifth leading cause of death worldwide by 2030 (2). Trauma accounts for approximately 30% of all Intensive Care Unit admissions (6). The burden of disease related to injuries, particularly road traffic injuries, interpersonal violence, war and self-inflicted injuries is expected to rise substantially by the year 2020 (7).
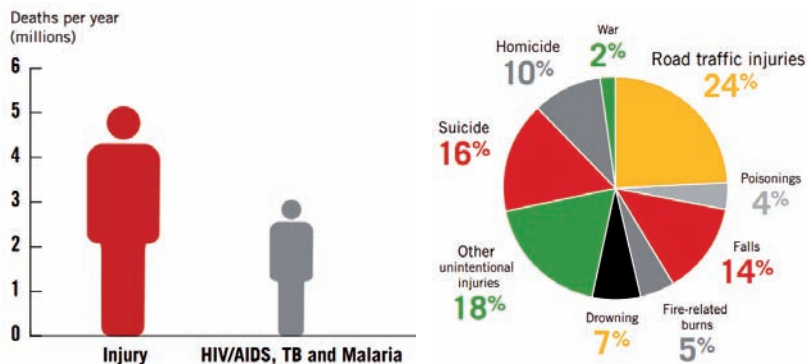
Figure 1. The magnitude and causes of injury mortality.
Reprinted with permission from the World Health Organization.

Donald Trunkey suggested that mortality from trauma follows a trimodal distribution (Figure 2) (8). The first peak of "immediate deaths" is caused by devastating injury to the brain, brain stem, upper spinal cord, the heart or one of the major blood vessels. These patients usually die at the scene of injury. The second peak, characterized as "early deaths" represents people who die within the first few hours after an injury most frequently as a result of exsanguination. These first few hours have been termed "the golden hour" (9). Most of these injuries can now be treated and modern trauma programs, such as Advanced Trauma Life Support (ATLS) from the American College of
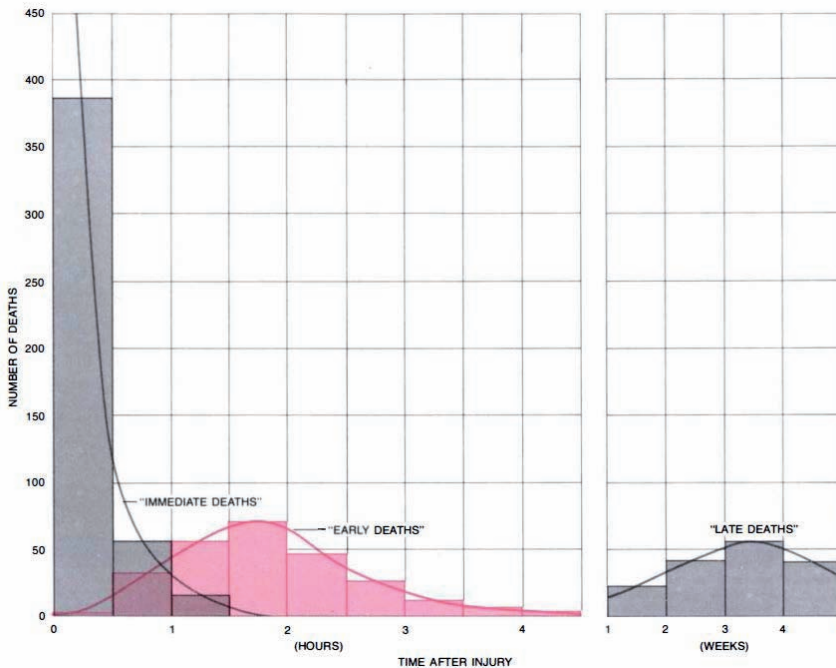
Figure 2. The original illustration of Donald D. Trunkey from *Scientific American* (1983) depicting the suggested trimodal distribution of trauma deaths based on a sample of 862 trauma deaths recorded over a two year period by Trunkey's group at San Francisco General Hospital (*reprinted with permission from Scientific American*).

Surgeons (ACS) and Definitive Surgical Trauma Course (DSTC) from the International Association for Trauma Surgery and Intensive Care (IATSIC), as well as specialized trauma hospitals have made an substantial impact on cognitive and clinical skills of doctors involved in the field of trauma and emergency care (10). The third peak, characterized as "late deaths" represents patients who die in days or weeks following the original trauma as a result of infection, sepsis or multiple organ failure (8). Trunkey stated that in this phase time is less of a factor than the quality of medical care and the extent of medical knowledge. And indeed the existence of this third peak has now been questioned by a number of authors claiming that progress in the field of Intensive Care Medicine has improved the treatment of multiple organ failure (11-13).

The prevalence of multiple organ failure in trauma patients is around 5% (14). Mortality in multiple organ failure increases with the number of organ systems affected with 32% mortality with two organ systems, 67% with three organ systems, and 90% mortality when four organ systems failed (14). Infectious posttraumatic complications such as sepsis and multiple organ dysfunction syndrome (MODS) remain important causes

for morbidity and mortality in patients who survive the initial trauma (15, 16). These complications increase the burden of trauma treatment cost to society.

In The Netherlands in 2012 more than 75000 injured patients were admitted to a hospital (with a mean Injury Severity Score of 9) of which around 4500 were directly admitted to an Intensive Care Unit (ICU). A total of 4870 patients with ISS greater than 15 were admitted to Dutch hospitals (17).

GENETIC VARIATION AND THE INFLUENCE ON DISEASE OUTCOME

The outcome of severe injury is determined by multiple factors. Age, co-morbidities and injury severity are well known factors but the genetic composition of the patient may well be one such factor (18-21). Genetic variations in the innate immune system of severely injured patients influence complement activation and the immune response by altered or absent protein synthesis and hence contribute to susceptibility and severity of infections and lead to prolonged hospital stay and increased cost. Genetic variability in pro- and anti-inflammatory cytokines may contribute to different clinical phenotypes in patients at high risk of critical illness (22). For example, variation in *TLR4*, a gene that codes for Toll-like Receptor 4 protein, may lead to prolonged stay at an ICU ward in patients with severe sepsis (23). Genetic variation in the innate immune system has been shown to be of importance in viral (24), bacterial (25), fungal (26) and protozoan (27) infections. There is increasing evidence for the role of genetic variation in the innate immune system on infectious complications in sepsis and trauma (18, 28-32).

SINGLE NUCLEOTIDE POLYMORPHISMS

Humans have 23 pairs of chromosomes and, on average, all humans are 99.9% similar to any other human in terms of DNA sequence. The remaining 0.1% accounts for all the differences between humans. These physical differences are known as 'polymorphisms'. The average number of nucleotide differences between a randomly chosen pair of humans is consistently estimated to lie between 1 in 1,000 and 1 in 1,500 (33, 34). This proportion is low compared with those of many other species, from fruit flies to chimpanzees, reflecting the recent origin of our species from a small founding population of approximately 10.000 individuals as recent as 200,000 years (35). The coding regions of DNA contain the approximately 20,000-25,000 human protein-coding genes responsible for the more than 20,000 known proteins (36). The coding regions - the exons are collectively referred to as the exome - take up less than 2% of all DNA. More than 98% of the human genome is composed of non-coding DNA (ncDNA) of which the function
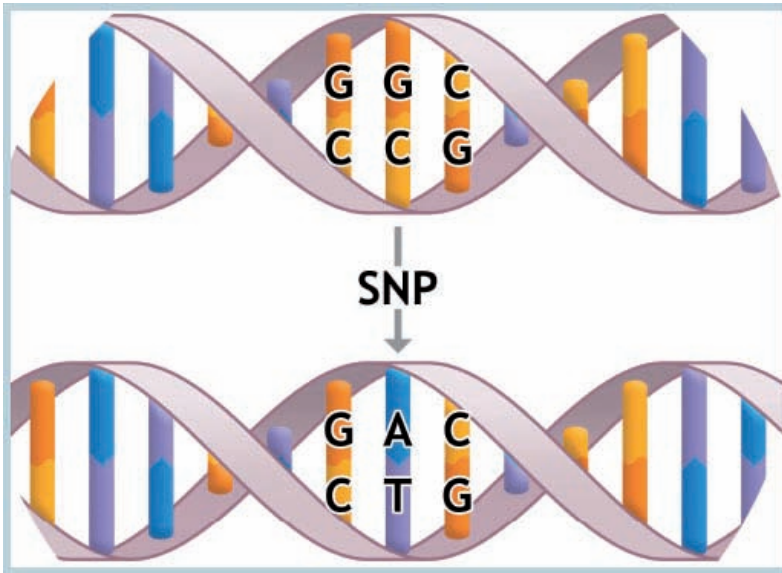
Figure 3. Single nucleotide polymorphisms are variations of a single nucleotide in the sequence of DNA

is partly unknown. Human diploid cells contain around six billion base pairs. These base pairs are pairs of nucleotides, the building blocks of DNA (A, C, T, and G).

Single Nucleotide Polymorphisms (SNPs; pronounced '*snip*,' plural *'snips'*) are variations of only one such nucleotide in the sequence of DNA. A typical genome differs from the reference human genome at 4.1 million to 5.0 million sites (37). More than 99,9% of all DNA variation is caused by SNPs and short indels making them the most common type of sequence variation but structural variants affect more bases affecting a total of 20 million base pairs in total (37). Most SNPs occur in non-coding regions of DNA. Because there are approximately three billion nucleotide base pairs in the haploid human genome, each pair of humans differs, on average, by two to three million base pairs.

SNPs in coding regions of DNA may have the potential to alter the amino acid sequence in a protein but as a result of degeneracy this is not always the case. Some proteins are coded by more than one codon. SNPs in coding regions are called *synonymous* if they do not affect the amino acid sequence and *non-synonymous* if they do influence the amino acid sequence of a protein. The non-synonymous SNPs can be divided into *missense* SNPs and *nonsense* SNPs.

Missense SNPs result in the transcription of a different amino-acid, changing the functionality of the resulting protein as is the case in *Factor V Leiden thrombophilia* (38) and *sickle cell disease* (39). A nonsense SNP results in the formation of a premature stopcodon

leading to a truncated, incomplete protein as is the case in β-*thalassemia* in Sardinia (40) and some forms of *cystic fibrosis* (41).

AIM AND OUTLINE OF THIS THESIS

This thesis aims to study the influence of sequence variations in the innate immune system on infectious outcome in a heterogeneous cohort of severely injured patients in a level 1 trauma center.

In **Chapter 1** the subject of late infectious complications following severe injury is introduced hypothesizing a role for genomic sequence variations in the development of such complications. A patient is presented in **Chapter 2** who survived the initial trauma but developed serious infectious complications . **Chapter 3** reviews all currently available literature that specifically describes the influence of different sequence variations on infectious complications in cohorts of trauma patients. Genetics of *MBL2*, a coding gene for a key protein in the innate immune system, is described in **Chapter 4** and **Chapter 5** describes epidemiology, evolutionary biology and the clinical effects of variations in *MBL2* on various micro-organisms and clinical conditions. **Chapter 6** describes the effects of sequence variations in *MBL2, MASP2* and *FCN2*, important genes in the Lectin Pathway of complement activation, in a prospectively analyzed cohort of trauma patients. In **Chapter 7** this same cohort is studied for effects on outcome of sequence variations in *TLR2, TLR4, TLR9* and *CD14*. A prediction model is suggested in **Chapter 8** combining the effects of different combinations of haplotypes on clinically relevant conditions.

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GACCTTAAAGTACTTAGTAAGGGA

Chapter 2

Multiple infectious complictions in a severely injured patient with single nucleotide polymorphisms in important innate immune response genes

Maarten W.G.A. Bronkhorst, Peter Patka, Esther M.M. van Lieshout

ABSTRACT

Trauma is a major public health problem worldwide. Infectious complications, sepsis, and multiple organ dysfunction syndrome (MODS) remain important causes for morbidity and mortality in patients who survive the initial trauma. There is increasing evidence for the role of genetic variation in the innate immune system on infectious complications in severe trauma patients. We describe a trauma patient with multiple infectious complications caused by multiple micro-organisms leading to prolonged hospital stay with numerous treatments. This patient had multiple single nucleotide polymorphisms (SNPs) in the *MBL2*, *MASP2*, *FCN2* and *TLR2* genes, most likely contributing to increased susceptibility and severity of infectious disease.

INTRODUCTION

We describe a trauma patient with multiple infectious complications caused by multiple micro-organisms leading to prolonged hospital stay with numerous treatments. This patient had multiple single nucleotide polymorphisms (SNPs) in the innate immune system, most likely contributing to increased susceptibility and severity of infectious disease.

SINGLE NUCLEOTIDE POLYMORPHISMS

Humans have 23 pairs of chromosomes and, on average, all humans are 99.9% similar to any other human in terms of DNA sequence. The remaining 0.1% account for all the differences between humans. These physical differences are known as 'polymorphisms'. The coding regions of DNA contain the approximately 20,000 human protein-coding genes. The coding regions take up less than 2% of all DNA. More than 98% of the human genome is composed of non-coding DNA of which the function is partly unknown. Human diploid cells contain around six billion base pairs. These base pairs are pairs of nucleotides, the building blocks of DNA (A, C, T, and G).

Single Nucleotide Polymorphisms (SNPs; pronounced '*snip*', plural '*snips*') are variations of only one such nucleotide in the sequence of DNA. Around 90% of all DNA variation is caused by SNPs making them the most common type of sequence variation. To date more than 60 million SNPs have been discovered in the human genome. Most SNPs occur in non-coding regions of DNA.

SNPs in coding regions of DNA may have the potential to alter the amino acid sequence in a protein but as a result of degeneracy this is not always the case; some proteins are coded by more than one codon. SNPs in coding regions are called *synonymous* if they do not affect the amino acid sequence and *non-synonymous* if they do influence the amino acid sequence of a protein. The non-synonymous SNPs can be divided into *missense* SNPs and *nonsense* SNPs. Missense SNPs result in the transcription of a different amino-acid, changing the functionality of the resulting protein as is the case in *Factor V Leiden thrombophilia* (1) and *sickle cell disease* (2). A nonsense SNP results in the formation of a premature stopcodon leading to a truncated, incomplete protein as is the case in β-*thalassemia* in Sardinia (3) and some forms of *cystic fibrosis* (4).

CASE REPORT

Patient A, a 57 year old mechanic with a medical history of occasional use of cocaine and of gradually worsening vision in the last five months as a result of optic nerve atrophy tripped over a low brick wall at work and fell about one meter on the back of his head. He was immediately found to be tetraplegic by the paramedics and was transferred to a level 1 trauma center. Clinically, the complete cord lesion was found to be on the level of C3 and C4. Computed tomography showed a congenital narrowing of the spinal canal at the level of C3 and C4 as well as an old fracture of the third thoracic vertebra (Figure 1). Magnetic resonance scanning showed hemorrhage in the myelum at the level of C3 and C4 as the reason for the tetraplegia (Figure 2). He was transferred to the Intensive Care Unit for ventilator support. On day 3 he developed acute respiratory distress syndrome (ARDS) and pneumonia in the right lower lobe, possibly as a result of aspiration, from which purulent sputum was removed repeatedly. The sputum grew Haemolytic *Streptococcus* group C, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Enterobacter cloacae* for which he was treated with piperacillin/tazobactam. On day 10 a percutaneous tracheostomy was used and weaning was possible. On day 20 patient was no longer dependent on ventilator support. He spent a total of 32 days on the Intensive Care Unit and 76 days in the hospital before being discharged to a rehabilitation center. After three months he was admitted again, this time to the department of Internal Medicine, for fe-

Figure 1. Computed tomography showing congenital narrowing of the spinal canal at the level of C3 and C4. Also, an preexisting injury at the level of T2 and T3 can be seen.

Figure 2. MR shows bleeding in the myelum at the level of C3 and C4 causing the complete cord syndrome.

ver, diarrhea and productive cough. Radiology was suspect for pulmonary tuberculosis and *Mycobacterium tuberculosis* was eventually found in gastric contents. He was placed on tuberculostatic triple therapy. His diarrhea was explained by pseudomembranous colitis caused by *Clostridium* toxins. He was given metronidazol and was free of diarrhea after ten days. He developed deep venous thrombosis in the right subclavian vein ultimately leading to erysipelas and ulceration on the fingers from which *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Morganella morganii* and *Proteus mirabilis* were cultured. The urine was positive for *Klebsiella pneumoniae* and *Enterococcus faecalis*. The ulcers were treated surgically.

Because this patient participated in a scientific trial studying genetic variation in trauma patients, his genome was sequenced for several single nucleotide polymorphisms (SNPs). Several SNPs were found (see table 1): heterozygosity in *MBL2* exon 1 (*i.e.*, AC genotype), heterozygosity in *MBL2* promoter region Y-221X, (YX genotype) homozygosity for the minor allele in *MASP2* Y371D (DD genotype), heterozygosity in *FCN2* T236M (TM genotype), heterozygosity in *FCN2* A258S (AS genotype), homozygosity for the minor allele in *TLR2* T-16934A (AA genotype), and heterozygosity in *CD14* C-159T (CD genotype).

Table 1. Genotypes found in presented patient

Gene	SNP	OMIM	Cytogenic location	dbSNP ID	Genotype of our patient
MBL2 exon 1	p.Gly57Glu	154545	10q21	rs1800451	AC
MBL2 promoter	p.Y-221X	154545	10q21	rs7096206	YX
MASP2	p.Y371D	605102	1p36	rs12711521	DD
FCN2	p.A258S	601624	9q34	rs7851696	AS
TLR2	g.T-16934A	603028	4q31	rs4696480	AA

SNP: single nucleotide polymorphism. **OMIM**: Online Mendelian Inheritance in Man, an online catalog of human genes and genetic disorders. **dbSNP ID**: Single Nucleotide Polymorphism Database, a free public archive for genetic variation hosted by the National Center for Biotechnology Information (NCBI).

DISCUSSION

Mortality as a result of sepsis in the third peak of Trunkey (5) has not changed in recent times despite the improvements in treatments in the Intensive Care Unit leading to reduction in the incidence of sepsis itself (6). The onset of sepsis in trauma patients is of course multifactorial, but genetic variation at the level of the innate immune system is certainly one important contributing factor. SNPs in genes coding for important proteins in the innate immune system, such as the complement system, may produce low serum levels of these proteins or they may leave these proteins dysfunctional. Hence, the immune response and cytokine response to trauma and infection is reduced leading to increased susceptibility and severity of infectious complications. These complications cause prolonged hospital stay and increase the use of antibiotics, the number of complications, and the cost of care to society.

The patient described in this study was proband in a prospective study in severely injured trauma patients focusing on SNPs in the innate immune system and the influence on infectious complications. A number of SNPs were found in this patient (see Table 1).

In the lectin pathway of complement activation three important genes were studied: *MBL2, MASP2*, and *FCN2*. The *MBL2* gene encodes for mannose-binding lectin (MBL), a protein that is secreted by the liver as part of the acute-phase response and is involved in innate immune defense. The ligands for MBL are expressed by a wide variety of microorganisms, and binding of the protein leads to opsonisation of the pathogen as well as activation of the complement system. Genetic variation in this gene leads to a dramatic decrease in circulating serum MBL. Heterozygosity for variants in exon 1 (*i.e.*, an A0 genotype) conferred an increased risk for wound colonization and infection in severely injured patients (7). This had previously only been demonstrated in a murine model of burns (8). Also, the YX promoter genotype increased the risk of fungal colonization and infection in trauma patients (7). Presented patient carried an AC genotype

in exon 1 and an YX genotype in the promoter region. MBL activates the complement pathway through mannan-binding lectin serine protease 2 (MASP2). *MASP2* Y371D DD homozygosity significantly increased the risk for SIRS and septic shock in trauma patients (7). Moreover, a trend was noted for an increased risk of Gram-positive infections in patients with *MASP2* Y371D DD genotype. Above presented patient carried the *MASP2* Y371D DD genotype. *FCN2* encodes for Ficolin-2, previously termed L-ficolin, a protein which is mainly produced in the liver and has been shown to have carbohydrate binding and opsonic properties in the innate immune system. The homozygous *FCN2* A258S AS genotype increased the risk for developing septic shock in trauma patients (7). Also, wound colonization and infection were significantly increased. A trend was noted for Gram-negative infections. The patient presented in this article carried the *FCN2* A258S AS genotype.

Deficiencies in the lectin pathway have been linked to susceptibility of various pathogens, for example MASP deficient mice are highly susceptible to *Streptococcus pneumoniae* (9) and also children (10) and adults (11) have previously been shown to be highly susceptible to *S. pneumoniae* with deficiencies in MBL and MASP2. In children lectin pathway deficiencies may play a role in *Haemophilus influenzae* (12) but this was not found in a cohort of adults with community acquired pneumonia (13). The effect of MBL genotype on the susceptibility to *Mycobacterium tuberculosis* is controversial (14, 15). In burn injury patients (8) and cystic fibrosis patients (16) MBL deficiency plays an important role on susceptibility to *Pseudomonas aeruginosa* infection and colonization. The *FCN2* A258S polymorphism was previously shown to influence susceptibility to leprosy (17), influence colonization with *Pseudomonas aeruginosa* in cystic fibrosis patients (16) and influence renal transplant outcome (18).

As a membrane surface receptor, TLR-2 recognizes many bacterial, fungal, viral, and certain endogenous substances. The *TLR2* T-16934A polymorphism was previously linked to spontaneous bacterial peritonitis in liver cirrhosis patients (19), atopic dermatitis, asthma and wheezing (20-22) and sarcoidosis (23). The *TLR2* T-16934A genotype was studied in a trauma population by one author (24) who found that the *TLR2* T-16934A TA genotype increased the risk of a Gram-positive infection and SIRS. The *TLR2* T-16934A AA genotype seemed to protect against urinary infection, oddly. However, patient A carried the *TLR2* T-16934A AA genotype but developed positive urine cultures.

Infectious complications are multifactorial in origin. SNPs in the innate immune system contribute to susceptibility and severity of these infections and lead to prolonged hospital stay and increased cost. The presented patient demonstrates the clinical course of such complications that will be recognized by all surgeons and physicians. In the future we expect that initial genotyping will become routine workup (e.g. with DNA micro array chips) in all trauma patients to quantify the individual risk for developing infections and other complications. Patients identified to be at risk for developing infec-

tious complications can be prophylactically treated with antibiotics in an early stage or can be supplemented with plasma from mixed donors containing the deficient proteins. Substitution therapy with purified or recombinant proteins has also produced clinical results, for example in the case of MBL-deficiency (25-29). Further studies are needed in order to determine which genes affect this risk and to quantify their effect.

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GACCTTAAAGTACTTAGTAAGGGA

Chapter 3

Effects of sequence variation in innate immune response genes on infectious outcome in trauma patients: a comprehensive review

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ABSTRACT

Objective: Infectious complications, sepsis and multiple organ dysfunction syndrome (MODS) remain important causes for morbidity and mortality in patients who survive the initial trauma. Increasing evidence suggests that genetic variants, particularly Single Nucleotide Polymorphisms (SNPs), are critical determinants for interindividual differences in both inflammatory responses and clinical outcome in sepsis patients. Although the effect of SNPs on sepsis and MODS has been studied in many populations and diseases this review aimed to summarize the current knowledge on the effect of SNPs on infectious complication specifically in trauma patients.

Methods: review of available literature in PubMed database.

Results: The following genes have been studied in populations of trauma patients: *CD14, HMGB1, IFNG, IL1A, IL1B, IL1RN, IL4, IL6, IL8, IL10, IL17F, IL18, MBL2, MASP2, FCN2, TLR1, TLR2, TLR4, TLR9, TNF, LTA, GR, MYLK, NLRP3, PRDX6, RAGE, HSPA1B, HSPA1L, HSP90, SERPINE1, IRAK1, IRAK3, VEGFA, LY96, ANGPT2, LBP, MicroRNA and mtDNA*. In this review we discuss the genes of the Pattern Recognition Receptors (PRR), Signal Transducing Adaptor Proteins (STAP) and Inflammatory Cytokines of the innate immune system.

Conclusions: A number of genetic variations have so far been studied in cohorts of trauma patients. Studies are often unique and numbers sometimes small. No definitive conclusions can be reached at this time about the influence of specific sequence variations on outcome in trauma patients.

INTRODUCTION

Trauma is a major public health problem worldwide, ranking as the fourth leading cause of death. In 2010, there were 5.1 million deaths from injuries and the total number of deaths from injuries was greater than the number of deaths from HIV/AIDS, tuberculosis and malaria combined (3.8 million) (1, 2). Infectious complications, sepsis and multiple organ dysfunction syndrome (MODS) remain important causes for morbidity and mortality in patients who survive the initial trauma (3). Although the rate of MODS in trauma patients has diminished over the last decade, MODS-related mortality, intensive care unit stay, and mechanical ventilation duration have not changed significantly (4, 5). These complications increase the burden of cost to society.

The primary inflammatory insult determines the magnitude of systemic inflammation and subsequent immune exhaustion, which makes patients prone for septic complications. Both a proinflammatory and anti-inflammatory response appear to coexist in trauma patients, possibly leading to both additional tissue damage by the immune system as well as increased susceptibility for subsequent infections (6). The development of the systemic inflammatory response (SIRS) with liberation of proinflammatory cytokines is recognized as a part of the physiologic response to trauma. Tissue injury following trauma results in depressed cell-mediated immunity (especially T-cell) leading to an increased risk of infectious complications (7). Cytokine production varies between individuals, due to genetic background and certain allelic variants of cytokine genes; in particular, single nucleotide polymorphisms (SNPs) in coding regions of cytokine genes are associated with higher or lower cytokine production. Polymorphism may be considered as an important genetic risk factor for susceptibility to posttraumatic sepsis and a potential target for immunotherapy. Increasing evidence suggests that genetic variants, particularly SNPs, are critical determinants for interindividual differences in both inflammatory responses and clinical outcome in sepsis patients (8). Although the effect of SNPs on sepsis and MODS has been studied in many populations and diseases this review aims to summarize the current knowledge on SNPs in genes of the innate immune system in trauma patients only.

A literature search was performed in PubMed by using "genetic variation", "trauma", and "innate immunity" and synonyms as search string. The search was finalized by cross-checking references. Studies describing the effect of SNPs in innate immune response genes on infectious complications in trauma patients were included. An overview of the SNPs included is shown in Supplemental Table S1.

1. PATTERN RECOGNITION RECEPTORS AND COMPLEXES

1.1 Toll-Like Receptors and associated genes

Toll-Like Receptor 1 (TLR1)

Three SNPs in TLR1 were studied in trauma patients (Table 1) (9). The *TLR1* -7202G allele (rs5743551) and the *TLR1* 742AG(p.Asn248Ser) (rs4833095) were associated with increased risk of mortality in sepsis and Gram-positive sepsis, respectively.

Toll-Like Receptor 2 (TLR2)

Five SNPs in *TLR2* have been studied in a trauma population (Table 1).

The *TLR2* 19216T>C (rs3804099) CC genotype conferred a significantly higher risk of developing sepsis and higher MOD scores than those with a TT or TC genotype (10).

The *TLR2* p.R753Q SNP was studied by two authors (11, 12). McDaniel *et al*. found the AG genotype significantly more often in septic patients (62,5%) than in aseptic patients (25%) in African-American patients (not so in whites) (12). Bronkhorst *et al*. found no association with sepsis or mortality in a mixed ethnic cohort of 219 trauma patients (11).

For the *TLR2* -16934T>A the TA genotype increased the risk of a Gram-positive infection and SIRS in a trauma population by (11) .

Toll-Like Receptor 4 (TLR4)

SNPs in *TLR4* have been studied in trauma patients (11-15) and in burns patients (16-19) (Table 1). In trauma patients multiple SNPs in *TLR4* have been studied making comparison difficult (11-15).

The *TLR4* 896A>G (rs 4986790) was studied in four cohorts of burns patients. Three studies that used the same growing cohort used sepsis as endpoint (16, 17, 19) and two studies used mortality as endpoint (18, 19)cThe *TLR4* 896A>G was significantly associated with an increased risk for severe sepsis (16, 17). Shalhub could not confirm this (19). Moreover, no association with mortality was found (18, 19). Carriage of the TLR4 896G allele was associated with a decreased risk of complicated sepsis in trauma (15). The cosegregating *TLR4* p.D299G and *TLR4* p.T399I were studied in trauma patients by two authors (11, 12), both of whom were not able to demonstrate an association between genotype and infection or outcome of sepsis. Chen *et al*. studied the clinical relevance of five single nucleotide polymorphisms in *TLR4* (-2381A>G, -2242T>C, -1892G>A, -1837A>G, and -1418T>C) in patients with major trauma (13). Only in *TLR4* -2242T>C polymorphism higher sepsis morbidity rates and multiple organ dysfunction scores were found. Duan *et al*. prospectively studied the *TLR4* 11367G>C polymorphism in patients with major trauma (14). Patients with the C variant allele had significantly lower sepsis morbidity than those homozygous for the G allele. In addition, MOD scores in the

patients with trauma who carry the C allele were also significantly lower than those in the patients carrying the G allele.

Toll-Like Receptor 9 (TLR9)
Several SNPs in *TLR9* have been studied in trauma patients by two authors (Table 1) (11, 20).

