Polymorphisms in immune response genes in infectious diseases and autoimmune diseases

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Polymorphisms in Immune Response Genes in Infectious Diseases and Autoimmune Diseases

Polymorfismen in immuunrespons-genen in infectieziekten en auto-immuunziekten

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“Tell me, and I will forget.
Show me, and I may remember.
Involve me, and I will understand.”

- Confucius, 450 B.C.

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Chapter 1

General introduction

Background

The clinical presentation of infections caused by the same micro-organism is highly diverse. This is for example demonstrated for infections with *Neisseria meningitidis* and respiratory syncytial virus. Some patients infected with *N. meningitidis* develop meningitis, while others present with sepsis or even septic shock. Moreover, only part of the individuals colonized with *N. meningitidis* develops clinical disease indicating a different susceptibility between individuals to develop clinical disease. The disease phenotype in respiratory syncytial virus infection varies from a common cold to severe bronchiolitis which requires mechanical ventilation.(1) A similar variation is observed in autoimmune diseases: While some patients with rheumatoid arthritis (RA) have relatively mild disease, in others joint erosion develops rather early after diagnosis. Although part of these variations in susceptibility and severity of infection is explained by, for example, age or crowding, the reasons for this variation are not completely clear. Genetic heterogeneity is thought to play a role.
Genetic polymorphisms are known to contribute to the susceptibility and severity of infectious and autoimmune diseases and other complex diseases.(2) Currently in complex diseases, only 2-3 and in some maybe 5% of the heritability, which is the proportion of differences between individuals explained by genetic differences, is elucidated by genes actually found to be associated with this disease. A few examples of complex phenotypes related to genetic polymorphisms are listed in Table 1.

Epidemiology of genetic variation

The human genome consists of about 30,000-50,000 genes and the complete sequence is now available at www.ncbi.nlm.nih.gov. Multiple types of genetic variations have been described thus far (Table 2).(3) The research described in this thesis is limited to single nucleotide polymorphisms (SNPs). SNPs are present throughout the genome and occur about once every 100 to 300 bases, although 'hotspots' like the HLA encoding regions are present. Nearly 12 million unique reference SNPs are known today, of which approximately 6 million have been validated, and this number is still expanding on a daily basis. Little over 5 million of the SNPs are located in genes, of which most (3.8 million) are located in intronic regions, while 80,000 SNPs result in an amino acid substitution. (Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Centre for Biotechnology Information, National Library of Medicine. (dbSNP Build ID: 128, available at www.ncbi.nlm.nih.gov/projects/SNP, April 1, 2008).(4) An overview of SNP location and their potential effects is listed in table 3.
The nature of the SNP, it being a single nucleotide substitution, and the available techniques enable researchers to perform high throughput analysis in terms of both SNPs and samples.
Each SNP, if relevant, generally is thought to have only a minor contribution to

Table 1. Selected (examples of) SNPs with critical influence on major biological traits and complex diseases.

Species	Gene	SNP location	SNP	Consequence	Phenotype	Reference
Man *(Homo sapiens)*	*TCF7L2*[1]	Intron	rs7903146 C/T	-	Diabetes	(14)
		Intron	rs12255372 G/T	-		
	IRF5	Intron	rs2004640 G/T	Altered splicing	Systemic lupus erythematosus	(15)
		Exon	30 bp insertion/deletion	Domain change		
		PolyA signal sequence	rs10954213 A/G	RNA stability 3' UTR		
	CARD15 (NOD2)	Exon	3020insC = L980P	Frameshift, truncation	Crohn disease	(16, 17)
	CFH	Exon	Y402H	Amino acid substitutions	Age related macular degeneration	(18-21)
			I62V			
Dog (*Canis familiaris*)	*IGF1*	Haplotype of 20 SNPs	Haplotype B/I	Expression level	Body size	(5)
Great tit (*Parus major*)	*Drd4*	Coding region	830 C/T	Synonymous	Novelty seeking behaviour	(22)

[1]Abbreviations: *TCF7L2*, transcription factor 7-like 2: *IRF5*, interferon regulatory factor 5; *CARD15*, caspase recruitment domain-containing protein 15; NOD2, nucleotide-binding oligomerization domain protein 2; *CFH*, complement factor H; *IGF1*, insulin-like growth factor 1; *Drd4*, dopamine receptor D4 gene.

Table 2. Different types of genetic variation and their definition.[1]

Type of genetic variation	Definition	Reference
SNP	Single base pair variation present in > 1% of chromosomes in a certain population	(23-25)
Haplotype	Combination of alleles on a single chromatid that are statistically associated	(26)
Microsatellite	Sequences with variable numbers of 1-6 bp repeats, (total < 200 bp)	(25, 27, 28)
Minisatellite and variable number of tandem repeats (VNTRs)	Polymorphic sequence containing 20-50 copies of 6-100 bp repeats	(29-31)
Insertion/deletion	Deletion or insertion of a segment of DNA of variable size	(32, 33)
Multisite variant (MSV)	Single nucleotide variant with complex characteristics due to CNV or gene conversion	(34)
Intermediate-sized structural variant (ISV)	Gain or loss of a DNA sequence > 8 kb in size, also includes inversion breakpoints	(35)
Copy number variation (CNV)	DNA segments ≥ 1 kb, present in a variable copy number when compared to a reference genome	(36-38)
Copy number polymorphism (CNP)	As CNV with a frequency > 1%	
Large scale CNV (LCV)	CNV ~50 kb in size or greater	
Inversion	Segment of DNA present in the reverse orientation	(39)
Translocation	DNA fragment that is attached to a different chromosome	(39, 40)
Unbalanced rearrangements	Rearrangements which lead to a net gain or loss of DNA	

[1]Table modified from Feuk et al. with author permission.(3)

the phenotype. This is especially true in multifactorial traits and diseases such as inflammatory diseases, on which this thesis focuses. In addition to multiple genetic polymorphisms, other host- environmental- and microbial factors, are expected to influence the course of disease. However, there are prominent examples of apparently complex and important traits that are critically determined by genetic variations with modest effects (see Table 1 for examples). The textbook example is the T>A substitution located in codon 6 of the β-globin gene resulting in a valine instead of a glutamine in this molecule, known as Hemoglobin S, which is famous for resistance to malaria infection in heterozygous individuals. Recently a major role was found for genetic variation in a single gene encoding insulin-like growth factor (IGF) in determining the body size of dogs.(5) Although it could be argued that body size is not a complex trait, it certainly is a very important one in biological terms. This thesis covers complex diseases of which the phenotype is codetermined by multiple factors.

Table 3. SNP location and its possible effect.

SNP location	Possible effect
Promoter region	Increased or decreased expression
Exon	Silent, no amino acid substitution Aberrant protein synthesis Amino acid substitution: No effect on protein function Altered protein function
Intron	RNA splicing, RNA stability, mRNA expression level
3' UTR	RNA stability
Outside gene region	Possible effect on mRNA expression level

At the initiation of these studies we intended to use the Affymetrix micro-array technology to genotype multiple single nucleotide polymorphisms (SNPs) and to develop an immuno-array in collaboration with Affymetrix. Unfortunately, the original plan was ahead of its time and it was not possible to realize this within the time available for this project. However, over the five year period of this project, there have been enormous technical developments, which have now resulted in the availability of genome wide SNP arrays.

Selection of diseases studied

Diseases/phenotypes in our study were selected on the basis of presumed certain similarities in their pathogenesis. All diseases included had inflammatory components, thought to be elicited by common (innate) immune response pathways. These immune response pathways could be initiated by infection or colonization with bacteria or viruses, or be an effect of autoimmune response. The availability of well described, homogenous patients was a prerequisite for the inclusion of a cohort into the study, to minimize the chance of introducing bias as seen in poorly defined heterogeneous cohorts.
Within the (inter)national collaborations in this project we have selected the following diseases: **meningococcal infection**, **chronic hepatitis C virus** (HCV) **infection** and **recurrent acute otitis media** being infectious diseases; **rheumatoid arthritis** (RA) from the group of autoimmune diseases; and **Guillain Barré syndrome** (GBS) and **Barrett oesophagus** as intermediates between infectious and autoimmune disease. In addition we investigated ***S. aureus* nasal carriage** as being a risk factor for infection, and **sepsis after severe multiple trauma** as an inflammatory condition (Table 4). To determine allele frequencies in the general population, genotypes were also determined in a cohort of 463 healthy Caucasian blood donors.
For *S. aureus* nasal carriage and chronic hepatitis C virus infection, genetic variation in these pathogens was also included in the study, as we do recognize the importance of studying both host and microbe variation in the elucidation of the pathogenesis of infectious diseases.(6, 7) The interaction of the microbe and the host in a given environmental context will determine the outcome after exposure of the host to the microbe: asymptomatic colonization, infection, severity of infection, and outcome of infection.(8)

Selection of the genetic variations studied

The candidate gene approach was used to select genes of interest. This method starts with the assumption, or concrete knowledge from functional studies, that a certain gene is likely to be involved in the pathogenesis of the selected disease. Since we aimed to analyze several infectious and autoimmune diseases in parallel, we assessed what could be common denominators in inflammatory diseases.

Table 4. Diseases evaluated in this thesis.

	Disease studied	Classification	Microbial involvement	Candidate disease mechanisms
1	**Meningococcal disease**	infection, inflammation	*Neisseria meningitidis*	Infection, imbalance pro- and anti-inflammatory pathways, imbalance coagulation and fibrinolysis (41-43)
2	**Sepsis in trauma**	infection, inflammation	Gram negative microbes, *Staphylococcus aureus*, but usually culture-negative	Infection, imbalance pro- and anti-inflammatory pathways, imbalance coagulation and fibrinolysis (41-43)
3	**Guillain Barré syndrome**	infection, neuropathy, autoimmunity	Antecedent infection: *Campylobacter jejuni*, *Mycoplasma pneumoniae*, *Haemophilus influenzae*, CMV, EBV (44)	Molecular mimicry at B-cell level (45, 46)
4	**Rheumatoid arthritis**	autoimmune	Gut flora? (47, 48)	Imbalance pro- and anti-inflammatory pathways, impaired tissue integrity (49, 50)
5	**Barrett oesophagus**	infection, inflammation	Stomach content	Repeated tissue injury due to gastro-oesophageal reflux (51)
6	**Chronic HCV infection**	infection, inflammation	HCV	Infection, imbalance pro- and anti-inflammatory pathways, CD4/CD8 T-cell dysfunction (52)
7	**Recurrent otitis media**	infection, inflammation	*Streptococcus pneumoniae*, *H. influenzae*, *S. aureus*, *Moraxella catarrhalis*, viral infections(53)	Infection load, impaired immune response, impaired tissue integrity (54, 55)
8	***S. aureus* nasal carriage**	colonization	*S. aureus*	Impaired tissue integrity, staphylococcal evasion of immune response (complement) (56, 57)

Our choice was based on the literature available at that time and included genes involved in pro- and anti-inflammatory response, complement regulation, coagulation and fibrinolysis, apoptosis, pattern recognition and signalling pathways (Table 5). In addition, factors involved in tissue repair and integrity were included, since their role became more and more prominent in the past few years, as illustrated in celiac disease and atopic dermatitis.(9, 10)

Table 5. Genetic single nucleotide polymorphisms analyzed.

Gene	SNP
Pro-inflammatory	
TNFA[1]	A-863C (rs1800630), T-857C (rs1799724), G-376A (rs3093659), G-308A (rs1800629), G-238A (rs361525)
IL6	G-174C (rs1800795)
IL8	C781T intron (rs2227306)
CRP	C1184T (rs1130864), C2042T (rs1205), C2911G (rs3093068)
IL1B	C-511T (rs16944), C-31T (rs1143627), 3' UTR (rs3087261)*, promoter region C/T (rs3087258)*
Anti-inflammatory	
IL4	C-524T (rs2243250) (is identical to -590)
IL10	G-1082A (rs1800896), C-819T (rs3021097)
IL1RN	C2018T (rs419598)
Complement regulation	
C1INH	V480M (rs4926), T308S (rs1803212)*
CFH	Tyr402His (rs1061170)
Tissue integrity, coagulation and fibrinolysis	
PAI1	-675 4G/5G (rs1799889), insertion/deletion, G-504 A (rs2227632)*, A15T (rs6092)*, V17I (rs6090)*,H25P (rs2227647)*, R209H (rs2227669)*, T255N (rs2227685)*
Apoptosis	
NOS2a	S608L (rs2297518)
PARP	V762A (rs1136410), A188T (rs1805409)*, H613Q (rs1059011)*, C908Y (rs1059040)*
Host-pathogen interaction	
TLR2	Arg753Gln (rs5743708)
TLR4	D299G (rs4986790), T399I (rs4986791), V270G (is identical to V310G) (rs2770144)*
CD14	C-260T (rs2569190)
DEFA4	3'UTR A/G (rs736227)
DEFB1	Promoter region G/C (rs1800972)
LBP	C98G*, P436L*
IFN signaling pathway	
IFNAR1	L168V (rs2257167)
IFNAR2	V10F (rs7279064)
STAT2	I294M (rs2066807)

[1]Abbreviations: *TNFA*, tumour necrosis factor α; *IL*, interleukin; *CRP*, C-reactive protein; *IL1RN*, IL1 receptor antagonist; *C1INH*; complement component inhibitor-1; *CFH*, complement factor H; *PAI1*, plasminogen activator inhibitor; *NOS2A*, inducible nitric oxide synthase; *PARP*, poly (ADP-ribose) polymerase; *TLR*, Toll like receptor; *DEFA4*, α defensin 4; *DEFB1*, β defensin 1; *IFNAR*, interferon alpha receptor; *STAT2*, signal transducer and activator of transcription.
[2]Polymorphisms indicated by asterisks were excluded after initial assessment, either because they were not observed in our cohort, since technical problems occurred, or because of errors in prior publication.

The SNPs in these genes were selected because of their location in the promoter or coding region, because of their potential functional relevance. One SNP in *IL8* that is located in an intron was included because of previously described associations with viral infection.(11) Fourteen of the initially 45 included SNPs were subsequently excluded. Three of these were excluded for technical reasons. Two SNPs appeared to

be incorrectly described in the initial publication, and for the other ten the rare variant was not observed in our Caucasian reference population.(12) For some diseases additional genes were included because of a special interest and collaboration with several research groups.
The genotyping techniques used for this thesis were Single Base Extension and TaqMan allelic discrimination. These methods were selected because of their robustness, because they can be applied in a high throughput fashion using 384-well plates, and because of their ready availability for our project.
During the course of this project the scientific community required increasingly sound evidence in SNP research. This is reflected by the demand of increasing cohort sizes, testing in validation cohorts, and functional support.(13)

Functional assays in meningococcal disease

We performed a pilot study comparing the transcriptional profile in severe paediatric meningococcal sepsis patients with that of healthy matched individuals, using Affymetrix expression micro-arrays (HG U133 2.0). The aim of this part of the study was to identify biological pathways in the complex processes involved in the pathogenesis of meningococcal sepsis. In addition, in collaboration with the Department of Haematology of the Erasmus MC and with other (inter)national research groups, we investigated the expression of several proteins involved in coagulation and fibrinolysis, and inflammation.

(Inter)national collaboration

As mentioned several times above, collaboration between different departments was initiated to perform the research project that has led to this thesis. Primarily eight departments within the Erasmus University Medical Centre Rotterdam (Erasmus MC) were involved: Paediatrics, Immunology, Neurology, Gastroenterology, Rheumatology, Dermatology, Medical Microbiology and Infectious diseases, and Virology. This collaboration was started because of a mutual interest in the role of genetic variation in disease susceptibility and pathogenesis, and because the diseases of interest were considered to have certain similarities in their pathogenesis.
Additional Erasmus MC departments joined the collaboration, including the department of Internal Medicine, where most of the genotyping was performed, the department of Epidemiology and Biostatistics, and the department of Haematology and their (inter) national partners from Groningen, the Netherlands and Leuven, Belgium.
For specific diseases of interest, other (inter)national partners joined our collaboration. For RA these were the departments of Rheumatology from the Medical Centre Rijnmond-Zuid, and the Sint Franciscus Hospital, both in Rotterdam. These ensured the inclusion of large numbers of well defined RA patients. Together with the department of Gastroenterology and Hepatology of the Academic Medical

Centre Amsterdam, and their partners, we analysed a cohort of patients with Barrett oesophagus and other projects are still ongoing. Through the department of Gastroenterology of the Erasmus MC we were able to perform a genotyping study in a cohort of patients with HCV infection that was established in an international collaborative effort.
A long lasting collaboration with the department of Anaesthesiology, Intensive Care Medicine and Pain Therapy, University Hospital Giessen, Germany enabled us to perform a replication study, analysing a *PAI1* polymorphism in trauma patients. Mutual interest in pneumococcal infections and previous collaboration resulted in the genotyping of children with recurrent otitis media from the OMAVAX study, based in Hoofddorp and Utrecht, the Netherlands.
The Netherlands Reference Laboratory for Bacterial Meningitis, Academic Medical Centre Amsterdam provided valuable information regarding meningococcal serogroups.
Together with the group of professor Thierry Calandra from the Infectious Diseases service, Department of Medicine from the Centre Hospitalier Universitaire Vaudois, Switzerland, and their USA partners, and the department of Chemical Endocrinology, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands, we combined adult and paediatric patient cohorts to provide a more complete view of the role of macrophage migration inhibitory factor in sepsis.
Large numbers of well defined patients with meningococcal infection were included together with the Department of Paediatrics, Imperial College London St. Mary's Campus, London, United Kingdom and the UK Meningococcal Research group. Even more, international collaboration with groups in Singapore, Graz (Austria) and London has been initiated and a genome wide approach study is currently ongoing for the cohort(s) of patients with meningococcal disease.
Statistical and bioinformatics support for the genotyping studies was provided by the department of Medical Statistics, LUMC, Leiden University Medical Centre, the Netherlands, and by the Information and Communication Theory Group, Faculty of Electrical Engineering, Mathematics, and Computer Science, Delft University of Technology, Delft, the Netherlands.
The unique aspect of this collaboration was the participation of departments with expertise in the fields of infection, immunology, haematology, genetics, genetic epidemiology and bioinformatics, and the availability of well-defined patient cohorts. Both clinical and laboratory departments participated. The study described in this thesis was supported by a Revolving Fund Top Down grant of the Erasmus MC.

Outline of this thesis

The first part of the thesis elucidates several factors involved in (meningococcal) sepsis in children and adults. Chapter **2.1** reviews the role of genetic polymorphisms in meningococcal infection as known at the start of project start of this project. Chapter **2.2** illustrates the role of age gender and era on the outcome of

meningococcal sepsis patients admitted to the Paediatric Intensive Care Unit of the Erasmus MC-Sophia. Chapter **2.3** describes the current hypotheses and findings in the pathogenesis of sepsis and therapeutic trials, underscoring the importance of the balance between pro-inflammatory and anti-inflammatory actions. In chapters **2.4** and **2.5** the role of genetic polymorphisms in antimicrobial peptides, cytokines and coagulation factors, in susceptibility to and severity of meningococcal infection is demonstrated. In addition, in chapters **2.5** and **2.6** the functional relevance is demonstrated of thrombin activatable fibrinolysis inhibitor (TAFI) and its activation markers and the role of von Willebrand factor (VWF) and ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 motif) in paediatric meningococcal infection. In chapter **2.7**, the role of macrophage migration inhibitory factor (MIF) in adult and paediatric sepsis patients is supported by the association with severity and mortality. The transcriptional profiling study in meningococcal sepsis patients illustrating the involvement of multiple pathways is described in chapter **2.8**. The role of plasminogen activator inhibitor-1 (*PAI1*) 4G/5G polymorphism in multiple trauma patients is described in chapter **2.9**.

The second part of the thesis concerns inflammatory diseases that are now considered to be (partly) of autoimmune and (partly) of infectious origin. The role of SNPs in Guillain Barré syndrome (GBS), rheumatoid arthiritis (RA), Barrett oesophagus, and chronic HCV infection is assessed in chapters **3.1-3.4**, suggesting a role for polymorphisms in genes in pro- and anti-inflammatory pathways, and in molecules involved in tissue integrity as well as Toll like receptors in these diseases.

In chapters **4.1** and **4.2**, the third part of this thesis, the role of genetic polymorphisms in recurrence of acute otitis media is discussed, demonstrating, e.g., a possible role for impaired tissue repair in the susceptibility for recurrent otitis media.

Chapters **5.1-5.3** provide evidence for an effect of host cytokine and complement, and microbial genetic polymorphisms in *Staphylococcus aureus* nasal colonization.

Finally, in chapter **6.1** and **6.2**, the previous studies and future research perspectives in genetic association studies are discussed.

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Chapter 2

Genetic polymorphisms and their consequences in patients with (meningococcal) sepsis

Chapter 2.1

Host genetic determinants of *Neisseria meningitidis* infections

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Abstract

The clinical presentation of infections caused by *Neisseria meningitidis* is highly diverse. Some patients develop meningitis, while others present with sepsis or even septic shock. After invasion of the bloodstream by the bacteria, three main cascade pathways are activated. These are the complement system, the inflammatory response, and the coagulation and fibrinolysis pathway. These pathways do not act independently but are able to interact with each other. Genetic polymorphisms among components of these pathways have shown to be involved in the susceptibility, severity and outcome of meningococcal disease. We review knowledge of genetic variations associated with susceptibility to and severity of meningococcal infection. Complement deficiencies and defects in sensing or opsonophagocytic pathways, like the rare Toll-like receptor 4 single nucleotide polymorphisms (SNPs) and combinations of inefficient variants of Fcγ-receptors, seem to have the most important role in genetically determined susceptibility. Effect on severity has repeatedly been found for FcγRIIa and Plasminogen activator inhibitor type 1 (*PAI1*) polymorphisms. Outcome effects have been confirmed for SNPs in properdin deficiencies, *PAI1* and combination of the -511C/T SNP in *IL1B* and the +2018C/T SNP in *ILRN*. Conflicting results are found for the effect of the -308G/A promoter polymorphism in Tumour necrosis factor (TNF) A. These differences may reflect discrepancies in group definitions between studies or the influence of additional SNPs in the *TNFA* promoter, which can form haplotypes representing different cytokine production capacity. For several SNPs, the potential effect on susceptibility, severity or outcome has not yet been confirmed in an independent study.

Introduction

Despite ongoing improvement of treatment strategies in infections caused by *Neisseria meningitidis* the mortality rates are still very high, and range from 4-40%. The wide range indicates the different clinical presentations of infection. The disease spectrum varies from meningitis to sepsis and septic shock (Figure 1). Meningitis has a mortality rate of 4-6%, while in septic shock mortality rates up to 40% have been reported. Meningococcal lipopolysaccharide (LPS) is thought to be one of the major factors that induce the host response during bacterial invasion. This response is complex and involves, besides non-immunological clearing mechanisms such as cholesterol, the activation of three main cascade reactions (Figure 2). The first is the complement system that, apart from contribution to phagocytosis of the bacteria, functions as an inducer for the inflammatory reaction via C3a and C5a. The second cascade reaction is the coagulation and fibrinolysis pathway. Both are stimulated in meningococcal infection, which results in a prothrombotic stage. The third system is the inflammatory reaction mediated by different cytokines and chemokines among which Tumour necrosis factor (TNF)α and IL-1β play a central role in immunological

defence. One of the pathways leading to cytokine stimulation is triggered through interaction of bacterial molecules with Toll-like receptors (TLRs).

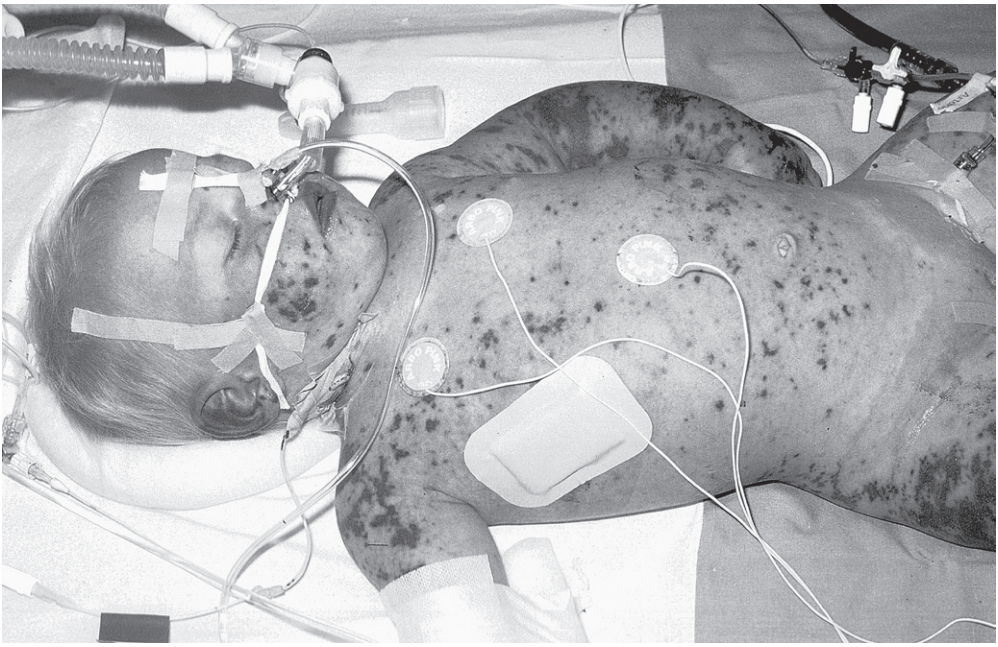

Figure 1. Petechial and purpuric rash in a child with meningococcal septic shock.

At this moment ten TLRs are identified in human beings, each recognizing specific known or unknown microbial molecules. TLR4 and TLR2, for instance, recognize meningococcal LPS, while TLR9 recognizes CpG-rich bacterial DNA.(1) Stimulation of TLRs leads to the transcription of NFκB, which in turn activates immune response genes leading to activation of dendritic cells and regulation of the adaptive immune response (Figure 3).(2) In patients with sepsis activation of these cascades are translated into a sudden onset of fever and petechial or purpuric rash followed by hypotension (Figure 1). In septic shock, disseminated coagulation and multiple organ failure develop. For extensive reviews of the pathophysiology see previous reports.(3, 4)

The broad range of clinical presentations of *N. meningitidis* infections raises the question of why some patients show very few clinical symptoms and others die within several hours after onset of symptoms. Variations in host genetic factors are known to contribute to differences in clinical response. Haralambous et al. quantified the host genetic component to meningococcal disease susceptibility, calculating the sibling risk ratio (λs) as the ratio of observed meningococcal disease cases among 845 siblings of 443 cases in white people in the UK to that expected. The overall λs they found was 30.3. Depending on the meningococcal disease onset within set time points of the index case λs varied between 8.2 and 11.9, suggesting that host

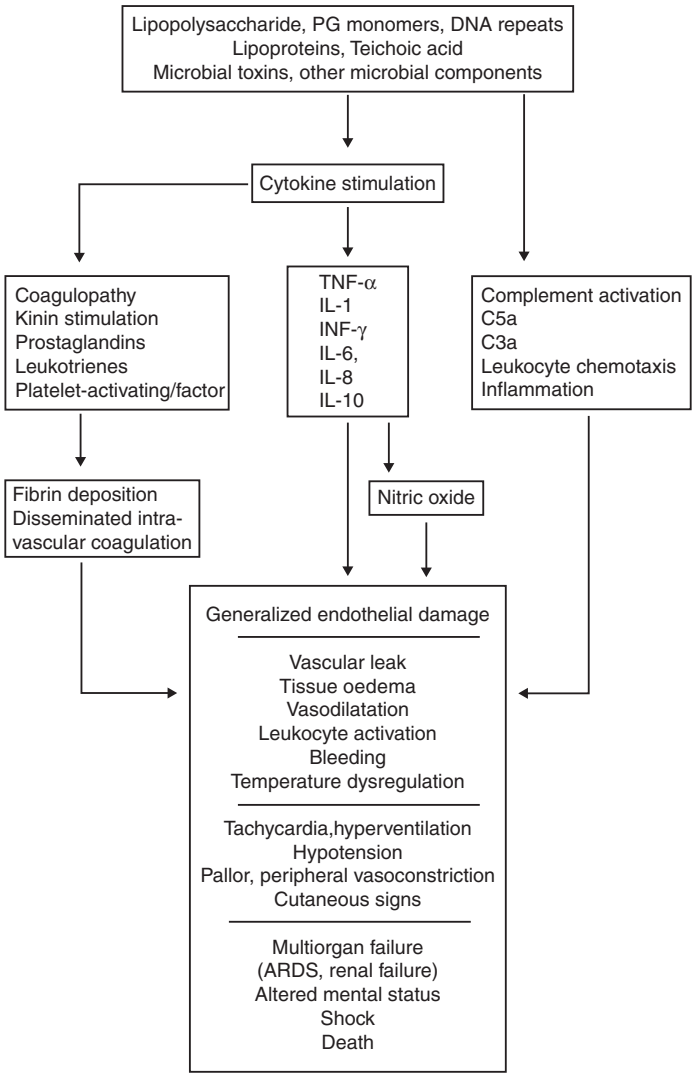

Figure 2. Inflammatory cascade initiated during meningococcal sepsis. The three main cascades involved in the pathophysiology of meningococcal infection. The factors depicted in bold are discussed in the text. Adapted from Rich et al.(84) ARDS, acute respiratory distress syndrome; IL, interleukin; INF, interferon; TNF, tumour necrosis factor.

genetic factors may contribute approximately one third of the total λs.(5)
Researchers currently focus on a wide variety of infections and other diseases. Genetic polymorphisms are relatively stable in the human population. It has been shown that these polymorphisms may affect susceptibility, severity and outcome of infectious disease. This review focuses on what is known of genetic variability in the susceptibility to and severity of meningococcal infections (Table 1).

Table 1. Genetic polymorphisms associated with meningococcal infection.

Pathway	Gene	Polymorphism	Su/Se/Ou[1]	OR[2]	Ref.	Comments
Innate immunity	*TLR4*	Asp299Gly	Su Su Se	No association OR 1.14 (0.86-1.52) OR 1.55 (0.70-3.44)	(10) (8)	
		Rare polymorphisms combined	Su	OR 27	(10)	
	TLR2	P631H	Su	No association	(10)	
		R753Q	Su	No association	(10)	
		Multiple SNPs (no aa change)	Su	No association	(10)	
	LBP[3]	Cys98Gly	Su Ou	Only in male patients Trend (low patient numbers)	(12)	No MD[4], sepsis in general
		Pro436Leu	Su Ou	No association Trend (low patient numbers)	(12)	No MD, sepsis in general
	BPI[3]	A645G	Su	No association	(12)	No MD, sepsis in general
		G545C	Su	No association	(12)	No MD, sepsis in general
		PstI in intron 5	Su	No association	(12)	No MD, sepsis in general
	ACE	284 bp insertion/deletion	Se Ou	> in DD 14% > risk of mortality	(16)	
	MBL[5]	Codon 52 (Arg/Cys) Codon 54 (Gly/Asp) Codon 57 (Gly/Glu)	Su Se	OR 6.5 (2.0-27.2) (homozygous) OR 4.5 (0.9-29.1) (homozygous) OR 1.7 (1.1-2.6) (heterozygous) OR 2.2 (1.1-4.3) (heterozygous) OR 2.0 (1.3-3.0) (he+ho) OR 2.4 (1.2-4.6) (he+ ho) Trend to less severe disease	(20) (20)	
	Properdin[6]	C2061T (stop exon 4) type I C2726T (stop exon 5) type I C3041G (stop exon 6) type I C2124T (Arg/Trp) type II G827A type II Codon 387 (Tyr/Asp) type III	Su Se Ou	RR 250 RR 4.7-15 Increased mortality	(30) (30) (30)	
	Factor D	Ser42 stop			(32)	Case report
	C3		Su	Increased		
	LCCD	Different defects	Su Se	Increased (57-71%) Decreased	(30)	
Acquired immunity	*FcγRIIa*	His131Arg	Se Su Se Su Se	His: sepsis Arg: meningitis No association Arg: OR 3.9 (1.0-16) OR 2.67 (1.09-6.53) Arg: OR 14	(34) (35) (35) (36) (31)	 Only 25 survivors of MD studied LCCD patients
	FcγRIIIa	Val158Phe	Se	Val: meningitis	(34)	Relatives of patient
	FcγRIIIb	NA1/NA2 (codon 65 and 82)	Su	No association	(36)	

Pathway	Gene	Polymorphism	Su/Se/Ou[1]	OR[2]	Ref.	Comments
	Combination	RR-FF-NA2/2	Su	OR 2.6 (1.1-6.3)	(34)	Relatives of patient
		RR-NA2/2	Su	OR 13.9	(38, 39)	LCCD patients
			Su	No association	(39)	Properdin deficient individuals
Coagulation / Fibrinolysis	*tPA*	Alu repeat insertion/deletion	Su	No association	(40)	
			Se	No association	(40)	
	PAI1	4G/5G insertion/deletion	Su	No association	(45, 46)	
			Ou	RR 4/4G 2.0 (1.0-3.8)	(45)	
			Se	OR 4/4G 5.9 (1.9-18)	(46)	Relatives of patient
			Ou	No association	(46)	Relatives of patient
			Su	No association	(47)	
			Se	4/4G 2 fold increase	(47)	Predicted mortality
			Se	R/R 4/4G 2.4	(47)	Vascular complications in survivors
			Ou	RR 2.7	(47)	In meningococcal sepsis patients
	Factor V	FV^{L} G1691A	Su	No association	(48)	
			Se	RR 3.1 (1.2-7.9) (heterozygous)	(48)	
			Ou	No association	(48)	
Cytokines	*TNFA*	G-308A	Su	No association	(56)	No MD, sepsis in general
			Se	RR 1.6 (1.1-2.3)	(56)	
			Ou	RR 2.5 (1.1-5.7)	(56)	Relatives of patients
			Ou	No association	(54)	
			Ou	No association	(57)	
		G-238A	Ou	No association	(54)	Relatives of patients
	TNFB	NcoI B1+, B2-	Ou	B2 increased mortality	(64)	No MD, sepsis in general
			Ou	OR 3.47	(63)	No MD, sepsis in general
	IL10	A-1082G	Su	Non random distribution	(34)	In combination with FcγRIIa
	IL6	G-174C	Su	No association	(68)	
			Se	OR 3.06 (1.25-7.49) GG vs. GC or CC	(68)	
			Ou	OR 2.64 (1.12-6.22) GG vs. GC and CC	(68)	
			Su	No association	(69)	No MD, sepsis in general
			Se	No association OR 0.73 (0.32-1.71)	(69)	No MD, sepsis in general
			Ou	OR 0.11 (0.02-0.57) GG vs. GC and CC	(69)	No MD, sepsis in general
		3 promoter polymorphisms	NA[5]		(70)	
	IL1RN	86 bp repeat in intron 2/ A2 or T2018C	Su (A2)	No association	(67)	
			Se	No association OR 1.0 (0.69-1.47)	(67)	
			Ou	No association OR 1.01 (0.49-2.09)	(67)	
			Su	Increased in A2	(63)	No MD, sepsis in general
			Ou	No association	(63)	No MD, sepsis in general
	IL1B	C-511T	Ou	1/1 vs. 1/2 OR 3.39 (1.39-8.29) 2/2 vs. 1/2 OR 7.35 (2.51-21.45)	(57)	
			Su	No association	(58)	
			Survival	OR 2.05 (1.1-3.79) in model	(58)	
	IL1B and *ILRN*	-511 C/T +2018T	Survival	OR 7.78 (1.05-59.05)	(57)	
		-511Cor C/T + 2018C or C/T	Survival	OR 0.61 (0.38-0.99)	(58)	

[1]Su=effect on susceptibility, Se=effect on severity, Ou=effect on outcome -relative risk (RR) for death are given. NA=not available.
[2]OR=Odds ratio -any number > 1 indicates more severe disease to be associated with the rare allele, unless stated otherwise.
[3]*LBP* and *BPI* polymorphisms have not been analysed in meningococcal infection, only in sepsis, without further specification.
[4]MD=meningococcal disease.
[5]The *MBL* variants were combined because of the effect of compound heterozygous individuals. Homozygous: variant allele at two *MBL* loci; heterozygous: a variant allele at one position and the common allele for both other loci.
[6]Effects of properdin deficiencies are combined for all variants.

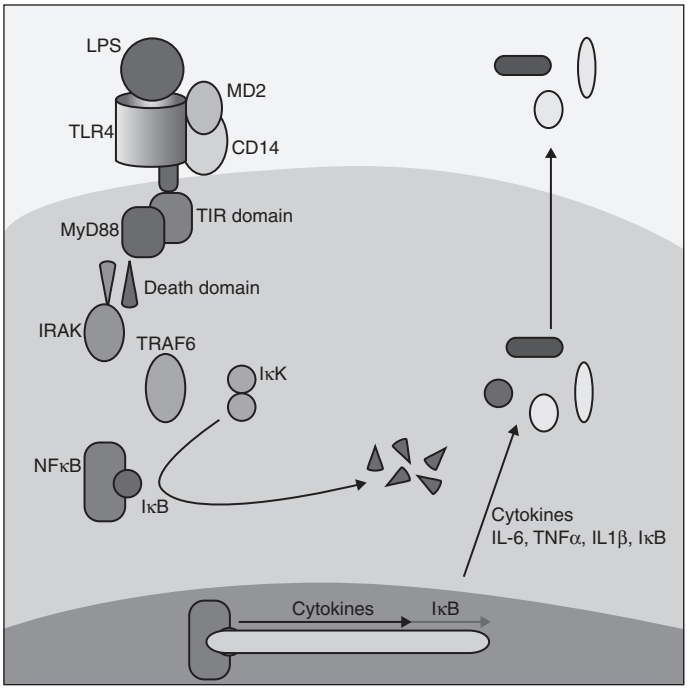

Figure 3. Activation of cytokines through interaction of LPS with Toll-like receptor 4. IRAK, interleukin-1 receptor-associated kinase; MyD, myeloid differentiation factor; TIR, Toll/interleukin-1 receptor; TRAF, TNF receptor-associated factor.

Search strategy and selection criteria

Data for this review were identified through Pub Med searches. Search terms were “meningococ* and polymorph*”, “meningococ* and (disease* or infection*)”, “meningococ*” and “meningococ* and (susceptib* or severity)”. Individual genes were searched for by their specific names. English language papers only were reviewed.

Innate immunity

Toll-like receptors. Innate immunity has a very important role in the first recognition of invading pathogens. This recognition was, until recently, believed to be non-specific. However, elucidation of the function of TLRs has shown otherwise. TLRs sense different microbial molecules, covering a range of pathogens that cause infections in the host. C3H/HeJ mice, having a point mutation in *tlr4* that abolishes LPS responses, are hypo-responsive to Gram-negative infections.(6, 7) The common Asp299Gly polymorphism in TLR4 alters the extracellular domain of the receptor, and airway epithelial cells of patients carrying this polymorphism are hypo responsive to

LPS. However, no association has been seen between the Asp299Gly polymorphism and the susceptibility or severity of meningococcal infection.(8) Smirnova et al. reported an excess of rare aminoacid polymorphisms in TLR4.(9) None of these rare variants was individually over-represented in a UK patient population with systemic meningococcal disease. When the individuals with a rare variant of TLR4 were combined, a significant excess was seen in the patient group compared to healthy controls. These results suggest that a deficiency in sensing meningococcal LPS increases risk of disease.(10)

No significant association was seen for polymorphisms in *TLR2* and meningococcal infection.(10) TLR2 recognizes other bacterial components, such as lipopeptides and peptidoglycan.(6) So far no polymorphisms in *TLR9* in relation to meningococcal disease have been studied. If a relation exists it is likely to contribute to a different extent in populations with different ethnical background considering the varying allele frequencies reported.(11) TLRs recognize ligands in the presence of CD14 and MD2 (TLR4) and activation of the diverse TLRs results in the activation of different pathways, leading to NFκB activation. Polymorphisms in the factors in these pathways might also indicate different phenotypes.

LPS binding protein and bactericidal/permeability increasing protein. Besides non-immunological clearing of LPS by alkaline phosphatase and cholesterol, LPS binds to LPS binding protein (LBP) to form a complex that binds TLR4 via CD14. In a recent study the Cys98Gly and Pro436Leu polymorphisms of LBP did not show correlation with the susceptibility or severity of bacterial sepsis in people but a relationship was found with the outcome of bacterial sepsis in men. It has been proposed that these polymorphisms might in part explain the worse outcome of bacterial infections reported in men. In the same study polymorphisms in bactericidal/permeability increasing protein (BPI) have also been assessed. BPI is another protein binding to LPS and inhibiting LPS-induced host cell responses. The A645G, the G545C silent polymorphism, nor the *Pst*I site in intron 5 was associated with outcome and severity of bacterial sepsis. However, in this study the limited amount of data prohibited the analysis of Gram-negative versus Gram-positive infections, which in our opinion introduces a bias with respect to LPS-related susceptibility. This might have limited the sensitivity in detecting an association in the latter study.(12) Confirmation of the findings in an independent study taking into account the nature of the causative bacteria is warranted.

Angiotensin converting enzyme. Angiotensin Converting Enzyme (ACE) is associated with proinflammatory response. The absence of a 284 base-pairs (bp) marker in the *ACE* gene (D allele) is associated with higher circulating ACE activity compared with presence of this marker (I allele).(13) The DD genotype has shown to be related to higher tissue activity and associated with venous thrombo-embolism.(14, 15) Harding et al. have shown that in children with meningococcal disease the DD genotype is associated with higher Glasgow Meningococcal Septicaemia Prognostic Scoring, higher inotropic support and ventilation, higher predicted risk of mortality and longer paediatric-intensive-care-unit stay, compared with the II and ID genotype. The DD genotype is associated with increased disease severity, and, although not

significant, a twofold increase in mortality has been reported. The genotype distribution was demonstrated to be similar for patients and healthy controls, suggesting no effect of *ACE* polymorphism on susceptibility to meningococcal disease. The reported association with disease severity might be explained by an increased proinflammatory response in DD individuals due to increased ACE concentrations. Unfortunately, no data on ACE concentrations are available in this study.(16)

Complement

Mannose binding lectin. Complement activation forms a significant part of the innate immunity. Besides beneficial effects, harmful effects have also been attributed to complement activation. The severity of hypotension is in part regulated by complement activation. At present, three activation pathways are considered (Figure 4). First, the classical pathway, which is activated by antibody-antigen interactions and by C-reactive protein.(17) Second, the alternative pathway of innate immunity, which is activated through interaction of C3 with factor B and factor D to generate C3b. Third the additional innate activation pathway, which is activated through mannose binding lectin (MBL). The two pathways of the innate immunity do not require antibodies. All three pathways converge in a common final pathway.

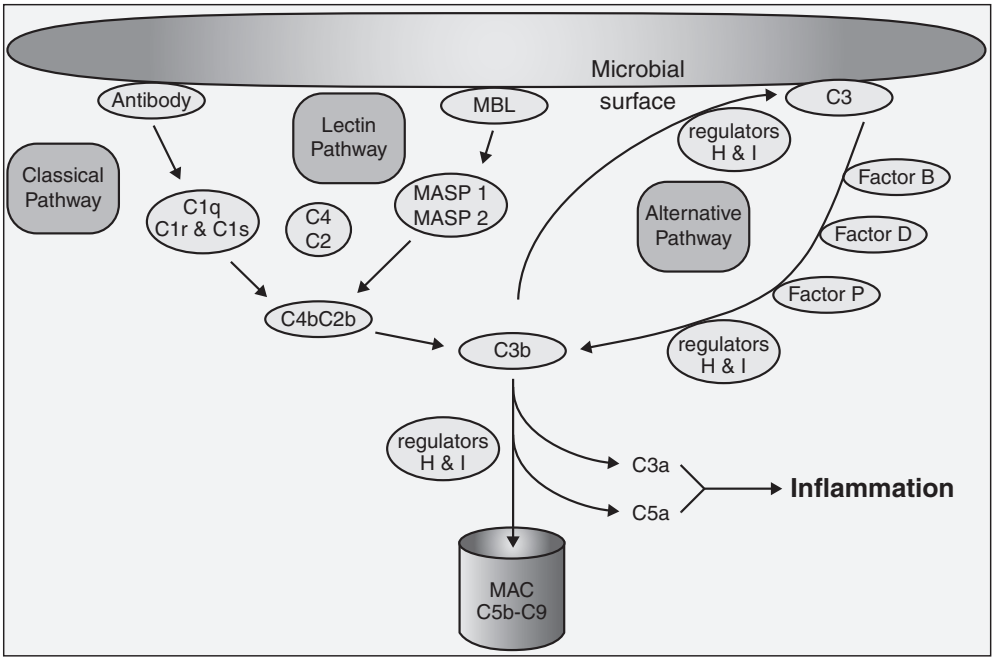

Figure 4. Three pathways of complement activation leading to a common late pathway, which generates the membrane attack complex (MAC) and inflammatory mediators C3a and C5a. Adapted from Hibberd et al.(18) MASP, mannose-binding-lectin-associated serine protease.