Chen *et al.* studied the effect of five polymorphisms in TLR9 in 557 consecutive Han Chinese patients with severe multiple blunt trauma injuries (20). Median ISS was 25 and 37.9% of patients developed sepsis. The rs187084 (−1486A>G), rs352140 (2848C>T) and rs352162 (6577T>C) SNPs were significantly associated with TLR9-mediated TNF-α production. Patients with a minor allele of the rs187084, rs352139 or rs352162 poly-morphism had a higher sepsis morbidity rate. Of these three SNPs, only the rs352162 polymorphism was significantly associated with MOD score, showing a recessive effect.

Bronkhorst *et al.* studied TLR9 (-1486T>C and -1237T>C) in a cohort of 219 severely injured patients and found -1486T>C to cause a trend toward reduced prevalence of Gram-positive bacteria and fungi for this SNP (p = 0.060), but no significant association with SIRS, sepsis, or septic shock (11).

Cluster of Differentiation 14 (CD14)
The effects of *CD14* -159C>T promoter SNP were studied in burns patients (16-19, 21-23) and in severely injured trauma patients (11, 24-26) in Chinese (22-24, 26) and mixed ethnic populations (Table 1) (11, 16-19, 21, 25). Comparison of results is complicated by the fact that different outcome parameters were used, including wound cultures, SIRS, sepsis, severe sepsis, MODS and mortality. Sepsis and MODS occurred more frequently in both burns and trauma patients with variant genotype in some reports (17, 22-24, 26) but was not influenced by genotype in other reports (11, 16, 19, 25). Remarkably, in some studies sepsis was associated with the C-allele whereas in other studies sepsis was associated with the T-allele (17,22). One can only speculate about the origin of this contrast which may be explained by differences in ethnicity of the study population. Mortality risk was increased by *CD14* -159C variant genotype in burns patients (18, 21) but this effect was not found in another study (19). Differences in total body surface area (TBSA) of burns as well as ethnic demographic baseline characteristics may contribute to these opposing findings.

The effects of *CD14* -1145G>A in trauma patients were studied in Chinese trauma patients (24, 26). In both studies, with a total of 211 trauma patients, the -1145G allele conferred an increased risk of sepsis and MODS.

Myeloid differentiation-2/ Lymphocyte antigen 96 *(LY96)*

Zeng *et al.* studied 726 unrelated Han Chinese patients with major trauma for *MD2* (27). A total of 37 SNPs were identified in *MD2*. Thirty five of them constructed three haplotype blocks. Sepsis developed in around 40% of patients. Only the rs11465996 was shown to be significantly associated with the risk of development of sepsis and MODS in major trauma patients. Patients carrying the variant G allele revealed significantly higher sepsis morbidity rate and MOD scores.

Gu *et al.* studied *MD2* -1625C>G in 105 severely injured patients of whom 40% developed sepsis (28). The MODS scores in trauma patients carrying G allele at position -1625 were significantly higher than those carrying C allele. Moreover, trauma patients carrying G allele appeared to have higher risk of sepsis compared to those carrying C allele. Sepsis morbidity was significantly different between subject with C and G alleles.

Lipopolysaccharide Binding Protein *(LBP)*

Zeng *et al.* used haplotype tagging to study SNPs in *LBP* in two independent cohorts of major trauma patients recruited from southwest and eastern China (29). Of the nine known SNPs in *LBP o*nly the rs2232618 (p.F436L) was significantly associated with higher susceptibility to sepsis and MOD. Patients carrying the variant C allele revealed significantly higher sepsis morbidity rate and MOD scores when compared to patients carrying the T allele.

1.2 Lectin Pathway Proteins

Mannose-Binding Lectin (MBL2)

Heterozygosity for the variants in exon 1 (A/0) conferred an increased risk of wound colonization and infection in severely injured patients (30). This had previously only been demonstrated in a murine model of burns (31). Also, the YX promoter genotype increased the risk of fungal colonization and infection in trauma patients (30).

MBL-Associated Serine-Protease 2 (MASP2)

MASP2 p.Y371D DD homozygosity increased the risk of SIRS and septic shock in trauma patients significantly (30). Moreover, a trend was noted for an increased risk of Gram-positive infections in patients with DD genotype. For the *MASP2 p.*D120G genotype polymorphism no statistically significant differences were found for all endpoints although, strikingly, fungi, positive blood cultures and septic shock were only found in DD patients (22.2%, 15.5%, and 17.9%, respectively). Another striking, yet non-significant, finding was that only 8.3% of DG patients developed sepsis versus 37.7% in DD patients (p=0.060).

Ficolin 2 (FCN2)

The homozygous *FCN2* p.A258S AS genotype increased the risk of developing septic shock in trauma patients (30). Also, wound colonization and infection risks were significantly increased. A trend was noted for Gram-negative infections.

No significant associations between the *FCN2 p.*T236M genotype and infectious events were found. Positive blood cultures developed in 25.0% of patients with a variant MM genotype, versus only 11.3% of patients with the common TT genotype but this difference was not statistically significant in a multivariate model.

1.3 Other Receptors

Receptor for Advanced Glycation Endproducts (RAGE)

A total of 728 unrelated patients with major trauma was studied by Zeng *et al*. and genotyped for *RAGE (32)*. Sepsis occurred in around 40% of patients with median time between trauma to sepsis being 6 days. From different genetic variants selected in this study, only the *RAGE* -429T>C (rs1800625) was shown to be significantly associated with the risk of development of sepsis and MODS in major trauma patients. The patients carrying the variant C allele revealed a significantly lower sepsis morbidity rate and MOD scores, when compared with those carrying the T allele. Moreover, *in vitro* LPS-induced TNF-α production was significantly lower in patients with the variant C allele than in those with wild T allele.

NOD-like Receptor Family, Pyrin Domain Containing 3 (NLRP3)

Zhang studied six SNPs in the *NLRP3* gene of 718 Chinese patients with major blunt trauma with a mean ISS of 22.5 (33). 40% of patients developed sepsis with a mean time to sepsis of 7 days. The *NLRP3* -1017G>A polymorphism (rs2027432), although it was found in only three patients with AA variant homozygotes in this study cohort, was significantly associated with higher risk of MODS. In addition, the *NLRP3* 5134A>G (rs12048215) polymorphism was significantly associated with a lower sepsis morbidity rate, showing 26.4% in GG versus 44% in AA. Data from multiple logistic regression analyses further indicated that the patients with the rs12048215 polymorphism had a lower risk of developing sepsis after adjusting for possible confounders. The rs2027432 polymorphism was significantly associated with higher IL-1β levels.

Glucocorticoid Receptor (GR)

Duan *et al*. studied a cohort of 95 severe trauma patients with a mean ISS of 27 (34). It appeared that the *BclI* mutation in the *GR* gene was not associated with posttraumatic sepsis or organ dysfunction.

2. SIGNAL TRANSDUCING ADAPTOR PROTEINS

Interleukin-1 Receptor-Associated Kinase 1 (IRAK1)
Sperry et al. studied a cohort of 321 patients with a median ISS of 16 for the T>C substitution (rs1059703) at position 1595 in exon 12 of *IRAK1* which results in a non-synonymous mutation (p.L532S) (35). They found this SNP to be a very strong independent predictor of post-trauma multiple organ failure and mortality

*Interleukin-1 Receptor-Associated Kinase 3 (**IRAK3**)*
Meyer *et al.* genotyped 474 patients with acute lung injury (ALI) from a prospective critically ill trauma patients cohort study for 25 candidate genes using the IBC chip (36). The incidence of ALI their cohort was 30%. *IRAK3* was found to be associated with ALI in patients from African descent.

3.   INFLAMMATORY CYTOKINES

3.1 Interleukins

Interleukin-I (IL1A, IL1B, IL1RN)
IL1A
In a cohort of 308 Han Chinese trauma patients with ISS>16 the *IL1A* -889C>T TT genotype had the highest risk of sepsis and produced the lowest serum levels of Il-1α (Table 1) (37).

IL1B
Carrying an *IL1B-Taq-1* 3953C>T CT genotype in combination with the *IL10* -592A>C AC genotype predisposed to acute respiratory failure in Caucasian trauma patients (N=216; ISS>16) (p=0.003) (Table 1) (38).

   The *IL1B* -1470G>C was studied in two overlapping cohorts of severely injured Han Chinese patients from the same hospital (37, 39). Chinese trauma patients carrying the major -1470G allele were more likely to develop sepsis than those with the minor -1470C allele in both studies.

   The *IL1B* -511T>C (rs16944) was studied in the previously overlapping cohorts of 238 and 308 Han Chinese patients with severe trauma (37, 39). The CC genotype conferred a statistically significant increase in the risk of sepsis. In a Caucasian cohort of 119 multiple trauma patients *IL1B* -511T>C variation was not found to confer any effect on sepsis (38). The *IL1B* SNP most studied is the -31C>T (16-19, 21, 37, 39). In mixed-ethnic burns patients from the USA (TBSA>15%) this SNP seems to be no relevant risk factor for the

Table 1. Overview of association with outcome for SNPs in the *TLR, CD14, IL,* and *TNF* genes of trauma patients

Gene	Number of SNPs studied	Number of patients studied	SIRS	Sepsis	Septic Shock	MODS	Mortality	References
TLR1	3	1498	-	+	+	+	↑ (3 SNPs)	(9)
TLR2	5	697	↑ (1 SNP)	↑ (2 SNPs)	+	↑ (1 SNP)	+	(10-12)
TLR4	8	1925	+	↑ (1 SNP) ↓ (2 SNPs)	↑ (1 SNP)	↑ (1 SNP) ↓ (1 SNP)	+	(11-19)
TLR9	5	776	+	↑ (3 SNPs)	+	+	+	(11, 20)
CD 14	2	1428	+	↑ (2 SNPs)	↑ (1 SNP)	↑ (2 SNPs)	↑ (1 SNP)	(11, 16-19, 21-26)
IL1A	1	308	-	↑ (1SNP)	-	↑ (1 SNP)	-	37
IL1B	4	1462	+	↑ (3 SNPs)	+	+	+	(16-18, 37-40, 45)
IL1RN	2	961	-	+	+	-	+	(40, 41)
IL4	1	308	-	↑ (1 SNP)	-	+	-	(37, 42)
IL6	3	1931	-	↑ (1 SNP) ↓ (1 SNPs)	+	+	↓ (1 SNPs)	(12, 16-19, 36-38, 43, 44, 46-48)
IL8	1	97	-	+	+	+	+	(49)
IL10	3	1953	-	↑ (2 SNPs)	+	↑ (1 SNP)	↓ (3 SNPs)	(12, 36-38, 43, 50-55)
IL17F	1	71	-	-	+	+	+	(43)
IL18	2	134	-	+	+	+	+	(12, 56)
TNF	3	2548	-	↑ (1 SNP)	+	+	↑ (1 SNP)	(12, 15-19, 21, 37, 58-62)

development of sepsis nor for mortality (16-19, 21). In Han Chinese multiple trauma patients, however, the *IL1B* -31C>T major CC genotype seemed to protect against sepsis (30.3% and 37.9%) following major trauma (37, 39).

IL1RN

In one study the effect of *IL1RN* variant 2 variable number tandem repeat (VNTR) poly-morphism was studied in patients with traumatic brain injury (TBI) (Table 1) (40). *IL1RN* VNTR allele 2 carriers were more likely to have hemorrhagic events after TBI. In another study in severe trauma patients a *IL1RN* SNP 130T>C (rs315952), distinct from the well-described VNTR SNP, was associated with decreased risk of ARDS (41).

Interleukin-4 (*IL4*)

Two studies from the same hospital with overlapping patient cohorts reported the influ-ence of *IL4* -589T>C genotype in a cohort of 308 Chinese severe trauma patients with a mean ISS of 25.5 (Table 1) (37, 42). A total of 48.4% of patients developed sepsis. The frequency of the TC heterozygous genotype in the sepsis group (37.6%) was significantly higher than in nonsepsis group (25.2%). There was a significant influence of the minor C allele. No relationship was observed between *IL4* -589T>C and MODS in these major trauma patients.

Interleukin-6 (*IL6*)

The *IL6* -174G>C (rs1800795) was studied in three cohorts of burns patients (16-19, 43), six cohorts of trauma patients (12, 38, 44-47) and a cohort of traumatic brain injury (TBI) patients (Table 1) (48). Only two out of these articles described an increased risk of sepsis with presence of the minor -174C allele (17, 45). In a cohort of TBI patients the GG genotype was found significantly more frequently in the survivor group than in non-surviving patients (48).

Chinese trauma patients carrying the *IL6* -572G>C CC genotype had significantly more sepsis morbidity than with a CG or GG genotype (37, 46). A small Bosnian cohort how-ever failed to demonstrate any influence of this SNP (47).

Interleukin-8 (*IL8*)

The effect of *IL8* -251A>T on the development of ARDS was studied in one cohort of 97 blunt trauma patients of whom 23 developed ARDS (Table 1) (49). The allele and genotype distribution of the polymorphism in this cohort did not exhibit a significant association with the development of ARDS or mortality. Patients with the AA genotype showed a significantly longer duration of mechanical ventilation compared to patients with the *IL8* -251TT genotype.

Interleukin-10 (*IL10*)

The effects of *IL10* -592A>C in trauma patients have been described in seven studies (Table 1) (12, 37, 38, 50-52). Three studies (12, 50, 51) found conflicting results of genetic variation in this gene on outcome. Schröder *et al*. found an increased risk for MODS in -592AC genotypes. Huebinger *et al*. found that carriage of the minor -592A allele was associated with a decreased risk of mortality. McDaniel *et al*. found that patients carrying the *IL10* ACC/ATA low producing genotypes were at a lower risk of developing sepsis.

IL10 -819C>T was studied in five cohorts of trauma patients (12, 37, 43, 50, 52). Three studies describe an effect on outcome (12, 37, 50). Huebinger *et al*. found that the minor -819T allele was significantly associated with a decreased risk of mortality. McDaniel *et al*. found that patients carrying the *IL10* ACC/ATA low producing genotypes were at a lower risk of developing sepsis. In a cohort of Chinese trauma patients (where C appeared to be the minor allele) it was shown that this C allele conferred a decreased risk of sepsis (37).

IL10 -1082G>A was studied by ten authors (12, 36-38, 43, 51-55). Six authors observed effects on outcome (12, 36, 38, 52-54). McDaniel *et al*. (12) found that patients who carried the *IL10* ACC/ATA low producing genotypes were at a lower risk of developing sepsis. Zeng *et al*., however, found that patients with the major A allele had significantly higher risk of sepsis (52). Jin *et al*. (54) as well as Schroeder *et al*. (38) described a reduced risk of ARDS and acute respiratory failure in GG genotypes. In contrast, Gong *et al*. found the -1082GG genotype to be associated with an increased risk of ARDS in patients younger than 52 years old.

Interleukin-17F (*IL17F*)

Accardo Palumbo *et al*. studied the effect of 7488T>C (His161Arg)(rs763780) in *IL17* in a cohort of burns patients (Table 1) (43). At the third day, burn patients had a very significant increase in IL-17 plasma levels. However, there were no statistically significant differences in *IL17* genotype distributions among patients that did or did not developed sepsis.

Interleukin-18 (*IL18*)

McDaniel *et al*. were unable to demonstrate a significant effect of SNPs in *IL18* in trauma patients (Table 1) (12). Stassen *et al*. studied *IL18* -137G>C and *IL18* -607C>A in 69 trauma patients (56). Although the individual SNPs were not associated with outcome, patients carrying both the -607CA genotype and a -137GC genotype (CA/GC) had a significantly reduced risk of sepsis. These data suggest that *IL18* genetic variability may play a role in the predisposition for the development of postinjury sepsis.

3.2 Other Inflammatory Cytokines
High-Mobility Group Box 1 (*HMGB1)*

Three *HMGB1* polymorphisms -1514T>C, 2179C>G and 6850G>A were studied in a cohort of 556 Han Chinese patients with major trauma. A total of 39.7% of patients developed sepsis. The *HMGB1* 2179C>G variant GG genotype predisposed to the occurrence of sepsis (p=0.003) and MODS (P=0.011) in trauma patients (57). With respect to the other 2 SNPs, there were no significant differences in sepsis morbidity rates and MOD scores.

Interferon-γ (*IFNG*)

In a mixed-ethnic cohort of 68 trauma patients (ISS > 15) of whom 42–50% developed sepsis (12) the *IFNG* 841T>A AA genotype protected against sepsis in African American patients, whereas this was not clear for Caucasian patients. The authors suggest that the carriage of the AA genotype could cause faster elimination of the pathogens (12). In an other cohort of 308 Han Chinese trauma patients (ISS>16) the *IFNG* 541T>A polymorphism was unrelated to sepsis or MOD (37).

Tumor Necrosis Factor (*TNF*)

Three SNPs in *TNF* have been studied in trauma and burns patients by nine authors (Table 1) (12, 16-19, 21, 37, 58-62).

The TNF -308G>A (rs1800629) was described in burns patients by two authors in five studies (16-19, 21) and in trauma patients in eight studies (12, 15, 19, 37, 58-62). Increased risk of sepsis and of mortality has been observed by seven authors (16, 17, 19, 37, 58, 61, 62) but was not seen by four authors (12, 15, 18, 21, 60). Moreover, Gill *et al.* demonstrated in a cohort of trauma patients that the A allele was significantly associated with the risk of microchimerism after allogenic transfusion of cells (59).

The *TNF* -238G>A (rs361525) was studied in trauma patients by one author (62). There was no influence of -238G>A variation on sepsis outcome in a cohort of 152 severely injured patients.

Also, the *TNF* -376G>A (rs1800750) was studied in trauma patients by one author (62). There was no influence of -238G>A variation on sepsis outcome outcome in a cohort of 152 severely injured patients.

Lymphotoxin-α (*LTA*)

Effects of variation in lymfotoxin-α *LTA* 252A>G (rs909253) (previously known as *TNF*-β NcO1) was studied in trauma patients in five manuscripts (45, 58, 60, 61, 63). Three authors observed an effect on clinical outcome (60, 61, 63) and two did not (45, 58).

Majetschak *et al*. found that severe posttraumatic sepsis was significantly increased in patients homozygous for the allele *TNFB2* (presently termed the A allele) (63). Three

years later, Majetschak again found that patients developing severe sepsis after trauma were significantly more likely to be homozygous for *TNFB2* and this time also homozygous for *TNFB1* (presently termed the G allele) (60). Menges *et al*. also found that carriage of the G allele (*TNFB1*) conferred an increased risk of developing sepsis (61). Hildebrand *et al*. (45) and Duan *et al*. (58) found no effect on sepsis morbidity.

CONCLUSION

Severe injury or multiple trauma (the so-called 'first hit') evokes a systemic inflammatory response in trauma patients. In uncomplicated cases this response is temporary and predictable to a certain extent. If the initial hit however is big enough it may produce a Systemic Inflammatory Response Syndrome (SIRS). The following emergency damage-control surgery and later definitive surgical procedures (the 'second hit') may further exhaust the immune system potentially leading to immune paralysis causing the Compensatory Anti-inflammatory Response Syndrome (CARS). Several mechanisms contribute to the development of SIRS such as hormonal, metabolic, hemodynamic, immunological, cell-mediated and ischemia/reperfusion processes (64).

The outcome following major trauma is thus determined by many factors of which sequence variation in the human genome may well be one such factor. A number of genes have been studied so far but these studies are generally unique and numbers are often small. Outcome parameters of studies, as shown in this review are sometimes different making pooling of results or comparison complicated. Nevertheless, some single nucleotide polymorphisms clearly appear to exert an effect on the outcome.

Identifying patients at risk of developing infectious complications may improve their outcome by targeted treatments such as antibiotic prophylaxis, substitution therapy or plasma transfusions. But unfortunately too little information is currently available to draw firm conclusions. Further research in this field is necessary. Since systemic response to trauma is a complex and polygenic phenotype, more genes will have to be studied in larger cohorts to determine their exact influence on outcome in severely injured patients. State-of-the-art techniques like exome sequencing and whole genome SNP arrays should be used in future studies in order to identify relevant sequence variations in other immune response genes and signalling pathways as well.

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Supplemental Table S1. Summary of SNPs studied in populations of trauma patients

Gene	OMIM	Cytogenetic Location	SNP	dbSNP ID	References
Pattern Recognition Receptors and Complexes					
TLR1	601194	4p14	-7202A>G	rs5743551	(9)
			742A>G	rs4833095	(9)
			1804G>T	rs5743618	(9)
TLR2	603028	4q31	-15607A>G	rs1898830	(10)
			19216T>C	rs3804099	(10)
			22215T/G	rs7656411	(10)
			p.R753Q	rs5743708	(11, 12)
			p.R753Q	rs5743708	(11, 12)
			-16934T>A	rs4696480	(11)
TLR4	603030	9q33	-2381A>G	rs2737190	(13)
			-2242T>C	rs10116253	(13)
			-1892G>A	rs10983755	(13)
			-1837A>G	rs1927914	(13)
			-1418T>C	rs10759932	(13)
			11367G>C	N.A.	(14)
			896A>G	rs4986790	(11, 12, 15-19)
			1196T>C	rs4986791	(11, 12)
TLR9	605474	3p21	-1486T>C	rs187084	(11, 20)
			2848C>T	rs352140	(20)
			6577T>C	rs352162	(20)
			g.6808A>G	rs352139	
			-1237T>C	rs5743836	(11)
CD 14	158120	5q31	-159C>T	rs2569190	(11, 16-19, 21-26)
			-1145G>A	rs2569191	(24, 26)
LY96	605243	8q21	-1625C>G	rs11465996	(27, 28)
LBP	151990	20q11	26877T>C	rs2232618	(29)
MBL2	154545	10q21	Codon 52	rs5030737	(30)
			Codon 54	rs1800450	(30)
			Codon 57	rs1800451	(30)
MASP2	605102	1p36	p.Y371D	rs12711521	(30)
			p.D120G	N.A.	(30)
FCN2	601624	9q34	p.A258S	rs7851696	(30)
			p.T236M	rs17549193	(30)
RAGE	600214	6p21	-407 to -345	63bp ins/del	(32)
			570G>A	rs2070600	(32)
			-374T>A	rs1800624	(32)
			-429T>C	rs1800625	(32)

Supplemental Table S1. Summary of SNPs studied in populations of trauma patients (continued)

Gene	OMIM	Cytogenetic Location	SNP	dbSNP ID	References
NLRP3	606416	1q44	-1017G>A	rs2027432	(33)
			5134A>G	rs12048215	(33)
hGR/NR3C1	138040	5q31	Bcl I C>G	rs41423247	(34)
Signal Transducing Adaptor Proteins					
IRAK1	300283	Xq28	1595 T>C	rs1059703	(35)
IRAK3	604459	12q14	15SNPs	Ht Block 1	(36)
Inflammatory Cytokines					
IL1A	147760	2q13	-889C>T	rs1800587	(37)
IL1B	147720	2q13	3953C>T	rs1143634	(38, 45)
			-1470G>C	N.A.	(37, 39)
			-511T>C	rs16944	(37-40)
			-31C>T	rs1143627	(16-18, 37, 39)
IL1RN	147679	2q13	VNTR	rs315952	(40)
			C>T	rs315952C	(41)
IL4	147780	5q31	-589T>C	rs2243250	(37, 42)
IL6	147620	7p15	-174G>C	rs1800795	(12, 16-19, 36, 38, 43, 44, 46-48)
			-572G>C	rs1800796	(37, 46, 47)
			-597G>A	rs1800797	
IL8	146930	4q13	-251A>T	rs4073	(49)
IL10	124092	1q32	-1082G>A	rs1800896	(12, 36-38, 43, 51-55)
			-819C>T	rs1800871	(12, 37, 43, 50, 52)
			-592C>A	rs1800872	(12, 37, 38, 43, 50-52)
IL17F	606496	6p12	7488T>C	rs763780	(43)
IL18	600953	11q23	-137G>C	rs187238	(12, 56)
			-607C>A	rs1946518	(12, 56)
TNF	191160	6p21	-308G>A	rs1800629	(12, 15-19, 21, 37, 58-62)
			-238G>A	rs361525	(62)
			-376G>A	rs1800750	(62)
LTA	153440	6p21	252A>G	rs909253	(45, 58, 60, 61, 63)
IFNG	147570	12q15	874T>A	rs2430561	(12, 37)
HMGB1	163905	13q12	-1514T>C	rs1412125	77
			2179C>G	rs2249825	(57)
			6850G>A	rs1045411	(57)
Other Genes not belonging to the Innate Immune System					
MYLK	600922	3q21	p.P21H	rs28497577	(65)
			p.S147P	rs9840993	(65)

Supplemental Table S1. Summary of SNPs studied in populations of trauma patients (continued)

Gene	OMIM	Cytogenetic Location	SNP	dbSNP ID	References
				rs4678047	(65)
PRDX6	602316	1q25		43 SNPs	(66)
HSPA1B	603012	6p21	1538A>G	N.A.	(67)
HSPA1L	140559	6p21	2437C>T	rs2075800	(67)
HSP90B1	191175	12q23	-144C>A	rs9472238	(68)
SERPINE1	173360	7q22	-688	rs1799768	(18, 69)
VEGFA	192240	6p21		Ht Block 1	(36)
ANGPT2	601922	8p23	127635T>A	rs1868554	(70)
			135709T>C	rs2442598	
mtDNA		mtDNA	T4216C		(71)
MicroRNA		stem-loop 37/5p +22	G>C	rs4919510	(72)

Supplemental Table S2. Detailed overview of association with outcome for SNPs in the *TLR*, *CD14*, *IL*, and *TNF* genes of trauma patients

Gene	SNP	dbSNP ID	Author	Year	Population	N	SIRS	Sepsis	Septic Shock	MODS	Mortality	References
TLR1	-7202A>G	rs5743551	Thompson	2013	Whites	1498	-	+	+	+	↑ G allele	(9)
	742A>G	rs4833095	Thompson	2013	Whites	1498	-	+	+	+	↑ G allele	(9)
	1804G>T	rs5743618	Thompson	2013	Whites	1498	-		+	+	↑ T allele	(9)
TLR2	-15607A>G	rs1898830	Chen	2011	Han Chinese	410	-	+	+	+	-	(10)
	19216T>C	rs3804099	Chen	2011	Han Chinese	410	-	↑ C allele	-	↑ C allele	-	(10)
	22215T/G	rs7656411	Chen	2011	Han Chinese	410	-	+	-	+	-	(10)
	p.R753Q	rs5743708	Bronkhorst	2013	Mixed Ethnic	219	+	+	+	+	+	(11)
		rs5743708	McDaniel	2007	Mixed Ethnic	68	-	↑ AG	-	-	-	(12)
	-16934T>A	rs4696480	Bronkhorst	2013	Mixed Ethnic	219	↑ AA	+	+	+	+	(11)
TLR4	-2381A>G	rs2737190	Chen	2010	Han Chinese	303	-	+	-	+	-	(13)
	-2242T>C	rs10116253	Chen	2010	Han Chinese	303	-	↑ C allele	-	↑ C allele	-	(13)
	-1892G>A	rs10983755	Chen	2010	Han Chinese	303	-	+	-	+	-	(13)
	-1837A>G	rs1927914	Chen	2010	Han Chinese	303	-	+	-	+	-	(13)
	-1418T>C	rs10759932	Chen	2010	Han Chinese	303	-	+	-	+	-	(13)
	11367G>C	N.A.	Duan	2009	Han Chinese	132	-	↓ C allele	-	↓ C allele	-	(14)
	896A>G	rs4986790	Bronkhorst	2013	Mixed Ethnic	219	+	+	+	+	+	(11)
			McDaniel	2007	Mixed Ethnic	68	-	+	-	-	-	(12)
			Shalhub	2009	Whites	598	+	↓ A allele	+	+	+	(15)
			Barber	2004	Mixed Ethnic	159	-	+	↑ G allele	-	-	(16)
			Barber	2006	Mixed Ethnic	228		+	↑ G allele			(17)
			Barber	2008	Mixed Ethnic	149	-	+	+	+	+	(18)
			Shalhub	2009	Mixed Ethnic	69	-	+	+	+	+	(19)
	1196T>C	rs4986791	Bronkhorst	2013	Mixed Ethnic	219	+	+	+	+	+	(11)
		rs4986791	McDaniel	2007	Mixed Ethnic	68	-	+	-	-	-	(12)

Supplemental Table S2. Detailed overview of association with outcome for SNPs in the *TLR*, *CD14*, *IL*, and *TNF* genes of trauma patients (continued)

Gene	SNP	dbSNP ID	Author	Year	Population	N	SIRS	Sepsis	Septic Shock	MODS	Mortality	References
TLR9	-1486T>C	rs187084	Bronkhorst	2013	Mixed Ethnic	219	+	+	+	+	+	(11)
			Chen	2011	Han Chinese	557	-	↑G allele	+	+	-	(20)
	2848C>T	rs352140	Chen	2011	Han Chinese	557	-	+	+	+	-	(20)
	6577T>C	rs352162	Chen	2011	Han Chinese	557	-	↑C allele	+	+	-	(20)
	g.6808A>G	rs352139	Chen	2011	Han Chinese	557	-	↑G allele	+	+	-	(20)
	-1237T>C	rs5743836	Bronkhorst	2013	Mixed Ethnic	219	+	+	+	+	+	(11)
CD 14	-159C>T	rs2569190	Bronkhorst	2013	Mixed Ethnic	219	+	+	+	+	+	(11)
			Barber	2004	Mixed Ethnic	159	-	+	+	-	-	(16)
			Barber	2006	Mixed Ethnic	228	-	+	↑C allele	-	-	(17)
			Barber	2008	Mixed Ethnic	149	-	+	+	+	↑C allele	(18)
			Shalhub	2009	Mixed Ethnic	69	-	+	+	+	+	(19)
			Barber	2007	Mixed Ethnic	223	-	+	+	+	↑C allele	(21)
			Dong	2010	Chinese	35	-	↑T allele	+	-	-	(22)
			Dong	2009	Chinese	77	-	↑T allele	-	↑T allele	-	(23)
			Gu	2010	Han Chinese	105	-	↑T allele	-	↑T allele	-	(24)
			Heesen	2010	Unknown	58	-	+	+	+	+	(25)
			Liu	2011	Chinese	106	-	-	-	↑T allele	-	(26)
		rs2569191	Gu	2010	Han Chinese	105	-	↑G allele	-	↑G allele	-	(26)
			Liu	2011	Chinese	106	-	+	+	↑G allele	-	(24)
IL1A	-889C>T	rs1800587	Gu	2010	Han Chinese	308	-	↑T allele	-	↑C allele	-	(37)
IL1B	3953C>T	rs1143634	Schroeder	2008	Caucasian	100	+	+	+	-	+	(38)
			Hildebrand	2005	Unknown	97	+	+	+	-	+	(45)
	-1470G>C	N.A.	Gu	2010	Han Chinese	308	-	↑G allele	-	+	-	(37)
			Wen	2010	Han Chinese	238	–	↑G allele	+	+	-	(39)
	-511T>C	rs16944	Gu	2010	Han Chinese	308	-	↑C allele	-	+	-	(37)

Supplemental Table S2. Detailed overview of association with outcome for SNPs in the *TLR*, *CD14*, *IL*, and *TNF* genes of trauma patients (continued)

Gene	SNP	dbSNP ID	Author	Year	Population	N	SIRS	Sepsis	Septic Shock	MODS	Mortality	References
			Schroeder	2008	Caucasian	100	-	-	-	-	+	(38)
			Wen	2010	Han Chinese	238	–	↑ C allele	+	+	-	(39)
			Hadjigeorgiou	2005	Greek	183	-	-	-	-	-	(40)
	-31C>T	rs1143627	Barber	2004	Mixed Ethnic	159	-	+	+	-	-	(16)
			Barber	2006	Mixed Ethnic	228	-	+	+	-	-	(17)
			Barber	2008	Mixed Ethnic	149	-	+	+	+	+	(18)
			Gu	2010	Han Chinese	308	-	↑ T allele	-	+	-	(37)
			Wen	2010	Han Chinese	238	–	↑ T allele	+	+	-	(39)
IL1RN	VNTR	rs315952	Hadjigeorgiou	2005	Greek	183	-	-	-	-	-	(40)
	C>T	rs315952C	Meyer	2013	European	778	-	+	+	-	+	(41)
IL4	-589T>C	rs2243250	Gu	2010	Han Chinese	308	-	↑ C allele	-	+	-	(37)
			Gu	2011	Han Chinese	308	-	↑ C allele	-	-	-	(42)
IL6	-174G>C	rs1800795	McDaniel	2007	Mixed Ethnic	68	-	+	-	-	-	(12)
			Barber	2004	Mixed Ethnic	159	-	+	+	-	-	(16)
			Barber	2006	Mixed Ethnic	228	-	+	+	-	+	(17)
			Barber	2008	Mixed Ethnic	149	-	+	+	+	+	(18)
			Shalhub	2009	Mixed Ethnic	69	-	+	+	+	+	(19)
			Meyer	2012	Mixed Ethnic	474	-	-	-	+	+	(36)
			Schroeder	2008	Caucasian	119	-	-	-	-	+	(38)
			Accardo	2012	Unknown	71	-	+	+	+	+	(43)
			Heesen	2002	Caucasian	57	-	+	+	+	-	(44)
			Gu	2008	Han Chinese	105	-	-	-	+	-	(46)
			Jeremic	2014	Unknown	47	-	+	+	+	+	(47)
			Dalla Libera	2011	Unknown	77	-	-	-	+	↓ G allele	(48)
	-572G>C	rs1800796	Gu	2010	Han Chinese	308	-	↑ C allele	-	+	-	(37)

Supplemental Table S2. Detailed overview of association with outcome for SNPs in the *TLR*, *CD14*, *IL*, and *TNF* genes of trauma patients (continued)

Gene	SNP	dbSNP ID	Author	Year	Population	N	SIRS	Sepsis	Septic Shock	MODS	Mortality	References
			Gu	2008	Han Chinese	105	-	↓ G allele	-	+	-	(46)
			Jeremic	2014	Unknown	47	-	+	+	+	+	(47)
	-597G>A	rs1800797	Gu	2008	Han Chinese	105	-	-	-	-	-	(46)
IL8	-251A>T	rs4073	Hildebrand	2007	Unknown	97	-	+	+	+	+	(49)
IL10	-1082G>A	rs1800896	McDaniel	2007	Mixed Ethnic	68	-	↑ G allele	-	-	-	(12)
			Meyer	2012	Mixed Ethnic	474	-	-	-	+	+	(36)
			Gu	2010	Han Chinese	308	-	+	-	+	-	(37)
			Schroeder	2008	Caucasian	100	-	-	-	-	+	(38)
			Accardo	2012	Unknown	71	-	↑ G allele	+	+	+	(43)
			Schröder	2004	Unknown	119	-	+	-	+	+	(51)
			Zeng	2009	Han Chinese	308	-	↑ A allele	+	+	+	(52)
			Gong	2006	Caucasian	211	-	-	-	-	↓ G allele	(53)
			Jin	2012	Chinese	29	-	-	-	-	↓ G allele	(54)
	-819C>T	rs1800871	McDaniel	2007	Mixed Ethnic	68	-	+	-	-	-	(12)
			Gu	2010	Han Chinese	308	-	↑ T allele	-	+	-	(37)
			Accardo	2012	Unknown	71	-	-	+	+	+	(43)
			Huebinger	2010	Mixed Ethnic	265	-	+	-	+	↓ T allele	(50)
			Zeng	2009	Han Chinese	308	-	+	+	+	+	(52)
	-592C>A	rs1800872	McDaniel	2007	Mixed Ethnic	68	-	+	-	-	-	(12)
			Gu	2010	Han Chinese	308	-	+	-	+	-	(37)
			Schroeder	2008	Caucasian	100	-	+	-	-	+	(38)
			Accardo	2012	Unknown	71	-	-	+	+	+	(43)
			Huebinger	2010	Mixed Ethnic	265	-	+	-	+	↓ A allele	(50)
			Schröder	2004	Unknown	119	-	+	-	↑ AC	+	(51)
			Zeng	2009	Han Chinese	308	-	+	+	+	+	(52)

Supplemental Table S2. Detailed overview of association with outcome for SNPs in the *TLR*, *CD14*, *IL*, and *TNF* genes of trauma patients (continued)

Gene	SNP	dbSNP ID	Author	Year	Population	N	SIRS	Sepsis	Septic Shock	MODS	Mortality	References
IL17F	7488T>C	rs763780	Accardo	2012	Unknown	71	-	-	+	+	+	(43)
IL18	-137G>C	rs187238	McDaniel	2007	Mixed Ethnic	68	-	+	-	-	-	(12)
			Stassen	2003	Mixed Ethnic	66	-	+	+	+	+	(56)
	-607C>A	rs1946518	McDaniel	2007	Mixed Ethnic	68	-	+	-	-	-	(12)
			Stassen	2003	Mixed Ethnic	66	-	+	+	+	+	(56)
TNF	-308G>A	rs1800629	McDaniel	2007	Mixed Ethnic	68	-	+	-	-	-	(12)
			Barber	2004	Mixed Ethnic	159	-	↑ A allele	+	-	-	(16)
			Barber	2006	Mixed Ethnic	228	-	↑ A allele	+	-	+	(17)
			Barber	2008	Mixed Ethnic	149	-	+	+	+	+	(18)
			Shalhub	2009	Mixed Ethnic	69	-	↑ A allele	+	+	+	(19)
			Shalhub	2009	Mixed Ethnic	598	-	+	+	+	+	(15)
			Barber	2007	Mixed Ethnic	223	-	+	+	+	+	(21)
			Gu	2010	Han Chinese	308	-	↑ A allele	-	+	-	(37)
			Duan	2011	Han Chinese	306	-	↑ A allele	+	+	+	(58)
			Gill	2008	Unknown	59	-	-	-	-	-	(59)
			Majetschak	2002	Unknown	70	-	+	+	-	+	(60)
			Menges	2008	Unknown	159	-	↑ A allele	+	+	↑ A allele	(61)
			O'Keefe	2002	Unknown	152	-	↑ A allele	+	+	↑ A allele	(62)
	-238G>A	rs361525	O'Keefe	2002	Unknown	152	-	+	+	+	+	(62)
	-376G>A	rs1800750	O'Keefe	2002	Unknown	152	-	+	+	+	+	(62)

+: outcome parameter was studied

-: outcome parameter was not studied

↑↓: genotype was positively/negatively associated with outcome parameter

GACCTTAAAGTACTTAGTAAGGGA

Chapter 4

Mannose-Binding Lectin

Maarten W.G.A. Bronkhorst, Lee H.Bouwman

INTRODUCTION

Mannose-binding lectin (MBL), also known as mannan-binding protein (MBP), is a protein that specifically binds or crosslinks carbohydrates. Lectins are involved in complement activation via the lectin pathway. The complement system provides immediate defense against infection and has proinflammatory effects; it is considered part of both the innate and adaptive immune systems.

This topic will discuss the genetic defects and polymorphisms of the *MBL2* gene and the function of the MBL protein. The diagnosis of MBL deficiency, diseases associated with both low and high levels of MBL, and other aspects of the complement system are discussed separately. (See "Mannose-binding lectin deficiency" and "Complement pathways" and "Overview and clinical assessment of the complement system" and "Regulators and receptors of the complement system".)

THE MBL GENE

In many animals there are two mannose-binding lectin (MBL) genes, MBL1 and *MBL2* that code for a functional product. However, in humans the MBL1 gene, MBL1P1 is a pseudogene (ie, it does not produce a functional protein) [1]. The two MBL genes are most likely the result of a gene-duplication event [2].

The gene encoding functional MBL in humans, *MBL2* is located on the long arm of chromosome 10 (10q11.2-q21) [3]. The *MBL2* gene is also known as collectin subfamily member 2 or *COLEC2* [4].

The human MBL protein is encoded by four exons. The *MBL2* gene also contains an extra exon about 1 kb upstream of exon 1, named exon 0, which may initiate transcription of the *MBL2* gene [5]. Gene expression is primarily regulated by several consensus elements in the promoter region [4].

The normal allele is called allele A. To date, three point mutations have been identified, all in exon 1 [6-8]:

- Allele B – Codon 54, GGC to GAC (Gly_Asp)
- Allele C – Codon 57, GGA to GAA (Gly_Glu)
- Allele D – Codon 52, CGT to TGT (Arg_Cys)

The wild-type is denoted *MBL2* genotype A/A and the pooled MBL variants (B, C, and D) are denoted *MBL2* genotype 0/0 (where the "0" stands for one of the variant alleles).

The MBL variant proteins are unstable and probably have a shorter half-life in the circulation [9-11]. In addition, all mutations in exon 1 interfere with the formation of

higher order oligomers [12]. The variant MBL has a lower molecular weight and does not bind mannan efficiently nor activate complement. (See 'The MBL protein' below.)

In addition to the three mutations in exon 1, there are several other polymorphic sites located in the MBL promoter region that are associated with decreased MBL serum concentration. These include single nucleotide polymorphisms (SNPs) located at position -550 (H/L variant), and -221 (Y/X variant), both G to C nucleotide substitutions. Another polymorphic site is located at position +4 of the 5'-untranslated portion of the *MBL2* gene (P/Q variant, C to T). The promoter haplotypes, HY, LY, and LX are associated with high, medium, and low levels of MBL serum concentrations, respectively. In addition, a low producing LX haplotype in the homozygous state seems to downregulate the basal expression of MBL as effectively as a single structural variant [13,14]. The molecular mechanisms resulting in different MBL serum levels associated with the *MBL2* gene promoter types have not yet been elucidated [12,13,15].

Four common haplotypes have been identified on a normal A chromosomal background: HYPA, LYPA, LYQA, and LXPA, with high, high-intermediate, intermediate, and low promoter activity. The variant alleles are associated with HYPD, LYPB, and LYQC [4,13,15]. Thus, seven common haplotypes are found. Other haplotypes have been described, but are rare due to linkage disequilibrium [16]. The serum level of MBL is strongly associated with the individual's haplotype.

THE MBL PROTEIN

Animal lectins that are dependent upon the presence of calcium ions are named C-type lectins. Members of the C-type family include selectins, hepatic type II receptors, type I transmembrane proteins, collectins (collagen-like lectins), and lecticans.

The collectins include three serum proteins (mannose-binding lectin, bovine conglutinin, and bovine collectin 43) and two lung surfactant proteins (SP-A and SP-D) [17-19]. These proteins play important roles in innate immunity [20-22]. (See "An overview of the innate immune system", section on 'Collectins'.)

The mannose-binding lectin (MBL) protein consists of multimers of an identical 25 kDa polypeptide chain synthesized by the liver [4]. Three polypeptide chains form a triple helix within the collagen-like region. These structural subunits form covalent oligomers via disulfide bonds at the N-terminal end. MBL in serum primarily consists of trimers and tetramers of 9 and 12 polypeptides respectively, but the oligomers can range from dimers to hexamers [4]. These higher-order oligomers are necessary for the function of MBL and the interaction with MBL-associated serine proteases (MASPs) [23,24].

MBL binds carbohydrates in the presence of $Ca2+$ through the C-terminal carbohydrate-recognition domain (CRD) [17,18,25]. The CRD is able to form bonds with hydroxyl

groups on specific ligands, including mannose, N-acetyl-d-glucosamine (GlcNAc), N-acetyl-mannosamine, fucose, and glucose. These carbohydrates are found on pathologic microorganisms, including bacteria, fungi, parasitic protozoans, and viruses. CRD also recognizes molecular structures of dying host cells, including nucleic acids, mitochondria, and the metalloproteases, meprin-alpha and -beta [4,26]. Carbohydrates that are found on mammalian glycoproteins, such as D-galactose and sialic acid, have no affinity for MBL. Thus, MBL is able to bind to microbes or unwanted material and trigger the activation of the lectin pathway, but it avoids recognition of self.

THE LECTIN PATHWAY

The complement system can be activated through three pathways: the classical pathway, the alternative pathway, and the lectin pathway (figure 1). The lectin pathway has an activation scheme similar to that of the classical pathway, but lectins substitute for antibodies and an associated protease replaces C1. Mannose-binding lectin (MBL) and related collectins bind sugar residues on the microbial surface; their associated proteases (MASPs) subsequently cleave C4 and C2. The complement activation pathways are discussed in greater detail separately. (See "Complement pathways".)

MBL DEFICIENCY

Overall, individuals generally benefit from normal levels of mannose-binding lectin (MBL). However, in some cases low levels appear to be advantageous. The association of both decreased and elevated serum levels of MBL, due to different polymorphisms of the *MBL2* gene and its promoter, with a variety of diseases is discussed in detail separately. (See "Mannose-binding lectin deficiency".)

SUMMARY

- Mannose-binding lectin (MBL) is a protein involved in complement activation via the lectin pathway (figure 1). The complement system provides immediate defense against infection and has proinflammatory effects. (See 'Introduction' above and 'The lectin pathway' above.)
- Decreased levels of MBL can result from mutations in the *MBL2* gene or its promoter. (See 'The MBL gene' above.)

- MBL binds to carbohydrates on microbes or unwanted material and triggers the activation of the lectin pathway, but it does not recognize self carbohydrates. (See 'The MBL protein' above and 'The lectin pathway' above and "Complement pathways".)

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Chapter 5

Mannose-Binding Lectin Deficiency

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INTRODUCTION

Mannose-binding lectin (MBL), also known as mannan-binding protein (MBP), is a protein that is involved in complement activation via the lectin pathway (figure 1). The complement system provides immediate defense against infection and has proinflammatory effects.
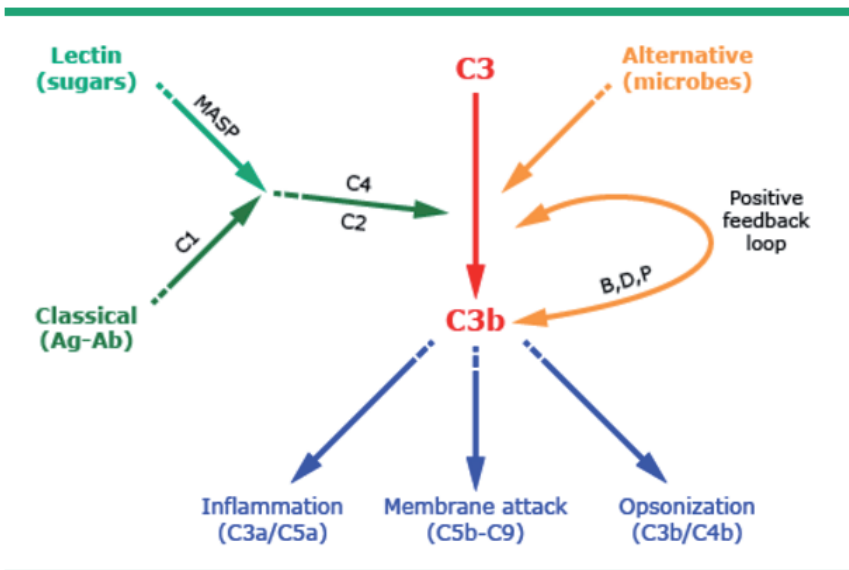

Figure 1. Pathways of complement activation

MBL deficiency is defined as a serum level <500 ng/mL. It is a laboratory finding that does not necessarily equate to a clinical disorder. MBL deficiency is associated with a large and heterogeneous group of disease processes. However, subnormal levels are also found in healthy people. To date, there is no consensus on the clinical relevance of MBL deficiency or its treatment.

This topic reviews the genetics, epidemiology, diagnosis, and management of MBL deficiency and also reviews diseases associated with both low and high levels of MBL. Genetic defects and polymorphisms of the *MBL2* gene, the function of the MBL protein, and other disorders of the complement system are discussed separately. (See "Mannose-binding lectin" and "Inherited disorders of the complement system" and "Acquired disorders of the complement system".)

EPIDEMIOLOGY

People with mutations of both mannose-binding lectin 2 (*MBL2*) alleles constitute between 1 and 65 percent (median 5 percent) of the population, depending upon the ethnic group [1,2]. Heterozygosity, or the presence of mutations in one *MBL2* allele, is found in more than 30 percent of the population in most ethnic groups evaluated. Thus, up to 40 percent of most populations may have single nucleotide polymorphisms in one or both *MBL2* alleles [3,4]. (See "Mannose-binding lectin", section on 'The MBL gene'.)

PATHOPHYSIOLOGY

Genetics

The normal or wild-type MBL allele is called allele A. Three point mutations have been identified, all in exon 1, designated alleles B, C, and D. As a group, the variant alleles are also called allele or genotype 0. There is an autosomal pattern of inheritance. However, the MBL serum phenotype is gene-dose dependent, so inheritance cannot be defined as dominant or recessive. The level of functional MBL is decreased by as much as 90 percent, even in heterozygotes. The effect of the D allele in heterozygotes is less dramatic than that of the B or C alleles. (See "Mannose-binding lectin", section on 'The MBL gene'.)

The B variant mutation occurs at a gene frequency of approximately 25 percent in Eurasian populations but is virtually absent in sub-Saharan West Africa [5]. The C variant is absent among Asians and American Indians, is rare among Caucasians, but is commonly seen in sub-Saharan African populations, with a frequency of 50 to 60 percent [6]. The D allele occurs with lower frequency than the B or C alleles and appears to be confined to Caucasians and northern East African populations [7].

In addition to the three mutations in exon 1, there are several other single nucleotide polymorphisms (SNPs) located in the *MBL2* promoter region that are associated with decreased MBL serum concentration (variants H/L, Y/X, and P/Q). There are many possible combinations of exon 1 mutations and promoter SNPs. However, only seven common haplotypes are found in humans:

- Promoter haplotypes associated with a normal ("wild-type") exon 1 haplotype include HYPA, LYPA, LYQA, and LXPA, which have high, high-intermediate, intermediate, and low promoter activity, respectively.
- The genotype 0 variant haplotypes include HYPD, LYPB, and LYQC.
- The LXPA haplotype is seen in 24 percent of Caucasians and, as such, is the most common cause of MBL deficiency in this group (either LXPA homozygous or LXPA plus a variant haplotype).

- LYPB is the most common variant haplotype in Asians and Caucasians (22 and 12 percent, respectively) but is rare in Africans [8].
- LYQC is the haplotype most often found in Africans (24 percent) but is uncommon in Caucasians and Asians.

Normal homozygotes (genotype A/A) generally have MBL levels above 1000 ng/mL, with levels up to 10,000 ng/mL [8,9]. Heterozygous individuals (A/0 genotype) have approximately one-sixth to one-eighth the normal functional level of MBL, with levels generally ranging from 500 to 1000 ng/mL. Individuals homozygous for variant *MBL2* alleles (genotype 0/0) usually have levels <50 ng/mL. Similar levels are seen in patients with the promoter variants LXP and normal A allele (LXPA haplotype) on one gene and the B, C, or D allele on the other. One patient was reported with undetectable MBL (<20 ng/mL) [10]. The combination of mutations in exon 1 and the promoter region results in a dramatic variation in MBL concentrations of up to 1000-fold in apparently healthy individuals.

High worldwide prevalence of MBL deficiency or low protein-producing alleles appears to be caused exclusively by human migration and genetic drift, indicating that *MBL2* variation does not have a strong effect on population fitness [11]. However, other data suggest that MBL deficiency may have an evolutionary advantage. The diverse role of MBL in innate immunity, particularly its effects on infectious disease susceptibility, may have led to selection for the heterozygous states of *MBL2*. There are several processes by which MBL heterozygosity may be advantageous in an evolutionary sense, including protection against adverse effects of various infectious diseases and lethal manifestations of atherosclerosis, a disease which now seems to have a more ancient history than previously assumed [12]. In addition, high MBL production (normal genotype A) is associated with preterm birth compared with intermediate MBL production (variant genotype B) and low MBL production (variant genotype C) [13].

Physiology

Circulating MBL in the newborn, measured in umbilical cord blood, is of fetal, not maternal, origin [14]. Levels in term infants are two-thirds that of their mothers and reach adult levels in approximately one month.

Plasma levels of MBL are stable over time in individuals [15]. No circadian variation is described. Physical exercise does not affect levels [16]. MBL is reported to behave like an acute-phase reactant, although levels increase only approximately 1.5-fold one to two weeks after the inciting event and show variation between individuals [17]. Persons with low levels of MBL do not reach serum levels of wild-type individuals during an acute-phase response.

CLINICAL MANIFESTATIONS

Many individuals suffer no sequelae from MBL deficiency due to different polymorphisms of the *MBL2* gene and its promoter because of redundancies in the immune system [18]. Increased susceptibility to infection is generally seen in patients with MBL deficiency when additional factors that compromise the immune system are present [8]. However, some individuals with insufficient serum MBL levels who are otherwise immunocompetent are more likely to have recurrent infectious disease and poorer outcomes from infection. It is possible that another part of the immune system that overlaps with MBL is functioning at the low end of normal in these individuals, and this lower level of function is not sufficient to make up for the MBL deficiency [19].

The role of MBL in autoimmune disorders is less clear cut. Overall, individuals generally benefit from normal levels of MBL. However, in some cases, low levels appear to be advantageous [20]. This dual effect is called heterosis.

Infection

The innate immune system constitutes the principal defense against infection when the adaptive immune response is either immature or compromised. In certain individuals, deficiency of MBL is associated with an increased frequency of pyogenic infections, especially encapsulated bacteria, due to defective opsonization. Low levels of MBL are associated with increased susceptibility to and severity of certain infections. (See "An overview of the innate immune system", section on 'Collectins'.)

In immunocompromised hosts

An increased incidence of infectious disease is seen in immunocompromised individuals and immunologically immature neonates and infants who are also MBL deficient. Patients may be immunocompromised for a number of reasons, including immunologic immaturity, acute critical illness, chronic disease, chemotherapy, transplantation, major surgery, and other immunodeficiencies.

An opsonization defect has been described in infants and young children with MBL deficiency [19,21-25]. These patients present with recurrent upper respiratory tract infections (URIs), chronic diarrhea, and failure to thrive. Another study found an increased risk of acute URIs in older infants with MBL insufficiency [26]. However, the data in children are contradictory regarding the possible association of MBL levels with sepsis [27] and lower respiratory symptoms [28]. Concomitant specific antibody deficiency is common in children with MBL deficiency [29]. These patients have more frequent episodes of otitis media, pneumonia, and sepsis and are more likely to receive prophylactic antibiotics and/or immune globulin replacement therapy than children with MBL deficiency alone. (See "Specific antibody deficiency".)

MBL levels are inversely correlated with the frequency of lower respiratory tract infections and bronchiectasis in patients with common variable immunodeficiency. MBL deficiency may also be found in combination with immunoglobulin G2 (IgG2) deficiency in infants and young children who present with recurrent infections. (See "IgG subclass deficiency" and "Common variable immunodeficiency in children"and "Clinical manifestations, epidemiology, and diagnosis of common variable immunodeficiency in adults".)

MBL deficiency is associated with an increased rate of infections in patients who have undergone solid organ [30-34] or hematopoietic cell transplantation [35] or who have undergone major gastrointestinal surgery for malignant disease [36]. In patients with cystic fibrosis, MBL deficiency is associated with earlier colonization with *Pseudomonas*, more rapid decline in lung function, and earlier death secondary to end-stage lung disease. (See "Cystic fibrosis: Genetics and pathogenesis".)

The effect of MBL deficiency in critically ill patients or in those receiving chemotherapy is variable. A meta-analysis of studies of critically ill patients admitted to the intensive care unit (ICU) found that MBL variant genotypes and low MBL levels were associated with increased risk of sepsis [37]. However, a European study of 1839 patients admitted to critical care units with sepsis caused by community-acquired pneumonia or peritonitis and 477 controls found no increased risk of sepsis-related mortality at 28 days or six months [38]. In most studies of patients receiving chemotherapy, MBL deficiency was associated with more prolonged episodes of febrile neutropenia and serious infections, such as bacteremia [8,39,40]. However, this association was not seen in patients who were severely neutropenic or who had acute myeloid leukemia, most likely because the significant impairment in phagocytosis in these patients blunted any effect of MBL deficiency [8,41,42].

In people with no other identified immunodeficiency

MBL deficiency may be clinically relevant in some individuals who do not have other predisposing factors. Low MBL levels are associated with increased susceptibility to meningococcal disease, tuberculosis, and Legionnaires' disease; recurrent tonsillitis, malaria, and vulvovaginitis; and poor immune response to influenza vaccination [43-54], although higher levels are associated with an increased risk of leprosy [55,56]. Low levels of MBL are also associated with more severe disease and worse outcomes with certain infections, including community-acquired pneumonia [57], invasive pneumococcal disease [58-61], *Escherichia coli*-induced pyelonephritis [62], *Chlamydia trachomatis* genital tract infections [63], and hepatitis B virus infection [43,55,64,65]. Results regarding the role of MBL in hepatitis C infection are inconsistent [66-69].

A higher prevalence of severe MBL deficiency (MBL level ≤50 ng/mL) was seen in one case-control study of patients with a history of recurrent and/or severe infections, particularly respiratory tract infections [70]. This difference persisted even in the absence

of concomitant immunodeficiency. However, the existence of other cofactors that influence the clinical phenotype in patients with MBL deficiency was suspected in this study for several reasons. Patients with severe MBL deficiency and recurrent and/or severe infections had a broad spectrum of clinical severity. No correlation was seen between severe MBL deficiency and severity of infection. Onset of recurrent and/or severe infections was not until adolescence or adulthood despite presumed longstanding MBL deficiency. In addition, a number of control subjects with no significant infection history had severe MBL deficiency.

The role of MBL in human immunodeficiency virus (HIV) infection and progression has been debated for several years. MBL is able to bind to the envelope protein of the HIV-1 virus (gp120) because it is highly glycosylated with N-linked carbohydrates [71,72]. Thus, it is conceivable that MBL-mediated complement activation could facilitate the immune response directed against HIV infection. However, reports on the effects of MBL on HIV infection and progression are contradictory. Results on the effect of MBL on HIV disease progression have ranged from finding no effect [73-75] to a negative effect of high MBL and a beneficial effect of low MBL [76,77]. However, subsequent literature seems to point towards a beneficial effect of high MBL levels and a negative effect of low MBL [78-91].

Autoimmunity

In the presence of MBL deficiency, chronic inflammatory conditions may be more severe. Low MBL serum levels and *MBL2* genetic polymorphisms with corresponding impaired MBL function appear to affect several autoimmune diseases, although the data are sometimes inconsistent.