MBL first forms trimers, which in turn further assemble to multimers. The MBL multimers bind to sugars and LPS on the bacterial surface. This complex then activates the MBL-associated serine proteases (MASP) 1 and MASP2. MASPs activate C4 of the typical pathway.

Three allelic variants of *MBL* have been described in codons 52, 54 and 57 of exon 1. In codon 54 and 57 a mutation causes the disruption of the axial glycine repeats (Gly-Xaa-Yaa) resulting in aberrant trimers. These trimers cannot form correct multimers and are unstable. Via an unknown mechanism, the codon 52 mutation also results in unstable trimers. Heterozygotes for the variant alleles show a decrease in MBL serum concentrations to 10% of normal, while in people homozygous for any of the three mutations concentrations drop to 1% of normal.(18, 19) In children the variant alleles were associated with the susceptibility to meningococcal infection. Patients homozygous for the variant alleles showed a trend towards slightly less severe disease than heterozygous and wild-type individuals. This observation, however, did not reach statistical significance.(20) The finding that low serum MBL is associated with susceptibility to meningococcal infection was supported by the report of three cases in a single family by Bax et al.(21) Unfortunately they did not provide information on the genotypes.

C3. C3 has a central position in complement activation. All three complement pathways described lead to activation of C3, resulting in the fragments C3a and C3b. C3a, together with C5a, is a potent proinflammatory and chemoattractant molecule. C3b functions as an opsonin and, when bound to the C3 convertase, forms the C5 convertase that produces C5a and C5b. C5b initiates the late events in the complement pathway resulting in the formation of the membrane-attack complex (MAC). The MAC creates pores in the pathogen cell membrane causing bacterial death. Deficiency of C3 results in compromised opsonisation and phagocytosis of bacteria and, hence, in increased susceptibility to meningococcal and other bacterial infections.(22) However, excessive activation of the complement system was shown in children with septic shock related to outcome, severity of disease and capillary leakage.(17)

Properdin. Another important factor of the alternative complement pathway is properdin, also called factor P, which is coded on the short arm of the X chromosome. (23) It is a positive regulator acting through stabilization of the C3 convertase C3b,Bb. Properdin deficiencies are associated with increased risk for meningococcal infection. Three different variants of properdin deficiency have been described. Type I deficiency is characterised by absence of the properdin protein in the plasma. In type II a low but detectable properdin plasma concentration is reported whereas in type III a normal concentration of a dysfunctional variant protein is found.(24) The molecular basis for the deficiencies is heterogeneous. Type I deficiency is caused mainly by a mutation resulting in a premature stop codon in exon 4-6.(25-27) In type II two distinct mutations have been described, C2124T (Arg/Trp) and G827A in exon 4 and intron 3, respectively.(26) In type III a single base substitution, T>G in exon 9 causing an amino acid substitution, Tyr387Asp, results in the abolishment of convertase stabilizing function.(28)

All three properdin deficiency variants are associated with increased susceptibility to infection with *N. meningitidis*. The severity of disease differs between individuals with properdin deficiency and, in addition, depends on the serotype of the meningococcal strain involved. In general an increased disease severity and poor outcome is observed. Mortality rates of 75% and more recently 33% have been reported.(29, 30) Differences with respect to meningococcal disease in properdin-deficient individuals can also be explained by the contribution of additional genetic factors. Spath et al. found in their group of nine properdin-deficient individuals that those patients with a positive history for meningococcal disease invariably lacked the G2m(n) IgG allotype, while in all other individuals this allotype could clearly be detected. Lack of the G2m(n) allotype is known to be associated with poor antibody responses to T-independent antigens and possibly lower IgG2 concentrations. Meningococcal LPS can elicit an IgG2 response.(25) Another explanation for the clinical differences may be different inactivation patterns of the X chromosome in heterozygous female patients.

Late complement deficiency and factor D. Patients having late complement deficiency (LCCD, C5-C9) are known to have recurrent Gram-negative bacterial infections. Failure to form a MAC underlies this increased susceptibility. These infections result in milder disease severity than reported for the total patient population, suggesting adverse effects of complement activation during meningococcal infections. Fcγ receptor polymorphisms have shown to affect disease susceptibility in LCCD individuals (See below).(31) Complement D deficiency was seen in members of a family with severe *N. meningitidis* infections. The alternative pathway for complement activation was impaired. This pathway, by contrast with the classical pathway, seems to be prominent in meningococcal infection.(32, 33)

The genetic variations described so far in complement factors appear to contribute mainly to susceptibility and not severity of meningococcal disease, underlining the importance of the serum bactericidal capacity in the host defence against meningococcal infection.

Aquired immunity

FCγ Receptors. Fcγ receptors (FcγRs) belong to a heterogeneous family of receptors and are grouped in three classes (FcγRI, II and III). Three subtypes of receptors responsible for IgG-mediated signalling (FcγRIIa, FcγRIIIa, FcγRIIIb) are thought to be important in host defence against meningococci. FcγRIIa is located on leukocytes and mononuclear macrophages and is sensitive to IgG2 and IgG3. Two alleles are known to differ at aminoacid position 131 because of single point mutation (SNP) in exon 4. The 131R (arginine) allotype confers lower interaction efficiency towards IgG2 and IgG3 than the 131H (histidine) allotype. Two allotypes known for FcγRIIIa, 158Phe and 158Val, show different binding of IgG1, IgG3 and IgG4, as interaction with FcγRIIIa158V is stronger. FcγRIIIb is expressed on neutrophils and has the neutrophil antigen (NA) polymorphism representing 4 aminoacids substitutions in the membrane-distal loop of the receptor. FcγRIIIb-NA2 binds IgG1 and IgG3 less

efficiently than FcγRIIIb-NA1.(34) One can imagine that differences in the efficiency to bind IgGs result in different host response effectiveness and thereby variable susceptibility to and/or severity of disease.

A study in survivors of meningococcal disease and first-degree relatives of survivors and non-survivors showed no differences in FcγR distribution. The FcγRIIaR131 allele was more often seen in meningitis patients compared with sepsis patients. The FcγRIIIa-V158 allelic frequency was markedly increased in relatives of meningitis patients compared with relatives of patients presenting with haemodynamic instability. The RR-FF-NA2/2 frequency that represents the least efficient FcγR combination, and is responsible for diminished phagocytosis, was tripled in first-degree relatives compared with healthy non-related controls. These data suggest an association between FcγR haplotype and susceptibility to and severity of meningococcal disease.(34) In contrast to Van der Pol et al., Domingo et al. have found the FcγRIIa-131Arg/Arg genotype to be more common in patients with sepsis than in patients with meningitis with or without sepsis. Differences in genotype distribution between patients and controls were not reported. Meningococcal serotypes were distributed similarly among different genotypes. Complications were more frequent in patients homozygous for FcγRIIa-131Arg, but long-term sequelae were found equally in patients with different genotypes. A prognostic score of 1 or more, indicating severe disease according to the Barcelona Meningococcal Disease Surveillance Group, was seen more often in patients homozygous for FcγRIIa-131Arg. No mortality differences were reported.(35) In a retrospective study Bredius et al. saw that the R/R genotype is present more often in survivors of meningococcal disease than in healthy controls (OR 2.67). This finding suggests a role for the FcγRIIa polymorphism in susceptibility to meningococcal disease. Because of the retrospective nature of the study, no data on genotypes for non-survivors are available, hence effects on severity of disease remain unknown.(36) In a recent study, Smith et al. were unable to find an association between FcγRIIa and FcγRIIIb polymorphisms and meningococcal disease in a patient population in Western Norway.(37) However, the small sample size, 50 patients and 100 healthy controls, although well defined, may have prevented them from finding an association.

Additionally, Platonov et al. have studied the FcγRIIa-131 polymorphism in 29 Russian LCCD patients and 107 healthy geographically matched controls. Genotype distribution was similar for patients and controls. Patients who contracted their first meningococcal disease episode under the age of 10 had mainly the FcγRIIa-131His/His genotype while in patients with the first disease period above the age of 10 years the FcγRIIa-131Arg/Arg genotype was more prevalent. Overall the R/R and R/H genotypes were associated with more severe disease as compared to the H/H genotype. This effect was most pronounced for the disease episodes in patients above 10 years of age.(31) The combination of R/R and NA2/NA2 in a group of 15 Dutch LCCD patients increased susceptibility to meningococcal disease (OR 13.9).(38) This relation could not be observed in a group of 15 properdin-deficient patients.(39)

Coagulation

Activation of coagulation and fibrinolysis is the result of the acute inflammatory response as mentioned above. The prothrombotic endothelium surface results from cytokine activation. Subsequently, tissue factor production results in activation of the extrinsic pathway of coagulation and the production of platelet-activating factors. The fibrinolytic system is initially activated but is subsequently inhibited. This results in a marked imbalance in coagulation and fibrinolysis resulting in a net procoagulant state and ultimately disseminated intravascular coagulation. This leads to deposition of fibrin, the formation of microthrombi and bleeding. Multiorgan failure and death are the most severe clinical outcomes of this imbalance.

Tissue-type plasminogen activator. Tissue-type plasminogen activator (t-PA) is a serine protease that converts plasminogen into its active form plasmin, which in turn leads to fibrinolysis. Impaired t-PA function leads to insufficient lysis of thrombi. Differences in t-PA production or impaired function could therefore affect the severity of meningococcal disease. An insertion/deletion polymorphism of an Alu element in *TPA* considered to affect the basal levels of t-PA has been investigated in relation to meningococcal disease. No association could be seen between the different alleles, and the severity or outcome of meningococcal disease.(40) The authors have regarded the admission to an intensive care unit (ICU) as a criterion for severe meningococcal disease. In our opinion this only holds partially true, since not all patients admitted to the ICU actually develop septic shock. This approach might have prevented them from finding significant differences between the study populations. Although the levels of t-PA during meningococcal septic shock do not differ much from baseline, the 4G/5G polymorphism in plasminogen activator inhibitor type 1 (*PAI1*, see below) might have prevented finding an association with t-PA.(41) Further research regarding polymorphisms in *TPA* in combination with polymorphisms in other factors involved in coagulation and fibrinolysis with respect to meningococcal disease is therefore required.

PAI-1. PAI-1 is responsible for the inhibition of fibrinolysis both directly and indirectly through inhibition of t-PA (Figure 5). In turn, PAI-1 is inhibited by activated protein C. In septic shock, laboratory findings show decreased concentrations of all coagulation factors especially protein C.(41) The gene encoding PAI-1 has several polymorphic loci, including a 3'-HindIII site, a CA(n) repeat in intron 3 and a 4G/5G insertion deletion polymorphism at -675 in the promoter. PAI-1 activity has shown to be significantly higher in control subjects homozygous for the 4G allele than in those homozygous for the 5G allele.(42) The production of PAI-1 mRNA after IL-1 stimulation seemed to be greater in HepG2 cells bearing the 4G allele.(43) In patients with meningococcal sepsis the levels of PAI-1 are positively related to severity of disease, outcome, cytokine levels, acute phase proteins and coagulation parameters.(41) In non-survivors the PAI-1 concentrations were shown to be 1.9 times higher for the same TNF-α concentrations than in survivors.(44) TNF-α induces PAI-1 production. In children with meningococcal disease a relation has been observed between genotype, PAI-1 concentrations and outcome of disease. The patients with

the 4G homozygous genotype had higher PAI-1 concentrations and had an increased risk of death. No association was observed with severity of disease.(45)
A similar study by Westendorp et al. has shown a higher incidence of the 5G/5G genotype among relatives of patients with meningitis. Patients whose relatives were carriers of the 4G/4G genotype had a six-fold higher risk of developing septic shock than meningitis.(46) In a recent study including 510 UK paediatric patients, 210 parents and 155 UK Caucasian controls, no association was seen between the 4G/5G polymorphism and susceptibility. In patients with meningococcal sepsis the relative risk of death was 2.9 for the 4G homozygous patients compared with the combined 4G/5G and homozygous 5G genotypes. In survivors the risk of developing vascular complications was significantly higher in the patients homozygous for the 4G allele.(47)

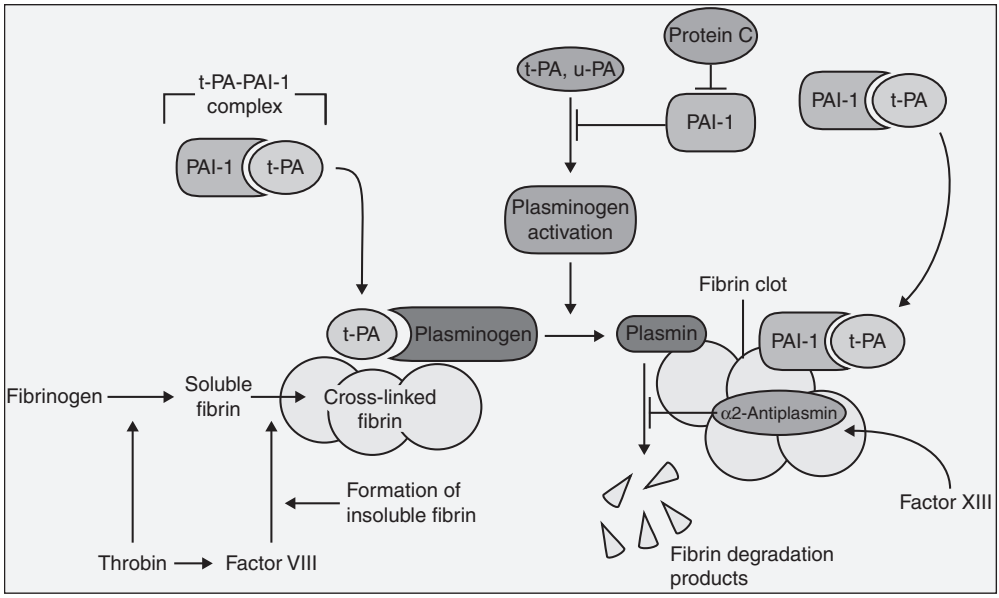

Figure 5. The action of Protein C, PAI-1 and t-PA on fibrinolysis. u-PA, urokinase-type plasminogen activator.

Factor V Leiden. Factor V Leiden (FV^L) is associated with thrombotic events, and is therefore a candidate for involvement in the development of meningococcal purpura fulminans. A study comparing children with meningococcal disease, healthy controls, and parents of children with meningococcal disease did not reveal an association with the FV^L mutation and susceptibility. Patients heterozygous for the mutation showed increased complications, as assessed by requirement for skin grafting, referral to plastic surgeon, and/or amputation. A significant effect on mortality has not been reported.(48)

Protein C and thrombomodulin. To prevent hypercoagulability, anticoagulants serve to guard the balance between coagulation and fibrinolysis. Activated protein C, together with its cofactor protein S, inactivates the procoagulant factors V and VIII. PAI-1 is also inhibited by protein C. Protein C is initially expressed as an inactive protein and for activation the interaction with thrombin, thrombomodulin and endothelial protein C receptor is required. In severe meningococcal sepsis low concentrations especially of protein C are seen, and the expression of thrombomodulin is down regulated.(41, 49) However, administration of unactivated protein C to two patients in a study by Faust et al. did not result in detectable concentrations of activated protein C. Low expression rates of thrombomodulin and protein C receptor on the intact vascular endothelium in skin biopsy samples of patients with meningococcal sepsis compared with normal controls might explain this finding, since low levels of activator molecules result in impaired activation of protein C. In-vitro studies using plasma spiked with activated protein C showed recovery of activated protein C function, which ruled out the presence of an inhibitory factor in the plasma.(49) Impairment of the protein C activation in patients with meningococcal sepsis results in a procoagulant state and formation of thrombotic lesions. On the other hand, a placebo-controlled study using protein C concentrate showed activation of protein C and a dose dependent effect on coagulation parameters.(50, 51) A third effect of (activated) protein C is the down regulation of the inflammatory response: decreased production of cytokines and decreased adherence of leukocytes to the endothelial wall, which makes this molecule extremely important during the sepsis process.(52) Until now there have been no studies investigating the role of protein C, thrombomodulin and thrombin polymorphisms in meningococcal disease, although the clinical importance of these molecules does warrant further investigation.

Cytokines

TNF-α. TNF-α has a central role in the activation of the inflammatory response, and concentrations are raised in all patients with meningococcal disease. TNF-α activates adhesion promoting receptors and ligands enabling leukocytes to cross the endothelium and reach the site of inflammation. Other inflammatory mediators such as INF-γ, IL-6 and IL-8 are activated by TNF-α. A procoagulant state is promoted by the induction of tissue factor release. TNF-α blood levels are positively associated with disease severity, coagulopathies and outcome.(53) Westendorp et al. have found higher TNF-α response in ex vivo blood samples after stimulation with endotoxin in patients who had experienced a moderately severe disease course compared to patients with a mild course. The TNF-α response was low again in survivors of fulminant disease. Similar experiments using blood from monozygotic twins showed that a 60% heritability for TNF-α production.(54) On admission for meningococcal disease, patients who did not survive had initial TNF-α concentrations three times higher than those in survivors with a clinical disease presentation of similar severity.(55)

The G-308A polymorphism in the *TNFA* promoter region has shown to be associated with outcome of meningococcal disease. The *TNF2* allele (-308A) is associated with an increased risk of death in children.(56) Despite sufficient power this could not be confirmed in a separate older patient group.(57) Since the mortality risk in meningococcal disease is significantly affected by age, this might explain the reported differences between the two studies.(58) No association has been reported for the G-308A polymorphism and susceptibility of meningococcal disease.(56) Opinions differ on the role of this polymorphism and the severity of disease.(54, 56, 59, 60) The G-308A and the G-238A polymorphisms did not show a relation with TNF production capacity.(54) Huizinga et al. reported that on ex-vivo simulation of whole blood with endotoxin individuals with the -238 G/A genotype had a significantly lower TNF-α production compared to donors with the -238 G/G genotype. No differences were found in TNF-α production for the different -308 and -376 promoter genotypes. (61) In a study performed by Uglialoro et al., who identified three additional SNPs at positions -574(G/A), -856(C/T) and -862(C/A), no relation was seen between these and formerly described SNPs and TNF-α production.(62) These results were obtained using cloned promoters and a reporter gene. In each construct only one of the rare variants was present and other polymorphic sites were represented by the common allele. Unfortunately, the possible cumulative effect of SNPs on the TNF-α production was not accounted for.(63, 64) It is clear that TNF-α production is affected by genetic factors in meningococcal sepsis, but the relative importance of the known polymorphisms is still under discussion.

Interleukin 1 family. The interleukin 1 family consists of both pro-inflammatory and counter-inflammatory members. The genes *IL1A* and *IL1B* encode the pro-inflammatory proteins IL-1α and IL-1β, respectively. The IL-1 receptor antagonist (IL-1RA) represents the anti-inflammatory component. IL-1RA can bind the IL-1 receptor without inducing signal transduction. Several polymorphisms are known in the IL-1 family. Five alleles of *IL1RN*, the gene coding IL-1RA, are known to differ by a variable number repeat of 86 bp in intron 2.(65) This repeat contains transcription factor binding sites. The *IL1RNA2* allele has two repeats and is in linkage disequilibrium with SNP T8006, also known as T2018C.(57, 66) The A2 allele has shown to be associated with susceptibility to severe sepsis in patients in a surgical intensive care unit.(63) Carrol et al. have shown no significant correlation between the presence of the A2 allele and disease susceptibility, severity, or risk of dying in 144 meningococcal infection cases in a tertiary children's hospital. In 70 % of cases the diagnosis was confirmed microbiologically. However, a trend suggesting less severe diseases in the presence of the A2 allele was reported.(67)

Read et al. have investigated whether variants of the IL-1 and TNF gene families are associated with severe manifestations of meningococcal infection. All patients included had microbiologically proven *N. meningitidis* infection. A significant association has been seen between the outcome and the *IL1B* C-511T polymorphism. Patients homozygous for either the common or the rare allele had increased odds ratios for death, compared to heterozygous individuals. The combination of heterozygosity of *IL1B* -511 together with homozygosity of the common allele of *IL1RN* at +2018 was

significantly associated with survival. This implies an interaction between the *IL1B* -511 and *IL1RN* 2018 polymorphisms. In this study no association between the *TNF* -308 genotype and fatal outcome has been shown.(57) The relationship between allelic variants of the IL-1 gene cluster and meningococcal disease has been confirmed in a second and larger study by Read et al. in England and Wales. All patients in this study had microbiologically confirmed meningococcal infection. Patients carrying the common allele at the *IL1B* -511 site were more likely to survive. Patients who carried the rare allele at *IL1RN* 2018 in addition to the common *IL1B* allele were less likely to survive than those who did not carry the rare allele. This association was still present after correction for effects of age, infecting serogroup and socio-economic status. No difference was found in genotype frequencies between patients and healthy controls.(58) These data suggest that the IL-1 genotype influences the outcome of meningococcal disease.

Interleukin 6. Interleukin 6 (IL-6) is a major pyrogen and is responsible for the induction of hepatic acute phase proteins and antibody production by B cells. IL-6 blood concentrations are increased in meningococcal infection and IL-6 concentrations were seen to be significantly higher in non-survivors compared with survivors.(44) The G-174C polymorphism in the promoter region of *IL6* is associated with poor outcome in meningococcal infection. Carriers of the G allele had an increased risk of death (OR 3.06).(68) In a second study, in which the role of the G-174C polymorphism in sepsis caused by different bacteria other than *N. meningitidis*, in patients admitted to a surgical ICU was investigated, the reverse relation was observed: the G/G genotype was associated with improved survival, while the non-survivors did have significantly higher IL-6 plasma concentration. IL-6 concentrations showed no correlation with the G-174C genotype. Again no difference was seen between genotype distributions between patients with and without sepsis and between patients and healthy controls, suggesting the lack of association between the G-174C SNP and susceptibility to sepsis.(69) The contradictory results regarding the outcome of sepsis might be explained by the fact that this polymorphism is part of a complex haplotype associated with differences in IL-6 production. The -579G, -572G, -373A9/T11, -174G haplotype shows higher transcription of IL-6 in an ECV304 cell line after IL-1 induction than the other polymorphism combinations. This study clearly shows that different polymorphisms can have an influence on transcription, but they are not functioning independently.(70) When assessing the contribution of the G-174C polymorphism in meningococcal disease, the complete haplotype should be considered. Another important matter in studying the relation of IL-6 levels and genotypes is the timeframe between onset of endotoxemia and measurement of IL-6 concentrations. Il-6 and other cytokines have been shown to reach a peak level at different time points after controlled injection with endotoxin before concentrations decline. Depending on the time of sampling one will find different IL-6 concentrations, which makes the identification of a possible relation between genotype and cytokine concentration very difficult in the non-controlled setting.(71) On the other hand, IL-6 is one of the few cytokines, which remains elevated in non-survivors in a murine sepsis model.(72) Furthermore IL-6 seems to be directly related to myocardial dysfunction,

which makes IL-6 an important target for research.