It is generally assumed that the recognition of self-determinants is confined to the adaptive immune system and that the innate immune system plays little role in autoimmunity. However, evidence is growing that the innate immune system may contribute to autoimmunity, either by priming or promoting aggressive immune responses [92,93].

A major pathophysiologic concept of autoimmunity is impaired apoptotic cell clearance. MBL facilitates the clearance of apoptotic cells [94,95]. Membrane carbohydrates are altered when a cell goes into apoptosis, leading to increased expression of fucose and N-acetyl-glucosamine [96,97]. Redistribution or clustering of glycoproteins expressed on apoptotic cells may enable MBL to bind to these carbohydrates, thereby facilitating clearance [98,99].

Rheumatoid arthritis

The data on the effect of MBL levels and rheumatoid arthritis are variable, depending upon patient factors and outcomes studied [100-106]. Early disease onset and findings linked with poor prognosis, including reactive systemic amyloidosis and increased joint erosions and inflammation, are generally associated with low MBL levels [100-105].

(See "Investigational biologic markers in the diagnosis and assessment of rheumatoid arthritis" and "HLA and other susceptibility genes in rheumatoid arthritis".)

However, a dose-dependent association was seen between *MBL2* expression potential (indicated by structural genotypes and promoter polymorphisms) and disease activity and physical disability in anticyclic citrullinated peptide-positive patients with newly diagnosed rheumatoid arthritis [107]. This finding is in accord with previous findings of increased serum concentrations of MBL in patients with rheumatoid arthritis [108].

MBL deficiency does not increase susceptibility to juvenile idiopathic arthritis (JIA), but it is associated with a younger age at onset of polyarticular JIA [109]. On the other hand, MBL-deficient children with oligoarticular JIA are more likely to go into remission than MBL-sufficient children.

Systemic lupus erythematosus

There are mixed reports regarding the relationship between MBL deficiency and systemic lupus erythematosus (SLE), although, on balance, low MBL levels are associated with SLE [110-112]. (See "Epidemiology and pathogenesis of systemic lupus erythematosus".)

Autoantibodies against MBL are more commonly elevated [113] and MBL levels lower [114] in patients with quiescent rather than active SLE. Anti-MBL autoantibodies in SLE patients can influence functional activity of MBL and have a significant role in SLE disease pathogenesis [115]. MBL-downregulating promoter polymorphisms were associated with juvenile-onset SLE and higher risk of cutaneous manifestations and cardiopulmonary complications [67]. Low serum MBL levels also predisposed patients with SLE to more major infections, particularly bacterial infections [68,114]. In addition, SLE patients with MBL-deficient genotypes were more likely to have cardiovascular disease [69] and increased intima-media thickness of the common carotid artery [116], although these findings may be due to the association of this genotype with antiphospholipid antibodies. High MBL expression genotypes were associated with an increased risk of renal disorders in children with SLE [114].

In contrast, one study found that there was no increase of severe MBL deficiency in SLE, nor an association between low MBL levels and infection [71]. Another study found that MBL genotype status did not affect the course or outcome of the disease [72].

Celiac disease

The MBL O/O genotype is associated with celiac disease and an increased risk of developing secondary autoimmune diseases [117].

Diabetes

Data regarding the association between MBL levels and early onset of diabetes mellitus (DM) are conflicting. One study found higher levels of MBL in children with type 1 DM

than in siblings without diabetes or in healthy controls [118]. Conversely, another study found that individuals who developed type 1 DM during childhood and adolescence were more likely to possess a variant allele than healthy controls [119]. The association between MBL levels and diabetic microvascular complications is discussed below. (See 'Cardiovascular disease' below.)

Ischemia/reperfusion injury

Tissue damage and impaired organ function as a result of ischemia/reperfusion (I/R) injury remain major hurdles in solid organ transplantation. MBL may contribute to the pathogenesis of inflammation-induced vascular damage both in the transplanted organ and in the recipient's native blood vessels [120]. The hypoxic state to which an organ is subjected during organ harvesting, transport, and implantation results in activation of various immunologic events [121]. The complement system plays an important role in mediating tissue injury after oxidative stress. Activation and deposition of complement on the vascular endothelium occurs following oxidative stress [122,123]. Tissue injury after I/R is significantly reduced by complement inhibition [92,124]. Complement activation via the lectin pathway occurs following oxidative stress, indicating that inhibition of MBL could be a novel approach in reducing I/R damage [125].

MBL depositions are observed early after transplantation of ischemically injured kidneys [126]. In addition, high MBL levels are associated with a more severe form of rejection leading to graft loss in kidney, heart, and lung transplantation [127-129]. Graft and patient survival following simultaneous pancreas and kidney transplantation (SPKT) is significantly better in recipients with MBL gene polymorphisms associated with low MBL levels. These findings identify higher levels of MBL as a potential risk factor for graft and patient survival in solid organ transplantation.

Infection risk in solid organ transplantation patients is discussed above. (See 'In immunocompromised hosts' above.)

Cardiovascular disease

MBL binds to intracellular components of endothelial cells during I/R injury, activating the lectin pathway [93,125]. The association between MBL levels and cardiovascular disease is variable, with some outcomes more commonly associated with high levels and others with low levels.

In a prospective study performed in patients with severe carotid atherosclerosis, the degree of early restenosis was significantly higher in patients homozygous for the normal *MBL2* genotype (allele A) compared with patients with *MBL2* variant genotypes [130].

After cardiac surgery, patients with MBL deficiency did not develop multiple organ dysfunction syndrome, unless they had been transfused with fresh frozen plasma

(containing MBL from donors), suggesting that sustained MBL deficiency is a favorable status for patients undergoing cardiac surgery [131]. However, in another study, patients expressing the combined *MBL2* LYQA high-secretor haplotype had a significantly higher incidence of postoperative myocardial infarction after primary coronary artery bypass graft surgery than patients who do not express this haplotype [132].

High MBL levels were also associated with an increased risk of ischemic heart disease and myocardial infarction [133] and risk of both cardiovascular death and death from all causes [134] in a cohort of Danish patients with rheumatoid arthritis. However, a large Norwegian health study showed that variant *MBL2* haplotypes causing MBL deficiency were associated with a twofold higher risk of myocardial infarction in a young to middle-aged Caucasian population [135].

A major source of mortality and morbidity in DM is microvascular complications. A substantial portion of patients with diabetes develops diabetic nephropathy and retinopathy. Higher levels of MBL are associated with diabetic microvascular complications. Several studies show an association between an increased risk of developing renal failure and high MBL-producing genotypes in patients with diabetes [136-138], although this relationship is inconsistent [139]. The exact immunologic processes involving the role of MBL in the pathogenesis of diabetic nephropathy are still unclear. In contrast, high MBL levels may predict a decreased likelihood of myocardial infarction in patients with diabetes [140].

Cerebrovascular disease

MBL and the lectin pathway of complement activation may play a role in the pathogenesis of stroke. MBL in cerebrospinal fluid (CSF) is predominantly brain derived, most likely from the leptomeningeal cells. There is a negligible physiologic connection between the MBL fractions in CSF and serum [141]. In a murine model, MBL null mice were protected from transient and permanent ischemic injury [142]. In addition, polyman2, a synthesized mannosylated molecule that binds to MBL, improved neurologic deficits and infarct volume when given up to 24 hours after brain ischemia was induced in wild-type mice. A study in human stroke patients found that serum MBL levels were significantly higher acutely in ischemic stroke patients compared with normal controls, and serum levels of MBL correlated with the stroke score [143].

Cancer

The MBL genotype appears to influence oncologic disease. Low MBL-producing variants are associated with increased risk of gastric cancer and more advanced phenotypes [144-146]. In addition, MBL-deficient oncologic patients that require surgery have a higher risk of developing postoperative complications. However, the opposite is seen with promoter polymorphisms. The X allele of the Y/X promoter polymorphism

(resulting in lower MBL levels) is associated with improved lung cancer survival [147]. Promoter polymorphisms that result in higher MBL levels are associated with increased susceptibility to human papillomavirus infection [148] but not increased risk for cervical cancer [149]. MBL deficiency, defined as an MBL level <200 ng/mL, was associated with decreased event-free survival in pediatric oncology patients [150]. MBL substitution therapy improved the outcome in patients with advanced pancreatic cancer [151].

Trauma

Complement activity is virtually abolished immediately following severe trauma. Plasma MBL levels are inversely correlated with injury severity [152]. Serum levels of MBL nadir at 12 hours posttrauma, with levels subsequently rising to more than threefold the levels seen in controls at 10 days posttrauma [153]. Severely injured patients with single nucleotide polymorphisms (SNPs) in *MBL2* are significantly more susceptible to having positive cultures, infectious complications, systemic inflammatory response syndrome (SIRS), and septic shock than patients with an *MBL2* wild-type genotype [154].

DIAGNOSIS

Mannose-binding lectin (MBL) deficiency is generally defined as a serum level <500 ng/mL or MBL activity below 200 units/mL, depending upon the assay used. However, physiologically relevant MBL levels may vary depending upon the associated disease state, with reported cutoffs ranging from 100 to 1000 ng/mL [39,40,155,156]. In addition, serum concentrations of lectin pathway components, including MBL, vary depending upon age of the patient [157]. Thus, some groups have defined MBL deficiency as partial (>50 to 1000 ng/mL) or severe (≤50 ng/mL) in adults [70,158,159], and others suggest a cutoff of <100 ng/mL for MBL deficiency in children [29]. Antigenic measurement of MBL serum concentration is becoming a widely available diagnostic test. However, there is no consensus on the relevance of MBL deficiency or its treatment. Thus, there are no standard guidelines regarding which patients to test.

Indications for testing

The authors suggest performing an evaluation of all arms of the complement system (classical, alternative, and lectin pathways) in individuals with recurrent and/or severe respiratory tract infections and in patients immunocompromised for other reasons, such as transplantation patients with recurrent infections who have decreased cellular immunity due to immunosuppressive drug therapy, since these are the patients most likely to benefit from the antibiotic prophylaxis and treatment. Testing includes total hemolytic complement (CH50), alternative pathway hemolytic (AH50), and MBL assays. This testing

is reviewed in greater detail separately. (See "Overview and clinical assessment of the complement system".)

There is no role for routine testing of MBL genotype or serum concentration in children with chronic wet cough [160].

Laboratory evaluation

Impairment of the lectin pathway is almost always due to MBL defects. In addition, there is a strong correlation between MBL serum concentration and MBL activity. Thus, antigenic measurement of MBL serum concentration is an appropriate screening test for MBL deficiency. However, this method will not detect rarer causes of impairment of the lectin pathway due to mutations in the mannan-binding lectin-associated protease (MASP) or ficolin genes. The activation assay is an alternative test that can detect impairment due to MBL, MASP, or ficolin defects [161]. Separate testing for opsonic activity is not necessary, since it is always low in cases of MBL deficiency. (See "Inherited disorders of the complement system", section on 'Lectin pathway deficiencies'.)

Further evaluation by functional tests or genotyping is usually not necessary. Testing only by genotyping is not recommended, because MBL levels can differ by 10-fold in individuals with identical genotypes for all of the known MBL variants [159]. In addition, patients with low levels of MBL can be missed by genotyping for only the exon 1 variants but not the promoter polymorphisms (eg, patients with the LXPA haplotype would be grouped with those with normal MBL levels). The other components of the lectin pathway, the MASPs and ficolins, can be analyzed if the *MBL2* gene is normal. (See 'Genetics' above.)

TREATMENT

General management

Patients with recurrent infections should be treated similarly to other patients with complement deficiencies. This includes prompt treatment with antibiotics with each febrile infection and possible prophylactic antibiotics, as is done with patients who are postsplenectomy or who have symptomatic immunoglobulin A (IgA) deficiency. In addition, these patients should receive pneumococcal and meningococcal vaccines, with documentation of antibody responses. A trial of intravenous immune globulin (IVIG) may be indicated if infections are recurrent and severe and a plasma opsonic defect is identified. (See "Selective IgA deficiency: Clinical manifestations, pathophysiology, and diagnosis" and "Secondary immunodeficiency due to underlying disease states, environmental exposures, and miscellaneous causes" and "Inherited disorders of the complement system".)

MBL substitution therapy

Animal studies and phase-I/II human studies suggest that replacement therapy with plasma-derived MBL is safe. However, it is still an experimental treatment and not available commercially [155,162-165]. Recombinant human MBL protein (rhMBL), derived from a Chinese hamster ovary cell line, is untested and is also not commercially available [166].

One plasma-derived MBL substitution product previously available was administered intravenously and had a relatively short half-life [167]. The mean half-life of plasma-derived MBL is approximately 70 hours (range 15 to 115) in healthy adult volunteers and adult patients with *Staphylococcus aureus* septicemia and approximately 36 hours (range 24 to 67) in children with chemotherapy-induced neutropenia. In a study of 10 pediatric oncology patients with chemotherapy-induced neutropenia who received twice-weekly MBL infusions, all patients reached trough MBL levels of ≥1 mcg/mL, a level sufficient for opsonization in healthy individuals [168]. However, these patients all had suboptimal levels of opsonophagocytosis. Insufficient opsonic function may be due to additional complement deficiencies in these patients.

SUMMARY

- Mannose-binding lectin (MBL) is a protein involved in complement activation via the lectin pathway (figure 1). As part of the innate immune system, the complement system provides immediate defense against infection and has proinflammatory effects. (See 'Introduction' above.)
- Decreased levels of MBL can result from mutations in the *MBL2* gene or its promoter. People with mutations of both *MBL2* alleles constitute approximately 3 to 5 percent of the population. Heterozygosity is found in up to 30 percent of the population. (See "Mannose-binding lectin", section on 'The MBL gene' and 'Epidemiology' above.)
- MBL binds to carbohydrates on microbes or unwanted material and triggers the activation of the lectin pathway, but it does not recognize self-carbohydrates. (See "Mannose-binding lectin", section on 'The MBL protein' and "Mannose-binding lectin", section on 'The lectin pathway' and "Complement pathways".)
- Plasma levels of MBL are stable over time. Serum levels of MBL range from less than 20 up to 10,000 ng/mL. Deficiency of MBL is typically defined as a serum level <500 ng/mL. (See "Mannose-binding lectin", section on 'The MBL protein' and 'Physiology' above and 'Diagnosis' above.)
- Individuals generally benefit from normal levels of MBL. However, in some cases, low levels appear to be advantageous. (See 'Clinical manifestations' above.)

- In certain individuals, deficiency of MBL is associated with an increased frequency of pyogenic infections, especially encapsulated bacteria, due to defective opsonization. (See 'Infection' above.)
- In the presence of MBL deficiency, chronic inflammatory conditions may be more severe. MBL can also alter the response that affected individuals have to a variety of medical conditions. (See 'Autoimmunity' above.)
- The management of symptomatic patients include appropriate immunizations, measuring antibody responses to these vaccines, prompt use of antibiotics with infections, and possible use of prophylactic antibiotics. Intravenous immune globulin (IVIG) is an option if infections are refractory and severe and a plasma opsonic defect is present. MBL substitution therapy is generally unavailable and as yet untested. (See 'MBL substitution therapy' above.)

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GACCTTAAAGTACTTAGTAAGGGA

Chapter 6

Infection and sepsis in severely injured patients is associated with single nucleotide polymorphisms in *MBL2, MASP2* and *FCN2* within the lectin pathway

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ABSTRACT

Background. Infectious complications remain a serious threat to polytraumatized patients. Susceptibility and response to infection is, in part, heritable. The lectin pathway plays a major role in innate immunity. The aim of this study was to assess if single nucleotide polymorphisms (SNPs) in three key genes within the lectin pathway affect susceptibility to infectious complications in severely injured patients.

Methods. A prospective cohort of trauma patients admitted to a Level I Trauma Center between January 2008 and April 2011 was genotyped for SNPs in *MBL2* (Mannose-Binding Lectin 2), *MASP2* (MBL-Associated Serine Protease 2), and *FCN2* (Ficolin 2). Association of genotype with prevalence of infection was tested with chi-square and logistic regression analysis.

Results. A total of 219 patients were included, of which 112 (51%) developed a positive culture in either sputum, wounds, blood, or urine. Systemic Inflammatory Response Syndrome (SIRS) developed in 140 patients (64%), sepsis in 79 (36%), and septic shock in 37 (17%). Patients with a *MBL2* exon 1 variant allele were more prone to positive wound cultures (OR 2.50, p=0.025). A *MASP2* Y371D DD genotype predisposed for SIRS (OR 4.78; p=0.042) and septic shock (OR 2.53; p=0.003). A *FCN2* A258S AS genotype predisposed for positive wound cultures (OR 3.37; p=0.005) and septic shock (OR 2.18 p=0.011).

Conclusion. Severely injured patients with SNPs in *MBL2*, *MASP2 Y371D* and *FCN2 A258S* of the lectin pathway of complement activation are significantly more susceptible to positive culture findings and to infectious complications, SIRS and septic shock than patients with a common (wildtype) genotype.

INTRODUCTION

Trauma is the leading cause of death for young people. Early mortality due to exsanguination seems to have decreased (1), probably as a result of improvements in pre-hospital and early in-hospital care. Sepsis (3.1-17.0%) and multiple organ dysfunction syndrome (1.6-9.0%) remain unchanged as important causes of late trauma-related mortality (1). Severe sepsis and septic shock are responsible for 30-50% of all deaths in Intensive Care Units (2). Moreover, sepsis, multiple organ dysfunction syndrome, and infectious complications such as pneumonia and wound infection increase hospital costs (3). Prevention of infections and identifying patients at risk deserves full attention.

The lectin pathway of complement activation plays a critical role in innate immunity (Figure 1). Severe injury poses a serious challenge to the immune system (4). Dysregulation of the systemic inflammatory response to injury is central to the development of acute respiratory distress syndrome, multiple organ failure and sepsis (5). Levels of complement factor C3a are already markedly increased at the scene of trauma. Moreover, complement function decreases dramatically within four hours after trauma, and does not return to normal in as much as five days. Complement activation correlates with injury severity, reduced tissue hypoperfusion as measured with base deficit, and adverse clinical outcomes (6). The pathological basis for these conditions is attributed to immune dysfunction (7). An understanding of the mechanisms of innate immune activation and alarmins following trauma may lead to new therapeutic agents and improved patient survival (7).

The ability of the complement system to be activated depends on the genetic integrity of the coding genes (6). Genetic variation, particularly Single Nucleotide Polymorphisms (SNPs), in innate immunity genes may play a central role in development of posttraumatic complications (8). Key genes in the lectin pathway are *MBL2* (Mannose-Binding Lectin 2), *MASP2* (MBL-Associated Serine Protease 2), and *FCN2* (Ficolin 2). SNPs in all three genes and their promoter regions, have been associated with infectious diseases.

SNPs in exon 1 (codons 52, 54, and 55) of the *MBL2* gene, which encodes Mannose-Binding Lectin, cause transition from an A to a D, B, or C allele (which are all three known as 0 alleles). An A0 or 00 genotype as well as the presence of an YX promoter SNP results in a dramatic reduction in MBL concentration compared with the common AA genotype (9). MBL deficiency has been associated with an increased risk of infectious disease in, among others, pediatric and ICU patients (10).

MBL-associated serine-proteases (MASPs) are activated upon binding of MBL or ficolin to pathogen-associated molecular patterns located on the surface of bacteria or viruses. MASPs circulate in plasma in complexes with MBL and with ficolins 2 and 3 (11). Of the known MASPs MASP2 is the main initiator of the lectin complement pathway (12,13).

The *MASP2* D120G and Y371D SNPs have been associated with pneumococcal disease and to increase the risk of infectious complications after liver transplantation (14,15,16).

The *FCN2* gene encodes for L-ficolin (Ficolin-2), a pattern recognition molecule. The *FCN2* T236M and A258S SNPs have been shown to influence infectious outcome after liver transplantation (16), peritoneal dialysis (17), perinatal infection in babies and respiratory infections (18), but largely the functional impact of these SNPs is unknown (19).

There are many reports on MBL levels, genetic variations in immune genes and susceptibility to infections in different kinds of populations such as surgical patients, burn patients, or ICU/sepsis patients (20-22), but the relevance of SNPs in the lectin complement pathway coding genes (*MBL2, MASP2* and *FCN2*) in severely injured patients is unknown. The aim of this study was therefore to determine the influence of lectin complement pathway SNPs on the susceptibility and course of infectious complications in severely injured patients.

PATIENTS AND METHODS

Patients and Clinical Data

A prospective cohort of consecutive severely injured patients admitted to a Level I Trauma Center between January 2008 and April 2011 was studied. Eligibility criteria were an Injury Severity Score (ISS) (23) 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. After obtaining written informed consent from patient or proxy a blood sample was taken in an EDTA tube and stored at -80°C. The study was approved by the local medical research ethics committee and was registered in the Dutch Trial Registry under NTR1625.

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, SIRS within the first 24 hours of admission and developing sepsis (primary endpoint), septic shock and mortality during hospital admission. SIRS, sepsis, and septic shock were defined using international criteria (24). The following data were retrieved from electronic files: age at trauma, trauma mechanism, ISS score, length of stay in the ICU 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 Supplementary Table 1. SNP data were retrieved from Ensembl.org, National Center for Biotechnology Information (NCBI) GenBank, and NCBI SNP Database. All oligonucleotides (see Supplementary Table 2) were purchased from Eurogentec (Seraing, Belgium). Primers for *MBL2* amplify a 103-bp fragment overlapping the SNPs in exon 1. For the High Resolution Melting Analysis (HRMA) of all other SNPs 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), and resulted in 400-555 base pair amplicons. Oligonucleotides had melting temperatures (Tm) between 65.0-66.5°C (Tm calculated using the nearest neighbor 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 (25). Polymerase chain reactions (PCRs) were performed in a total volume of 10µL, containing 20ng genomic DNA, 5pmol of both gene-specific oligonucleotides, 2pmol dNTPs (Promega, Madison, WI), 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. Reaction mixtures for the detection of *MBL2* exon 1 SNPs contained 1pmol forward and 5pmol reverse oligonucleotide and 5pmol probe. 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, 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. Typical examples are shown in Figure 2.

All variant genotypes were confirmed by direct sequencing. PCR were performed in a total volume of 25µL, containing 50ng 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.

Statistical 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. The Hardy-Weinberg equilibrium is a principle stating that the genetic variation in a population will remain constant from one generation to the next in the absence of disturbing factors. In the χ^2 analysis the observed genotype frequencies are tested against the expected genotype based on the Hardy-Weinberg principle. A p-value <0.05 indicates deviation from theHardy-Weinberg equilibrium.

Data were analyzed using the Statistical Package for the Social Sciences, version 16.0 (SPSS, Chicago, Ill., USA). 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 <0.050 was considered to be statistically significant.

RESULTS

Patient Demographics

During the study period, 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 (72.6%) were admitted to the ICU with a median stay of 3 (P$_{25}$-P$_{75}$ 0-14) days. Trauma mechanisms are shown in table 1.

Table 1. Demographics of trauma mechanisms

Traffic accidents	48%
Falls from height	32%
Crush injury to thorax	6%
Street fighting	3%
Gunshot wounds	2%
Stabbing	2%
Horseriding accidents	2%
Miscellaneous accidents	5%

Miscellaneous injuries include collapsing buildings, helicopter crash, ice skating, kitesurfing, fireworks.

**Figure 1.** Schematic illustration of the Lectin pathway of complement activation
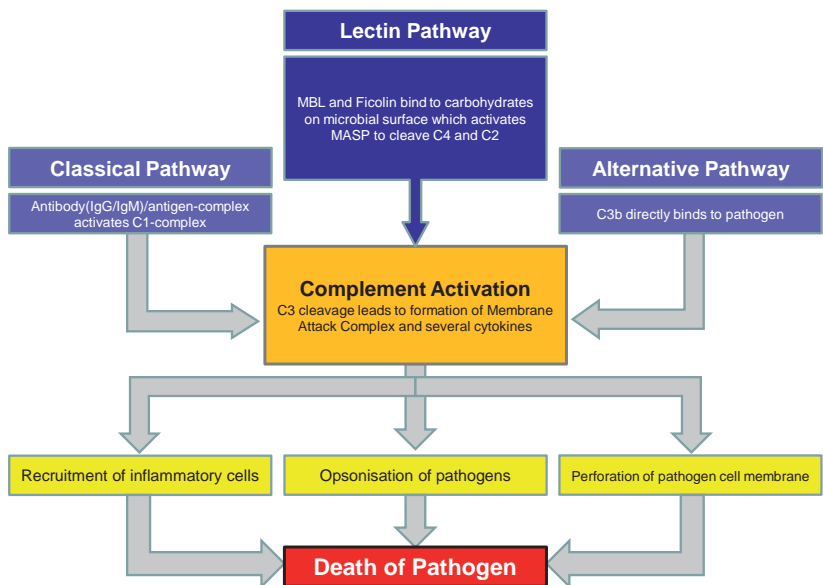
Genetic variation in *MBL2*, *MASP2*, and *FCN2* results in protein deficiency or diminished protein activity. This in turn reduces the ability to encounter pathogen attacks and increases the susceptibility to the onset and course of infections.
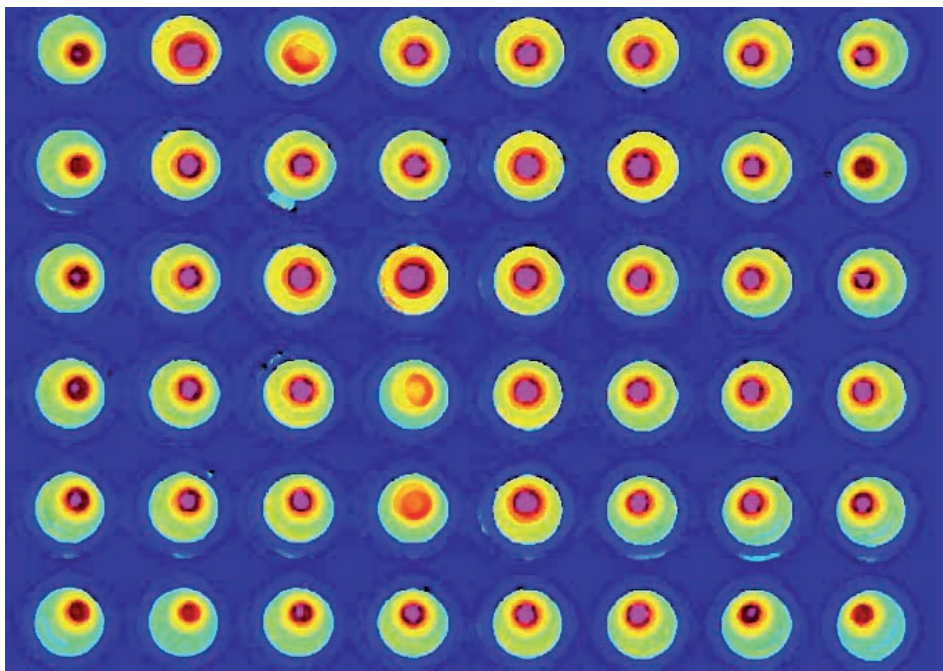

High-Resolution LightScanner CCD camera image of fluorescent LG-Green dye in a 96-wells plate at the start of High Resolution Melting Analysis
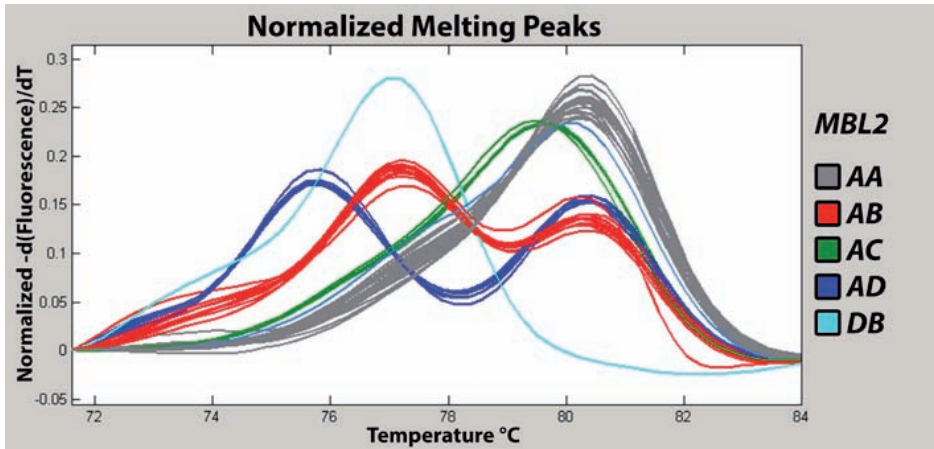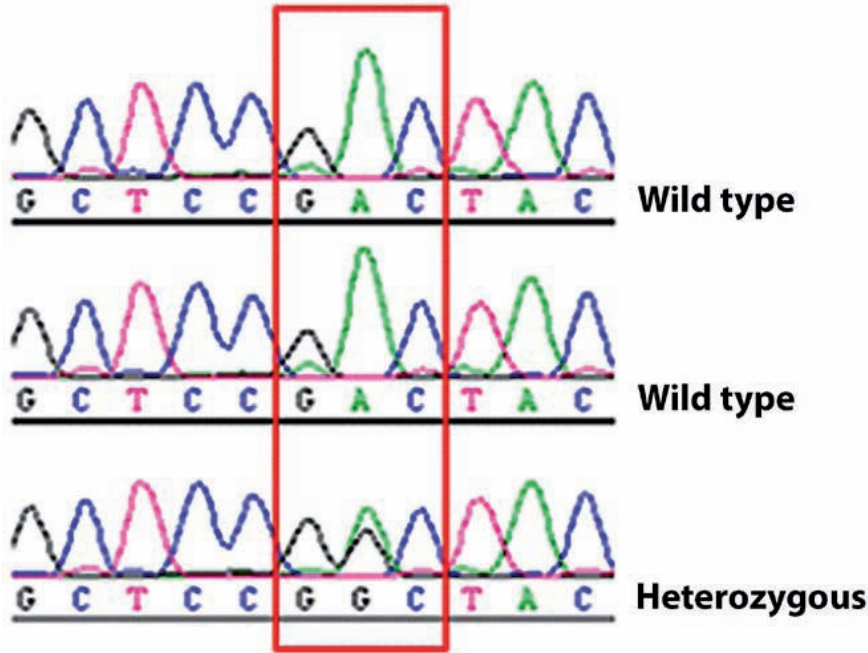
Example of Normalized Melting Peaks following High Resolution Melting Analysis (HRMA) showing different genotypes found in a 96 wells plate following PCR of *MBL2* exon1.
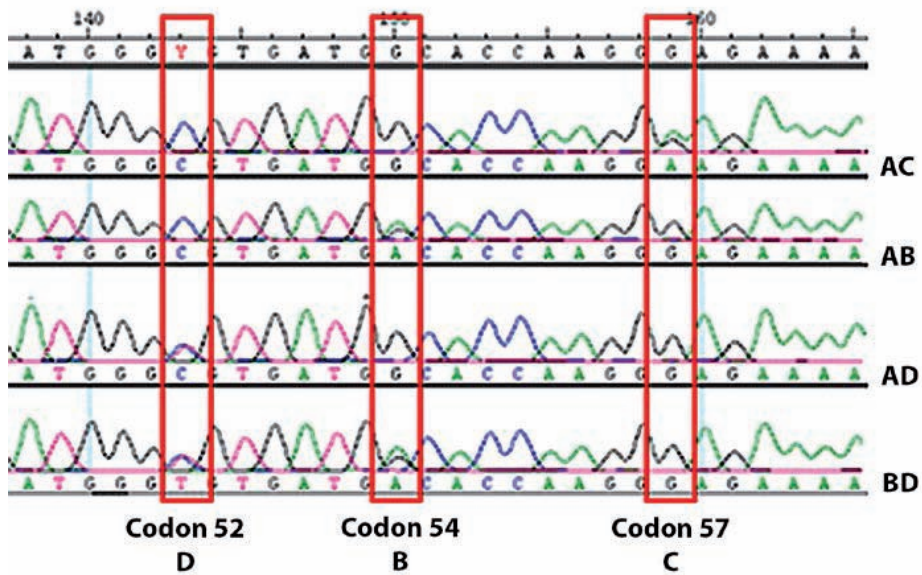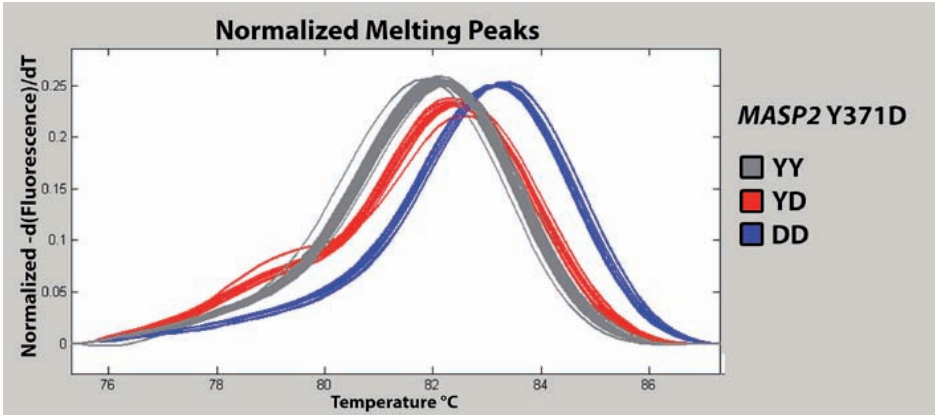

Example of sequencing of codons 52, 54 and 57 of *MBL2* exon 1 showing different mutations.
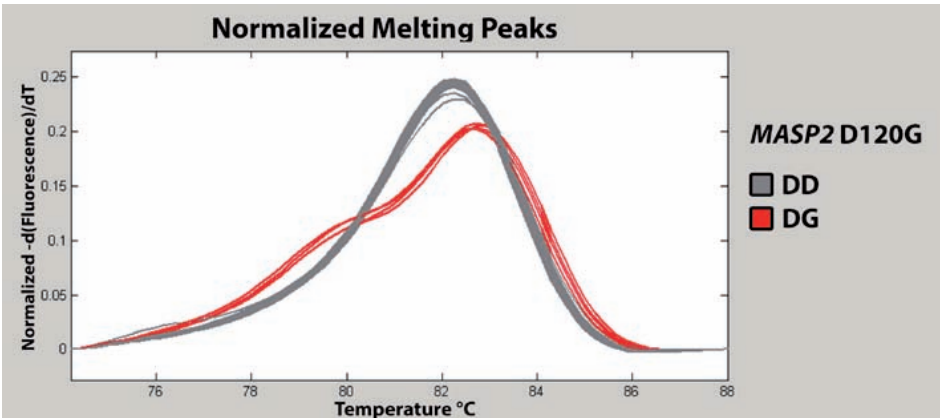
Example of Normalized Melting Peaks following High Resolution Melting Analysis (HRMA) showing different genotypes found in a 96 wells plate following PCR of MASP2 D120G.

Example of MASP2 exon 3 heterozygous D120G mutation detected by sequencing.

Example of Normalized Melting Peaks following High Resolution Melting Analysis (HRMA) showing different genotypes found in a 96 wells plate following PCR of MASP2 Y371D.
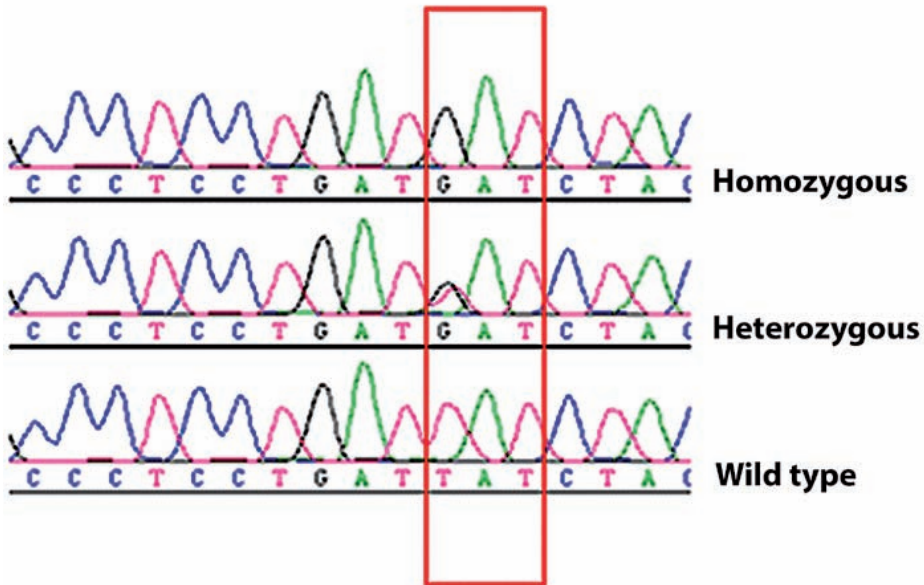
Example of MASP2 exon 9 heterozygous Y371D mutation detected by sequencing.

Genotype Frequencies

HRMA showed that 131 (59.8%) of all patients were wildtype for the *MBL2* exon 1 polymorphism, 72 (32.9%) patients carried the heterozygote A0 genotype, and 16 (7.3%) patients the homozygote 00 genotype. Of the A0 patients, 34 (15.5%) had an AB genotype, 13 (5.9%) AC, and 25 (11.4%) AD. The 00 group consisted of five BB, three BC, three BD, four CD, and one DD genotype. Seventy-four patients (33.8%) were heterozygous for the *MBL2* YX SNP, all other patients had the common YY genotype. For the *MASP2*

D120G SNP 207 (94.5%) patients had the common DD genotype; the remaining 5.5% percent were DG. For the *MASP2* Y371D SNP 145 (66.2%) were YY, 60 (27.4%) YD, and 14 (6.4%) DD. For the *FCN2* T236M SNP 106 (48.4%) had a TT, 97 (44.3%) a TM and 16 (7.3%) a MM genotype. For the *FCN2* A258S SNP 168 (76.7%) had an AA, 47 (21.5%) and AS, and 4 (1.8%) a SS genotype. Linkage disequilibrium was noted for the *MBL2* YX (χ^2=9.05; P=0.003) and *MASP* Y371D (χ^2=4.72; P=0.030); the genotype distribution of the other SNPs was in agreement with the Hardy-Weinberg equilibrium (χ^2=0.11-1.86; P=0.173-0.736).

Effect of Genotype on Infectious Complications

Table 2 shows an overview of infectious complications encountered separated by genotype. Overall, Gram-positive bacteria were detected in 79 patients (36%), Gram-negative bacteria in 84 (38%) and fungi in 46 (21%). Approximately half of the patients, 112 (51.1%), developed a positive culture; this was mostly in sputum in70 patients, but positive cultures were also found in wounds in 36 patients, blood (N=32), and urine (N=32). A total of 139 patients (63%) developed SIRS within 24 hours, of which 79 (57%) developed sepsis and 37 (27%) septic shock. Thirteen (5.9%) patients died.

Table 3 shows the results of the multivariable logistic regression models. Patients with the *MBL2* exon 1 heterozygous A0 variant were at significantly increased risk for developing positive wound cultures (Odds Ratio, OR, 2.51; 95% CI 1.12-5.62; p=0.025). Twenty-five percent of A0 patients developed a positive wound culture versus 11.5% of patients with the common AA genotype (Table 2). The risk of Gram-positive or Gram-negative organisms was unaltered by the *MBL2* exon 1 genotype. Patients with an A0 or 00 genotype had a consistently higher rate of SIRS, sepsis, and septic shock than patients with an AA genotype (Table 2); however, no statistically significant associations between *MBL2* exon 1 genotype and these endpoints were found in the multivariable analysis.

The presence of a *MBL2* YX promoter SNP was a significant risk factor for a fungal culture (OR 2.32; 95% CI 1.08-4.96; p=0.030; Table 3); in 27.0% of YX patients a fungus was cultured (mainly *Candida albicans* from pulmonary aspirates) versus 17.9% of YY patients. No other significant associations were found for the YX genotype.

For the *MASP* D120G genotype polymorphism we found no statistically significant differences for all endpoints although, strikingly, fungi, positive blood cultures and septic shock were only found in DD patients (22.2%, 15.5%, and 17.9%, respectively). Another striking, yet non-significant, finding was that only 8.3% of DG patients developed sepsis versus 37.7% in DD patients (p=0.060).

Carrying a *MASP2* Y371D DD variant genotype was a risk factor for developing SIRS within 24 hours after hospital admission (OR 4.78; 95% CI 1.06-21.59; p=0.042); SIRS developed in 78.6% of homozygous DD patients versus 58.6% of patients with a com-

mon YY genotype. Patients with the *MASP2* Y371D DD variant were also at significantly increased risk for developing septic shock (OR 2.53; 95%CI 1.12-4.33; p=0.003; Table 3); 35.7% of patients with a DD genotype developed a septic shock versus 23.3% in the DG and 12.4% in the YY group (Table 2). The prevalence of Gram-positive culture findings in patients with a *MASP2* Y371D DD genotype was 50% (versus 33% in the YY group), however this was not statistically significantly different (OR 3.19; 95% CI 0.95-10.71; p=0.060).

No significant associations between the *FCN2* T236M genotype and infectious events were found. A noteworthy finding was that positive blood cultures developed in 16.5% of patients with a TM variant genotype and in 25.0% of patients with a variant MM genotype, versus only 11.3% of patients with the common TT genotype; both were not statistically significant in the multivariate model (p=0.560 and p=0.092, respectively).

Heterozygosity for the *FCN2* A258S SNP was associated with increased risk of positive wound cultures (OR 3.37; 95%CI 1.45-7.85; p=0.005); positive culture findings were seen in 29.8% of AS patients versus 12.5% in AA patients. In addition, a *FCN2* A258S AS genotype significantly increased the risk of developing septic shock (27.7% in heterozygous AS patients versus 14.3% of AA patients; OR 2.18; 95%CI 1.30-4.78; p=0.011). Strikingly, the prevalence of Gram-negative bacteria in *FCN2* A258S AS patients was 48.9% (versus 34.5% in the AA group), yet this did not reach statistical significance (OR 1.87; 95% CI 0.93-3.77; p=0.079).

DISCUSSION

In this study the association between single nucleotide polymorphisms (SNPs) in three important genes (*MBL2, MASP2* and *FCN2*) of the innate immune system and the susceptibility of severely injured patients to positive culture findings and infectious complications has been investigated. The *MBL2* exon 1 A0 genotype was associated with increased rates of positive wound cultures, whereas the YX genotype of the *MBL2* promotor region increased the rates of colonization with fungi. The *MASP2* Y371D DD genotype put the patient at increased risk for SIRS and septic shock, and the *FCN2* A258S AS genotype was a risk factor for positive wound cultures and septic shock.

Trauma is the leading cause of death among young adults and infection is one of the major causes of late deaths among trauma patients. Prevention of infection in trauma patients is therefore of great clinical importance. Finding the association between different SNPs and infection risk is a first step in creating a genetic risk profile for infection susceptibility. In the future this may lead to the development of preventive measures for patients who are found to be at risk. The development of infectious complications is multifactorial in origin but genetic variation in the innate immune system may be one such important factor

The genotype distributions of *MBL2* (21,26,27), *MASP2* D120G (14), *MASP2* Y371D (16), *FCN2* T236M (16), and *FCN2* A258S (16,28) were in agreement with published data on European patients. All five genotypes were in agreement with the Hardy-Weinberg equilibrium, indicating that the allele and genotype frequencies of all SNPs in the population remain in equilibrium from generation to generation.

The *MBL2* exon 1 A0 genotype was significantly associated with an increased risk of developing a positive wound culture. This has been demonstrated before in a murine model of burn wounds (29) but is a novel finding in human trauma patients. Also, heterozygosity in the *MBL2* YX promoter region conferred a risk for developing fungal colonization (mainly *C. albicans*) with YX patients having a 10% higher frequency of fungi than patients with the common YY genotype. The *MBL2* exon 1 genotype seemed unrelated to Gram-positive or Gram-negative organisms as causative micro-organisms, while others found *MBL2* 0/0 to be associated with a higher incidence of Gram-positive bacteria (26). Although the percentage of patients with a *MBL2* A0 or 00 genotype that developed SIRS, sepsis, or septic shock was higher than in wildtype AA patients, this difference was not statistically significant. In a Danish ICU cohort study, the MBL 00 and to a lesser extent MBL A0 genotypes were significantly associated with mortality after sepsis (26).

In the current study only twelve patients carried a *MASP2* D120G DG genotype, which prevalence is similar as published before (14). In DG patients, a consistent trend was noted towards lower rates of infection, sepsis and septic shock as compared to DD patients, yet statistical power was too low to reach statistical significance. Lack of association with bacteraemia was also described for patients admitted to an ICU in Belgium (21). In that study, however, mortality was increased in patients with a *MASP2* D120G DG or GG genotype. The *MASP2* D120G polymorphism has also been associated with pneumococcal disease and to increase the risk of infectious complications after liver transplantation (14-16). As no relevant information is available, the importance of genetic variation in *MASP2* for injured patients yet has to be elucidated in a larger cohort.

In this study, the rare *MASP2* Y371D DD genotype increased the risk for developing SIRS and septic shock. in a univariate model in patients undergoing liver transplantation this association was not found (15). No further data on trauma and intensive care patients are available for this polymorphism.

For the *FCN2* T236M SNP we found no statistically significant association with any of the endpoints. In previous studies *FCN2* T236M was found to predispose to bacterial infection after liver transplantation (15), and to infection in patients on continuous ambulatory peritoneal dialysis (17), but no data on trauma or patients in the ICU are available.

The *FCN2* A258S AS genotype was significantly associated with positive wound cultures (13% to 25%) and with septic shock (6% to 25%) in our cohort. This SNP produces

Table 2. Demographic description of positive cultures and infectious complications by genotype

	Geno type	N (%)	Sputum Culture	Blood Culture	Urine Culture	Wound Culture	SIRS *	Sepsis *	Septic Shock *
Entire population		219	70 (32.0)	32 (14.6)	32 (14.6)	36 (16.4)	139 (63.5)	79 (36.4)	37 (16.9)
MBL2 **Exon 1****	**AA**	131	44 (33.6)	17 (13.0)	21 (16.0)	15 (11.5)	81 (61.8)	44 (33.6)	19 (14.5)
	A0	72	21 (29.2)	15 (20.8)	11 (15.3)	18 (25.0)	49 (68.1)	31 (43.1)	15 (20.8)
	00	16	5 (31.2)	0 (0.0)	0 (0.0)	3 (18.8)	9 (56.2)	4 (25.0)	3 (18.8)
MBL2 **YX**	**YY**	145	47 (32.4)	21 (14.5)	22 (15.2)	26 (17.9)	88 (60.7)	54 (37.2)	27 (18.6)
	YX	74	23 (31.1)	11 (14.9)	10 (13.5)	10 (13.5)	51 (68.9)	25 (33.8)	10 (13.5)
	XX	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
MASP2 D120G	**DD**	207	68 (32.9)	32 (15.5)	30 (14.5)	35 (16.9)	132 (63.8)	78 (37.7)	37 (17.9)
	DG	12	2 (16.7)	0 (0.0)	2 (16.7)	1 (8.3)	7 (58.3)	1 (8.3)	0 (0.0)
	GG	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
MASP2 Y371D	**YY**	145	45 (31.0)	17 (11.7)	22 (15.2)	19 (13.1)	85 (58.6)	48 (33.1)	18 (12.4)
	YD	60	20 (33.3)	14 (23.3)	7 (11.7)	13 (21.7)	43 (71.7)	24 (40.0)	14 (23.3)
	DD	14	5 (35.7)	1 (7.1)	3 (21.4)	4 (28.6)	11 (78.6)	7 (50.0)	5 (35.7)
FCN T236M	**TT**	106	32 (30.2)	12 (11.3)	18 (17.0)	19 (17.9)	69 (65.1)	36 (34.0)	15 (14.2)
	TM	97	31 (32.0)	16 (16.5)	13 (13.4)	16 (16.5)	61 (62.9)	36 (37.1)	18 (18.6)
	MM	16	7 (43.8)	4 (25.0)	1 (6.3)	1 (6.3)	9 (56.2)	7 (73.8)	4 (25.0)
FCN2 A258S	**AA**	168	53 (31.5)	23 (13.7)	22 (13.1)	21 (12.5)	107 (63.7)	58 (34.5)	27 (14.3)
	AS	47	17 (36.2)	9 (19.1)	9 (19.1)	14 (29.8)	30 (63.8)	19 (40.4)	13 (27.7)
	SS	4	0 (0.0)	0 (0.0)	1 (25.0)	1 (25.0)	2 (50.0)	2 (50.0)	0 (0.0)

* SIRS, sepsis and septic shock were only determined for patients admitted to the Intensive Care Unit.
** The wildtype is referred to as A, while the variant genotype is referred to as 0

the lowest serum levels of ficolin-2 (30). This SNP was found to be related to earlier onset of P. *aeruginosa* colonization in patients with cystic fibrosis (31) and to cytomegalovirus infection after liver transplantation (16), but largely the functional impact of this SNP is unknown (19).

The strength of this study is that we assessed the relevant SNPs in the lectin pathway as a whole. Some genotypes were found very infrequently. Although this study is among the largest studies assessing the contribution of genotype differences to infection susceptibility in trauma patients, the sample size is not large enough yet to answer all questions. For instance, it was not large enough to assess the roles of the genotypes studied

Table 3. Association between genotype and infections outcome in polytraumatized patients

Covariate		Sputum Culture	Blood Culture	Urine Culture	Wound Culture	SIRS	Sepsis	Septic Shock
MBL2 Exon 1	**A0**	0.73 (0.38-1.42)	1.54 (0.67-3.54)	0.99 (0.43-2.31)	**2.51*** (**1.12-5.62**)	1.24 (0.65-2.39)	1.28 (0.66-2.47)	1.34 (0.59-3.05)
	00	1.19 (0.35-4.10)	N.D.	N.D.	2.29 (0.49-10.81)	0.97 (0.30-3.14)	0.78 (0.20-3.05)	1.71 (0.35-8.46)
MBL2 Promoter	**YX**	1.04 (0.54-1.99)	1.13 (0.46-2.76)	0.72 (0.31-1.66)	0.86 (0.36-2.05)	1.61 (0.85-3.08)	0.92 (0.47-1.80)	0.85 (0.35-2.07)
MASP D120G	**DG**	0.35 (0.07-1.79)	N.D.	1.04 (0.19-5.75)	0.45 (0.05-4.35)	1.03 (0.29-3.66)	0.14 (0.01-1.35)	N.D.
MASP Y371D	**YD**	0.95 (0.47-1.90)	2.03 (0.83-4.97)	0.87 (0.33-2.30)	1.62 (0.70-3.77)	1.71 (0.85-3.44)	1.14 (0.57-2.31)	1.73 (0.73-4.12)
	DD	1.90 (0.54-6.64)	0.93 (0.11-8.26)	1.13 (0.25-5.09)	3.23 (0.80-13.04)	**4.78 *** (**1.06-21.59**)	2.82 (0.82-9.69)	**2.53 ***** (**1.12-4.33**)
FCN2 T236M	**TM**	0.91 (0.48-1.73)	1.31 (0.53-3.21)	0.87 (0.38-2.00)	0.85 (0.37-1.93)	0.81 (0.43-1.52)	1.12 (0.58-2.16)	1.05 (0.45-2.46)
	MM	1.71 (0.54-5.42)	3.49 (0.82-14.92)	0.32 (0.04-2.82)	0.44 (0.05-3.84)	0.67 (0.22-2.07)	1.80 (0.54-2.95)	3.09 (0.76-12.56)
FCN2 A258S	**AS**	1.34 (0.65-2.76)	1.70 (0.64-4.50)	1.23 (0.50-3.01)	**3.37***** (**1.45-7.85**)	1.07 (0.51-2.22)	1.41 (0.67-2.99)	**2.18 *** (**1.30-4.78**)
	SS	N.D.	N.D.	1.36 (0.10-19.10)	1.65 (0.13-21.69)	0.31 (0.04-2.75)	0.71 (0.07-7.39)	N.D.

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. N.D., not determined due to insufficient numbers.
Statistically significant data are indicated in boldface. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$.

for rare outcomes such as mortality. It also limits the evaluation of combinations of SNPs. Increasing the sample size will allow us to overcome these limitations. A final limitation of this study relates to the multitude of statistical tests applied in order to assess the relevance of several SNPs to multiple outcome measures. The authors acknowledge that not applying a multiple comparison correction may result in falsely accepting a spurious relation. However, a Bonferroni correction would be a too stringent correction and may result in falsely denying true effects. Presenting the 95% confidence intervals for all odds ratio's allows readers, who will mainly be interested in a specific gene or effect, to value the results.

In summary, the results of the current study clearly show that SNPs in *MBL2, MASP2* and *FCN2* influence the susceptibility to positive cultures and severity of infection in severely injured trauma patients. The development of infectious complications is multifactorial in origin but genetic variation in the innate immune system may be one such important factor. Finding the association between different SNPs and infection risk is a first step in creating a genetic risk profile for infection susceptibility. In the future this may lead to the development of preventive measures for patients who are found to be at risk. Substitution therapy would be one way to overcome deficiencies caused by genetic variation. MBL substitution therapy phase I trials have already shown interesting results (32-36).

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

Gene	SNP	dbSNP ID	Nucleotide transition (Accession code)	Amino-Acid transition (Accession code)	Location on gene / Domain
MBL2	D allele	rs5030737	5219C>T	Arg52Cys	Exon 1, collagenous region
	B allele	rs1800450	5226G>A	Gly54Asp	Exon 1, collagenous region
	C allele	rs1800451	5235G>A	Gly57Glu	Exon 1, collagenous region
	Y-221X	rs7096206	4776C>G (NG_008196.1)	N.A. (NP_000233.1)	Promoter, 5'untranslated region
MASP2	D120G	rs72550870	5620A>G (NG_007289.1)	Asp120Gly (NP_006601.2)	Exon 3, CUB1
	Y371D	rs12711521	21370T>G (NG_007289.1)	Tyr371Asp (NP_006601.2)	Exon 9, CCP
FCN2	T236M	rs17549193	11369C>T	Thr236Met	Exon 8, fibrinogen-like domain
	A258S	rs7851696	11434G>T (NG_011649.1)	Ala258Ser (NP_004099.2)	Exon 8, fibrinogen-like domain

SNP, Single Nucleotide Polymorphism;

Amino acids: Ala (A), Alanine; Arg (R), Arginine; Asp (D), Asparctic acid; Cys (C), Cysteine; Gly (G), Glycine; Glu (E), Glutamic acid; Met (M), Methionine; Ser (S), Serine; Thr (T), Threonine; Tyr (Y), Tyrosine

Supplementary Table 2. Oligonucleotide sequence for primers and probes used in *MBL2, MASP2* and *FCN2* genotyping

Name (Accession code)	SNP	Assay	Primer /probe	Start Position	Sequence
Calibrators	-	HRMA	Forward	-	5'- TTAAATTATAAAATATTTAT AATATTAATTATATA TATATAAATATAATA-Amine-C6-3'
			Reverse	-	5'- TATTATATTTATATATATAT AATTAATATTATAAA TATTTTATAATTTAA-Amine-C6-3'
MBL2	B, C, D	HRMA	Forward	5163	5'-CTGCAGTGATTGCCTGTAGC-3'
(NG_008196.1)			Reverse	5246	5'-GCCCAACACGTACCTGGTTC-3'
			Probe	5207	5'-GGCAAAGATGGGCGTGATGGC ACCAAGGGA-Amine-C6-3'
		Seq	Forward	5033	5'-TTCCCTGAGTTTTCTCACA-3'
			Reverse	5430	5'-GGCTGGCAAGACAACTATT-3'
	YX	HRMA	Forward	4755	5'-CCCATTTGTTCTCACTGCCA-3'
			Reverse	4777	5'-AAGACTATAAACATGCTTTC-3'
		Seq	Forward	4392	5'-CAGGGCCAACGTAGTAAG-3'
			Reverse	4886	5'-TTGTGACACTGCGTGACT-3'
MASP2	D120G	HRMA	Forward	5600	5'-GGACATTACCTTCCGCTCCG-3'
(NG_007289.1)			Reverse	5621	5'-ACGGCTTCTCGTTGGAGTAG-3'
		Seq	Forward	5313	5'-ACCTCTGCGAGTACGACT-3'
			Reverse	5733	5'-TGCCTGGCCTAAGACA-3'
	Y371D	HRMA	Forward	21350	5'-TTGACTGTGGCCCTCCTGAT-3'
			Reverse	21371	5'-CACTCGGCCACTGGGTAGAT-3'
		Seq	Forward	21171	5'-CTCGGCTTTTTAACCTTTC-3'
			Reverse	21695	5'-ACCATTTGGAATACATTGTGT-3'
FCN2	T236M	HRMA	Forward	11349	5'-CTGCACAGGAGATTCCCTGA-3'
(NG_011649.1)			Reverse	11370	5'-AGGACTGGTTGTTGTGGAAC-3'
	A258S	HRMA	Forward	11414	5'-ATCTTAACACCGGAAATTGT-3'
			Reverse	11435	5'-AGCTCCCTGAAACATCACAG-3'
	T236M, A258S	Seq	Forward	11186	5'-CAGCAGGGCAGTATTCAC-3'
			Reverse	11541	5'-TATCCTTTCCCCGACTTC-3'

HRMA, High-Resolution Melting Analysis.

Seq: Sequencing

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GACCTTAAAGTACTTAGTAAGGGA

Chapter 7

Single nucleotide polymorphisms in the Toll-Like Receptor pathway increase susceptibility to infections in severely injured trauma patients

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Schoemaker Award 2013
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Dr. J. Schoemaker
(1871-1940)

Dr. Jan Schoemaker was born in 1871 in Almelo, The Netherlands. His father was a surgeon. He attended the gymnasium in Kampen and Leiden and studied Medicine in Leiden. In 1895 he obtained his medical degree and in 1896 he defended his thesis called *Techniek van de darmnaad*. In 1902 he was appointed consultant surgeon at the gemeenteziekenhuis Zuidwal in Den Haag at which he worked for the rest of his career. He was a gifted general surgeon and especially famous internationally for his gastric surgery (the Schoemaker-Billroth modification) with surgical instruments he engineered himself. He was a (honorary) member of many (international) societies and was awarded many honours and prizes.

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 $<1 \times 10^9$ 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).