Interleukin 10. IL-10 is an important cytokine, particularly known for its anti-inflammatory action. It is produced by cells of the monocyte/macrophage lineage. It deactivates macrophages and inhibits activation of Th1 cells and expression of IFN-γ.

The pro-inflammatory TNF is also inhibited by IL-10. To estimate the heritability of IL-10 and TNF production a study in first-degree relatives of survivors and non-survivors of meningococcal disease has been undertaken. Basal levels of neither IL-10 or TNF production differed between relatives of survivors and non-survivors. However, after ex-vivo stimulation of whole-blood samples with *Escherichia coli* endotoxin, the IL-10 production in relatives of non-survivors was twofold higher than in relatives of survivors, while the reverse was seen for TNF production. Families with high IL-10 production had a 20-fold increased risk for fatal disease.(54) Additionally, in a twin study the heritability of IL-10 in monozygotic twins was found to be 75%.(54) In this study no data are available on *IL10* polymorphisms. Two CA-repeat microsatellites, IL-10.R and IL-10.G located at -4 kb and -1.2 kb upstream from the IL-10 transcription start site, are correlated with IL-10 production levels upon induction with LPS. IL-10.R3 was associated with low IL-10 production, while the IL-10.R2/IL-10.G14 haplotype was associated with the highest secretion levels.(73) Additional polymorphisms in the promoter region, A-3715T, T-3575A, G-2849A, A-2776G, C-2763A, C-2100A, G-2050A, G-1330A, G-1082A, C-819T and C-592A, have also been identified.(74, 75) The -3575, -2849, -2763 haplotype A-[G/A]-A was seen more often in low IL-10 producers, while the T-G-C haplotype was significantly associated with high IL-10 production. The high IL-10 production phenotype in the individuals carrying the distal TGC haplotype was independent of the proximal haplotype. In black Americans a significant difference between normal donors and SLE patients at -2763 was reported. The lack of effect from the -3537 SNP in this population might be explained by the different allele frequencies observed between black Americans and whites. The -3537T allele frequency was significantly higher in the first group.(75) In first-degree relatives of survivors and non-survivors of meningococcal disease Van der Pol et al. have found a non-random distribution of *IL10* -1082 genotype in combination with the FcγRIIa, compared with a healthy control population in which the combination was randomly distributed.(34) No data concerning the distribution of the *IL10* genotype in relatives of survivors versus non-survivors has been discussed. Hence, the role of *IL10* polymorphisms in meningococcal disease needs further investigation.

Discussion

Meningococcal disease comprises a complex pathophysiology resulting in a spectrum of disease presentation in affected individuals. The contribution of host genetic factors to the risk of meningococcal disease as determined in a sibling study by Haralambous et al. was suggested to be one-third of the total risk.(5)

Defective serum bactericidal activity has an important role in susceptibility to meningococcal infection. This is illustrated by the recurrent infections in complement-deficient individuals. The presence of specific antibodies, however, is of even more importance in conferring resistance to infection. Increase of antibody titers upon nasopharyngeal colonization with meningococci has been demonstrated.(76) A study in military recruits revealed that all individuals that developed meningococcal disease in their training period showed selective deficiency of antibodies to the causative agent in base line sera drawn at the time of enrollement in the army. These individuals were capable of initiating an immune response to meningococcal antigens as shown by the increase in antibody titers in convalescent sera. The increase in antibody titer was accompanied by a rise in bactericidal activity of the serum samples.(77) Absence of serotype-specific antibodies in recurrent meningococcal infection has been reported. (78) The fact that prevalence of meningococcal C disease declines after major vaccination campaigns in different European countries supports the importance of bacterial antibodies in susceptibility to disease.(79, 80)

Given the complex nature of the disease, assessing the contribution of host factors in infection requires a strict definition of the patient population. Patients with meningococcal meningitis might have different “susceptibility genotypes” than septic shock patients, since different pathophysiologic pathways are activated. Combining these study populations is therefore not advisable. Unfortunately, severity of disease is defined in different ways by distinct research groups. Also different subgroups are defined. This may in part explain the observed differences between studies analysing similar polymorphisms. In the analysis of the role of Fcγ receptors, for instance, Domingo et al. have excluded patients with complement deficiency.(35) This was not done by Van der Pol et al. and, hence, might have contributed to the differences observed between the two studies.(34) Within countries this problem is usually addressed by the introduction of severity scores like the Rotterdam score and the PRISM score for paediatric patients in the Netherlands and the United Kingdom, respectively.(81, 82) Although these scores do not differ essentially, subtle differences in clinical definitions (e.g. septic shock criteria) do occur. The same problems occur when comparing patients with sepsis of unknown microbiologic origin. Host response to Gram-positive microbes might differ from the response towards Gram-negative bacteria.(6). It must be noted, however, that in approximately 10% of the cases meningococcal infection cannot be proven with microbiological techniques, while the clinical presentation is typical.

In addition, it might be difficult to obtain patient groups of sufficient numbers to show an association between disease susceptibility, severity and outcome when investigating genotypes with extremely low allele frequencies. Concerning associations with disease susceptibility it is important to define an appropriate control cohort. The individuals of this cohort should be from the same geographic region as the patient cohort, to avoid confounding effects such as different disease prevalence, and socio economic status of the populations. While selecting the healthy controls it is advisable to obtain information on ethnical background, although we are aware that this is not always possible due to local guidelines of medical ethical committees. Ethnic

differences in the study population might interfere with the analysis of supposed associations. Some investigators have demonstrated that the contribution of ethnic differences to the variations in the human genome is limited to about 10% (between different continents), while the variation within a sample of individuals from the same region contributes 84.4% to the total variation.(83) However, it is also known that allele frequencies of certain polymorphisms show a different distribution among populations with distinct ethnical backgrounds.(11, 19, 75) Preferably, a control population with the same genetic and environmental background, which has also been exposed to meningococcal species, is considered. Studies including relatives of patients as controls, might overcome this problem. In such studies the reported and expected inheritance of the different alleles is compared. An issue that is not addressed in all studies is Hardy Weinberg Equilibrium (HWE). In the general population the genotypes should be in HWE. This means that the observed genotype frequencies are in accordance with those expected in Mendelian inheritance. If HWE is not reached this means that one of the genotypes (1/1, 1/2, or 2/2) is over- or underrepresented in comparison with the other genotypes. If this happens several problems might have raised. First, the selection of the control population is biased, giving a deviation of the expected genotype frequencies. Secondly, the disequilibrium may reveal a technical problem in the genotyping analysis in which one of the alleles is preferentially observed, skewing the distribution. Third, it is a coincidence, which is more likely to occur in small control populations.

Besides differences in host genetics, one also should consider confounders of microbial differences. Read et al. have found that patients infected with serogroup C meningococci were more likely to die of infection than those infected with other serotypes.(58) Such an event should be considered in a regression model. Unfortunately, this correction has not been implemented in all studies. In some studies this was not possible due to inclusion of patients with microbiologically unconfirmed meningococcal infection.

Another focus of investigation is the interaction of the polymorphisms and the cumulative effect on the course of meningococcal disease. These interactions might be either synergistic or counteracting. In the IL-1 family synergistic interactions have been observed for *IL1B* -511 and *IL1RN* 2018.(70) If SNP interactions are present a single polymorphism might explain only part of the differences observed, while in combination with an, or more than one, additional polymorphism, the relative contribution might be different. This might also be expected for polymorphisms in *PAI1* and *TPA*. The higher levels of PAI-1 in individuals homozygous for the 4G allele will lead to increased inhibition of t-PA. This results in a net prothrombotic state, which would also be expected from a low t-PA productive allele. Although an association has been found between the *PAI1* polymorphism and meningococcal disease, no relation was found for the *TPA* insertion-deletion polymorphism and the disease.(40, 45-47) The functional consequences for the *TPA* polymorphism are not clear and lack of association might indicate that the insertion deletion polymorphism is not involved in t-PA production. On the other hand, the *PAI1* 4G/5G polymorphism might interact with the *TPA* polymorphism and since no correction was made for this polymorphism

with known interaction with meningococcal disease, an association with *TPA* can be missed. However, the increase in PAI-1 concentrations in septic shock is much more extensive than the slight increase in t-PA, possibly overruling the effect of relatively small differences in t-PA concentrations. By combining multiple genes in the coagulation and fibrinolysis pathway an effect on disease severity can be estimated. Carriers of FV^{L}, who also have the alleles resulting in the combination of increased PAI-1 levels and decreased protein C, t-PA, endothelial protein C receptor, thrombin and thrombomodulin concentrations, will have the highest risk of developing vascular complications in meningococcal disease. Large patient cohorts are needed to ensure sufficient power for such interaction analyses. When polymorphisms are located on the same chromosome it is important to consider linkage disequilibrium. Linkage disequilibrium addresses the allelic association of pairs of polymorphic loci. If linkage disequilibrium is present this means that the alleles are not independently inherited, and it should be corrected for. Additionally, one must always consider the possibility that disease is not linked to the studied gene, but to another gene in linkage with the first.

When an association is found it is important to look at the clinical relevance of the association. Is the polymorphism associated with susceptibility to or severity of meningococcal disease? What is the contribution of the polymorphism and might it affect therapy? Polymorphisms associated with susceptibility only will not likely contribute to changes in therapeutic strategy once an individual has contracted the disease, but may influence vaccination strategies. Was the association seen in one study or was it seen repeatedly in unrelated patient cohorts? This fact is of special interest, since conflicting results have been found for some of the polymorphic loci. On the other hand, publication bias can also occur. It can be assumed that studies finding no association for a given polymorphism are less likely to be published.

Since the prevalence of meningococcal disease in individual medical centres is low and large patient cohorts are required to study the relevance of multiple polymorphisms in a reliable way, we advocate collaborative efforts between centres to increase statistical power. Data regarding the causative microbes, age and other possible confounders should be included in a regression analysis to obtain the ”true” relevance of the studied genetic variations. Reported associations should be confirmed in an independent patient cohort. The development of high-throughput molecular genetic techniques is of great importance in our efforts to unravel the immense complexity of the presumed genetic interactions.

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Chapter 2.2

Improved survival of children with sepsis and purpura: Effects of age, gender and era

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Abstract

Objective. To gain insight into factors that might affect results of future case-control studies, we performed an analysis of children with sepsis and purpura admitted to the Paediatric Intensive Care Unit (PICU) of Erasmus MC-Sophia Children's Hospital (Rotterdam, the Netherlands).
Patients and methods. Between 1988 and 2006, all 287 children consecutively admitted with sepsis and purpura were included in various sepsis studies. Data regarding age, gender, ethnicity, serogroup of *Neisseria meningitidis,* severity, therapy and survival were collected prospectively. These data were pooled into one database and analyzed retrospectively.
Results. The case fatality rate (CFR) from sepsis and purpura was 15.7%. During the study period survival improved significantly. Younger age was significantly associated with more severe disease and a higher CFR. Children under the median age of 3.0 years had an increased risk of case fatality (odds ratio 4.3, 95% CI 2.1-9.2; p<0.001). Gender was not associated with CFR. However, males did have higher Paediatric Risk of Mortality scores, fewer PICU-free days and more presence of shock. The course of sepsis and purpura was not related to ethnic origin. A causative organism was isolated in 84.3%. *N. meningitidis* was the major organism (97.5%). Although *N. meningitidis* serogroup B was observed more often in younger children, serogroups were not associated with severity or survival. During the study period, the use of inotropic agents and corticosteroids changed substantially (less dopamine and more dobutamine, norepinephrine and corticosteroids).
Conclusions. Age and gender are determinants of severity of paediatric sepsis and purpura. Survival rates have improved during the last two decades.

Introduction

Sepsis and purpura in children is a clinically distinct disease entity caused by high concentrations of microbes and their products. Since the introduction of a vaccine against *Haemophilus influenzae* type b more than 90% of the cases of sepsis and purpura in the western world are caused by *Neisseria meningitidis.*(1-3) The resulting disease entity is referred to as meningococcal sepsis.
Meningococcal sepsis in children develops when the initial host response to the infection becomes inappropriately amplified and dysregulated. Clinically, the onset is often insidious. After the development of the first petechiae, the patient rapidly deteriorates and may subsequently develop shock, disseminated intravascular coagulation (DIC) and ultimately organ failure. The severity of these symptoms requires immediate therapy.(4, 5) Despite recent advances in therapy, the case fatality rate (CFR) remains high and ranges from 4 to 40%.(1, 6-8) The incidence of disease is highest among young children (age 0 to 4 years) and adolescents.(1-3) In the Netherlands meningococcal sepsis occurs in 4.5 per 100,000 inhabitants

(2001). Due to the sudden increase in the incidence of meningococcal disease in 2001 a national vaccination campaign against serogroup C meningococci (2002) was implemented among children from 1 to 18 years of age.(9, 10)
In recent years, many studies have focused on the elucidation of the pathogenesis of sepsis. However, much about the epidemiology of sepsis in children is still unknown. In this paper, we seek to describe the epidemiology of sepsis and purpura in children referred to the Paediatric Intensive Care Unit (PICU) of Erasmus MC-Sophia Children's Hospital in Rotterdam, the Netherlands. The aim of this study was to analyze the variation in severity and survival of children with respect to age, gender, ethnicity and serogroup of *N. meningitidis*.

Methods

The study was conducted in accordance with the Declaration of Helsinki. Permission for the study was obtained from the medical ethics committee of Erasmus MC.
Participants. All children admitted with sepsis and purpura (and/or petechiae) to the PICU of the Erasmus MC-Sophia Children's Hospital since 1988 were included. A vast majority of the children were previously included in Rotterdam-based sepsis studies.(11-16) Data regarding the remaining children with sepsis and purpura were derived from PICU admission records. Informed consent was obtained from parents or legal guardians of all children that were included in this study. Children were considered to have sepsis when they presented with tachycardia, tachypnea and a body temperature of less than 36°C or greater than 38.5°C (rectal).(17) Prospective data on all children were collected at various time points in the course of the disease. Both laboratory parameters and disease severity scoring systems, like Paediatric Risk of Mortality (PRISM) score and predicted death rate (PDR) based on the Rotterdam score, were selected as markers of severity of disease.(18-20) Additionally, presence of DIC and presence of shock were recorded as markers of severity.(17, 19, 21) The number of PICU-free days was determined on day 28 after admission using the date of admission and the date of discharge. A non-survivor had 0 PICU free days.
All laboratory parameters, obtained at baseline from an arterial blood sample, were collected within 4 hours after admission to the PICU.
Ethnicity was determined by checking patient information, and if not specified, first and surname were checked and ethnicity was determined by means of the combined name method.(22) Ethnicity was categorized into Dutch Caucasian, Turkish, Moroccan, Hindustani, African descent and other.
Serogrouping of *N. meningitidis* isolates was performed at the Netherlands Reference Laboratory for Bacterial Meningitis Amsterdam using immunodiffusion with polyclonal antisera.(23)
Statistical analyses. Retrospectively, severity and survival of children with sepsis and purpura with respect to age, gender, causative organism and ethnicity was analyzed using SPSS 11.01 (SPSS Inc., Chicago, IL, USA). Clinical and laboratory parameters were included in the analysis only if they were determined in at least 90%

of all children.
Mann-Whitney *U* test, Student's *t* test, χ^2 test and Spearman correlation (r_s) were used when appropriate. When necessary, variables were log-transformed to obtain an approximately normal distribution. For these variables, geometric mean values and their 95% confidence intervals (CI) are depicted in text and tables. *P* values of less than or equal to 0.05 were considered statistically significant.

Results

Between August 1988 and June 2006, 287 children with sepsis and purpura were admitted to the PICU of the Erasmus MC-Sophia Children's Hospital. The overall CFR was 15.7% (45 children died). Median age at admission was 3.0 years (range 0.1-17.9 years) (Figure 1).

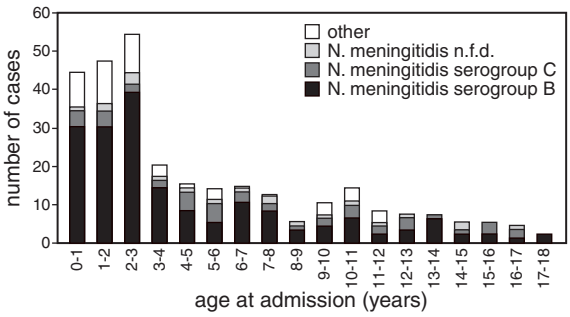

Figure 1. Distribution of age at admission in children with sepsis and purpura. The children are subdivided according to causative organism. N.f.d., not further defined.

Of the 287 children, 155 (54%) were male and 132 (46%) were female. The male-to-female ratio was 1.2. The majority of the children were Dutch Caucasians (73.8%). Laboratory parameters present at baseline in more than 90% of the children were base excess, lactate, C-reactive protein (CRP), fibrinogen, platelet count, leukocytes, and glucose.
Survival. Severity of illness was significantly less in survivors when compared to non-survivors, both in disease severity scoring systems and laboratory parameters (Table 1). Survival was significantly correlated with year of admission (p=<0.05, r_s 0.128), indicating that survival has improved significantly during the study period (Figure 2). Gender did not differ between survivors and non-survivors (p=0.15). The vast majority of fatal cases died of refractory septic shock (75.6%).
Age. Age was significantly correlated with PRISM-score (p<0.001, r_s -0.317), PDR (p<0.001, r_s -0.321), presence of DIC (p<0.001, r_s -0.245), base excess (p<0.001, r_s 0.313), CRP (p<0.05, r_s 0.161), fibrinogen (p<0.001, r_s 0.301), leukocyte count (p<0.001, r_s 0.284), thrombocyte count (p<0.01, r_s 0.184) and glucose levels (p<0.001, r_s 0.296).

Table 1. Comparison of disease characteristics between non-survivors and survivors.

		Survivors[1]	Non-survivors
Total number of children (%)		242	45
		(84.3)	(15.7)
Male-to-female ratio		1.1	1.7
Number of children with DIC (%)		174[2]	32[2]
		(75)	(97)
***N. meningitidis* serogroup**	**B (%)**	147 (74.2)	28 (73.7)
	C (%)	37 (18.7)	7 (18.4)
PRISM score		14[3]	23[3]
		(1 - 37)	(8 - 44)
Predicted death rate (%)[4]		3.1[3]	87.4[3]
		(0 - 100)	(1.1 - 100.0)
Base excess (mmol/L)		-7[3]	-13[3]
		(-23 - 4.4)	(-28 - 0.6)
Lactate (mmol/L)		3.7[3]	6.6[c]
geometric mean (95% CI)		(3.4 - 4.3)	(5.8 - 7.4)
C-reactive protein (mg/L)		106[3]	53[3]
		(10 - 334)	(6 - 226)
Fibrinogen (g/L)		2.8[3]	0.9[3]
		(0.3 - 6.8)	(0.2 - 5.4)
Platelet count (x10^3/µL)		126[3]	47[3]
		(15 - 475)	(13 - 202)
Leukocytes (x10^3/µL)		10.6[3]	4.7[3]
geometric mean (95% CI)		(9.5 - 11.9)	(3.7 - 6.0)
Glucose (mmol/L)		6.3[3]	4.3[3]
geometric mean (95% CI)		(5.9 - 6.8)	(3.6 - 5.3)

[1]Results represent median (min-max) unless stated otherwise
[2]p<0.01.
[3]p<0.001.
[4]Predicted death rate was based on the Rotterdam score. CI, confidence interval; DIC, Disseminated Intravascular Coagulation; PRISM, Paediatric Risk of Mortality.

This indicates that younger children had higher PRISM scores, higher PDR, more presence of DIC, lower base excess, lower CRP, lower fibrinogen, lower leukocyte count, lower thrombocyte count and lower glucose levels on admission.

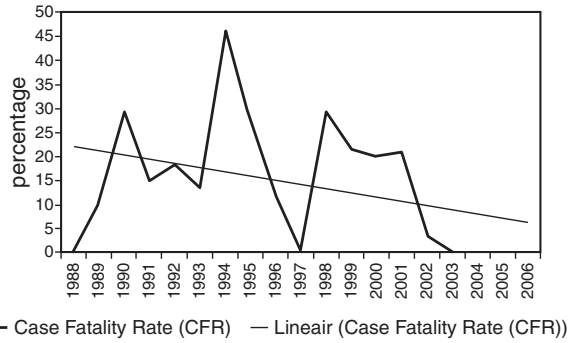

Figure 2. Case fatality rate (CFR) and CFR trend line during the study period.

Median age of children was 3.0 years (range 0.1-17.9 years). Children 3.0 years old or younger had a higher CFR (odds ratio 4.3; 95% CI 2.1-9.2, p<0.001) (Figure 3).

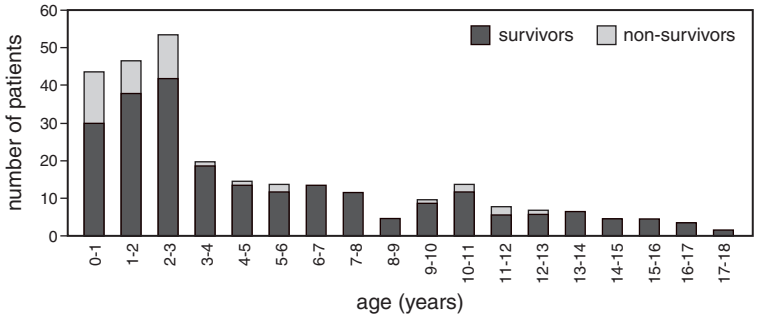

Figure 3. Distribution of age at admission among survivors and non-survivors of sepsis and purpura.

Gender. The median age did not differ significantly between males (2.8 years) and females (3.5 years) (p=0.16). Male patients had significantly fewer PICU-free days (p=0.04) and higher PRISM-scores (p=0.02) than females. Shock was slightly more often present in males than in females (89% versus 80%, p=0.04). CFR and other markers of severity of disease did not differ between males and females. Since males had higher PRISM scores, but no increased CFR, we analyzed the different variables determining the PRISM score. Of these variables, only a trend for lower glucose levels in males compared to females was observed (p=0.06).