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'-TTAAATTATAAAAATATTTATAATATTAATTATATA TATATAAATATAATA-Amine-C6-3'	50
			Reverse	-	5'-TATTATATTTATATATATAATTAATATTATAAA 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'-TTGGGGTTTCTAAGCTAT-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

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

Name (Accession code)	SNP	Assay	Primer	Start Position	Sequence	Amplicon size (base pairs)
			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.

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 χ2 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 χ2 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 ($\chi2$=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),

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)

Table 3. Demographic description of positive cultures and infectious complications by genotype (continued)

		N	Gram-Positive	Gram-Negative	Fungus	Sputum Culture	Blood Culture	Urine Culture	Wound Culture	SIRS *	Sepsis *	Septic Shock *	Mortality
	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.
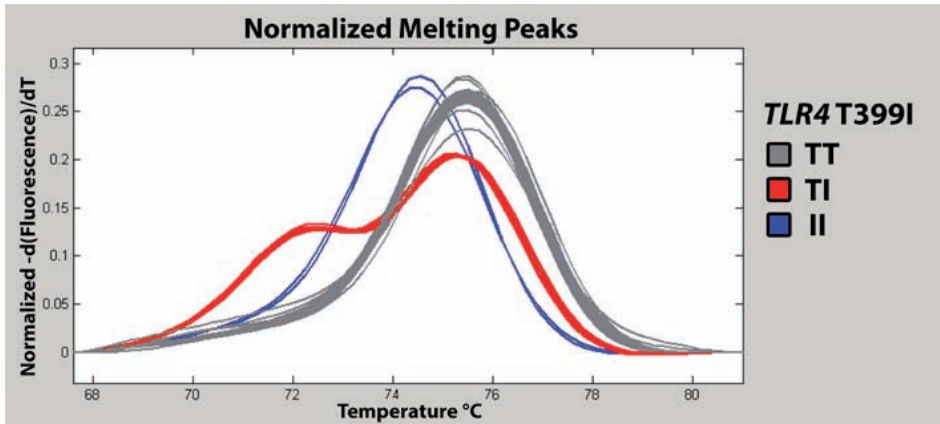
Example of Normalized Melting Peaks obtained with LightScanner following High Resolution Melting Analysis (HRMA) showing different genotypes found in a 96 wells plate following PCR of *TLR4* T399I.
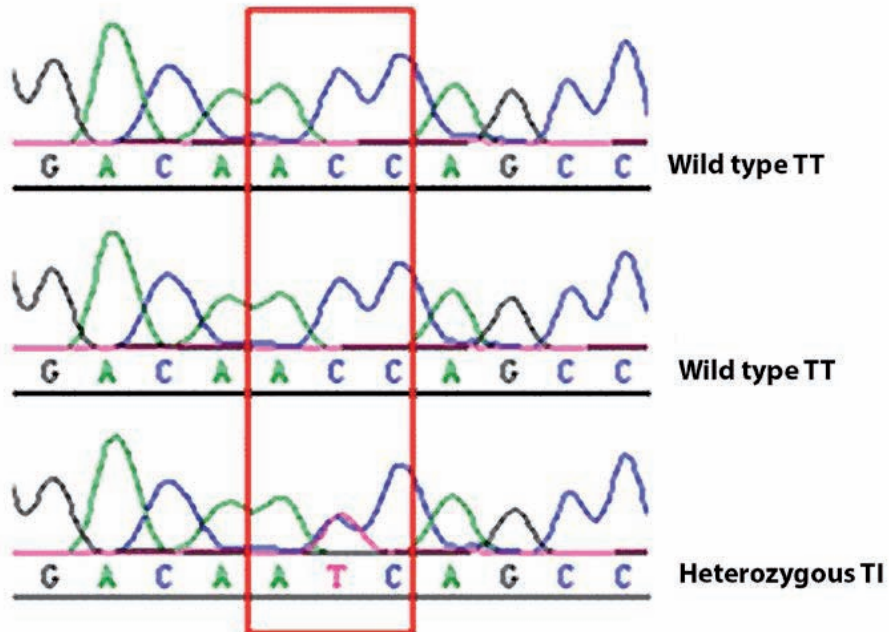
Example of sequencing of *TLR4* exon 3 which shows a heterozygous TI genotype at T399I.

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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GACCTTAAAGTACTTAGTAAGGGA

Chapter 8

Identifying trauma patients at risk for developing complications by analysis of genomic sequence variations; introducing a genetic risk score

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ABSTRACT

Introduction Trauma is the leading cause of death for people under 45 years of age. Infection and Multiple Organ Dysfunction Syndrome remain important causes of morbidity for those who survive the initial trauma. Identification of trauma patients at risk for developing infectious complications may improve outcome and cut costs. This study aimed to investigate the effects of genetic sequence variations in innate immune response genes on outcome in severely injured patients.

Methods From January 2008 to June 2015 severely injured trauma patients were included at a Level 1 University Trauma Center. DNA was extracted and Single Nucleotide Polymorphisms (SNP) in eight innate immune response genes (*MBL2*, *MASP2, FCN2, TLR2, TLR4, TLR9, CD14* and *NOD2*) were amplified by Polymerase Chain Reaction (PCR) and analyzed by High Resolution Melting Analysis (HRMA). Patients were prospectively monitored for positive culture findings, Systemic Inflammatory Response Syndrome (SIRS), sepsis, septic shock, and mortality.

Results A total of 410 severely injured trauma patients were included with a median ISS of 25. The risk of urine infection was influenced by SNPs in *CD14*, wound cultures by SNPs in *MBL2* and *FCN2*, SIRS by SNPs in *MASP2*, and septic shock and mortality by SNPs in *MASP2* and *TLR9.* A genetic risk model confirmed that a combination of specific SNPs could possibly be used as a prediction model for infectious complications in the future.

Conclusions Single Nucleotide Polymorphisms in specific innate immune response genes increase the risk of infectious outcome in severely injured trauma patients.

INTRODUCTION

Infectious complications threaten the recovery of severely injured trauma patients. The initial trauma, surgery (the 'second hit'), and admittance to a surgical ICU ward puts patients at increased risk of developing infectious complications resulting in complicated recovery, prolonged hospital stay, increased mortality risk, and increased costs to society (1, 2).

A number of risk factors have already been defined that put trauma patients at increased risk for developing complications. These include injury severity score (ISS) and Revised Trauma Score (RTS) (1, 3), Glasgow Coma Score (1, 2), age (2), gender (4), mechanical ventilation (5), multiple surgical procedures (5), multiple blood transfusions (5), comorbidity (1), and spinal cord injury (5).

The immune response between groups of trauma patients with and without late infectious complications differs already shortly after trauma (6). Sequence variations in specific innate immune response genes are considered to be variables in the development of these different immune responses which may increase the risk for infectious complications, Systemic Inflammatory Response Syndrome (SIRS), sepsis, septic shock, and Multiple Organ Dysfunction Syndrome (MODS) (7-13).

The aim of this study was to determine the influence of sequence variations in innate immune response genes on the risk of developing infectious complications in a prospective cohort of severely injured patients. Sequence variations were used to calculate genetic risk scores. Finally, a prediction model was developed in order to study if these genetic risk scores contributed to the identification of high-risk groups. Early identification of high-risk groups may decrease complications and lower hospital resource utilization.

MATERIALS AND METHODS

Patients and Data Collection

Materials and methods shall be discussed briefly since they were published before (12, 13). A prospective cohort of consecutive polytraumatised patients admitted to a Level I Trauma Center (Rotterdam, The Netherlands) between January 2008 and June 2015 was studied. Eligibility criteria were an Injury Severity Score (ISS) (3) of 16 or higher and aged 18 to 80 years old. 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 study was registered in the Dutch Trial Registry (NTR1625) before patient recruitment.

Cultures were taken on a standardised routine basis or at the discretion of the physician based upon clinical signs. Endpoints used were: positive cultures during hospital

stay, SIRS within the first 24 hours of admission, development of sepsis or septic shock, or mortality during hospital admission. SIRS, sepsis, and septic shock were defined using international criteria (14).

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.

Genotyping

SNPs were detected using High Resolution Melting Analysis (HRMA) as described before (15). Polymerase chain reactions (PCRs) were performed as described before (12, 13). 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 analysed with the LightScanner® Software using Call-IT 1.5.

All variant genotypes found were confirmed by direct sequencing. PCRs were performed as described before (12, 13). Amplicon purification and sequencing was performed by BaseClear (Leiden, The Netherlands). Results were analysed 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 analysed using the Statistical Package for the Social Sciences, version 16.0 (SPSS, Chicago, IL, USA).

HaploReg v4.1 (http://www.broadinstitute.org/mammals/haploreg/haploreg.php; accessed February 08, 2016) was used for exploring linkage disequilibrium (LD) between SNPs within the same gene in four continental populations (*i.e.* African, American, Asian, and European). A correlation coefficient (r^2) or D' value of 0.8 or larger was considered indicative of significant LD. In that case only one of the SNPs was used in the genetic risk score calculation.

Categorical variables were analysed 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.

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

Gene	SNP	dbSNP ID	Nucleotide transition (Accession code)	Amino-Acid transition (Accesion code)
MBL2	D allele	rs5030737	5219C>T	Arg52Cys
	B allele	rs1800450	5226G>A	Gly54Asp
	C allele	rs1800451	5235G>A	Gly57Glu
	Y-221X	rs7096206	4776C>G (NG_008196.1)	N.A. (NP_000233.1)
MASP2	D120G	rs72550870	5620A>G (NG_007289.1)	Asp120Gly (NP_006601.2)
	Y371D	rs12711521	21370T>G (NG_007289.1)	Tyr371Asp (NP_006601.2)
FCN2	T236M	rs17549193	11369C>T	Thr236Met
	A258S	rs7851696	11434G>T (NG_011649.1)	Ala258Ser (NP_004099.2)
TLR2	T-16934A	rs4696480	6686T>A	N.A.
	R753Q	rs5743708	25877G>A (NG_016229.1)	Arg753Gln (NP_003255.2)
TLR4	D299G	rs4986790	13843A>G	Asp299Gly
	T399I	rs4986791	14143C>T (NG_011475.1)	Thr399Ile (NP_612564.1)
TLR9	T-1486C	rs187084	52261031A>G	N.A.
	T-1237C	rs5743836	52260782A>G (NC_000003.11)	N.A.
CD14	C-159T	rs2569190	5371T>C (NG_023178.1)	N.A.
NOD2	R702W	rs2066844	19877C>T (NG_008508)	Arg702Trp (NP_071445.1)

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.

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. In the initial model, age, gender, injury severity score, and individual SNPs were added as covariates. Next, similar models were developed in which an overall or data-driven genetic risk score was calculated and added as covariate instead of the individual SNPs. A p-value of <0.05 was considered to be statistically significant. The added effect of the genetic risk score on discriminating between patients with versus without infectious complications was further visualised by generating Receiver Operator Curves (ROC) curves and by computing the Area Under the ROC (AUC).

Table 2. Oligonucleotide sequence for primers and probes used for genotyping

Name (Accession code)	SNP	Assay	Primer	Start Position	Sequence	Amplicon size (base pairs)
Calibrators	-	HRMA	Forward	-	5'-TTAAATTATAAAAATATTTATAATATTAATTATATATATATATAAATATAATA-Amine-C6-3'	50
			Reverse	-	5'-TATTATATTTATATATATATAATTAATAATTATATAAATATTTATAATTTAA-Amine-C6-3'	
MBL2	B, C, D	HRMA	Forward	5163	5'-CTGCAGTGATTGCCTGTAGC-3'	103
(NG_008196.1)			Reverse	5246	5'-GCCCAACACGTACCTGGTTC-3'	
			Probe	5207	5'-GGCAAAGATGGGCGTGATGGCACCAAGGGA-amine-C6-3'	
		Sequencing	Forward	5033	5'-TTCCCTGAGTTTTCTCACA-3'	416
			Reverse	5430	5'-GGCTGGCAAGACAACTATT-3'	
	YX	HRMA	Forward	4755	5'-CCCATTTGTTCTCACTGCCA-3'	42
			Reverse	4777	5'-AAGACTATAAACATGCTTTC-3'	
		Sequencing	Forward	4392	5'-CAGGGCCAACGTAGTAAG-3'	512
			Reverse	4886	5'-TTGTGACACTGCGTGACT-3'	
MASP2	D120G	HRMA	Forward	5600	5'-GGACATTACCTTCCGCTCCG-3'	41
(NG_007289.1)			Reverse	5621	5'-ACGGCTTCTCGTTGGAGTAG-3'	
		Sequencing	Forward	5313	5'-ACCTCGCGAGTACGACT-3'	436
			Reverse	5733	5'-TGCCTGGCCTAAGACA-3'	
	Y371D	HRMA	Forward	21350	5'-TTGACTGTGGCCCTCCTGAT-3'	41
			Reverse	21371	5'-CACTCGGCCACTGGGTAGAT-3'	
		Sequencing	Forward	21171	5'-CTCGGCTTTTAACCTTTC-3'	545
			Reverse	21695	5'-ACCATTTGGAATACATTGTGT-3'	
FCN2	T236M	HRMA	Forward	11349	5'-CTGCACAGGAGATTCCCTGA-3'	41

Table 2. Oligonucleotide sequence for primers and probes used for genotyping (continued)

Name (Accession code)	SNP	Assay	Primer	Start Position	Sequence	Amplicon size (base pairs)
(NG_011649.1)			Reverse	11370	5′-AGGACTGGTTGTTGTTGGAAC-3′	
	A258S	HRMA	Forward	11414	5′-ATCTTAACACCGGAAATTGT-3′	41
			Reverse	11435	5′-AGCTCCCTGAAACATCACAG-3′	
	T236M, A258S	Sequencing	Forward	11186	5′-CAGCAGGGCAGTATTCAC-3′	373
			Reverse	11541	5′-TATCCTTTCCCGACTTC-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′	

Table 2. Oligonucleotide sequence for primers and probes used for genotyping (continued)

Name (Accession code)	SNP	Assay	Primer	Start Position	Sequence	Amplicon size (base pairs)
	T-1237C	HRMA	Forward	52260762	5'-TATGAGACTTGGGGGAGTTT-3'	41
			Reverse	52260783	5'-TGTGCTGTTCCCTCTGCCTG-3'	
	T-1486C, T-1237C	Sequencing	Forward	52260931	5'-CTGACTGCTGGGTGTACAT-3'	508
			Reverse	52260925	5'-AAAGCCACAGTCCACAGA-3'	
CD14	C-159T	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'	
NOD2	R702W	Sequencing	Forward	19499	5'-AGCCCTGATGACATTTCTC-3'	531
(NG_007508)			Reverse	20013	5'-CTCCTCCTGCATCTCGT-3'	

HRMA, High-Resolution Melting Analysis.

RESULTS

Patient Demographics

From January 2008 to June 2015 410 patients were included. Of all patients, 307 (75%) were male (Table 3). The median age was 47 years (P_{25}-P_{75} 30-60) and the median Injury Severity Score (ISS) was 25 (P_{25}-P_{75} 18-29). The trauma mechanism was typically blunt (388 patients, 95%), with traffic injuries (206 patients, 50%) and falls from height (120 patients, 29%) being the most common mechanisms. Of penetrating injuries (22 patients, 5%) gunshot wounds (9 patients, 2%) and stab wounds (9 patients, 2%) were most common. Seventy percent of patients were admitted to the Intensive Care Unit (ICU) and stayed there for a median of three days (P_{25}-P_{75} 0-11). The median highest SOFA score measured during ICU stay was 8 (P_{25}-P_{75} 5-11) and 220 patients (54%) were mechanically ventilated during a median of 141 hours (P_{25}-P_{75} 26-340). A total of 22 (5%) patients died, mostly as a result of traumatic brain injury (TBI) (20 of 22; 91%).

Genotype frequencies

Of the 16 SNPs studied the percentage of non-wild type (heterozygous and homozygous variants combined) ranged from 5.4% for *MASP2* D120G and 7.3% for *TLR2* R753Q to 74.4% for *TLR2* T-16934A and 75.4% for *CD14* C-159T (Table 3). For two SNPs, *MBL2* YX and *MASP2* D120G, no homozygous variant genotypes were found. Apart from *MBL2* YX (χ^2 = 17.68, 1 d.f., p<0.001) and *MASP2* Y371D (χ^2 = 16.86, 1 d.f., p<0.001), all genotypes were in Hardy-Weinberg equilibrium.

For the SNPs in *TLR4*, the LD r^2 value was 0.88 and 0.98 for the American and European populations, respectively. The corresponding D' values were 0.94 and 1.00, respectively. In these populations, these SNPs were significantly linked. In African and Asian populations, no significant linkage was found (r^2<0.8).

Effect of individual genotypes on infectious complications

Both in univariate analysis (Table 4) and in multivariable logistic regression analysis (Table 5), significantly increased influence of genotype was found for urine culture (*CD14* C159T TT genotype; Odds Ratio (OR) 2.47; 95% CI 1.13-5.40; p=0.024), wound culture (*MBL2* exon 1 AO genotype; OR 2.15; 95% CI 1.15-4.01; p=0.016 and *FCN2* A258S AS genotype; OR 3.05; 95% CI 1.60-5.81; p=0.001), SIRS (*MASP2* Y371D YD genotype; OR 1.81; 95% CI 1.08-3.02; p=0.023), septic shock (*MASP2* Y371D DD genotype; OR 2.76; 95% CI 1.07-7.12; p=0.036 and *TLR9* T-1237C TC genotype; OR 2.91; 95% CI 1.54-5.52; p=0.001) and mortality (*MASP2* Y371D YD genotype; OR 3.98; 95% CI 1.44-10.97; p=0.008 and *TLR9* T-1237C TC genotype; OR 2.83; 95% CI 1.12-7.16; p=0.028).

Table 3. Patient, injury, and outcome characteristics

	No. of patients* (*n* = 410)
PATIENT CHARACTERISTICS	
Age (years)†	47 (30-60)
Male gender	307 (74.9%)
INJURY CHARACTERISTICS	
Trauma mechanism Traffic accident	206 (50.2%)
Fall from height	120 (29.3%)
Crush injury to thorax or abdomen	20 (4.9%)
Fall from standing height	15 (3.7%)
Gunshot wound	9 (2.2%)
Stabbing	9 (2.2%)
Object against head	8 (2.0%)
Assault	7 (1.7%)
Horseriding accident	5 (1.2%)
Boat accident	5 (1.2%)
Miscellaneous‡	6 (1.5%)
Blunt trauma	388 (94.6%)
AIS≥1 Head/neck	274 (66.8%)
Face	109 (26.6%)
Thorax	239 (58.3%)
Abdomen	132 (32.2%)
Extremity	212 (51.7%)
External	20 (4.9%)
Injury Severity Score†	25 (18-29)
OUTCOME CHARACTERISTICS	
ICU admission	289 (70.5%)
Length of ICU stay (days)†	3 (0-11)
Mechanical ventilation	220 (53.7%)
Duration of mechanical ventilation (hours) †	141 (26-340)
Lactate (mmol/L) <24 hours†	1.3 (0.9-2.3)
Highest level measured during admission†	2.6 (1.7-3.6)
SOFA score First score†	6 (4-8)
Highest score†	8 (5-11)
Positive culture findings Sputum (N=155 patients cultured)	114 (27.8%)
Blood (N=89 patients cultured)	65 (15.9%)
Urine (N=91 patients cultured)	59 (14.4%)
Wound (N=65 patients cultured)	52 (12.7%)

Table 3. Patient, injury, and outcome characteristics (continued)

	No. of patients* (n = 410)
Gram+ (overall)	138 (33.7%)
Gram- (overall)	159 (38.8%)
SIRS <24 hours	283 (69.0%)
Sepsis	140 (34.1%)
Septic shock	51 (12.4%)
Length of hospital stay (days)†	19 (12-33)
Mortality	22 (5.4%)
Time to death (days) †	13 (8-22)
Cause of death: Traumatic brain injury	20 (90.9%)
Arrhythmia	1 (4.5%)
Sepsis	1 (4.5%)

*Data are shown as number (%) unless indicated otherwise; †values are shown as median (P_{25}-P_{75}). ‡Includes explosion, helicopter crash, kite surfing, patient found with neurotrauma due to unknown cause, and train accident. ICU, intensive care unit.

In multivariable analysis, a reduced risk for developing sepsis was found for *TLR2* R753Q RQ genotype (OR 0.37; 95% CI 0.14-0.98; p=0.046). The univariate effect of *CD14* on mortality could not be confirmed in the multivariable analysis.

Effect of genetic risk score on infectious complications

Table 6 and Figures 1 and 2 show the effects of different genetic risk scores on development of infectious complications. In model 1 the effect of already known risk factors age, gender, and ISS was tested in a multivariable logistic regression model and plotted in a Receiver Operating Curve (ROC; Figures 1 and 2). For all infectious and clinical endpoints these risk factors increased the area under the curve (AUC) to well above 0.5 (Table 6). These curves were used as baseline reference values to compare two other models in which two different approaches were used for computing the genetic risk score.

In model 2 the overall genetic risk score is a summation of one point granted to each variant allele found. In this model the influence of all 14 genetic variants studied was included. The overall genetic risk score increased the risk of septic shock both when entered into the model alone (model 2A; OR 1.26; 95% CI 1.07-1.49; p=0.006) as well as in combination with the other known risk factors from model 1 (model 2B; OR 1.25; 95% CI 1.06-1.48; p=0.008). The significant effect of the overall genetic risk score on positive wound cultures (OR 1.18; 95% CI 1.00-1.69; p=0.049) is lost in Model 2B when combined with traditional risk factors (OR 1.18; 95% CI 1.00-1.38). The genetic risk score had no significant effect for the other infectious outcomes.

Table 4. Demographic description of positive cultures and infectious complications by genotype

		N	Gram-Positive	Gram-Negative	Sputum Culture	Blood Culture	Urine Culture	Wound Culture	SIRS *	Sepsis *	Septic Shock *	Mortality
Entire population		410	138 (33.7)	159 (38.8)	114 (27.8)	65 (15.9)	59 (14.4)	52 (12.7)	283 (69.0)	140 (34.1)	51 (12.4)	22 (5.4)
MBL2 exon 1	**AA**	240	75 (31.2)	91 (37.9)	71 (29.6)	34 (14.2)	34 (14.2)	**22 (9.2)**	164 (68.3)	74 (30.8)	24 (10.0)	12 (5.0)
	A0	142[A]	52 (36.6)	55 (40.8)	36 (25.4)	28 (19.7)	25 (17.6)	**25 (17.6)**	102 (71.8)	57 (40.1)	24 (16.9)	8 (5.6)
	00	28[B]	11 (39.3)	10 (35.7)	7 (25.0)	3 (10.7)	0 (0.0)	**5 (17.9)**	17 (60.7)	9 (32.1)	3 (10.7)	2 (7.1)
MBL2 promoter	**YY**	269	95 (35.3)	106 (39.4)	76 (28.3)	42 (15.6)	41 (15.2)	39 (14.5)	182 (67.7)	96 (35.7)	37 (13.8)	14 (5.2)
	YX	141	43 (30.5)	53 (37.6)	38 (27)	23 (16.3)	18 (12.8)	13 (9.2)	101 (71.6)	44 (31.2)	14 (9.9)	8 (5.7)
	XX	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)
MASP2 D120G	**DD**	388	129 (33.2)	147 (37.9)	109 (28.1)	63 (16.2)	55 (14.2)	48 (12.4)	268 (69.1)	133 (34.3)	51 (13.1)	20 (5.2)
	DG	22	9 (40.9)	12 (54.5)	5 (22.7)	2 (9.1)	4 (18.2)	4 (18.2)	15 (68.2)	7 (31.8)	0 (0.0)	2 (9.1)
	GG	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)
MASP2 Y371D	**YY**	264	89 (33.7)	105 (39.8)	67 (25.4)	41 (15.5)	42 (15.9)	32 (12.1)	**170 (64.4)**	86 (32.6)	26 (9.8)	9 (3.4)
	YD	112	38 (33.9)	41 (36.6)	36 (32.1)	20 (17.9)	11 (9.8)	17 (15.2)	**86 (76.8)**	42 (37.5)	18 (16.1)	10 (8.9)
	DD	34	11 (32.4)	13 (38.2)	11 (32.4)	4 (11.7)	6 (17.6)	3 (8.8)	**27 (79.4)**	12 (35.3)	7 (20.6)	3 (8.8)
FCN2 T236M	**TT**	198	62 (31.3)	76 (38.4)	54 (27.3)	25 (12.6)	28 (14.1)	27 (13.6)	133 (67.2)	70 (35.4)	22 (11.1)	8 (4.0)
	TM	179	68 (38.0)	73 (40.8)	50 (27.9)	35 (19.6)	28 (15.6)	24 (13.4)	128 (71.5)	60 (33.5)	24 (13.4)	12 (6.7)
	MM	33	8 (24.2)	10 (30.3)	10 (30.3)	5 (15.2)	3 (9.1)	1 (3.0)	22 (66.7)	10 (30.3)	5 (15.2)	2 (6.1)
FCN2 A258S	**AA**	325	103 (31.7)	122 (37.5)	87 (26.8)	50 (15.4)	45 (13.8)	**32 (9.8)**	225 (69.2)	105 (32.3)	38 (11.7)	17 (5.2)

Table 4. Demographic description of positive cultures and infectious complications by genotype (continued)

		N	Gram-Positive	Gram-Negative	Sputum Culture	Blood Culture	Urine Culture	Wound Culture	SIRS *	Sepsis *	Septic Shock *	Mortality
	AS	78	33 (42.3)	33 (42.3)	27 (34.6)	14 (17.9)	13 (16.7)	19 (24.4)	53 (67.9)	32 (41.0)	13 (16.7)	4 (5.1)
	SS	7	2 (28.6)	4 (57.1)	0 (0.0)	1 (14.3)	1 (14.3)	1 (14.3)	5 (71.4)	3 (42.9)	0 (0.0)	1 (14.3)
TLR2 T-16934A	TT	105	28 (26.7)	42 (40.0)	29 (27.6)	13 (12.4)	16 (15.2)	11 (10.5)	73 (69.5)	35 (33.3)	12 (11.4)	7 (6.7)
	TA	202	73 (36.1)	75 (37.1)	55 (27.2)	35 (17.3)	31 (15.3)	25 (12.4)	138 (68.3)	72 (35.6)	24 (11.9)	11 (5.4)
	AA	103	37 (35.9)	42 (40.8)	30 (29.1)	17 (16.5)	12 (11.7)	16 (15.5)	72 (69.9)	33 (32.0)	15 (14.6)	4 (3.9)
TLR2 R753Q	RR	380	128 (33.7)	151 (39.7)	107 (28.2)	62 (16.3)	57 (15.0)	49 (12.9)	263 (69.2)	134 (35.3)	49 (12.9)	22 (5.8)
	RQ	29	10 (34.5)	8 (27.6)	7 (24.1)	3 (10.3)	2 (6.9)	3 (10.3)	19 (65.5)	6 (20.7)	2 (6.9)	0 (0.0)
	QQ	1	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 (0.0)
TLR4 D299G	DD	362	120 (33.1)	140 (38.7)	101 (27.9)	57 (15.7)	53 (14.6)	46 (12.7)	247 (68.2)	122 (33.7)	45 (12.4)	19 (5.3)
	DG	47	18 (38.3)	19 (40.4)	13 (27.7)	8 (17.0)	6 (12.8)	6 (12.8)	35 (74.5)	18 (38.3)	6 (12.8)	3 (6.4)
	GG	1	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 (0.0)
TLR4 T399I	TT	361	119 (33.0)	139 (38.5)	101 (28.0)	56 (15.5)	52 (14.4)	46 (12.7)	247 (68.4)	123 (34.1)	45 (12.5)	19 (5.3)
	TI	47	18 (38.3)	19 (40.4)	13 (27.7)	9 (19.1)	6 (12.8)	6 (12.8)	35 (74.5)	17 (36.2)	6 (12.8)	3 (6.4)
	II	2	1 (50.0)	1 (50.0)	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	144	56 (38.9)	59 (41.0)	39 (27.1)	25 (17.4)	24 (16.7)	23 (16.0)	95 (66.0)	52 (36.1)	24 (16.7)	7 (4.9)
	TC	202	62 (30.7)	74 (36.6)	56 (27.7)	30 (14.9)	22 (10.9)	21 (10.4)	145 (71.8)	66 (32.7)	18 (8.9)	12 (5.9)
	CC	64	20 (31.2)	26 (40.6)	19 (29.7)	10 (15.6)	13 (20.3)	8 (12.5)	43 (67.2)	22 (34.4)	9 (14.1)	3 (4.7)

Table 4. Demographic description of positive cultures and infectious complications by genotype (continued)

		N	Gram-Positive	Gram-Negative	Sputum Culture	Blood Culture	Urine Culture	Wound Culture	SIRS *	Sepsis *	Septic Shock *	Mortality
TLR9 T-1237C	**TT**	299	103 (34.4)	112 (37.5)	81 (27.1)	46 (15.4)	45 (15.1)	38 (12.7)	201 (67.2)	103 (34.4)	**27 (9.0)**	**12 (4.0)**
	TC	99	32 (32.3)	42 (42.4)	29 (29.3)	19 (19.2)	13 (13.1)	12 (12.1)	73 (73.7)	33 (33.3)	**21 (21.2)**	**10 (10.1)**
	CC	12	3 (25.0)	5 (41.7)	4 (33.3)	0 (0.0)	1 (8.3)	2 (16.7)	9 (75.0)	4 (33.3)	**3 (25.0)**	**0 (0.0)**
CD14 C-159T	**CC**	101	32 (31.7)	36 (35.6)	29 (28.7)	15 (14.9)	**11 (10.9)**	12 (11.9)	68 (67.3)	30 (29.7)	13 (12.9)	**4 (4.0)**
	CT	194	60 (30.9)	70 (36.1)	49 (25.3)	30 (15.5)	**23 (11.9)**	22 (11.3)	141 (72.7)	67 (34.5)	19 (9.8)	**16 (8.2)**
	TT	115	46 (40.0)	53 (46.1)	36 (31.3)	20 (17.4)	**25 (21.7)**	18 (15.7)	74 (64.3)	43 (37.5)	19 (16.5)	**2 (1.7)**
NOD2 R702W	**RR**	374	124 (33.2)	145 (38.8)	107 (28.6)	58 (15.5)	55 (14.7)	45 (12.0)	259 (69.3)	158 (34.2)	48 (12.8)	21 (5.6)
	RW	34	14 (41.2)	14 (41.2)	7 (20.6)	7 (20.6)	4 (11.8)	7 (20.6)	23 (67.6)	11 (32.4)	3 (8.8)	1 (2.9)
	WW	2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)

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

A. 69 patients with AB, 21 with AC, and 52 with AD genotype.

B. 8 patients with BB, 5 with BC, 8 with BD, 5 with CD, and 2 with DD genotype.

All statistically significant results are indicated in boldface.

Table 5. Association of different genotypes with culture findings and clinical syndromes in polytraumatized patients

		N	Sputum Culture	Blood Culture	Urine Culture	Wound Culture	SIRS *	Sepsis *	Septic Shock *	Mortality
MBL2 exon 1	AA	240	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	AO	142	0.81 (0.50-1.31)	1.50 (0.86-2.63)	1.34 (0.75-2.38)	**2.15 (1.15-4.01)**	1.15 (0.72-1.63)	1.50 (0.96-2.35)	1.80 (0.97-3.34)	1.09 (0.42-2.85)
	OO	28	0.80 (0.32-2.00)	0.72 (0.20-2.55)	N.D.	2.08 (0.71-6.10)	0.68 (0.30-1.54)	1.06 (0.45-2.50)	1.01 (0.28-3.66)	1.80 (0.35-9.19)
MBL2 promoter	YY	269	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	YX	141	0.94 (0.59-1.50)	1.07 (0.61-1.88)	0.76 (0.41-1.39)	0.62 (0.32-1.21)	1.26 (0.80-1.98)	0.81 (0.52-1.28)	0.71 (0.37-1.37)	1.05 (0.41-2.67)
	XX	0	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
MASP2 D120G	DD	388	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	DG	22	0.67 (0.24-1.92)	0.44 (0.10-2.00)	1.52 (0.49-4.76)	1.45 (0.47-4.55)	8.88 (0.34-2.25)	0.78 (0.30-2.06)	N.D.	1.61 (0.30-8.55)
	GG	0	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
MASP2 Y371D	YY	264	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	YD	112	1.40 (0.86-2.30)	1.17 (0.64-2.12)	0.59 (0.29-1.21)	1.25 (0.66-2.37)	**1.81 (1.08-3.02)**	1.24 (0.77-2.00)	1.68 (0.87-3.25)	**3.98 (1.44-10.97)**
	DD	34	1.65 (0.75-3.62)	0.84 (0.28-2.55)	0.96 (0.37-2.52)	0.80 (0.23-2.79)	2.41 (1.00-5.80)	1.30 (0.60-2.80)	**2.76 (1.07-7.12)**	2.99 (0.65-13.78)
FCN2 T236M	TT	198	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	TM	179	1.04 (0.66-1.65)	1.69 (0.95-2.98)	1.20 (0.67-2.14)	0.92 (0.51-1.68)	1.22 (0.78-1.91)	0.93 (0.60-1.44)	1.19 (0.64-2.23)	2.10 (0.80-5.54)
	MM	33	1.11 (0.49-2.52)	1.22 (0.43-3.51)	0.58 (0.16-2.07)	0.19 (0.03-1.49)	1.00 (0.45-2.22)	0.76 (0.34-1.80)	1.55 (0.53-4.50)	1.43 (0.27-7.70)
FCN2 A258S	AA	325	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	AS	78	1.55 (0.90-2.66)	1.26 (0.65-2.44)	1.18 (0.59-2.34)	**3.05 (1.60-5.81)**	0.97 (0.57-1.66)	1.57 (0.93-2.65)	1.53 (0.76-3.08)	1.13 (0.35-3.63)
	SS	7	N.D.	0.87 (0.10-7.57)	1.041 (0.12-9.09)	1.54 (0.18-13.49)	1.02 (0.19-5.46)	1.42 (0.29-6.86)	N.D.	2.41 (0.21-27.01)

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Table 5. Association of different genotypes with culture findings and clinical syndromes in polytraumatized patients (continued)

		N	Sputum Culture	Blood Culture	Urine Culture	Wound Culture	SIRS *	Sepsis *	Septic Shock *	Mortality
TLR2 T-16934A	TT	105	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	TA	202	0.92 (0.57-1.67)	1.45 (0.73-2.91)	1.01 (0.52-1.96)	1.18 (0.56-2.53)	0.92 (0.55-1.55)	1.07 (0.64-1.79)	0.98 (0.47-2.08)	0.68 (0.24-1.91)
	AA	103	1.08 (0.58-2.00)	1.40 (0.63-3.07)	0.73 (0.32-1.64)	1.57 (0.69-3.60)	1.03 (0.57-1.88)	0.94 (0.52-1.72)	1.32 (0.58-3.00)	0.53 (0.14-1.97)
TLR2 R753Q	RR	380	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	RQ	29	0.70 (0.28-1.72)	0.51 (0.15-1.77)	0.44 (0.10-1.93)	0.74 (0.22-2.58)	0.76 (0.33-1.70)	**0.37 (0.14-0.98)**	0.45 (0.10-1.99)	N.D.
	QQ	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
TLR4 D299G	DD	362	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	DG	47	0.85 (0.42-1.72)	0.96 (0.41-2.21)	0.95 (0.38-2.37)	0.91 (0.36-2.30)	1.26 (0.62-2.55)	1.10 (0.57-2.11)	0.91 (0.37-2.40)	1.21 (0.32-4.64)
	GG	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
TLR4 T399I	TT	361	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	TI	47	0.85 (0.42-0.71)	1.13 (0.51-2.53)	0.947 (0.39-2.44)	0.90 (0.36-2.29)	1.24 (0.62-2.52)	0.97 (0.50-1.89)	0.94 (0.37-2.39)	1.22 (0.32-4.67)
	II	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
TLR9 T-1486C	TT	144	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	TC	202	1.01 (0.62-1.66)	0.85 (0.47-1.53)	0.57 (0.30-1.08)	0.64 (0.33-1.21)	1.40 (0.87-2.24)	0.88 (0.55-1.40)	0.53 (0.27-1.02)	1.15 (0.42-3.15)
	CC	64	1.16 (0.60-2.25)	0.90 (0.40-2.04)	1.17 (0.54-2.53)	0.80 (0.33-1.91)	1.10 (0.58-2.09)	0.93 (0.49-1.76)	0.86 (0.37-1.99)	0.69 (0.16-3.00)
TLR9 T-1237C	TT	299	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	TC	99	1.18 (0.71-1.98)	1.38 (0.76-2.53)	0.81 (0.41-1.59)	0.99 (0.49-2.00)	1.42 (0.85-2.38)	0.98 (0.60-1.62)	**2.91 (1.54-5.52)**	**2.83 (1.12-7.16)**

Table 5. Association of different genotypes with culture findings and clinical syndromes in polytraumatized patients (continued)

	N	Sputum Culture	Blood Culture	Urine Culture	Wound Culture	SIRS *	Sepsis *	Septic Shock *	Mortality
CC	12	1.58 (0.45-5.52)	N.D.	0.49 (0.06-3.86)	1.45 (0.30-7.03)	1.51 (0.39-5.79)	1.00 (0.29-3.52)	3.43 (0.84-13.94)	N.D.
CD14 C-159T									
CC	101	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
CT	194	0.79 (0.46-1.37)	1.00 (0.51-1.99)	1.08 (0.50-2.33)	0.96 (0.45-2.05)	1.27 (0.75-2.16)	1.17 (0.69-2.00)	0.72 (0.34-1.54)	1.85 (0.57-5.97)
TT	115	1.10 (0.61-2.00)	1.15 (0.55-2.41)	**2.47 (1.13-5.40)**	1.32 (0.60-2.93)	0.82 (0.46-1.46)	1.34 (0.74-2.41)	1.25 (0.58-2.70)	0.31 (0.05-1.85)
NOD2 R702W									
RR	374	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
RW	34	0.64 (0.27-1.53)	1.42 (0.58-3.46)	0.72 (0.24-2.15)	1.99 (0.80-4.90)	0.94 (0.44-2.01)	0.89 (0.41-1.94)	0.63 (0.18-2.18)	0.51 (0.06-4.11)
WW	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Multivariable logistic regression models were made for each individual outcome measure (*i.e.*, positive culture or infectious complication) as dependent variable. Age, gender, 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 was used as reference categories for gender.

[§]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; Ref, reference category.

Statistically significant data are indicated in boldface.

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

In model 3 only the genetic variants that had an effect in this study were taken into account. This data-driven genetic risk score sums the variant alleles only for the SNPs with p<0.1 in the multivariable analysis as can be seen in Table 5. Receiver Operating Curves for models 1 and 3 are shown in Figure 2. The data-driven genetic risk score alone statistically significantly increased the risk of SIRS (OR 1.35; 95% CI 1.08-1.69; p=0.007), sepsis (OR 1.33; 95% CI 1.06-1.66; p=0.014), and septic shock (OR 1.37; 95% CI 1.09-1.71; p=0.007). This influence is further increased slightly when combined with traditional risk factors SIRS (OR 1.42; 95% CI 1.23-1.78; p=0.003), sepsis (OR 1.34; 95% CI 1.06-1.69; p=0.014), and septic shock (OR 1.37; 95% CI 1.09-1.72; p=0.007) (model 3B).

DISCUSSION

Identifying trauma patients at risk for developing complications may improve outcome and lower the use for hospital resources. Outcome is determined by the magnitude of the trauma, the host response, and the medical care that is delivered to the patient. The host response to trauma depends on many factors but also seems to depend on the genetic integrity of the patient. Of the approximately 25,000 genes in the human genome only a few have been studied in human trauma patients and studies are often small and unique resulting in limitations to reach conclusions (16). This study aimed to evaluate the effects of SNPs at 14 loci in eight genes on seven different chromosomes. These innate immune response genes were chosen for their previously reported effects on infection in other diseases such as sepsis and liver transplantation (11, 13, 17-20).

Data showed that sequence variations in these innate immune response genes in the human genome conferred a risk for adverse outcome after severe trauma. Sequence variations in *MBL2* exon 1, *FCN2* A258S, and *CD14* C-159T increased the risk of developing infectious wound complications and urinary tract infection. *MBL2* deficiency is known to increase the risk for infections in a number of clinical situations but was never studied in a trauma population (21-25). The homozygous variant genotype *CD14* C-159T TT was associated with increased risk of urinary culture findings in this cohort. *MASP2* A258S has not been intensively studied clinically but in our unique cohort of trauma patients variation in this gene increased the risk for wound infections, which is a novel finding. Although a role for *CD14* was demonstrated in a murine model (26), a clinical relation in humans has not yet been established (27).

SIRS, sepsis, septic shock, and even mortality were strongly influenced by individual sequence variations in both *MASP2* Y371D and *TLR9* T-1237C in the present study. The MASP2 Y371D DD genotype increased the risk for SIRS, septic shock, and even mortality in a multivariable analysis. Carriage of the minor TLR9 T-1237C C allele conferred a risk for septic shock and mortality in this cohort. In a small series of orthopedic trauma patients

Table 6: Effect of genetic risk score of different genotypes with culture findings and clinical syndromes in polytraumatized patients

		Sputum Culture	Blood Culture	Urine Culture	Wound Culture	SIRS *	Sepsis *	Septic Shock *	Mortality
Model 1	AUC (95% CI)	**0.62 (0.56-0.68)**	**0.60 (0.52-0.68)**	**0.62 (0.54-0.69)**	**0.60 (0.52-0.68)**	**0.60 (0.54-0.66)**	**0.65 (0.59-0.71)**	**0.62 (0.53-0.71)**	**0.79 (0.70-0.88)**
Model 2A	OR (95% CI)	1.04 (0.92-1.17)	1.11 (0.96-1.29)	1.02 (0.87-1.19)	**1.18 (1.00-1.69)**	1.10 (0.98-1.23)	1.09 (0.97-1.22)	**1.26 (1.07-1.49)**	1.22 (0.96-1.54)
	AUC (95% CI)	0.52 (0.46-0.59)	0.56 (0.48-0.64)	0.50 (0.42-0.59)	**0.59 (0.50-0.67)**	0.55 (0.49-0.61)	0.55 (0.49-0.61)	**0.61 (0.53-0.70)**	**0.61 (0.70-0.88)**
Model 2B	OR (95% CI)	1.05 (0.93-.1.18)	1.11 (0.96-1.29)	1.01 (0.86-1.19)	1.18 (1.00-1.38)	1.10 (0.98-1.24)	1.09 (0.97-1.23)	**1.25 (1.06-1.48)**	1.22 (0.94-1.61)
	AUC (95% CI)	**0.62 (0.56-0.68)**	**0.61 (0.54-0.69)**	**0.62 (0.54-0.70)**	**0.63 (0.55-0.71)**	**0.61 (0.55-0.67)**	**0.65 (0.60-0.71)**	**0.65 (0.57-0.73)**	**0.81 (0.72-0.90)**
Model 3A	OR (95% CI)	1.19 (0.91-1.54)	1.31 (0.87-1.97)	1.16 (0.91-1.47)	1.18 (0.94-1.48)	**1.35 (1.08-1.69)**	**1.33 (1.06-1.66)**	**1.37 (1.09-1.71)**	1.34 (0.96-0.87)
	AUC (95% CI)	0.53 (0.48-0.61)	0.55 (0.48-0.63)	0.55 (0.47-0.63)	0.57 (0.48-0.65)	**0.58 (0.52-0.64)**	0.56 (0.51-0.62)	0.60 (0.51-0.69)	0.60 (0.47-0.72)
Model 3B	OR (95% CI)	1.25 (0.95-1.63)	1.31 (0.87-1.98)	1.14 (0.89-1.46)	1.18 (0.94-1.48)	**1.42 (1.23-1.78)**	**1.34 (1.06-1.69)**	**1.37 (1.09-1.72)**	1.37 (0.95-1.96)
	AUC (95% CI)	**0.63 (0.57-0.69)**	**0.62 (0.54-0.70)**	**0.63 (0.55-0.71)**	**0.63 (0.54-0.71)**	**0.64 (0.58-0.69)**	**0.66 (0.60-0.71)**	**0.65 (0.56-0.73)**	**0.79 (0.69-0.89)**

Multivariable logistic regression models were made for each individual outcome measure (*i.e.*, positive culture or infectious complication) as dependent variable.

Statistically significant data are indicated in boldface.

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

Model 1 contains age, gender, and ISS as covariates.

Model 2A contains the overall genetic risk score (*i.e.*, sum of all risk alleles studied) as covariate.

Model 2B contains age, gender, ISS, and the overall genetic risk score as covariates.

Model 3A contains a data-driven genetic risk score (*i.e.*, sum of all risk alleles for SNPs with a p<0.1 in the multivariable analysis; see Table 4) as covariate.

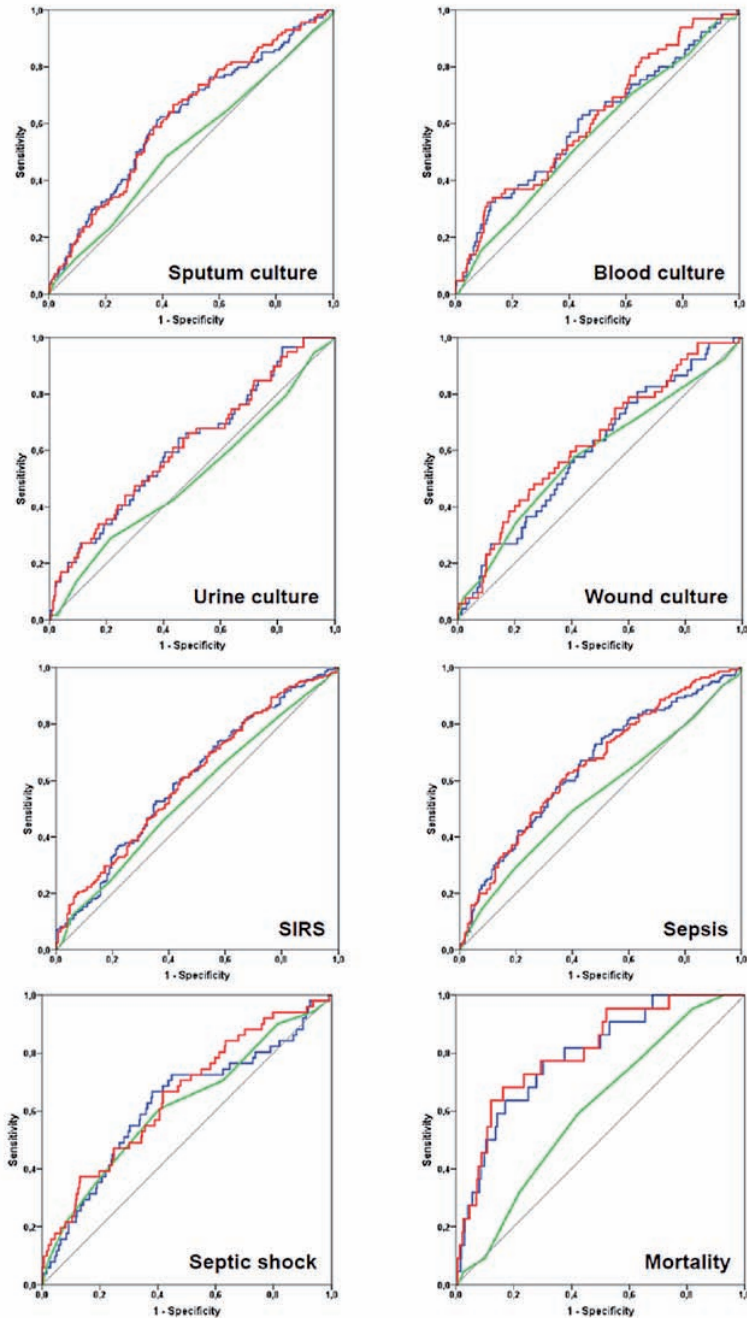Model 3B contains age, gender, ISS, and a data-driven genetic risk score as covariate.

Figure 1. Receiver Operator Characteristic (ROC) plotting the True Positive Rate (Sensitivity) against the False Positive Rate (1-Specificity) of the variables studied using the genetic risk score based on literature data

The blue line shows the standard risk based on know covariates (model 1). The green line refers to the genetic effect only (model 2A). The red line combines the genetic effect and standard risk (model 2B).
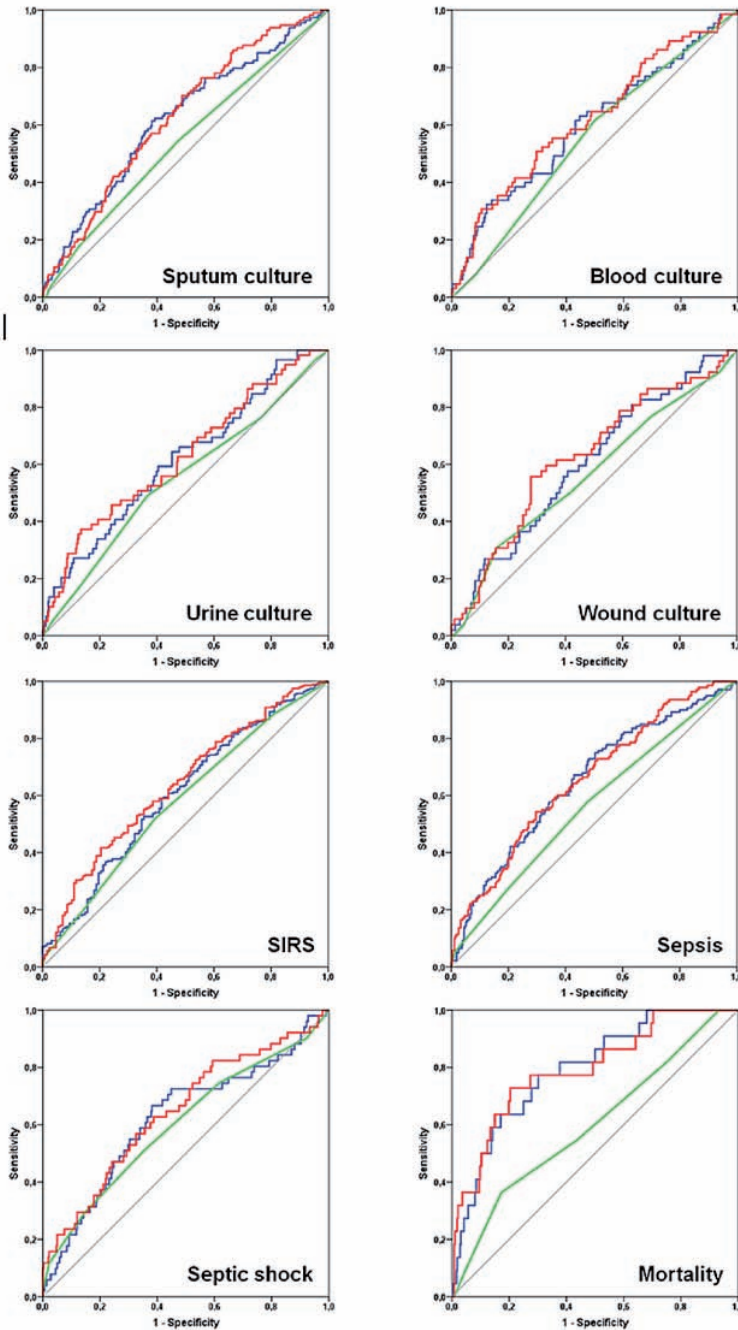
Figure 2. Receiver Operator Characteristic (ROC) plotting the True Positive Rate (Sensitivity) against the False Positive Rate (1-Specificity) of the variables studied using the genetic risk score based on study data The blue line shows the standard risk based on know covariates (model 1). The green line refers to the genetic effect only (model 3A). The red line combines the genetic effect and standard risk (model 3B).

it was found that patients carrying the TLR9 T-1237C C allele produced higher levels of cytokines shortly after trauma (19). Enhanced inflammation in reaction to physical injury was proposed to be related to certain genetic polymorphisms in *TLR9* (28). Thus, it may be that recognition of self-antigens released due to tissue damage might activate certain TLR9 variants, which may cause over-secretion of pro-inflammatory cytokines (19, 28).

By combining all relevant genes in a genetic risk model it was demonstrated that sequence variations in specific genes increase the risk of infectious complications in severely injured trauma patients. When compared with the influence of the three known risk factors age, gender, and ISS alone our risk model demonstrated that the AUC increased for all infectious endpoints indicating a significant effect.

In conclusion, many factors influence outcome of severely injured trauma patients, such as the magnitude of the trauma, the host response, and the medical care delivered. The host response is, in part, determined by the genetic integrity of the host. Sequence variations in selected host immune response genes confer a risk for developing infectious complications. By combining known candidate-gene SNPs in a genetic risk model the magnitude of this influence was demonstrated to equal that of other known risk factors. By using genome-wide association studies (GWAS) in the future the set of genes influencing this risk may even grow larger further improving the genetic risk model. With technical innovations reducing duration of GWAS analysis and cost reduction, clinical use of this risk model may well be feasible in the near future. Future research will have to focus on enlarging the cohorts of probands, using GWAS and exploring the influence of epigenetic factors.

ACKNOWLEDGMENTS

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Chapter 9

General Discussion

IDENTIFYING PATIENTS AT RISK

Identifying trauma patients at risk for developing infectious complications is important. It is something clinicians do every day in every hospital in the world. The reliable tools to do this are limited. As we have seen in the studies presented in this dissertation studies more than half of the patients who are admitted after trauma to an Intensive Care Unit (ICU) will develop some pattern of positive culture or infectious complication. These infections will increase the length of stay on an ICU department and will use resources and produce costs to society. The patient we presented in Chapter two spent a total of 32 days on the ICU and a total of 76 days in the hospital prior to transfer to a rehabilitation center. If infectious complications could have been foreseen or even prevented it would likely have made a considerable difference for the length of hospitalization for this patient. Most clinicians will probably recognize this category of patients who, for some reason, suffer more than average from infectious complications. Traditionally, estimating the risk for developing complications of a severely injured individual patient is done by using scoring systems like the Injury Severity Score, and taking into account age, gender, co-morbidity, medication, smoking habits, et cetera.

With the possibilities of genotyping and reports of associations between certain genotypes and outcomes the question rises if genotyping the trauma patient can be advantageous. Do genes exert any effect on outcome in trauma patients? What is known already in this field of science? And can genes that possibly predict the occurrence of complications be found? Which genes should we look for? And how do we find them? Traditionally, candidate gene analysis has been used in sepsis research to identify genes that influence outcome. Many of these studies are on sepsis patients only, for example patients suffering from pneumosepsis on an ICU. Clearly, associations can be found easiest in homogenous study populations, but it is unknown if these effects can also be found in real clinical situations such as the heterogeneous trauma population.

PREVENTION OF TRAUMA

Prevention of infectious complications may involve prevention of trauma itself and, additionally, improving medical and surgical care. The main causes for global trauma mortality are road traffic accidents, falls, suicide, and homicide [1], which, at least to certain degrees, may be influenced through road safety regulations, technical improvements in (self-driving) cars, programs for improvement through occupational health and safety regulations and other regulations and measures. In medical and surgical care prevention of infection could be optimized by hygiene protocols, use of antibiotics, the invention of new antibiotics, prevention of multi-drug resistant micro-organisms from

contaminating hospitals, and by identification of patients at increased risk for developing infectious complications.

THIRD PEAK OF TRUNKEY

Mortality rates were low in our cohort. Patients died only as the result of complications of neurotrauma. No patient died as a result of sepsis although many patients actually suffered from sepsis or septic shock. This may on the one hand be a result of improvements in modern ICU-treatment which have reached such a quality level that mortality from sepsis can be prevented. On the other hand this can be a reflection that the third peak of mortality of Trunkey no longer exists in modern hospitals, an observation which has been made before [2]. The third peak, however does still exist judging by the amount of patients suffering from infectious complications such as septic shock although mortality can now be prevented by modern resuscitation techniques, artificial ventilation support, and the use of antibiotics. However, the increasing numbers of antibiotic resistant micro-organisms are a serious threat to global public health and we can only hope that this mortality peak will not return in trauma patients in the future.

THE ROLE OF GENETIC VARIATION

The results of our studies indicate that genomic sequence variations influence outcome in severely injured patients to a measurable degree and that this effect can, to some extent, be predicted with a genetic risk score model with at least the same reliability as it can be predicted with traditional risk factors. By using a candidate-gene approach of only a few selected genetic loci in these genetic association studies we were able to demonstrate the proof-of-principle of genes being associated with increased rate of infections in a very heterogeneous cohort of trauma patients. We also define a specific subset of SNPs that may be used in a predictive risk model. This risk model however is composed of a relatively small subset of the around 25,000 human genes and its limited accuracy renders clinical application at this point premature. The genetic infection risk model will first have to be evaluated and probably expanded before it will become a reliable tool for clinicians. One way of expanding the subset of sequence variations would be by conducting a Genome-Wide Association Study (GWAS) which has not yet been done in trauma patients but which has recently produced some new genetic insights in ICU patients with pneumosepsis [3]. Both GWAS and candidate-gene analysis techniques have their advantages and disadvantages and reliability of GWAS itself depends among other parameters on well defined case and control groups and sufficient sample

size. Currently, in the available literature, trauma patient cohorts are small, the largest series describing around 600 or 700 patients [4-6]. Moreover, candidate-gene analysis and GWAS assume that common genetic variation plays a large role in explaining the heritable variation of common disease which actually may only be part of the answer since it can be hard to define single molecular functions or certain genes as primary causes of specific processes [7]. Genes seem not to be causal agents. Focus is shifting towards transcriptome profiling and understanding the influence of epigenetics which may also hold clues to the systemic response to trauma or the development of complications. Epigenetic programming of thousands of genes decides the fate of the organism during acute inflammation including sepsis [8]. The transcriptome and epigenome have not yet revealed their secrets in populations of sepsis patients or trauma patients. With genomics, transcriptomics, and epigenomics combined, the set of variables and spurious findings is potentially incomprehensibly large.

Recent technical advances make GWAS an interesting option for further research which should lead to the use of specific DNA microarray chips (a 'Trauma-SNP-chip') and may accurately identify patients at risk for developing complications at the bedside in the Emergency Department at reasonably low cost. A similar 70-gene signature test, called MammaPrint, is used by breast cancer surgeons to identify those patients who may benefit from chemotherapy [9, 10].