Ethnicity. The majority of the children were Dutch Caucasians (n = 211, 73.5%). Of the remaining 76 children, 12 were Turkish (4.2%), 16 were Moroccan (5.6%), 3 were Hindustani (1.1%), 7 were of African descent (2.5%), 7 were designated other (2.5%) and in 31 children ethnicity could not be determined (10.8%). No differences with respect to severity of disease or case fatality were found between the different ethnic groups.

Causative organism. A causative organism could be determined in 242 children (84.3%), with *N. meningitidis* being the major causative organism (n = 236 (97.5%),

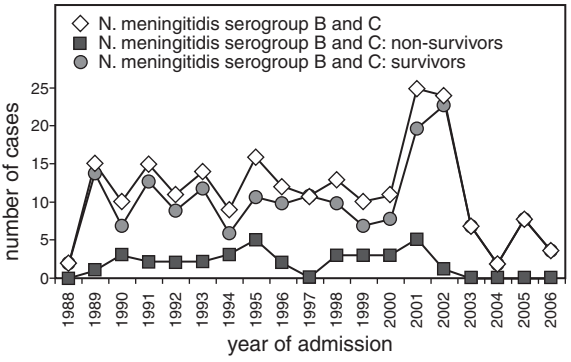

Figure 4. Number of children with sepsis and purpura due to N. meningitidis per year (since 1988), admitted to the PICU of Erasmus MC-Sophia Children's Hospital (Rotterdam, the Netherlands).

Figure 4). Of these 236, 175 (74.2%) were *N. meningitidis* serogroup B, 44 (18.6%) were serogroup C, and in 17 (7.2%) the serogroup was not determined (Tables 1 and 2). *Streptococcus pneumoniae* was the causative organism in 3 children, *Staphylococcus aureus* in 1 and *H. influenzae* in 2. Of the remaining 45 children, 43 children had clinical features of meningococcal sepsis.(3)

Table 2. Incidence of serogroup, serotype and serosubtype of N. meningitidis.

Serogroup	Serotype	Serosubtype	Number	Percentage
B	1	P1.4	4	1.8
		P1.16	4	1.8
		NT	3	1.4
		Other	1	0.5
	2A		3	1.4
	4	P1.4	57	26
		P1.6	3	1.4
		P1.7	3	1.4
		P1.9	4	1.8
		P1.10	4	1.8
		P1.15	5	2.3
		NT	28	12.8
		Other	13	5.9
	NT	P1.1	6	2.7
		P1.4	8	3.7
		NT	8	3.7
		Other	4	1.8
	Other		17	7.8
C	2A	P1.2	12	5.5
		P1.5	9	4.1
		P1.7	1	0.5
		NT	7	3.2
	2B	P1.1	1	0.5
		P1.2	7	3.2
	4	P1.4	3	1.4
	NT		2	0.9
	Other		2	0.9

NT, Non-typable.

For logistic reasons the causative organism could not be determined in 2 children. No differences with respect to survival, disease severity scoring systems and presence of shock were observed between *N. meningitidis* serogroups B and C. However, the median age of children with sepsis and purpura due to serogroup B was lower than that of the serogroup C-infected children (2.8 and 6.0 years respectively, $p<0.001$, Table 3). The distribution of serogroup, serotype and serosubtype of *N. meningitidis* in the positive cultures is depicted in Table 2.

Meningococcal C vaccination campaign and therapy. In 2001 and 2002, a sudden increase was noted in the incidence of meningococcal infection in the Netherlands. This was caused mainly by serogroup C *N. meningitidis*. The implementation of the meningococcal C vaccination campaign in July 2002 resulted in a sharp decline of

Table 3. Comparison of disease characteristics based on serogroup of N. meningitidis.

	N. meningitidis[1]	
	Serogroup B	**Serogroup C**
Total number of children	175	44
Age in years	2.8[2]	6.0[2]
	(0.1-17.9)	(0.1-16.5)
PRISM score	16	14
	(1-37)	(1-35)
Predicted death rate (%)[3]	8.9	4.9
	(0 - 100)	(0 - 100)
Number of children with DIC (%)	128	32
	(81)	(74)
Number of PICU-free days	24	25
	(0 - 28)	(0 - 27)
Base excess (mmol/L)	-8	-8.0
	(-21 - 4.4)	(-28 - 3)
Lactate (mmol/L)	4.2	3.5
geometric mean (95% CI)	(3.8 - 4.6)	(2.9 - 4.2)
C-reactive protein (mg/L)	82[4]	128[4]
	(6 - 287)	(20 - 326)
Fibrinogen (g/L)	2.4	2.8
	(0.2 - 6.8)	(0.3 - 6.6)
Platelet count (x10^3/µL)	110	113
	(15 - 475)	(13 - 336)
Leukocytes (x10^3/µL)	8.8[5]	12.2[5]
geometric mean (95% CI)	(7.6 - 10.1)	(9.9 - 15.0)
Glucose (mmol/L)	5.9	6.2
geometric mean (95% CI)	(5.4 - 6.5)	(5.5 - 6.9)

[1]Results represent median (min-max) unless stated otherwise.
[2]p<0.001.
[3]Predicted death rate was based on the Rotterdam score.
[4]p<0.01.
[5]p<0.05.
PRISM, Paediatric Risk of Mortality; DIC, Disseminated Intravascular Coagulation; PICU, Paediatric Intensive Care Unit.

the number of cases caused by serogroup C (Figure 4). Since 2003, there has not been a case of sepsis due to *N. meningitidis* serogroup C in our hospital. Parallel to this, the incidence of serogroup B has declined and is returning to the incidence level of before 1989. Before the national meningococcal C vaccination, 248 children in our study population were admitted with sepsis and purpura, whereas since the vaccination campaign 39 children have been admitted.

Remarkably, since the implementation of meningococcal C vaccination, no deaths have occurred in children with sepsis and purpura admitted to our PICU. The median age of the children did not differ significantly before and after vaccination (3.2 and 2.5 years, respectively, p=0.23, Table 4). Glucose levels were significantly lower in the patient group before compared to the patient group after the vaccination campaign (p<0.05). Children admitted before the vaccination campaign had significantly fewer PICU-free days and more presence of DIC (both p<0.05). The PRISM score was not significantly different between patients groups before and after the Meningococcal C vaccination campaign. In addition, since 2002, treatment of children with meningococcal sepsis at our PICU has changed due to implementation of international guidelines.(8) After the vaccination campaign, more children were

treated with corticosteroids (18 (9.3%) before versus 15 (42.9%) after; p<0.001) and more children were mechanically ventilated (128 (51.8%) before versus 28 (71.8%) after; p<0.05) (Table 4).

Table 4. Comparison of disease characteristics between children with sepsis and purpura before and after the national Meningococcal C vaccination campaign (July 2002).

	Before MenC vaccination[1]	After MenC vaccination
Total number of children (%)	248 (86.4)	39 (13.6)
Case fatality (%)	45[2] (18.1)	0[2] (0)
Age in years	3.2 (0.1 - 17.9)	2.5 (0.3 - 13.1)
Number of children with DIC (%)	186[3] (79.5)	20[3] (62.5)
Number of PICU-free days	24[3] (0 - 28)	25[3] (0 - 27)
PRISM	15 (1 - 44)	20 (2 - 37)
Predicted death rate (%)[4]	5.6 (0 - 100)	8.1 (0 - 100)
Base excess (mmol/L)	-7.7 (-28 - 4.4)	-8 (-18 - -2)
Lactate (mmol/L) geometric mean (95% CI)	4.1 (3.8 - 4.4)	4.0 (3.3 - 4.8)
C-reactive protein (mg/L)	93 (6 - 326)	84 (25 - 334)
Fibrinogen (g/L)	2.5 (0.2 - 6.8)	3.2 (0.3 - 6.4)
Platelet count (x10^3/µL)	110 (13 - 475)	135 (25 - 227)
Leukocytes (x10^3/µL) geometric mean (95% CI)	8.9 (7.9 - 10.0)	12.1 (9.4 - 15.6)
Glucose (mmol/L) geometric mean (95% CI)	5.7[3] (5.3 - 6.2)	7.2[3] (6.2 - 8.2)

[1]Results represent median (min-max) unless stated otherwise.
[2]p<0.01.
[3]p<0.05.
[4]Predicted death rate was based on the Rotterdam score.
DIC, Disseminated Intravascular Coagulation; PICU, Paediatric Intensive Care Unit; PRISM, Paediatric Risk of Mortality.

In addition, year of admission was significantly correlated with the use of dobutamine (p<0.001, r_s 0.262), dopamine (p<0.001, r_s -0.218), norepinephrine (p<0.001, r_s 0.329) and corticosteroids (p<0.001, r_s 0.245), but not with the use of epinephrine. This indicates that during the study period the use of dobutamine, norepinephrine and corticosteroids significantly increased in the treatment of sepsis and purpura while the use of dopamine significantly decreased (Figure 5).

Discussion

In this mono-centre cohort study of 287 children between 0 and 18 years with sepsis and purpura, we found that younger children had more severe disease and an increased risk of case fatality. The CFR of sepsis and purpura has improved in recent

years despite comparable disease severity on admission. Male patients had higher PRISM scores and fewer PICU-free days. However, the CFR did not differ between males and females. Ethnicity did not influence disease severity and survival. The serogroups of *N. meningitidis* were not related to severity or survival.

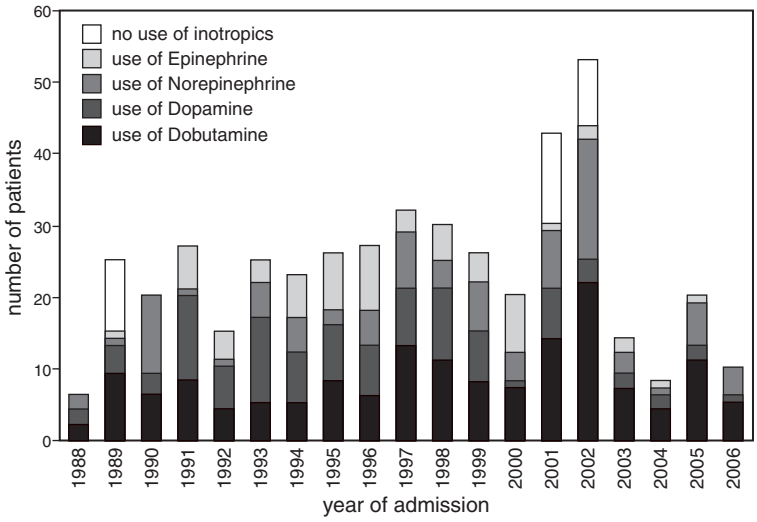

Figure 5. Use of inotropic agents during the study period 1988 to 2006. Some patients received more than one inotropic agent; therefore the number of patients in this figure exceeds the number of patients in this study (n = 287).

Children with sepsis and purpura admitted to the PICU of the Erasmus MC-Sophia Children's Hospital account for approximately 25% of all paediatric sepsis cases in the Netherlands and therefore may provide a representative sample of cases in the Netherlands. (National PICU registry, unpublished data) In addition, Rotterdam covers an area in the Netherlands (that is the southwest of the Netherlands) in which meningococcal disease used to occur frequently.

In this large cohort of paediatric sepsis and purpura, low age was significantly associated with increased severity of disease, higher incidence of DIC and increased CFR. Half of the children in our population were younger than 3 years of age. A comparison with the literature showed that incidence rates indeed drop after infancy and then increase again slightly during adolescence.(1, 9, 24) The increased CFR and the more severe disease in younger children may result from the still developing immune, coagulation and stress response systems in young children and therefore the relative inability to induce an effective immune response to a high load of micro-organisms such as *N. meningitidis*.(13, 19)

The CFR due to sepsis and purpura was 15.7% over the past two decades. This is in accordance with other large studies reporting case fatality rates of 10.4 to 20%.(7, 24-26) It must be noted that Jensen et al. and Sharip et al. studied meningococcal disease, not specifically paediatric sepsis and purpura.(7, 24)

N. meningitidis was the causative organism of sepsis and purpura in the vast majority of cases. Martin et al. also found that Gram-negative bacteria were the predominant causative organisms of sepsis in the US between 1979 and 1987.(27) In our study, the incidence of disease due to serogroup B was much higher than that due to serogroup C. *N. meningitidis* and serogroup B was seen more often in younger children compared to serogroup C. No differences with respect to severity of illness scores and CFR were observed between serogroup B and C. Erickson and de Wals suggested a more severe course of serogroup C infections, indicated by increased mortality due to serogroup C (14%) compared with serogroup B (7%).(28) Spanjaard et al. found a CFR in meningococcal sepsis caused by serogroup B of 8.1% compared with 7.1% in serogroup C.(29) However, Erickson and de Wals studied both meningitis and sepsis in all culture-proven cases of *N. meningitidis*, and Spanjaard et al. studied all culture-proven cases including adults in the Netherlands, whereas we studied paediatric cases of sepsis and purpura.(28, 29)

Since the implementation of the meningococcal C vaccination in July 2002, there has not been a fatal case of sepsis and purpura in our PICU. Because severity of disease before and after the implementation did not differ between the two groups, the increased survival may have resulted from improved treatment strategies.(8) International treatment guidelines were implemented at that time, health care workers received additional training, and public awareness increased, resulting in a decreased patient delay. Furthermore, we observed a change in the choice of inotropic agents used since 2002. It must be noted that the number of children included since 2002 is low. However, these observations do warrant further research in a prospective study. Gender was not associated with CFR from sepsis and purpura, although males did have significantly more severe disease, based on the PRISM score and fewer PICU-free days, compared to females. Bindl et al. found a male-to-female ratio of 1.7 in sepsis patients aged 1 week to 8 years with severe sepsis and septic shock, whereas we observed a male-to-female ratio of 1.2.(30) However, in those cases caused by *N. meningitidis*, which is the major causative organism in our study, males and females were equally represented among non-survivors. Watson et al. and Martin et al. also found a predisposition for male gender in sepsis, but they did not specify the male-to-female ratio in sepsis caused by *N. meningitidis*.(26, 27)

Due to the small number of children in the different ethnic groups, we may not have been able to detect differences between the different ethnic groups with respect to severity or case fatality of sepsis and purpura as of yet. In addition, during the 18-year study period the dynamics of the Dutch population (especially in Rotterdam) underwent changes, which may not be reflected in this study. Rosenstein et al. proposed a predisposition for sepsis in children of African descent.(1) Sharip et al. found an age-adjusted increased risk of case fatality in individuals of African descent compared to Caucasians and other ethnic groups.(24)

A possible limitation of our study may be that the serotypes of *N. meningitidis* were not determined in all children with meningococcal sepsis. Due to the rapidly progressive nature of this disease, it is possible that we did not include a number of the most severe cases because of case fatality before admission or referral to the Erasmus MC-Sophia. On the other hand, the fact that only children with sepsis and

purpura admitted to the PICU were included, this may have resulted in a skewed representation of all children with sepsis and purpura (that is, children with relatively mild disease admitted to a general ward).

Conclusion

The CFR in this study was 15.7%. Age was the most important predictor of severity and case fatality of sepsis and purpura. Male gender was associated with higher PRISM scores and fewer PICU-free days, but no differences in CFR were seen. *N. meningitidis* was the causative organism in the vast majority of cases. No differences between *N. meningitidis* serogroup B and C with respect to disease severity scores and case fatality were observed. Ethnicity was not associated with the course of sepsis and purpura. In future studies investigating effects on severity and survival of sepsis and purpura, age and gender should be taken into account. The possible effect of different choice of inotropic agents warrants further investigation. Also, other possible differences between male and female sepsis patients should be investigated. With the changing demography in the Netherlands (especially in the Rotterdam area) differences between ethnic groups require further examination.

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Chapter 2.3

Sepsis syndrome in children: Can we do better?

M. Emonts and R. de Groot

Hot topics in infection and immunity in children, Adv Exp Med Biol. 2004;549, Chapter 10:63-70.
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Introduction

Sepsis is a significant healthcare problem both in industrialised and developing countries. In the United States the incidence of disease is 56 and 240 per 100.000 per year in children and adults respectively. In adults underlying disease is present in 83%. Approximately 50% of the children have underlying diseases. Annual total costs of sepsis in the United States are estimated at $2 billion in children and $17 billion in adults.(1, 2) The major pathogens causing severe sepsis and septic shock are *N. meningitidis*, *S. pneumoniae*, *Group A* α- *and* β- *haemolytic Streptococci* and *Staphylococcus aureus*. *Group B Streptococci* play an important role in neonatal sepsis, while *Capnocytophaga canimorsus* is occasionally reported after dog bites. Before the vaccination era, *Haemophilus influenzae* was a major cause of sepsis in infants and young children.

Pathogenesis of sepsis

The severity of sepsis is partly dependent on virulence characteristics of the pathogen. Initially the innate immune system plays a key role in the recognition of microbial molecules. Signalling through Toll-like receptors (TLRs), of which ten different types have been described, each recognizing specific molecules, results in activation of NFκB.(3, 4) NFκB is a transcription factor that induces cytokine expression and activation of immune cells from both the innate and the specific lineages. In meningococcal infection lipopolysaccharide (LPS) of the bacterial cell wall is the major mediator of TLR4 and TLR2 activation (Figure 1). In addition, LPS induces extensive activation of the complement cascade and also, signals through the recently discovered TREM-1 receptor on myeloid cells.(5) This results in the recruitment of macrophages, opsonophagocytosis and lysis of pathogens. LPS levels in patients with meningococcal sepsis are associated with severity of disease and mortality. Another important pathway in the pathophysiology of sepsis is the tissue factor mediated activation of coagulation. In combination with attenuation of fibrinolysis due to high levels of plasminogen activator inhibitor (PAI)-1, an anti-fibrinolytic mediator, this results in a net procoagulant state.
In patients with sepsis the endothelium becomes activated and apoptosis is induced. This leads to containment of infection, when only a local inflammatory response is present. However, in systemic infection generalised endothelial dysfunction develops manifesting itself by an inappropriate vascular tone, increased vascular permeability, endothelial cell apoptosis and diffuse intravascular coagulation.(6-8)

Different models explaining disease

The immune response in (meningococcal) sepsis evolves over time and is

characterised by an early and a late phase. Different models have been proposed seeking to increase the understanding of the disease process. Hotchkiss et al. delineate three patterns of immune response in patients with sepsis. Initially healthy persons with meningococcal sepsis undergo a vigorous inflammatory response followed by the secondary development of a hypoimmune state with a predominant activation of anti-inflammatory mediators. The authors also refer to another pattern of immune response mainly occurring in adults with severe underlying diseases. These patients have a diminished hyperimmune response or a predominant hypoimmune response on pathogens or their products.(7) Anti-inflammatory therapies may be detrimental in subjects with a hypoimmune response as has been suggested from work in animal models. Interestingly cytokine and endotoxin levels in patients with meningococcal sepsis decrease rapidly after the initiation of antibiotic and intensive care therapies. The hypoimmune state in these patients may be present within several hours instead of days as suggested by Hotchkiss and colleagues.

Netea et al. propose an alternative model. In the first contact with the host, microbes either induce a large amount of pro-inflammatory cytokines, leading to effective killing and recovery of the patient, or induce a poor initial cytokine response with an ineffective host defence. In the latter case, the microbial load expands greatly,

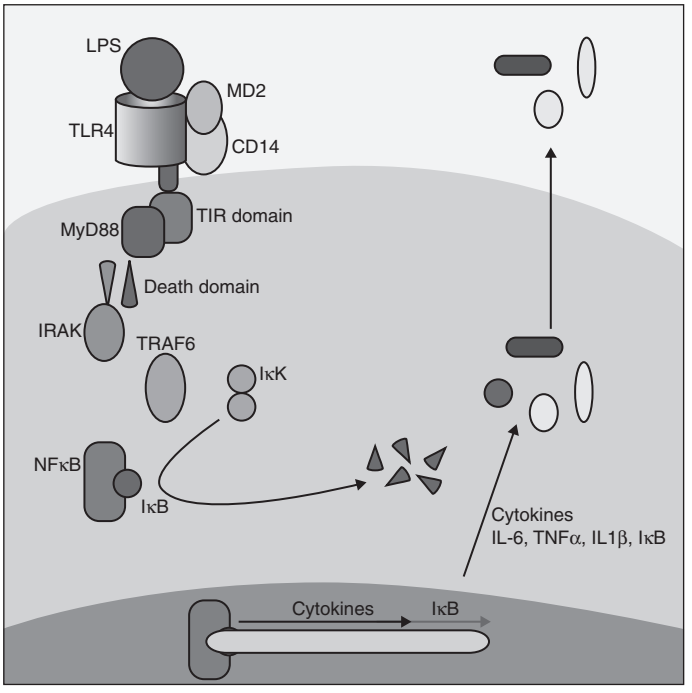

Figure 1. Activation of the TLR4 receptor pathway through interaction with LPS. Interaction of TLR4 with LPS results in the stimulation of a cascade causing activation of NFκB. NFκB is a transcription factor that is involved in the activation of transcription of numerous cytokines and IκB. IκB inhibits the function of NFκB.

leading to massive production of cytokines and septic shock. Immune paralysis and death may follow.(9) However, there are many patients especially adults who initially have a low cytokine response and who do not show an increase of host defence mechanisms at any point of time. In these patients failure to induce an immune response possibly contributes to disease.
Different mechanisms are involved in the transfer from a pro- to anti-inflammatory immune pattern. A shift from Th1 to Th2 response results in an increase of anti-inflammatory cytokines. Second, anergy, defined by a decreased Th1 function without increased Th2 cytokine production, can occur. This may be induced by apoptotic cells, which have an immunosuppressive effect. Stress mediated endogenous release of glucocorticoids may induce apoptosis. Low levels of CD4 T cells, B cells and dendritic cells have been observed in the spleen of patients with sepsis. This observation is consistent with anergy. In addition, loss of MHC class II and costimulatory molecule expression on macrophages can prevent appropriate antigen presentation resulting in an impaired immune response.(7)

The central role of protein C

The severe deficiency of protein C is a critical component in the development of thrombosis in patients with purpura fulminans. Protein C is an anticoagulant that is normally present in an inactive form. For activation, thrombomodulin, thrombin and the endothelial protein C receptor are required. The serum levels of these factors are significantly decreased in children with sepsis. After activation, protein C needs its cofactor protein S to conduct its anticoagulatory function, through inhibition of factor Va and factor VIIIa. Plasminogen activator inhibitor 1 (PAI-1) inhibits fibrinolysis through inhibition of plasmin formation. Inhibition of PAI-1 by protein C therefore results in increased fibrinolysis.
Activated protein C also decreases inflammation by inhibition of thrombin generation and the reduction of apoptosis (Figure 2).(7) Children with meningococcal sepsis have extremely low protein C concentrations on hospital admission, which are associated with an increased risk for morbidity and mortality.
Bernard et al. examined the efficacy and safety of recombinant human activated protein C (APC) in the treatment of severe sepsis in 1690 adults. Patients at high risk for bleeding were excluded. Patients received either a placebo or APC at 24 µg per kilogram of body weight per hour for a duration of 96 h. The investigators observed a significant decrease in mortality in the APC treated group versus the placebo group (30.8 to 24.7%). The risk of bleeding was slightly higher in the APC treated patient group (3.5% vs. 2.0%, p=0.06).(10)
Faust et al. investigated the activation process of protein C in children with meningococcal septic shock using plasma and skin biopsy samples. They found a lower expression of the endothelial protein C receptor and the endothelial thrombomodulin in patients with meningococcal sepsis than in controls, both in vessels with and in vessels without thrombosis. Plasma levels of thrombomodulin were increased while

plasma levels of protein C antigen, protein S antigen, and antithrombin antigen were lower in patients compared to controls. Administration of protein C to two patients did not lead to increased APC levels.(11)

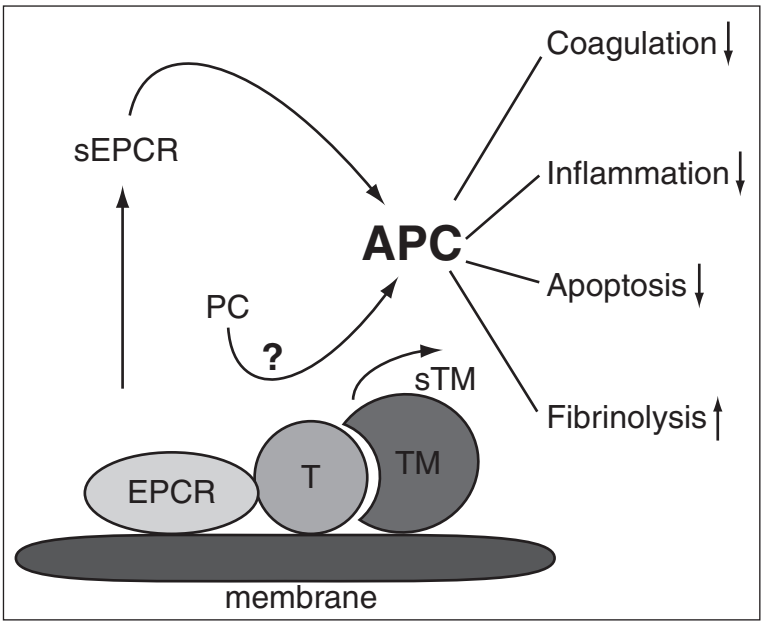

Figure 2. Actions of Protein C. EPCR, Endothelial protein C receptor; T, Thrombin; TM, Thrombomodulin; PC, protein C; APC, activated protein C; PS, protein S. Activated protein C has several functions. First, it decreases coagulation through inhibition of fVa and fVIIIa. Protein C also decreases inflammation and apoptosis. A fourth effect of activated protein C is the increase of fibrinolysis through inhibition by PAI-1.

In contrast, in a phase II/III study including 38 children in three treatment groups and one placebo group, de Kleijn et al. did find activation of protein C after administration of protein C concentrate.
The dosage levels used in the three treatment groups were 50, 100 and 150 IU/kg respectively. The treatment regimen was 3 days, 4 times daily or 4 days, 2 times daily in a bolus injection. Protein C levels were measured at different time points before and after infusion. Initially a dose-dependent increase in protein C was observed followed by a decline to 0.5-1 U protein C per ml in 6 hours. APC levels increased upon infusion of protein C concentrate. Additionally a dose dependent effect on coagulation parameters was observed.(12)
The data generated in adults and in children all point to a crucial role of APC in the pathogenesis of (meningococcal) sepsis and justify further phase 3 prospective randomised studies with PC or APC in children with meningococcal sepsis.

Anti-inflammatory and other therapies in patients with severe sepsis

In addition to the use of (activated) protein C many other therapies have been investigated mainly in adults with sepsis (Table 1).

Table 1. Anti-inflammatory and other therapies in patients with severe sepsis.

Therapy	Effect[1]
Corticosteroids	+/-
Anti-endotoxin antibodies (HA-1A, BPI)	-
TNF-antagonists	-
IL-1 receptor antagonists	-
PAF antagonists	-
Recombinant GCSF	-
Ibuprofen	-
Activated protein C	+
TFPI, AT3	-
Insulin	+
Early therapy to treat cellular oxygen deficit	+

[1]A + indicates a positive effect of therapy in the patient group. A – indictes no effect of therapy in the patient group. A +/- indicates conflicting results between different studies.

Physiologic dosages of corticosteroids have been administered to critically ill patients with a positive result, but careful patient selection has to take place to identify those who are most likely to benefit.(13) High doses of corticosteroids do not improve survival in sepsis patients and may even result in secondary infections. Intensive insulin therapy has recently been shown to improve survival and lower morbidity in critically ill adults.(14) The mechanism of the protective function of insulin may be the recovery of the phagocytic function of neutrophils which is impaired due to hyperglycaemia. Another potential mechanism is the anti-apoptotic effect of insulin. Anti-TNF-α antibodies have been successful in animal sepsis models in which TNF-α was used to induce sepsis. In adults and children with sepsis there was no beneficial effect. Levin et al. studied the effect of $rBPI_{21}$, a recombinant N-terminal fragment of BPI which has LPS neutralizing capacities, in a phase III, randomized clinical trial in children with severe meningococcal sepsis. Patients receiving the drug had significantly less severe complications. A trend for decreased mortality was observed, but this was not significant.(15) However, it is likely that the ability to observe potential drug efficacy was decreased because of the rapid progression of meningococcal sepsis, and delays in study drug administration imposed by transport and study procedures.

In adults, early goal-directed therapy, aiming at balancing oxygen delivery with oxygen demand, resulted in a decreased mortality (46.5 vs. 30.5%) compared to the standard treatment, in severe sepsis patients.(16)

Data from Dutch investigators show that 50% of the deaths in patients with meningococcal sepsis occur within the first 12 h after admission to a hospital or an intensive care unit, whilst 80% of all deaths occur within the first 18 h. This indicates that further improvements in the outcome of therapy can only be made in these first

hours after hospital admission. This is confirmed by the fact that improvements in initial management of patients with meningococcal disease at referring hospitals, use of mobile intensive care service, and centralization of care resulted in a decreased mortality rate from 23% in 1992/93 to 2% in 1997 in a specialist unit in the United Kingdom. Severity of disease remained the same during the decade of the study. (17)

Genetic polymorphisms and association with susceptibility, severity and outcome of meningococcal sepsis

Complement deficiencies and defects in sensing or opsonophagocytic pathways, like the rare *TLR4* single nucleotide polymorphisms (SNPs) and combinations of inefficient variants of Fcγ-receptors, appear to play a major role in genetically determined susceptibility.(18-22) Individuals encountering a pathogenic meningococcal strain for the first time, not having specific antibodies are at risk for development of a severe meningococcal infection. *FCGR2a* and *PAI1* polymorphisms clearly have a significant effect on the severity of meningococcal infections. (23-25) SNPs in *properdin*, *PAI1* and combinations of the -511C/T SNP in *IL1B* and the +2018C/T SNP in *ILRN* have also significant effects on outcome.(23, 24, 26, 27) Conflicting results were found for the effect of the -308G/A promoter polymorphism in *TNFA*.(26, 28, 29) This may reflect differences in group definitions between studies or the influence of additional SNPs in the *TNFA* promoter that, like *IL6*, can form haplotypes representing different cytokine production capacity.(30) For several SNPs, a supposed effect on susceptibility, severity or outcome of disease has not yet been independently confirmed.

PAI1 4G/5G promoter polymorphism: An example of a clinically relevant genetic marker for disease

PAI-1 is responsible for the inhibition of fibrinolysis both directly and indirectly through inhibition of t-PA. The gene encoding PAI-1 has several polymorphic loci, including a 3'-HindIII site, a CA(n) repeat in intron 3 and a 4G/5G insertion deletion polymorphism at -675 in the promoter. PAI-1 activity has shown to be significantly higher in control subjects homozygous for the 4G allele than in subjects homozygous for the 5G allele. (31) The production of PAI-1 mRNA after IL-1 stimulation appeared to be higher in HepG2 cells bearing the 4G allele.(32) In patients with meningococcal sepsis the levels of PAI-1 are related to severity of disease, outcome, cytokine levels, acute phase proteins and coagulation parameters.(33) In non-survivors PAI-1 levels are 1.9 times higher for the same TNF-α levels than in survivors.(34) TNF-α induces PAI-1 production. In children suffering from meningococcal disease a relationship has been observed between genotype, PAI-1 levels and outcome of disease. The subjects with the 4G homozygous genotype had higher PAI-1 levels and an increased risk of death. No association was observed with severity of disease.(23)

A similar study performed by Westendorp et al. showed a higher incidence of the 5G/5G genotype among relatives of patients with meningitis. Patients whose relatives were carriers of the 4G/4G genotype had a six-fold higher risk to develop septic shock than meningitis.(25) In a recent study including 510 UK paediatric patients, 210 parents and 155 UK Caucasian controls, no association was found between the 4G/5G polymorphism and susceptibility. In patients with meningococcal sepsis the relative risk of death was 2.9 for the 4G homozygous patients compared to the combined 4G/5G and homozygous 5G genotypes. In survivors the risk of developing vascular complications was significantly higher in the patients homozygous for the 4G allele.(24)

In adult patients suffering from sepsis after severe trauma an association was found between mortality and the *PAI1* 4G/5G polymorphism and polymorphisms in *IL6*, *TNFB* and *IL1B*. Patients with a combination of alleles that individually were associated with survival had the lowest mortality risk while, patients having multiple "bad outcome" alleles had increasing mortality risk, being the highest in patients having only mortality associated alleles.

These findings in combination with the data from Haralambous et al. suggest that host genetic make up contributes over one third to the susceptibility to meningococcal disease and imply that genetic studies in patients with sepsis may contribute toward improvements in research, diagnosis and care.(35)

Conclusions

The question asked in our title was "Sepsis syndrome in children: Can we do better?" And if so, how can we improve the case fatality rate (CFR) in children with sepsis?

Improved health care delivery certainly may result in a reduction of the CFR from paediatric meningococcal disease from 23% to 2% as has been shown by the infectious disease and intensive care groups in St Mary's Hospital in London.(17) Early goal-directed therapy also reduces CFR from sepsis in adults.(16) Therefore, a better selection of patients and a more rapid intervention by experimental therapies may potentially further improve the CFR. Different interventions thus far have been associated with conflicting results. One must however consider that sepsis is a heterogeneous disease in which many interlinking pathways are involved. Parallel intervention in multiple components of the inflammatory and coagulation pathways may lead to better results. Also targeting of non-redundant components such as apoptosis factors or NFκB may in the future be beneficial. However, this approach may also invoke unwanted effects due to the wide spectrum in gene activation for which NFκB is responsible. Use of genetic diagnostic tests can help to identify patients who would benefit most from expensive experimental drugs such as protein C. Finally, there is an urgent need for clinical trials in children to evaluate the use of APC or PC.

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Chapter 2.4

Polymorphisms in *PARP, IL1B, IL4, IL10, C1INH, DEFB1,* and *DEFA4* in meningococcal disease in three populations

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Abstract

Objective. The pathogenesis of meningococcal infections involves activation of the complement system, pro- and anti-inflammatory mediators, antimicrobial peptides and apoptosis. We hypothesize that variations in genes encoding these products are involved in the susceptibility to and severity of paediatric meningococcal infections.
Patients and methods. Polymorphisms in *PARP*, *C1INH*, *IL4*, *IL10* and *IL1B*, *DEFA4* and *DEFB1* were analyzed in two independent Caucasian case control cohorts from the UK and the Netherlands and in a family based TDT cohort from the UK
Results. In the UK case control cohort the *DEFB1* -44 G/G homozygous genotype was overrepresented in patients with meningococcal disease compared to the G/C and C/C genotypes when combined (OR 1.57, 95% CI 1.12-2.20). The TDT analysis did not confirm this, but did find an association and linkage of the *IL4* -524 and the *C1INH* 480 polymorphisms with susceptibility to meningococcal infection. Haematological failure was present more often in UK patients with the *DEFB1* -44 G/G genotype compared to the C allele carriers (OR 2.17, 95% CI 1.22-3.85).
Conclusions. Additional studies are necessary to elucidate the conflicting results obtained for the *DEFB1*, *IL4* and *C1INH* polymorphisms and their role in susceptibility to and severity of meningococcal disease.

Introduction

Meningococcal infection is characterized by an imbalance between pro- and anti-inflammatory processes. The hyper-inflammatory response may result in (multiple) organ dysfunction and even death. The European case fatality rate is approximately 8%.(1) Different pathways including molecules involved in microbial recognition, pro- and anti-inflammatory cytokines, the complement system, apoptosis related molecules and coagulation and fibrinolysis are involved in the immune response against meningococci. Increasing interest has developed in the role of host genetic polymorphisms in infectious diseases including meningococcal infection.(2)
Human β defensins are thought to be broad acting antimicrobial peptides that are constitutively expressed in epithelial cells.(3) In addition to antimicrobial action, chemotactic activity has been reported.(4) Since β defensin 1 expression is reported to be increased upon stimulation with lipolysaccharides (LPS) a major component of the meningococcal cell wall, genetic variation in the gene encoding β defensin 1 (*DEFB1*) may contribute to the susceptibility to meningococcal infections.(5) In addition α defensins are known for their antimicrobial action. They are expressed by neutrophils. One of these peptides, α defensin 4, was reported to also inhibit ACTH induced corticosteroid expression in rats.(6) Adrenal insufficiency is known to play a role in severe meningococcal sepsis.(7)
The complement system is important in the defence against meningococcal infections as may be illustrated by the increased susceptibility to infection in patients with

deficiencies of complement factors.(8) Over-activation of the complement cascade is prevented by the serine protease C1 inhibitor (C1-Inh), which is an important molecular regulator of the human complement system. This multifunctional protein also inhibits activity of contact system proteases, such as kallikrein and blood coagulation factor XII, and is involved in endothelial binding of leukocytes.(9-11) Recently we observed that a genetic polymorphism resulting in a valine to methionin substitution at amino acid position 480, was associated with *S. aureus* nasal carriage.(12) The role of this complement component polymorphism in meningococcal disease has not been studied.

Upon activation of the immune system a large variety of cytokines, both pro-inflammatory (TNF-α, IL-6 and IL-1β) and anti-inflammatory (IL-10, IL-4) are produced, resulting in a net pro-inflammatory action. Previously, the *IL4* -524 C allele was found to be associated with decreased transcriptional activity in reporter gene studies and carriage of the C allele is associated with a significantly lower serum concentration of IL-4.(13, 14) In addition, polymorphisms in the *IL10* promoter region have been associated with altered expression levels.(15) Apoptosis is thought to play a role in the immune and inflammatory response in sepsis.(16) Poly (ADP-ribose) polymerase (PARP) plays a role in inflammation and apoptosis and is involved in maintaining genomic stability of cells upon oxidative stress.(17) The *PARP* 762Ala variant has recently been found to be associated with reduced activity after H_2O_2 exposure.(18) We hypothesized that polymorphisms in promoter or coding regions of *DEFB1*, *DEFA4*, *C1INH1*, *IL4*, *IL10*, *IL1B* and *PARP* may be involved in meningococcal infection susceptibility and severity. We investigated this hypothesis in three independent populations from the United Kingdom (UK) and the Netherlands.

Methods

Participants. Data from three separate cohorts of patients with meningococcal disease were collected and combined.

Imperial College (IC) cohort. Children with meningococcal disease who were admitted to the St.Mary's Hospital between 1992 and 2002 were eligible for the study (n = 439; 364 Caucasian, 75 non-Caucasian). Ethical approval was obtained. Children were enrolled after informed consent was given by their parents or guardians. Patients were diagnosed with meningococcal meningitis, sepsis or a combination of sepsis and meningitis. Disease severity was measured using disease severity scoring systems including the platelet and neutrophil counts (PN) product, the revised paediatric index of mortality (PIM2) score and its derived predicted death rate, and the Glasgow meningococcal septicaemia prognostic score (GMSPS).(19-21) In addition, failure of organ functions was assessed as previously described and outcome, alive or dead, was recorded.(22) Ethnicity, gender and age at onset of disease were recorded.

Meningitis Research Foundation (MRF) cohort. UK survivors of meningococcal disease (n = 104; 101 Caucasian, 3 non-Caucasian) were enrolled via the MRF between 1996 and 1999. There was no overlap between the IC and MRF cohort.

Erasmus MC Sophia (EMC) cohort. This cohort consisted of 101 paediatric patients with meningococcal sepsis (85 Caucasian, 16 non-Caucasian) admitted to the paediatric intensive care unit (PICU) of the Erasmus MC Sophia (Rotterdam, the Netherlands) between 1988 and 2005. These patients had been enrolled in meningococcal sepsis studies.(7, 23-26) All studies have been approved by the ethical committee of the Erasmus Medical Centre and informed consent was obtained from parents or guardians. Inclusion criteria (tachycardia, tachypnea, rectal body temperature < 36°C or > 38.5°C and petechiae) were in accordance with recommendations of the International Paediatric Sepsis Consensus Conference.(22) Clinical data, severity scores (PRISM and Rotterdam score based predicted death rate) and laboratory parameters were collected at study entry and at several time-points during the course of disease, as previously defined.(27, 28) The number of PICU free days was determined on day 28 after admission.

UK Caucasian controls. Healthy Caucasian, age matched unrelated contacts of patients at the time of meningococcal disease onset were collected throughout the UK (n = 242). Controls were enrolled via both the hospital and the MRF patients. For family based transmission disequilibrium test (TDT) analysis blood for DNA extraction was also collected from parents of patients.

Dutch Caucasian controls. Caucasian controls (n = 463) were derived from the Dutch Blood bank Sanquin and included healthy adult donors from the south west region of the Netherlands.

DNA isolation and genotyping. DNA was isolated from blood samples using standard techniques (QIAamp DNA blood Mini, Midi and Maxi kits, Qiagen, Crawley, UK). Single base extension analysis was used to genotype *PARP* Val762Ala (T/C; rs1136410), *C1INH* Val480Met (G/A; rs4926), *IL4* C-524T (rs2243250), *IL10* G-1082A (rs1800896), *IL10* C-819T (rs3021097) and *IL1B* C-31T (rs1143627) (http://www.ncbi.nlm.nih.gov/SNP/). In brief, the genomic region of interest was amplified using PCR. After purification a single base extension was performed using a primer ending one nucleotide prior to the single nucleotide polymorphism (SNP) location. Up to three SNPs were analyzed in one multiplex assay. A poly-T-tail attached to the primer combined with the use of a Liz size marker served to distinguish SNPs in the multiplex analysis.

Two polymorphisms in 3' UTR region of α-defensin *(DEFA4)* A/G (rs736227) and in the promoter region of human β defensin (*DEFB1)* G-44C (rs1800972) were genotyped using Taqman analysis. Primer and probe sequences are available on request. All genotypes were annotated independently by two investigators who were blinded for the clinical data. Only when identical genotypes were assigned by the investigators was the individual included in the analysis. This resulted in a different number of individuals for the various polymorphisms analysed.

Study design and statistical analysis. Both susceptibility and severity analyses were performed. Susceptibility to meningococcal infection was assessed comparing the Caucasian IC and MRF (combined together as "UK cohort"), with the UK control samples and Caucasian EMC patients with the Dutch controls. Non-Caucasian UK samples were only included in the family based TDT. In the TDT test an additional

100 patients (26 from IC and 74 from MRF) and relatives were included of whom ethnicity was unknown, this resulted in a total of 148 nuclear families available for TDT analysis. Severity analysis was performed on the IC and EMC Caucasian cohorts separately (n= 280 and 85, respectively).

Susceptibility analyses. Three independent analyses, two case control (UK and Dutch) and a family based TDT, were carried out. Case control studies consisted of Caucasian individuals only, whereas the TDT analysis included both Caucasian and non-Caucasian families. There was no overlap in patients and controls between case control and TDT analysis. Verification of Hardy-Weinberg equilibrium of genotypes was performed using the χ^2 test (1df). The case-control analyses were performed using SPSS 11.5. In the TDT analysis the transmission rate of the separate alleles from the parents to the affected offspring was calculated in 148 nuclear families using FBAT v 1.7.3.(29) A significant increase in the transmission of an allele above the expected indicates association and linkage to meningococcal disease, and is more powerful at locating true variants that have a role in disease susceptibility than case control studies where limited numbers of cases and controls are used.

Severity analysis. In the IC cohort clinical diagnosis (sepsis, meningitis, or mixed disease), actual mortality, predicted mortality (from PIM score) and the presence of organ failure were analyzed with respect to the different polymorphic genotypes.(22) In the EMC cohort only patients admitted to the Paediatric Intensive Care Unit (PICU) requiring an arterial line were included. No analysis on differing clinical diagnosis was possible as no patients with meningitis only were included in the cohort. Predicted mortality (from the Rotterdam score), actual mortality, PRISM score, the number of PICU free days at day 28 after admission, need of ventilation, and presence of shock or disseminated intravascular coagulation were analyzed with respect to the different polymorphic genotypes. All analyses were performed using SPSS 11.5. Odds ratios were determined using logistic regression and nonparametric analyses were carried out using Kruskal-Wallis and Mann-Whitney *U* tests. *P* values smaller than 0.05 were considered significant.

Results

Comparison of UK Caucasian patients collected in the IC and MRF cohorts showed no statistical difference in the genotype distribution of the polymorphisms tested (data not shown). For this reason these two cohorts were combined for susceptibility analysis in the UK samples.

Susceptibility to meningococcal disease. Only the *DEFB1* promoter genotype distribution was significantly different between patients and controls in the UK samples (Table 1). The *DEFB1* -44 G/G genotype was overrepresented in patients compared to the G/C and C/C genotypes combined (OR 1.57, 95% CI 1.12-2.20). No significant differences between patients and controls were observed for any of the polymorphisms tested in the Dutch samples.