The studies presented in this thesis have limitations. Although the volume of the cohort studied is at the positive end of all studies published its size has limitations. Also, the cohort is heterogeneous in many ways: it consists of multiple ethnicities and it is known that genetic variation is strongly related to ethnicity as, for example, is the case with *MBL2*. Ethnicity was not recorded in this study.

THERAPEUTIC OPTIONS

What are the options once we have successfully identified a patient at risk for developing complications? Currently there is no specific treatment available that targets sepsis pathophysiology. Until then, one option would be not to offer treatments that would further increase the likelihood of complications such as aggressive surgery or the use of central venous catheters. One other option would be to prophylactically start antimicrobial therapy assuming that the predicted causative organisms are not resistant to available antibiotics. Also, substitution of specific proteins rendered unavailable or dysfunctional by the genetic variation can be considered. One specific solution could be the transfusion of plasma donated by multiple donors of which probably some donors contributed without the genetic variation. Also, human plasma-derived proteins can be used to substitute the missing or dysfunctional proteins of the patient such as has been

studied already in the case of improved opsonofagocytosis following substitution of plasma derived MBL in a pediatric population of MBL deficient cancer patients [11, 12].

FUTURE PERSPECTIVES

Future studies will have to focus on genomic sequence variations, transcription profiling and epigenetics. Enlarging or combining existing cohorts of participating trauma patients will be another important step. Introducing GWAS to further identify and increase the number of relevant SNPs will improve the accuracy of the prediction model. Much the same as pharmacogenetics may help in tailoring the choice for the right drug for a patient and much the same as the mammaprint SNP-set may guide difficult decisions to use chemotherapy, the trauma SNP-set may prove to be of help in personalized medicine. Finally, cooperation between all relevant research departments may boost research in this expanding largely untrodden field combining genetics, transcription profiling and epigenetics ultimately hoping for clues to therapeutically influence sepsis pathology following trauma.

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Chapter 10

Summary

Chapter 1 explains the aims and scope of this thesis. Millions of people die every year as a result of injuries. This accounts for 10% of the world's deaths, 32% more than the number of fatalities that result from malaria, tuberculosis, and HIV/AIDS combined. A quarter of these deaths are caused by road traffic accidents and one third are the cause of violence (homicide, suicide and war casualties). A number of injured patients will die at the accident scene or in the first few hours after trauma (the so-called Golden hour). But when victims have survived these first few hours and have made it to an Intensive Care Unit (ICU) recovery can be compromised by conditions such as Systemic Inflammatory Response Syndrome (SIRS), Acute Respiratory Distress Syndrome (ARDS), sepsis, septic shock or mortality. Even when death can be prevented, infectious complications can give rise to increased morbidity and cost of care. The outcome of trauma is determined by the severity of the injury, by the quality of medical or surgical care delivered and by host factors. Known host risk factors are age, gender and co-morbidity. But it is also known that genomic sequence variations can influence the course and outcome of a disease. The aim of this thesis was to study the associations between genomic sequence variations in severely injured patients and infectious outcome parameters during hospital stay.

In **Chapter 2** a trauma patient is presented to illustrate the clinical framework of this thesis. A 57 year old construction worker tripped over a low brick wall and fell on the back of his head from approximately 1 meter height. He was immediately found tetraplegic by the paramedics at the scene of trauma as the result of a cord lesion at the level of C3 and C4. During hospital stay he developed multiple infectious complications caused by a number of different micro-organisms. Acute Respiratory Distress Syndrome (ARDS), later a pneumonia was treated with a number of antibiotics. During his stay he was also diagnosed with tuberculosis. Later, he was treated for pseudomembranous colitis and he developed deep venous thrombosis in the right subclavian vein with erysipelas and ulceration of the fingers from which multiple micro-organisms were cultured. Urine cultures were positive. This patient appeared to have a number of genomic sequence variations in important innate immune response genes: AC variant genotype in *MBL2* exon 1, YX variant genotype in *MBL2* promoter region -221, DD homozygous minor allele variant genotype in *MASP2* Y371D, TM variant genotype in *FCN2* T236M, AS variant genotype in *FCN2* A258S, AA homozygous minor allele variant genotype in *TLR2* T-16934A, and CT variant genotype in *CD14* C159T.

Chapter 3 is a comprehensive review summarizing all genes that have so far been studied for an effect on infectious complications in cohorts of trauma patients. A total of 44 SNPs in more than 35 genes have been studied. SNPs in Pattern Recognition Receptors (PRR), Signal Transducing Adapter Proteins (STAP), and inflammatory cytokines in the

innate immune system are discussed. Different SNPs in *TLR 2, TLR4, TLR9, CD14, IL1A, IL1B, IL4, IL6, IL10* and *TNF* are reported to influence SIRS, sepsis or septic shock. Since the published studies are often unique and have limited sample sizes, no definitive conclusion can be drawn at this stage.

Chapter 4 discusses the protein Mannose-Binding Lectin (MBL) as well as the *MBL2* gene that codes for its production on chromosome 10. MBL is a key protein in the Lectin Pathway, one of the pathway for complement activation together with the classical pathway and the alternative pathway. MBL is synthesized in the liver and it circulates in plasma in the form of trimers. The Carbohydrate Recognition Domain (CRD) of MBL binds to specific ligands present on bacteria, viruses, fungi and protozoa. Following recognition of MBL (as a Pattern Recognition Receptor/ PRR) of such a ligand (as a Danger Associated Molecular Pattern/ DAMP) it associates with Mannose-Binding Lectin Serine Protease (MASP) to activate the complement system. Activation of the complement system ultimately leads to the formation of the Membrane Attack Complex (MAC) that can destroy pathogenic micro-organisms.

A number of sequence variations (single nucleotide polymorphism; SNP) are known in exon 1, the promoter region and in the 5'-untranslated region (UTR) of *MBL2*. Three SNPs in exon 1 are known for their potential to produce unstable proteins with shorter plasma half-lives of MBL. Also the formation of functional oligomers (mainly trimers) is impaired in these variant peptides. The normal, wildtype allele is called A and the three variant alleles in exon 1 are called B, C, and D respectively. Two polymorphic loci are know in the promoter region: -550 (H/L variant) and -221 (Y/X variant). On position +4 in the 5'-UTR the P/Q variant can be found. By combination of all these allele variants 7 haplotypes can be found in humans (HYPA, LYPA, LYQA, LXPA, HYPD, LYPB, and LYQC) which can be associated with high, intermediate or low concentrations of circulating plasma MBL.

Chapter 5 describes the clinical consequences of such a MBL deficiency. Depending on ethnicity sequence variations in *MBL2* occur in 1% to 5% of individuals. For example, the B-allele is not found in Sub-Saharan West Africa, and the C-allele is not found in Asians and American Indians but occurs frequently in Sub-Saharan populations. Heterozygosity occurs in about 30% in most populations. Mutations in *MBL2* can reduce circulating plasma MBL with 90% depending on haplotype. High worldwide prevalence of MBL deficiency or low protein-producing alleles appears to be caused exclusively by human migration and genetic drift, indicating that *MBL2* variation does not have a strong effect on population fitness. However, other data suggest that MBL deficiency may have an evolutionary advantage. The diverse role of MBL in innate immunity, particularly its effects on infectious disease susceptibility, may have led to selection for the heterozygous states of *MBL2*.

Most people experience no consequences of MBL deficiency because of redundancies in the immune system such as the alternative pathway and classical pathway of complement activation. Increased susceptibility to infection is generally seen in patients with MBL deficiency when additional factors that compromise the immune system are present. It is possible that another part of the immune system that overlaps with MBL is functioning at the low end of normal in these individuals, and this lower level of function is not sufficient to make up for the MBL deficiency. Low levels of MBL are also associated with more severe disease and worse outcomes with certain infections, including community-acquired pneumonia, invasive pneumococcal disease, *Escherichia coli*-induced pyelonephritis, *Chlamydia trachomatis* genital tract infections, and hepatitis B virus infections. Low MBL levels are associated with increased susceptibility to meningococcal disease, tuberculosis, and Legionnaires' disease; recurrent tonsillitis, malaria, and vulvovaginitis; and poor immune response to influenza vaccination. In this chapter further associations are discussed between MBL-deficiency and auto-immunity, rheumatoid arthritis, systemic lupus erythematodes, celiac disease, ischemia/reperfusion-syndrome, cardiovascular disease, cerebrovascular disease, cancer and trauma.

Mannose-binding lectin (MBL) deficiency is generally defined as a serum level <500 ng/mL. It is suggested to perform an evaluation of all arms of the complement system (classical, alternative, and lectin pathways) in individuals with recurrent and/or severe respiratory tract infections and in patients immunocompromised for other reasons, such as transplantation patients with recurrent infections who have decreased cellular immunity due to immunosuppressive drug therapy, since these are the patients most likely to benefit from the antibiotic prophylaxis and treatment. Testing includes total hemolytic complement (CH50), alternative pathway hemolytic (AH50), and MBL assays.

Patients with recurrent infections should be treated similarly to other patients with complement deficiencies. This includes prompt treatment with antibiotics with each febrile infection and possible prophylactic antibiotics, as is done with patients who are postsplenectomy or who have symptomatic immunoglobulin A (IgA) deficiency. In addition, these patients should receive pneumococcal and meningococcal vaccines, with documentation of antibody responses.

Animal studies and phase-I/II human studies suggest that replacement therapy with plasma-derived MBL is safe. However, it is still an experimental treatment and not available commercially. Recombinant human MBL protein (rhMBL), derived from a Chinese hamster ovary cell line, is untested and is also not commercially available.

Chapter 6 describes a prospective cohort of 219 severely injured trauma patients treated in a Level 1 Trauma Center. All patients were genotyped for a total of 6 SNPs in *MBL2, FCN2* and *MASP2* using high-resolution melting analysis (HRMA). Association

of genotype with prevalence of positive culture findings (in urine, wounds, blood and sputum) as well as clinical parameters SIRS, sepsis, septic shock and mortality was tested by χ2 and logistic regression analysis. More than 50 per cent developed a positive culture from sputum, wounds, blood or urine. A systemic inflammatory response syndrome (SIRS) developed in 64% of patients, sepsis in 36% and septic shock in 17%. Patients with a *MBL2* exon 1 variant allele were more prone to positive wound cultures. A *MASP2* Y371D DD genotype predisposed to SIRS and septic shock. A *FCN2* A258S AS genotype predisposed to positive wound cultures and septic shock.

Chapter 7 studies this same prospective cohort of patients who were now genotyped for a total of 7 SNPs in *TLR2*, *TLR4*, *TLR9*, and *CD14* using HRMA. Association of genotype with prevalence of positive culture findings (in urine, wounds, blood and sputum) as well as clinical parameters SIRS, sepsis, septic shock and mortality was tested by χ2 and logistic regression analysis.. More than 50% developed a positive culture finding 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 and SIRS. Trends were noted for *TLR9* and *CD14* but did not reach statistical significance. Sepsis and septic shock were unrelated to any of the SNPs studied.

Chapter 8 describes a prospective cohort of 410 severely injured trauma patients in whom a total of 16 SNPs in eight immune reponse genes (*MBL2, MASP2, FCN2, TLR2, TLR4, TLR9, CD14* en *NOD2)* on seven different chromosomes were studied for associations with infectious complications. Mean ISS was 25 and most patients (95%) sustained blunt trauma of which road traffic accidents (50%) and falls from height (29%) were the most common trauma mechanisms. A total of 70% of patients were admitted to an ICU. The median highest SOFA score was 8 points. More than 50% of patients needed mechanical ventilation for a median duration of 141 hours. In univariate and multivariable analysis statistically significant positive associations were found for individual genotypes for urine culture (*CD14* C159T TT genotype), wound culture (*MBL2* exon 1 AO genotype and *FCN2* A258S AS genotype), SIRS (*MASP2* Y371D YD genotype), septic shock (*MASP2* Y371D DD genotype and *TLR9* T-1237C TC genotype) and mortality (*MASP2* Y371D YD genotype and *TLR9* T-1237C TC genotype). Then, different genetic risk score models were tested in a multivariable logistic regression analysis and plotted in a receiver operator curve (ROC). It was shown that the genetic risk score model that was composed of clinically relevant genes is able to predict SIRS, sepsis and septic shock as accurate as the use of traditional risk factors age, gender and ISS.

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Chapter 11

Samenvatting

In **Hoofdstuk 1** is het doel van dit proefschrift uiteengezet. Miljoenen mensen sterven jaarlijks wereldwijd aan de gevolgen van trauma. Een kwart van die sterfgevallen ontstaat in het verkeer en een derde door geweld (moord, zelfmoord en oorlogsslachtoffers). Een deel van de slachtoffers overlijdt ter plaatse van het ongeval of binnen enkele uren (Golden hour). Wanneer slachtoffers de eerste uren hebben overleefd en een Intensive Care Unit (ICU) hebben weten te bereiken kan het beloop gecompliceerd raken door onder meer Systemic Inflammatory Response Syndrome (SIRS), Acute Respiratory Distress Syndrome (ARDS), sepsis, septische shock of overlijden. Ook als het overlijden kan worden afgewend zorgen infectieuze complicaties voor veel morbiditeit en zorgkosten. De uitkomst van trauma wordt bepaald door de ernst van het trauma, factoren in de patiënt en door de geleverde medische zorg. Bekende risicofactoren van de patiënt zijn leeftijd, geslacht en comorbiditeit. Ook is bekend dat variatie in het genoom van de patiënt invloed kan hebben op de uitkomst van vele ziekten. In dit proefschrift is onderzocht of genetische variatie bij ernstig gewonde traumapatiënten de infectieuze uitkomst gedurende de ziekenhuisopname beïnvloedt.

In **Hoofdstuk 2** wordt het klinisch kader van de vraagstelling in dit proefschrift geïllustreerd aan de hand van een casus van een traumapatiënt. Een 57-jarige bouwvakker struikelde over een muurtje en viel ongeveer 1 meter achterover op zijn hoofd. Hij was meteen tetraplegisch ten gevolge van een laesie in het ruggenmerg op niveau C3 en C4. Tijdens de opname ontwikkelde patiënt Acute Respiratory Distress Syndrome (ARDS) en een pneumonie waarvoor hij met meerdere antibiotica werd behandeld. Er werd tuberculose vastgesteld en een pseudomembraneuze colitis werd behandeld. Ook ontwikkelde hij een diep veneuze thrombose in de rechter vena subclavia met erysipelas en ulceratie van de vingers waarin meerdere micro-organismen werden gekweekt. De urinekweek was ook positief.

Bij analyse bleek deze patiënt verschillende mutaties te hebben in belangrijke immuunresponsgenen, genen in het aangeboren immuunsysteem: AC genotype in *MBL2* exon 1, YX genotype in *MBL2* promoter regio -221, homozygoot variant genotype DD in *MASP2* Y371D, heterozygoot genotype TM in *FCN2* T236M, heterozygoot genotype AS in *FCN2* A258S, homozygoot variant genotype AA in *TLR2* T-16934A en heterozygoot genotype CT in *CD14* C-159T.

In **Hoofdstuk 3** is onderzocht welke genen tot nog toe onderzocht zijn op een effect op infectieuze complicaties in populaties van traumapatiënten. Uit een diepgaande literatuurstudie blijkt dat SNPs in meer dan 35 genen onderzocht zijn. De Pattern Recognition Receptors (PRR), Signal Transducing Adapter Proteins (STAP) en Inflammatory Cytokines van het aangeboren immuunsysteem worden besproken. Het blijkt dat de

verschillende studies vaak uniek zijn en niet zelden kleine patiëntenaantallen hebben zodat vooralsnog geen eenduidige conclusies kunnen worden getrokken.

In **Hoofdstuk** 4 wordt het eiwit Mannose-Binding Lectin (MBL) besproken evenals *MBL2*, het gen op chromosoom 10 dat codeert voor de productie van MBL. MBL is onderdeel van het Lectine pad, een van de drie bekende paden om het complementsysteem te activeren. Er zijn genetische variaties (Single Nucleotide Polymorphism; SNP) bekend in exon 1, de promoter regio en de de 5'-UTR (untranslated region) van *MBL2*. In exon 1 van *MBL2* zijn drie SNPs bekend die leiden tot productie van instabiele eiwitten met een kortere plasma halfwaardetijd van MBL. Bovendien lijkt de vorming van functionele oligomeren door deze variante eiwitten verstoord. Het normale allel wordt A genoemd en de drie variante allelen respectievelijk B, C en D. In de promoter regio bestaan eveneens meerdere polymorfe loci: -550 (H/L variant), -221 (Y/X variant) en in de 5'-UTR op positie +4 (P/Q variant). Aldus zijn door combinatie zeven haplotypen bekend bij de mens (HYPA, LYPA, LYQA, LXPA, HYPD, LYPB, en LYQC) die geassocieerd zijn met hoge, intermediaire of lage concentraties van circulerend MBL. MBL wordt gesynthetiseerd in de lever en circuleert in plasma in de vorm van trimeren. Het CRD (Carbohydrate Recognition Domain) van MBL bindt aan specifieke koolhydraten die voorkomen op bacteriën, virussen, schimmels en protozoa. Na herkenning van MBL (als Pattern Recognition Receptor - PRR) van een van dergelijke koolhydraten (als Danger Associated Molecular Pattern - DAMP) ontstaat, na associatie met MASP (Mannose-Binding Associated Serine Protease), activatie van het complement systeem dat uiteindelijk leidt tot productie van het MAC (Membrane Attack Complex) dat verdere destructie van het pathogene micro-organisme tot gevolg heeft.

In **Hoofdstuk 5** worden de klinische gevolgen beschreven van MBL-deficiëntie. Afhankelijk van etniciteit komen *MBL2* mutaties voor bij 1% tot 65% van de individuen. Het B-allel komt bijvoorbeeld niet voor in sub-Sahara West-Afrika. Het C-allel daarentegen komt niet voor in Aziaten en Amerikaanse Indianen maar komt veel voor in sub-Sahara populaties. Heterozygotie komt in ongeveer 30% voor in de meeste populaties. Mutaties kunnen het circulerend MBL in plasma met 90% verlagen afhankelijk van het haplotype. Het algemeen voorkomen van MBL deficiëntie op aarde lijkt voornamelijk door migratie en genetic drift te zijn veroorzaakt hetgeen erop wijst dat *MBL2* variatie niet veel invloed heeft op de fitness van een populatie. De pluriforme rol van MBL in het aangeboren immuunsysteem heeft mogelijk geleid tot de hoge frequentie van heterozygotie en daarom wordt ook wel beweerd dat hierdoor een selectievoordeel heeft kunnen ontstaan.

De meeste mensen ondervinden geen gevolgen van een MBL-deficiëntie vanwege de aanwezigheid van alternatieve routes om het aangeboren immuunsysteem te activeren

zoals de zogenaamde klassieke route en de alternatieve route. Wanneer andere factoren de beschikbaarheid van immuunpotentieel bedreigen kunnen symptomen ontstaan zoals wanneer het verworven immuunsysteem eveneens gecompromitteerd is en er vatbaarheid ontstaat voor bacteriële infecties, voornamelijk door ingekapselde bacteriën.

De ernst van bepaalde infecties lijkt toegenomen in geval van MBL-deficiëntie zoals bij community aquired pneumonie, infectie met pneumococcen, pyelonefritis door *E. coli* en de kans op occlusie van de tuba fallopii door *Chlamydia*. Maar ook lijkt juist de vatbaarheid voor bepaalde infecties verhoogd zoals onder meer het geval is bij meningococcen, tuberculose, legionella en lepra.

In dit hoofdstuk worden verdere associaties besproken tussen MBL-deficiëntie en auto-immuniteit, rheumatoide arthritis, systemische lupus erythematodes, coeliakie, ischemie/reperfusie-syndroom, cardiovasculaire aandoeningen, cerebrovasculaire aandoeningen, kanker en trauma.

De diagnose 'MBL-deficiëntie' wordt gesteld als serumwaarden dalen onder 500 ng/ml. Geadviseerd wordt om alle paden van het complementsysteem te testen (klassiek, alternatief en lectine) bij patiënten met recidiverende luchtweginfecties en bij patiënten bij wie de cellulaire immuniteit onderdrukt wordt met immunosuppressiva, zoals transplantatiepatiënten met recidiverende infecties, omdat deze patiënten meest waarschijnlijk baat hebben bij een therapie. CH50, AH50 en serum MBL kunnen worden bepaald.

De behandeling bestaat uit het snel starten met antibiotische therapie bij eerste tekenen van symptomen zoals bij de behandeling van andere complement-deficiënties. Ook kan substitutie van het MBL eiwit worden overwogen. Dit eiwit wordt gewonnen uit humaan plasma maar recombinant humaan MBL (rhMBL) is ook in een ovariële cellijn van hamsters geproduceerd. Toediening is veilig gebleken en in vroege studies bij neonaten lijkt éénmalige substitutie te leiden tot een fysiologische plasmawaarde gedurende drie dagen.

Hoofdstuk 6 beschrijft een prospectief cohort van 219 ernstig gewonde patiënten die in een Level 1 traumacentrum zijn behandeld. Bij al deze patiënten zijn de genotypes bepaald voor *MBL2*, *FCN2* en *MASP2*. Er werd gezocht naar associaties tussen de respectievelijke genotypen en het ontstaan van infectieuze complicaties. Meer dan 50% van de patiënten ontwikkelde een positieve kweek in sputum, bloed, urine of wond. Zo'n 63% van patiënten ontwikkelde een SIRS, 36% een sepsis en 17% een septische shock. Patiënten met een variant allel in *MBL2* exon 1 hadden meer kans op een positieve wondkweek. Het genotype *MASP2* Y371D DD gaf een verhoogde kans op SIRS en septische shock. *FCN2* A258S AS verhoogde de kans op positieve wondkweken en op septische shock.

In **Hoofdstuk 7** wordt in hetzelfde prospectieve cohort van 219 ernstig gewonde patiënten gekeken naar associaties tussen SNPs in *TLR2*, *TLR4*, *TLR9*, *CD14* en de kans op het ontstaan van infectieuze complicaties. Het Toll-like Receptor (TLR) pathway speelt een belangrijke rol in het aangeboren afweersysteem en herkent meerdere pathogen-associated molecular patterns (PAMPs) op bacteriën, schimmels, virussen en protozoa. CD14 is een centraal pattern recognition receptor (PRR) in het aangeboren afweersysteem en heeft interactie met TLR als co-receptor voor de detectie van verschillende PAMPs. Het bleek dat *TLR2* T-16934A TA genotype de kans op het ontstaan van een gram-positieve infectie vergrootte evenals op SIRS. Voor *TLR4*, *TLR9* en *CD14* werden geen statistisch significante associaties gevonden.

In **Hoofdstuk 8** wordt een prospectief cohort van 410 ernstig gewonde patiënten beschreven bij wie in totaal 16 SNPs in 8 immune respone genes (*MBL2, MASP2, FCN2, TLR2, TLR4, TLR9, CD14* en *NOD2*) op 7 verschillende chromosomen worden onderzocht op associaties met infectieuze complicaties. De mediane ISS was 25 en de meeste (95%) patiënten maakten stomp trauma door waarvan verkeersongevallen (50%) en een val van hoogte (29%) de meest voorkomende traumamechanismen waren. In totaal werd 70% van de patiënten opgenomen op een ICU waar de mediane hoogste SOFA score 8 bedroeg. Meer dan 50% van de patiënten werd beademd voor een mediane duur van 141 uur. In univariate en multivariate analyse werden statistisch significante associaties van individuele genotypes gevonden voor urinekweek (*CD14* C159T TT genotype), wondkweek (*MBL2* exon 1 AO genotype en *FCN2* A258S AS genotype), SIRS (*MASP2* Y371D YD genotype), septische shock (*MASP2* Y371D DD genotype en *TLR9* T-1237C TC genotype) en mortaliteit (*MASP2* Y371D YD genotype en *TLR9* T-1237C TC genotype). Vervolgens werden verschillende modellen van genetische risicoscore onderzocht, in een multivariabele logistische regressie-analyse en geplot in een receiver operator curve (ROC), op een voorspellende waarde voor het ontwikkelen van infectieuze complicaties. Het blijkt dat deze genetische risicoscore een voorspellende waarde heeft voor SIRS, sepsis en septische shock die juist groter is dan de voorspellende waarde van de traditionele risicofactoren leeftijd, geslacht en ISS.

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Chapter 12

Dankwoord

Mijn dank gaat in de eerste plaats uit naar alle **patiënten** die hebben deelgenomen aan dit onderzoek. Zij, of hun familieleden, hebben in de moeilijkste perioden van hun leven een beslissing moeten nemen over deelname aan een wetenschappelijk onderzoek. Ik hoop dat er een klein steentje bij is gedragen aan verbetering van de uitkomst van zorg voor ernstig gewonde patiënten.

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Ik dank alle studenten en master-studenten die mij hebben geholpen tijdens alle fasen van dit onderzoek:

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Chapter 13

Curriculum vitae auctoris

The author of this thesis was born 1971 in Nijmegen. After graduating from secondary school at the Jacobus College in Enschede he studied Medicine at the Rijksuniversiteit Maastricht in 1990 at the newly opened Academisch Ziekenhuis Maastricht (azM). In 1993 he worked at the Hand Unit of the Department of Orthopaedic and Trauma Surgery at the Royal North Shore Hospital, Sydney, Australia (professor Michael A. Tonkin, Dr. Douglas J. Wheen). After returning to Maastricht he joined the Renal Preservation Laboratory (professor dr. G. Kootstra, dr. M.H. Booster) at the Department of Surgery at azM. For a few years he was involved in research, machine perfusion and transportation of donor kidneys in Europe. In 1998 he graduated from Medical School at the University of Maastricht and subsequently started as a resident at the Department of Surgery, Westeinde Ziekenhuis, Den Haag (dr. B.C. de Vries). In 1999 he continued his surgical residency at the Leyenburg Ziekenhuis, Den Haag (dr. C.M.A. Bruijninckx). Formal surgical training started at Leyenburg Ziekenhuis in 2001 and was continued at the Leids Universitair Medisch Centrum, Leiden in 2005 (professor dr. O.T. Terpstra, professor dr. J.F. Hamming). With the completion of a one-year differentiation in Vascular Surgery (professor dr. J.F. Hamming) he qualified as a surgeon as from 2007. That same year he became a fellow (CHIVO) in Trauma Surgery at the Erasmus MC in Rotterdam (professor dr. P. Patka). Here he developed the ideas for this present thesis, together with dr. L.H. Bouwman, that were made possible thanks to a grant by the OTC-foundation. Under the supervision of dr. E.M.M. van Lieshout he spent a few years collecting DNA from polytraumatised patients eventually to build a large database. For the studies conducted in Chapter 7 he was awarded the Schoemaker Prijs 2013 by the Association of Surgeons of The Netherlands. As from 2008 he is a consultant surgeon at the Bronovo Ziekenhuis, Den Haag. In 2009 the author passed the European Board of Surgery Qualifications (EBSQ) in Berlin, Germany (professor dr. H-J. Oestern) to become a Fellow of the European Board of Surgeons. In 2010 he was admitted as a Fellow of the Royal College of Surgeons of England (mister J. Black, MD FRCS FRCP). The surgeons of Bronovo and MC Haaglanden have been working in a partnership since 2012 only to see both hospitals merge into the Haaglanden Medisch Centrum (HMC) as from 2016. He is a member of a number of national and international societies and his scientific contributions currently produce a Hirsch-index of 13. The author is happily married to Janneke and they live in Wassenaar with daughters Eline and Frederieke and sons Hugo and Maurits.

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Chapter 15

PhD Portfolio

PHD PORTFOLIO

SUMMARY OF PHD TRAINING AND TEACHING

Name PhD Candidate: **M.W.G.A. Bronkhorst**	Promotor: **Prof. Dr. P. Patka**
Erasmus MC Department: **Surgery**	Supervisor: **Dr. E.M.M. van Lieshout**

1. PhD training

	Year	Workload (Hours/ECTS)
General courses		
- ATLS Generic Instructor Course	2011	1
- Teach the Teacher	2015	1
Specific courses (e.g. Research school, Medical Training)		
- Basiscursus Stralingsbescherming	2010	2
Presentations		
- EBJIS Heidelberg (D)	2010	1
- DKOU Berlin (D)	2010	1
- AAST Boston (USA)	2010	1
- ECTES Milano (I)	2011	1
(Inter)national conferences		
- Traumadagen	2008-2016	6
- Chirurgendagen	2008-2016	6
- ECTES Budapest (H)	2008	1
- ECTES Brussel (B)	2010	1
- ECTES Frankfurt (D)	2014	1

2. Teaching

	Year	Workload (Hours/ECTS)
Lecturing		
- AO National Faculty Amsterdam (NL)	2013	1
- AO National Faculty Sint Michielsgestel (NL)	2014	1
- AO National Faculty Leiden (NL)	2015	1
Supervising practicals and excursions, Tutoring		
- ATLS Instructor	2011-2016	4
- AO Table Instructor Davos (CH)/ Oisterwijk (NL)	2010-2011	2
Supervising Master's theses		
M.A.Z. Lomax	2010	2
N. Ruiter	2010	2
N.D.A. Boye	2011	2
L.M. Rietveld	2011	2