The TDT found no increased transmission of either allele (n = number of informative

nuclear families) of the *PARP* 762 (n = 28), *IL1B* -31 (n = 72), *IL10* -1082 (n = 62), *IL10* -819 (n = 40), *DEFA* (n = 72) and *DEFB1* (n = 69) polymorphisms, indicating no linkage or association to meningococcal disease susceptibility. However, the *IL4* -524 C allele was transmitted more often from parents to affected patients than expected (Z=2.60, p=0.009, 70 informative transmissions: C allele transmitted 57 times). In addition the *C1INH* 480 Val allele was transmitted more often from parents to affected patients than expected (Z=2.53, p=0.01, 132 informative transmissions: Val allele transmitted 101 times). These results indicate evidence for both association and linkage of the *IL4* -524 and the *C1INH* 480 polymorphisms with susceptibility to meningococcal infection.

Table 1. Analysis of SNPs in MRF and IC patient cohorts.

SNP	Caucasian UK samples Controls n (%)	Cases n (%)	p[1]	OR crude	Caucasian Dutch samples Controls n (%)	Cases n (%)	p[1]	OR crude
				Val/Val vs. Val/Ala and Ala/Ala				Val/Val vs. Val/Ala and Ala/Ala
***PARP* 762**								
Val/Val	137 (70)	278 (75)	0.41	1.29 (0.88-1.89)	299 (67)	61 (73)	0.52	1.33 (0.79-2.23)
Val/Ala	55 (28)	88 (24)			140 (31)	22 (26)		
Ala/Ala	4 (2)	5 (1)			10 (2)	1 (1)		
				C/C vs. C/T and T/T				C/C vs. C/T and T/T
***IL4* -524**								
C/C	164 (78)	283 (73)	0.31	0.75 (0.50-1.11)	339 (74)	63 (74)	0.28	0.98 (0.58-1.66)
C/T	44 (21)	99 (25)			112 (24)	18 (21)		
T/T	2 (1)	7 (2)			9 (2)	4 (5)		
***C1INH* 480**								
Val/Val	120 (56)	206 (53)	0.54	1.19 (0.61-2.35)	255 (55)	51 (60)	0.69	1.45 (0.49-4.30)
Val/Met	80 (37)	160 (41)		1.39 (0.70-2.78)	176 (38)	30 (35)		1.24 (0.41-3.77)
Met/Met	16 (7)	23 (6)		1	29 (6)	4 (5)		1
***IL1B* -31**								
C/C	22 (11)	41 (11)	0.75	1	57 (13)	10 (12)	0.84	0.88 (1.41-1.87)
C/T	94 (45)	162 (42)		0.93 (0.52-1.65)	214 (47)	37 (44)		0.86 (0.53-1.42)
T/T	92 (44)	182 (47)		1.06 (0.60-1.89)	185 (41)	37 (44)		1
***Il10* -1082**								
G/G	56 (27)	89 (24)	0.61	0.79 (0.48-1.29)	123 (27)	21 (26)	0.55	1.18 (0.59-2.34)
G/A	108 (52)	200 (53)		0.92 (0.60-1.41)	219 (48)	44 (54)		1.38 (0.76-2.53)
A/A	44 (21)	89 (24)		1	117 (26)	17 (21)		1
***IL10* -819**								
C/C	122 (61)	234 (62)	0.70	1.41 (0.63-3.16)	283 (62)	49 (62)	0.97	0.92 (0.37-2.33)
C/T	68 (34)	131 (35)		1.41 (0.62-3.24)	145 (32)	24 (30)		0.88 (0.33-2.34)
T/T	11 (6)	15 (4)		1	32 (7)	6 (8)		1
***DEFA4* 3' UTR**								
A/A	109 (49)	170 (45)	0.56	0.95 (0.56-1.61)	114 (48)	32 (49)	0.37	0.69 (0.31-1.54)
A/G	85 (38)	161 (43)		1.15 (0.67-1.98)	98 (41)	22 (34)		0.55 (0.24-1.28)
G/G	28 (13)	46 (12)		1	27 (11)	11 (17)		1
				G/G vs G/C and C/C				G/G vs G/C and C/C
***DEFB1* -44**								
G/G	132 (58)	263 (68)	0.03	1.57 (1.12-2.20)	143 (61)	35 (54)	0.55	0.73 (0.42-1.28)
G/C	82 (36)	105 (27)			81 (35)	27 (42)		
C/C	14 (6)	17 (4)			9 (4)	3 (5)		

[1]χ^2.

Severity of meningococcal disease.

UK patients (IC and MRF cohorts). A total of 280 Caucasian patients of whom 140 (50%) were male were included in the severity analysis. The median age (min-max) was 3.13 (0.08-19.83) years. Twelve (4.3%) patients died. In the UK samples the *DEFB1* -44 G/G genotype was underrepresented in non-survivors. However, this

did not reach significance (OR 0.33, 95% CI 0.10-1.09 when compared to C allele carriers) (Table 2). Haematological failure (defined as a platelet count < 80,000/mm^3, or an international normalized ratio > 2: disseminated intravascular coagulation) was present more often in patients with the *DEFB1* -44 G/G genotype compared to the C allele carriers (OR 2.17, 95% CI 1.22-3.85) (Table 3).(22) No significant differences in genotype distributions were observed between patients with and without circulatory failure or between patients with sepsis and patients with meningitis or a combination of the two (data not shown).

Table 2. Genotype distributions in survivors and non-survivors.

SNP	Caucasian UK samples Survivor n (%)	Non-survivor n (%)	p[1]	OR crude	Caucasian Dutch samples Survivor n (%)	Non-survivor n (%)	p[1]	OR crude
				Val/Val vs. Val/Ala and Ala/Ala				Val/Val vs. Val/Ala and Ala/Ala
***PARP* 762**								
Val/Val	176 (72)	9 (75)	0.90	1.14 (0.30-4.35)	54 (73)	7 (70)	0.90	0.86 (0.20-3.67)
Val/Ala	63 (26)	3 (25)			19 (26)	3 (30)		
Ala/Ala	4 (2)	0 (0)			1 (1)	0 (0)		
				C/C vs. C/T and T/T				C/C vs. C/T and T/T
***IL4* -524**								
C/C	185 (74)	10 (83)	0.75	1.73 (0.37-8.11)	57 (76)	6 (60)	0.05	0.47 (0.12-1.87)
C/T	60 (24)	2 (17)			16 21)	2 (20)		
T/T	4 (2)	0 (0)			2 (3)	2 (20)		
								Val/Val vs. Val/Met and Met/Met
***C1INH* 480**								
Val/Val	137 (55)	7 (58)	0.79	1.17 (0.36-3.77)	45 (60)	6 (60)	0.74	1.00 (0.26-3.85)
Val/Met	102 (41)	4 (33)			26 (35)	4 (40)		
Met/Met	12 (5)	1 (8)			4 (5)	0 (0)		
				C/C and C/T vs. T/T				C/C and C/T vs. T/T
***IL1B* -31**								
C/C	30 (12)	1 (8)	0.93	0.91 (0.29-2.90)	10 (14)	0 (0)	0.36	1.21 (0.31-4.64)
C/T	102 (41)	5 (42)			31 (42)	6 (60)		
T/T	120 (48)	6 (50)			33 (45)	4 (40)		
***Il10* -1082**								
G/G	68 (28)	3 (25)	0.79	1.28 (0.21-7.92)	20 (28)	1 (10)	0.44	0.23 (0.02-2.48)
G/A	120 (49)	7 (58)		1.69 (0.34-8.40)	38 (53)	6 (60)		0.74 (0.16-3.35)
A/A	58 (24)	2 (17)		1	14 (19)	3 (30)		1
				C/C vs. C/T and T/T				C/C vs. C/T and T/T
***IL10* -819**								
C/C	145 (59)	6 (50)	0.74	0.69 (0.22-2.20)	46 (67)	3 (30)	0.07	0.21 (0.05-0.91)
C/T	89 (36)	5 (42)			18 (26)	6 (60)		
T/T	11 (5)	1 (8)			5 (7)	1 (10)		
***DEFA4* 3' UTR**								
A/A	114 (47)	3 (25)	0.32	0.32 (0.05-1.99)	27 (49)	5 (50)	0.94	0.83 (0.14-5.07)
A/G	106 (43)	7 (58)		0.79 (0.16-4.06)	19 (35)	3 (30)		0.71 (0.10-5.03)
G/G	24 (10)	2 (17)		1	9 (16)	2 (20)		1
				G/G vs G/C and C/C				G/G vs G/C and C/C
***DEFB1* -44**								
G/G	171 (68)	5 (42)	0.05	0.33 (0.10-1.09)	31 (56)	4 (40)	0.38	0.52 (0.13-2.04)
G/C	70 (28)	5 (42)			21 (38)	6 (60)		
C/C	10 (4)	2 (17)			3 (7)	0 (0)		

[1] χ^2.

Dutch patients (EMC cohort). Eighty-five patients were included. The median (min-max) age was 3.12 (0.26-15.48) years and 53 (62%) were male. The *IL10* -819 C/C genotype was underrepresented in non-survivors compared to the C/T and T/T genotypes combined (OR 0.21, 95% CI 0.05-0.91) (Table 2). In contrast to the UK patients, no significant difference was observed in the incidence of disseminated intravascular coagulation between the *DEFB1* -44 genotypes (Table 3).

No significant difference was observed in either cohort between the genotypes for respiratory failure and severity scores (data not shown).

Table 3. Genotype distribution in patients with meningococcal disease with and without disseminated intravascular coagulation.

SNP	Caucasian UK patients			Caucasian Dutch patients		
	No DIC[1] n (%)	DIC n (%)	p[2]	No DIC n (%)	DIC n (%)	p[2]
***DEFB1* -44**						
G/G	37 (54)	128 (72)	0.02	12 (44)	23 (61)	0.36
G/C	29 (42)	42 (24)		14 (52)	13 (34)	
C/C	3 (4)	9 (5)		1 (4)	2 (5)	

[1]DIC disseminated intravascular coagulation.
[2]χ^2.
G/C and C/C versus G/G OR (95% CI) for UK patients and Dutch patients was 2.17 (1.22-3.85) and 1.29 (0.71-5.21), respectively.

Discussion

In this study we assessed the possible role of genetic polymorphisms in genes involved in the pathophysiology in meningococcal infection susceptibility and severity.
In the UK cohort an association was observed for the *DEFB1* -44 G/G genotype and susceptibility for meningococcal disease in a case control study. This genotype was also associated with DIC in the UK Caucasian patients. These associations could not be confirmed in the UK TDT and the Dutch studies. This may be due to insufficient informative meiosis and thus a lack of statistical power, specifically in the small Dutch population, for which the number of available genotypes is insufficient to observe an OR less than 2. In addition, the excessive transmission from parent to affected offspring of the *IL4* -542 C allele and the *C1INH* 480 Val allele were not found in the case control studies of both UK and Dutch samples. These discrepancies may be the result from either false negative or false positive findings. With regard to *DEFB1* promoter polymorphism and susceptibility of disease, the genotype distribution in the smaller Dutch cohort in fact, shows overrepresentation of the -44 G/G genotype in controls, pointing into the opposite direction. This does, however, not exclude a possible association as was postulated by Lin et al., who introduced an explanatory hypothesis for the flip-flop phenomenon, in which the other allele of a marker is observed to be the risk allele in subsequent studies, explaining reverse associations through differences in linkage disequilibrium patterns and interaction with other risk factors.(30) For the *IL4* and *C1INH* polymorphisms the cause of the discrepancy is less clear since the genotype distributions are rather similar. If an effect is present, we would expect it to be very limited. In addition, it must be noted that the *P* values presented do not include correction for multiple testing. When we corrected for this, none of the observed associations remained significant. In addition, Dutch patients all had sepsis or septic shock, while part of the UK patients presented with only meningitis, which usually is presented by somewhat milder symptoms. This may be an important confounder and have contributed to the inconsistent observations. *IL4*

polymorphisms have been associated with atopy, asthma and severe respiratory infections.(31-33) In addition, the *IL4* -524 C/C genotype was previously associated with lower *IL4* expression.(13) The role of IL-4 in systemic infection seems crucial since IL-4 deficient mice are more susceptible to shock induced with a combination of staphylococcal enterotoxin B and D-galactosamine than wild type mice.(34) In adult sepsis patients IL-4 mRNA levels were decreased when compared to patients with bacteraemia or controls.(35)

In this study we did not observe an association in any of the populations for the *PARP* 762 Val/Ala, *IL1B* -31 T/C, *DEFA4* 3'UTR A/G, and *IL10* -1082 G/A polymorphisms with regard to susceptibility to and severity of meningococcal infection. These results do, however, not imply that these genes are not at all involved in meningococcal disease. The *IL1B* -31 T/C polymorphism is known to be in linkage disequilibrium with the *IL1B* -511 C/T polymorphism that previously, in combination with polymorphisms in the IL1 receptor antagonist (*IL1RN*), has been associated with outcome of meningococcal infection.(36, 37) Read *et al* discussed the hypothesis that altered inhibition of the *IL1B* transcription due to difference of LPS binding to the promoter region might be one explanation of the association reported for the *IL1B* polymorphism at position -511. The discrepancy between these and our findings might be explained by a possible linkage of the *IL1B* -511 polymorphism with a disease locus other than the *IL1B* -31 C/T polymorphism. In addition, the number of non-survivors in both our UK and Dutch cohort were small, which may have prohibited finding an association in our study. Although in the Dutch cohort the *IL10* -819 C/C genotype was underrepresented in non-survivors, this was not confirmed in the UK cohort. Additional studies with large numbers of patients are required to analyse the relevance of this finding, since a small change in numbers in the Dutch cohort as it is presented would very likely alter the results.

The *IL10* -1082 G/A polymorphism has previously been reported by van der Pol *et al*. to be significantly associated with susceptibility to meningococcal disease in combination with a polymorphism in FcγRIIa.(38) This study compared observed genotype distributions to those expected in healthy controls and first-degree relatives of affected patients, but the *IL10* -1082 polymorphism was not analyzed separately.

In conclusion, we found no association between polymorphisms in *PARP*, *IL1B*, and *DEFA* and meningococcal infection susceptibility or severity. We observed an association of a SNP in *DEFB1* with susceptibility and severity in the UK cohort. This was not confirmed in the Dutch cohort. This discrepancy may be the result of either a false positive or false negative association. The association of the *IL4* -542 C and the *C1INH* 480 Val allele with susceptibility to meningococcal infection observed in the family based TDT analysis was also not confirmed in the additional cohorts. Additional functional and genetic association studies using large numbers of individuals, ensuring increased statistical power, are necessary to elucidate the conflicting results obtained for the *DEFB1*, *IL4*, *IL10* and *C1INH* polymorphisms and their role in susceptibility to and severity of meningococcal disease.

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Chapter 2.5

Thrombin activatable fibrinolysis inhibitor is associated with severity and outcome of severe meningococcal infection in children

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Abstract

Objective. In paediatric meningococcal sepsis, an imbalance between coagulation and fibrinolysis and proinflammatory action play major roles. We hypothesized that thrombin activatable fibrinolysis inhibitor (TAFI) and/or TAFI activation markers are involved in the pathogenesis of meningococcal sepsis.
Patients and methods. Children with severe meningococcal sepsis (n = 112) previously included in Rotterdam-based trials participated in this study. Clinical and laboratory parameters and severity scores were assessed. TAFI and TAFI activation markers were determined: TAFI activation peptide (TAFI-AP) and (in)activated TAFI (TAFIa(i)). The -438G/A, Ala147Thr, and Thr325Ile polymorphisms were genotyped.
Results. TAFI levels were significantly decreased in patients with meningococcal disease at admission compared to the convalescence state. TAFI was decreased in patients with septic shock vs. those with no shock. TAFI-AP levels were increased in patients with disseminated intravascular coagulation (DIC) vs. patients without DIC. TAFI-AP and TAFIa(i) were significantly increased in non-survivors vs. survivors. TAFI-AP levels and TAFI-AP/TAFI ratio were also strongly correlated to severity scores and laboratory parameters. The *TAFI* 325Ile/Ile genotype was overrepresented in patients with DIC.
Conclusions. Activation markers of TAFI were associated with the occurrence of DIC and mortality in meningococcal sepsis patients. A determination of TAFI, TAFI-AP and TAFIa(i) is required to enable coherent interpretation of the role of TAFI in disease.

Introduction

The observed clinical phenotype in meningococcal infection is thought to be determined by the activation of inflammatory, coagulation and fibrinolysis pathways. While meningococcal infection results in a ‘mild’ sepsis with petechiae in some patients, others develop septic shock and/ or disseminated intravascular coagulation (DIC). This, in combination with ischemia, may result in multi-organ failure. Although improved treatment possibilities have resulted in a decreased mortality over the past 15 years, up to 2% of meningococcal infections is still lethal in the developed countries. (1) A significant number of patients suffer from long-term sequelae including minor or major amputations. Thrombin activatable fibrinolysis inhibitor (TAFI) was described to be a potent inhibitor of fibrinolysis *in vitro*.(2, 3) TAFI is a glycoprotein that is present in the plasma as a proenzyme and is activated by thrombin/thrombomodulin or plasmin by a single cleavage at Arg-92. This results in the activation peptide (TAFI-AP) and in the exposure of the substrate binding site of activated TAFI (TAFIa). TAFIa is thermolabile (half-life of 8-15 minutes at 37°C) as a result of a spontaneous conformational conversion into an inactive form (TAFIai) which is prone to further proteolytic cleavage. TAFIa exerts its anti-fibrinolytic function by removing the

carboxy-terminal lysine residues of partially degraded fibrin. These lysine residues are required for binding of plasminogen and tissue-type plasminogen activator (t-PA) and efficient plasmin formation. Removal of the lysine residues results in decreased binding and activation of plasminogen and induces inhibition of fibrinolysis.(4, 5) Still, although plasma of TAFI-deficient mice fails to prolong clot lysis time *in vitro*, no differences are observed in *in vivo* markers of haemostasis and endotoxin-induced DIC between wild-type and TAFI-deficient mice.(6, 7)

Exuberant activation of TAFI could result in decreased degradation of thrombi and therefore in exacerbation of multi-organ failure. In addition, *in vitro* and animal studies have revealed that TAFIa also has an anti-inflammatory role by inactivating C5a, resulting in decreased vascular permeability and risk of septic shock.(8, 9) Furthermore, TAFI-deficient mice appear to be protected from sepsis-induced liver injury.(7) Which of these functions dominates is currently unknown. In studies on adults with sepsis, TAFI levels were decreased.(10, 11) The cause of this decrease, activation or consumption of TAFI was not revealed, because TAFI activation markers were not determined.(10) Knoefler et al. observed no differences in TAFI levels in children of different age groups with and without hematological malignancies.(12)

A variety of single nucleotide polymorphisms (SNPs) have been described for the TAFI gene. However, it remains rather unclear to what extent genetic variations in *TAFI* contribute to the disease severity.(13)

In this study we investigated the levels of TAFI, the activation markers TAFI-AP and TAFIa(i), and *TAFI* polymorphisms in a cohort of paediatric patients with severe meningococcal infection. We analyzed whether these parameters were associated with survival, the presence of shock as a marker for inflammatory activity, and the presence of DIC as marker for a possible involvement in fibrinolysis. We also analyzed the possible correlations of TAFI/TAFI activation markers with other coagulation and fibrinolysis related factors as well as inflammatory and endocrine factors.

Methods

Participants. Patients consecutively admitted to the paediatric intensive care unit (PICU) of the Erasmus University Medical Centre-Sophia (Rotterdam, the Netherlands) who previously participated in Rotterdam-based meningococcal sepsis studies between 1988 and 2005 were eligible for the current study (n = 279).(14-19) Children were enrolled in the study after obtaining approval of the medical ethical committee and informed consent from the parents or guardians. The 112 patients from whom DNA and/or citrate blood was available were included. Clinical inclusion criteria were presentation with tachycardia, tachypnea, body temperature < 36°C or > 38.5°C (rectal) and petechiae.(20) Prospective data on all patients were collected at various time-points. Both laboratory parameters and disease severity scoring systems, such as the PRISM-score, predicted death rate based on the Rotterdam score. and the presence of DIC, were selected as markers of severity of disease.(21-23) DIC was scored on admission and during the first 24 h, as some patients do not

meet the criteria at first, but do develop DIC during the first hours in the PICU.
All laboratory parameters were obtained at baseline from an arterial blood sample that was collected within 4 h after admission to the PICU (t = 0, n = 67). Additionally, blood was sampled at t = 1 day for TAFI measurements (n = 58). For some of the patients, blood was also drawn 0.5 days after admission (n = 28) and 90 days after admission (n = 13). Differences in the number of patients at the different time points are the result of inclusion of patients in several different studies, which did not all include a blood sample being drawn at t = 0.5 days. Additionally, no more blood samples were drawn when an arterial line was no longer available, when a patient was transported back to the referral hospital, or when a patient died. At t = 90 days, according to regulations of the medical ethical committee, blood could only be drawn from cooperative children.
N. meningitidis serotyping. The *N. meningitidis* serogroup was determined in cultured isolates at the Netherlands Reference Laboratory for Bacterial Meningitis Amsterdam using immunodiffusion with polyclonal antisera.(24)
TAFI concentration. Citrate blood was drawn and the plasma stored at -80°C until TAFI concentrations were determined without prior thawing. Three sandwich-type ELISAs (i.e. MA-T12D11/MA-T30E5-HRP, MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-17D7-HRP) were used to quantify the plasma levels of TAFI, TAFI-AP and TAFIa(i), respectively.(25) To obtain pooled human plasma, blood samples (n = 21 blood donors) were taken on 4% citrate according to the guidelines of the blood transfusion centre (Red Cross, Leuven, Belgium) and plasma was pooled. Either non-activated pooled human plasma (MA-T12D11/MA-T30E5-HRP ELISA) or activated pooled human plasma (MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-17D7-HRP ELISAs) was used as a standard (1:40 dilution of plasma in PTAE buffer (PBS pH 7.4 containing 0.002% Tween 80, 1 g/L BSA and 5 mM EDTA), followed by serial two-fold dilutions up to 1:2560). For preparation of the standard for the MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-17D7-HRP ELISAs, pooled human plasma was incubated with thrombin (20 nM), thrombomodulin (5 nM) and $CaCl_2$ (17 mM) in Hepes buffer at 37°C for 15 min. The reaction was stopped by addition of H-D-Phe-L-Prolyl-L-arginine chloromethylketone (PPACK, 30 μM final concentration).
Plasma samples derived from patients were diluted 1:160 (MA-T12D11/MA-T30E5-HRP ELISA), 1:80 (MA-T12D11/MA-T18A8-HRP ELISA) and 1:80 (MA-T30E5/MA-17D7-HRP ELISA). All values of TAFI and TAFI activation markers are expressed relative to the levels in the pooled human plasma.
Determination of levels of cytokines, coagulation factors and endocrinological factors. Plasma or serum levels of different parameters were determined previously. (14, 15, 18, 26)
DNA isolation and genotyping. DNA isolation from whole blood was performed with column methods using standard protocols (Qiagen). Bi-allelic discrimination with Taqman analysis was used to determine the *TAFI* -438 G/A (rs2146881), Ala147Thr (505G/A, rs3742264), and Thr325Ile (1040C/T, rs1926447) genotypes in Caucasian patients only (n = 82). Primer sequences are available upon request from the corresponding author.

Statistical analysis. Statistical analysis was performed using SPSS 11.0. Binomial variables were analyzed using Pearson's χ^2 test (2df) or Fisher's exact test when appropriate. For continuous variables the Mann-Whitney *U* test was used.
Spearman's correlation was determined to assess the relation between TAFI levels and severity scores or laboratory parameters. TAFIa(i) and TAFI-AP levels were log transformed resulting in a near normal distribution. These log transformed levels were analyzed in relation to different outcome variables, severity scores, and genotypes using the Student's *t*-test. Geometric mean and 95% CIs are depicted.(27) A comparison of genotype frequencies was made between outcome variables such as survival, need of ventilation and presence of DIC. Probability (*P*) values < 0.05 were considered to be statistically significant. No correction was made for multiple testing.

Results

In total, 112 patients were included in this study. Sixty-nine (62%) were males and the median (range) age was 2.9 years (0.1-16.1). Septic shock was observed in 96 (87%) patients. The median (range) PRISM score was 19 (1-43). Twelve patients did not survive (11%; Table 1).

Table 1. Patient characteristics.

	Non-survivor n = 12	Survivor n = 100	p[1]
Male gender (%)	9 (75.0)	60 (60.0)	0.37
Median age (min-max)	0.83 (0.46-9.43)	3.57 (0.12-16.11)	0.000
Presence of shock (%) n = 111	12 (100)	84 (84.8)	0.36
Mechanical ventilation (%) n = 110	12 (100)	55 (56.1)	0.003
Presence of DIC[2] (%)	12 (100)	48 (48)	<0.001
PRISM score[3] (min-max) n = 100	31.5 (23-43)	18.0 (1-37)	0.000
Predicted death rate[4] (min-max) n = 104	95.9 (55.1-99.9)	5.7 (0.0-96.3)	0.000
Meningococcal Serogroup			
B (%)	9 (90.0)	63 (80.8)	
C (%)	1 (10.0)	15 (19.2)	0.68

[1]Fisher's Exact test or Mann-Whitney *U* test were performed when appropriate.
[2]DIC, disseminated intravascular coagulation; PRISM, paediatric risk of mortality.
[3]Related to the first six hours.
[4]Based on the Rotterdam score.

On admission 60 (54%) patients met the criteria for DIC, whereas an additional 16 (14%) patients fulfilled the DIC criteria during the first 24 h of their stay in the PICU. In 95 (85%) patients blood cultures were positive for *N. meningitidis*. Serotype B was found in 72 of these, while 16 had serotype C. In the remaining seven, no serotype was determined. Additionally, *N. menigitidis* specific polymerase chain reaction (PCR) was positive in two patients. The remaining 15 patients had possible meningococcal infection based on clinical criteria.(28)
TAFI levels and survival. Plasma at t = 0 was available for 57 survivors and 10 non-survivors. At t = 0.5 and 1 day, plasma was available from three and two non-

survivors, respectively. Other patients died in the period between blood sampling. At t = 0.5 and 1 day, plasma was available from 25 and 56 survivors, respectively. At admission, levels of TAFI and TAFIa(i) were strongly decreased in the total group of patients with meningococcal infection (Table 2).

Table 2. TAFI, TAFI-AP en TAFIa(i) levels in plasma of paediatric patients with meningococcal sepsis.

Time point (days)	0	0.5	1	90
	n = 67	n = 28	n = 58	n = 13
TAFI[1]	26.5 (10.6)	36.5 (13.5)	40.1 (11.0)	91.1 (16.9)
TAFI-AP[2]	100 (80-126)	84 (61-115)	77 (66-90)	123 (100-150)
TAFIa(i)[2]	61 (53-70)	73 (60-90)	59 (52-67)	137 (108-173)
TAFI-AP/TAFI[3]	2.9 (0.8-47.5)	2.1 (0.9-19.4)	1.7 (0.8-8.6)	1.4 (0.9-3.1)
TAFIa(i)/TAFI[3]	2.6 (0.7-11.0)	2.2 (0.7-4.7)	1.5 (0.5-3.6)	1.6 (0.8-2.6)

[1]Mean (sd) % relative to pooled normal plasma.
[2]Geometric mean (95% CI) % relative to pooled normal plasma.
[3]Ratio, median (range).

TAFI-AP levels were normal at admission, but decreased to about 75% of normal reference levels during the first day. In survivors, TAFI levels returned to normal 90 days after admission (Table 2). In subgroup analysis, non-survivors and survivors had similar levels of TAFI at all time points (Figure 1A).

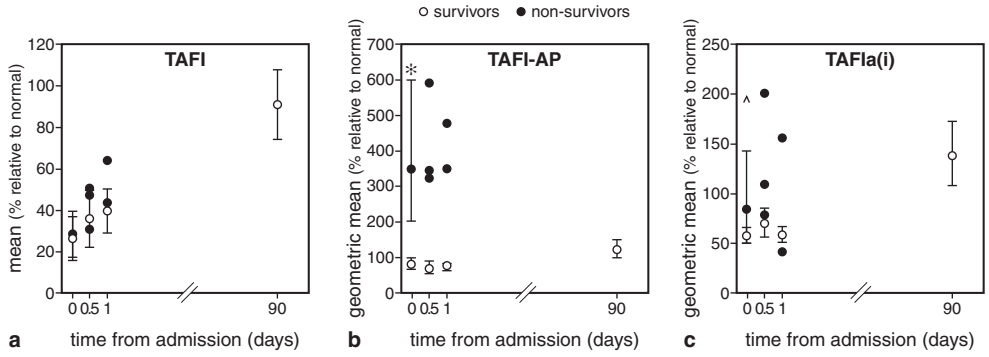

Figure 1. Plasma TAFI and activation marker levels in survivors and non-survivors.
Geometric mean or mean plasma TAFI levels (% relative to normal controls) on admission and after 0.5 and 1 and 90 days as listed. Error bars represent SD for TAFI and 95% CI for TAFI AP and TAFIa(i). Numbers of survivors at these time points are 57, 25, 56 and 13, respectively. The numbers of non-survivors at these time points are 10, 3 and 2, respectively. As only very few non-survivors were left at t = 0.5 and 1 day individual levels are depicted for these patients, and statistical analysis was performed only on admission. 1A. TAFI levels; 1B. TAFI-AP levels; 1C. TAFIa(i) levels. *, p<0.001; ^, p=0.047.

The TAFI-AP levels were significantly higher in non-survivors than in survivors on admission (p<0.001, Figure 1B). In addition, TAFI-AP levels were three to six times increased at other time points in non-survivors compared to survivors and no overlap was present; however, because of the small number of patients in the

non-survivor group, no statistical analysis could be performed. In survivors, TAFI-AP levels were similar to the normal reference levels at all time points. On admission, the TAFIa(i) levels were significantly higher (p=0.047) in non-survivors than in survivors, corroborating an enhanced TAFI activation in the non-survivor group (Figure 1C).

TAFI levels and DIC and septic shock. Patients with septic shock showed lower TAFI levels compared to the small group of patients without septic shock, while no differences were observed for the TAFI activation markers (Figures 2A-C).

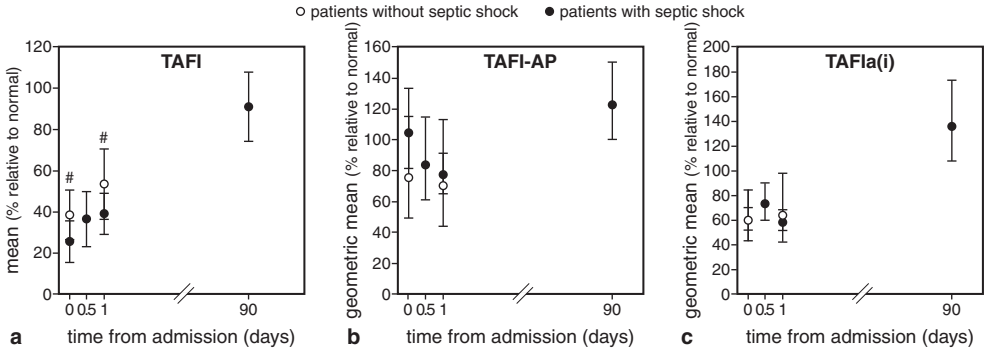

Figure 2. Plasma TAFI and activation marker levels in patients with and without shock.
Geometric mean or mean plasma TAFI levels (% relative to normal controls) on admission and after 0.5 and 1 and 90 days as listed. Error bars represent SD for TAFI and 95% CI for TAFI AP and TAFIa(i). The numbers of patients with shock at these time points are 61, 28, 54 and 13, respectively. The numbers of patients without shock at the time points 0 and 1 day are 5 and 4, respectively. Data regarding shock were present for 66 out of 67 patients for whom TAFI and activation levels were determined at t = 0.
2A. TAFI levels, #, p<0.01; 2B. TAFI-AP levels; 2C. TAFIa(i) levels.

On admission, TAFI levels were slightly lower in patients with DIC than in patients without DIC (Figure 3A). TAFI-AP levels were significantly increased at t = 0, 0.5 and 1 day in patients with DIC versus no DIC on admission, while no difference was observed on admission with regard to TAFIa(i) level (Figures 3B and 3C). When the presence of DIC was defined within the first 24 h after admission, similar results were obtained (data not shown).

Some of the patients received protein C concentrate after t = 0 (n = 26, 21, 19 and 9 of the patients for whom TAFI levels are available at t = 0, 0.5, 1 and 90 days, respectively) because they participated in a randomized clinical trial. Because this may have influenced the results, analyses were also performed excluding these patients. The only difference observed was for the TAFIa(i) levels 1 day after admission. TAFIa(i) levels were significantly higher in the patients receiving protein C concentrate when compared to those receiving placebo (geometric mean (95% CI) 72.1% (56.2-92.6%) and 54.4% (32.4-91.2%), respectively; p=0.03), but this difference was already present on admission when no study medication had yet been administered (83.1% (66.9-103.3%) vs. 49.8% (42.6-58.1%), respectively (p<0.001)). When comparing placebo groups and groups who received different dosages of protein C concentrate (low, moderate or high), only at t = 0 a significant

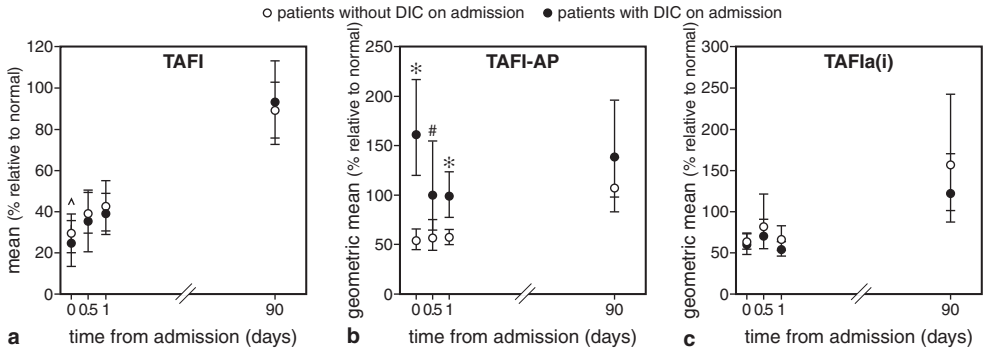

Figure 3. Plasma TAFI and activation marker levels in patients with and without DIC on admission. Geometric mean or mean plasma TAFI levels (% relative to normal controls) on admission and after 0.5 and 1 and 90 days as listed. Error bars represent SD for TAFI and 95% CI for TAFI AP and TAFIa(i). The numbers of patients with DIC at these time points are 38, 19, 32 and 7, respectively. The numbers of patients without DIC at these time points are 29, 9, 26 and 6, respectively.
3A. TAFI p=0.05 at t = 0; 3B. TAFI-AP levels; 3C. TAFIa(i) levels. *, p<0.001; #, p<0.01, ^, p=0.05.

difference was observed between the patients receiving high dosages of protein C and the placebo group (p=0.004, after Bonferroni correction). At this point, however, no protein C concentrate had been administered to any of the patients.
The *TAFI* 325Ile/Ile genotype was overrepresented in patients with DIC on admission when compared to the 325Thr/Thr genotype (crude OR (95% CI) 10.5 (1.3-88), adjusted for age OR (95% CI) 13.7 (1.5-123). No other significant differences or associations were observed in *TAFI* genotype distributions.
TAFI activation markers: correlation with other clinical parameters. No correlation was observed between TAFI-AP and TAFIa(i) at any of the time points. On admission, TAFI-AP was negatively correlated with age (n = 67, Spearman's Rho -0.44, p<0.001). No correlation was observed between age and convalescent TAFI-AP levels three months after admission. In contrast, TAFIa(i) convalescent levels positively correlated with age (n = 13, Spearman's Rho 0.747, p=0.003), while no correlation was observed on admission. TAFI levels showed no correlation with age at any time point. TAFI-AP levels were positively correlated with PRISM score (n = 67, Spearman's Rho 0.529, p<0.001) and the predicted death rate calculated from the Rotterdam score (n = 66, Spearman's Rho 0.584, p<0.001).
We hypothesized that the ratio between activation markers such as TAFI-AP and TAFI might give a more proper indication of TAFI activation. TAFI was considerably more decreased in the acute phase than its activation markers as reflected by the increased TAFI-AP/TAFI and TAFIa(i)/TAFI ratio's (Table 2). However, a large variation between patients was found. The TAFI-AP/TAFI ratio was significantly increased in non-survivors compared to the survivors at all time points (median (min-max) survivors 2.8 (0.8-47.5), 2.0 (0.9-8.8) and 1.7 (0.8-8.6) at t = 0, 0.5 and 1 day; non-survivors 15.8 (2.6-44), 7.3 (6.4-19.4) and 7.7 (7.5-7.9) at t = 0, 0.5 and 1day, p<0.001, p=0.008 and p=0.02, respectively). A trend for higher TAFI-AP/TAFI ratio

was observed for patients with septic shock compared to those without septic shock. Patients with DIC on admission had increased TAFI-AP/TAFI ratio's at t = 0, 0.5 and 1 day (median (min-max) with DIC 7.4 (1.5-47.5), 2.5 (1.1-19.4) and 2.4 (1.2-8.6) at t = 0, 0.5 and 1 day; without DIC 1.8 (0.8-6.9), 1.5 (0.9-2.5) and 1.3 (0.8-2.6) at t = 0, 0.5 and 1 day, p<0.001 for t = 0 and 1 day and p=0.005 for t = 0.5 day). No significant difference was observed in the ratios in the convalescence period. In addition, the TAFI-AP/TAFI ratio was negatively correlated with age (Spearman's Rho -0.474, p<0.001), while it was positively correlated with PRISM score (Spearman's Rho 0.622, p<0.001) and the predicted death rate calculated from the Rotterdam score (Spearman's Rho 0.691, p<0.001).

TAFI levels in relation to other laboratory parameters. Strong correlations were observed between TAFI-AP and the TAFI-AP/TAFI ratio and other laboratory parameters, including cytokines and other coagulation factors. TAFI-AP levels were positively correlated with lactate and procalcitonin levels, while they were negatively

Table 3. Spearman correlation coefficients of TAFI levels with other laboratory parameters on admission.

Laboratory parameter	n	Spearman's Rho (p) TAFI	TAFI-AP	TAFIa(i)	TAFI-AP/TAFI
General factors					
Lactate	67	-	0.454 (<0.001)	-	0.391 (0.001)
C-reactive protein	66	-	-0.363 (0.003)	-	-0.390 (0.001)
Procalcitonin	35	-	0.542 (0.001)	-	0.596 (<0.001)
Cholesterol	29	0.603 (0.001)	-	0.569 (0.001)	-0.401 (0.031)
Cytokines					
IL-1β[1]	29	-	0.755 (<0.001)	0.466 (0.01)	0.638 (<0.001)
IL-1RA	36	-	0.801 (<0.001)	-	0.803 (<0.001)
TNF-α[1]	23	-	0.698 (<0.001)	0.589 (0.003)	0.637 (0.001)
sTNFR	36	-	0.660 (<0.001)	-	0.675 (<0.001)
IL-6	35	-	0.860 (<0.001)	-	0.836 (<0.001)
IL-8	35	-	0.783 (<0.001)	-	0.774 (<0.001)
IL-10	36	-	0.485 (0.003)	-	0.539 (0.001)
MIF[2]	66	-	0.580 (<0.001)	-	0.667 (<0.001)
Coagulation/Fibrinolyis					
Platelet count	67	0.246 (0.045)	-0.490 (<0.001)	-	-0.626 (<0.001)
Prothrombin	36	0.655 (<0.001)	-	0.501 (0.002)	-0.422 (0.01)
TAT	35	-	0.658 (<0.001)	0.331 (0.052)	0.674 (<0.001)
Protein C	36	0.638 (<0.001)	-	-	-0.422 (0.010)
Activated protein C	36	-	0.764 (<0.001)	-	0.742 (<0.001)
PAI-1	35	-	0.684 (<0.001)	-	0.727 (<0.001)
D-dimer	36	-	0.699 (<0.001)	-	0.796 (<0.001)
PAPc	35	-	0.687 (<0.001)	-	0.758 (<0.001)
sTM	36	-	0.530 (0.001)	-	0.560 (<0.001)
Factor V	14	-	-0.662 (0.01)	-	-0.741 (0.002)
Adrenal function					
ACTH	59	-	0.618 (<0.001)	-	0.604 (<0.001)
Cortisol	59	-	-0.356 (0.006)	-	-0.484 (<0.001)
Cortisol/ACTH ratio	57	-	-0.611 (<0.001)	-	-0.637 (<0.001)
Glucose	67	-	-0.372 (0.002)	-	-0.404 (0.001)

[1]Only patients for whom cytokine levels exceeded the detection limit of 5 pg/mL were included in the analysis.

[2]MIF, macrophage migration inhibitory factor; TAT, thrombin-antithrombin III complexes; PAI-1, plasminogen activator inhibitor-1; PAPc, plasmin-2antiplasmin complexes; sTM, soluble thrombomodulin.

A '-' indicates non significant (Spearman's correlation coefficients).

correlated with platelet counts and levels of coagulation factor V and C-reactive protein (CRP; Table 3). In addition, TAFI-AP levels were positively correlated with cytokine levels and most coagulation/fibrinolysis markers. With regard to adrenal function as a measure for anti-inflammatory action, a positive correlation was observed with adrenocorticotropic hormone (ACTH), while a negative correlation was observed between TAFI-AP and cortisol, cortisol/ACTH ratio and glucose. TAFIa(i) level was positively correlated with prothrombin, thrombin-antithrombin III complexes (TAT), cholesterol and cytokines TNF-α and IL-1β. TAFI-AP/TAFI ratio was correlated to the different laboratory markers in a similar way as TAFI-AP (Table 3).

Discussion

In our study we observed strongly decreased TAFI levels in both survivors and non-survivors of meningococcal sepsis in a paediatric cohort. Both markers of activation, and specifically TAFI-AP were strongly increased in non-survivors compared to survivors. It must be noted that most non-survivors, as expected, died within the first 12 h after admission, leaving only a few patients in the non-survivor group at 0.5 and 1 day. For this reason, results after t = 0 should be interpreted with caution. TAFI levels were slightly more decreased in patients with DIC compared to those without DIC. TAFI-AP levels were increased in patients with DIC. In contrast, TAFIa(i) levels were similar for patients with and without DIC. This may be explained by the differences in the half-life of TAFI and its fragments in the circulation, which may prohibit the detection of differences between these fragments for the various clinical groups, specifically in meningococcal sepsis, which is known for its rapid onset and deleterious course. TAFI-AP may therefore be a more stable marker of activation. Also the TAFI-AP/TAFI ratio, reflecting the relative activation, was increased both in patients with DIC and in non-survivors. All levels were expressed as percentage of pooled human plasma from healthy adult blood donors. No paediatric healthy controls were available. In our study TAFI levels on admission were not correlated with age. This confirms the observation of Knoefler et al. who showed that TAFI levels were not significantly different between children of different age groups.(12) In addition, TAFI levels at t = 90 days, when patients were fully recovered, normalized to approximately 100% of reference samples.

The association of TAFI-AP with DIC was illustrated by the negative correlation with platelet count and factor V, which are decreased in DIC because of extensive consumption, and by the positive correlation with plasminogen activator inhibitor-1 (PAI-1), Thrombin-antithrombin (TAT) complexes, activated protein C and D-dimer. Both TAFI and PAI-1 are known to inhibit fibrinolysis as a result of interference with plasmin formation.(2-4, 29, 30) Exuberant activation of TAFI and increase of PAI-1 might result in decreased degradation of thrombi and could therefore increase multi-organ failure by promoting formation of multiple microthrombi. Our results indicate that increased activation of TAFI indeed is associated with a more severe clinical disease state reflected by the low survival rate in patients with the highest level of TAFI-AP.

However, the cause or effect of that increased severity remains to be established. Another major feature of meningococcal septic shock is an increased vascular permeability, resulting in oedema and persistent hypovolemia and organ dysfunction.(31) In animal models TAFIa was shown to inactivate C3a and C5a, factors responsible for the increased vascular leaking that is seen in septic shock patients.(8, 9) As the most severely ill patients have the highest C3a levels the decrease of TAFI levels in our study in patients with septic shock compared to those without may also result from an increased activation.(32) However, no difference in TAFI-AP or TAFIa(i) levels were observed. When only TAFI was considered, one might reason that activation of TAFI was even increased in patients with septic shock in contrast to what would be expected from the animal studies described above. It must be noted, however, that the patients in our study admitted to a PICU were only included if an arterial line was *in situ* because of the blood needed for the analyses. This implies that these patients were severely ill and the use of inotropic medication was at least considered and in most cases administered. Indeed, data on TAFI levels were only available in five and four patients without shock at t = 0 and 1 day, respectively.

Supportive treatment of meningococcal sepsis has changed over the time period in which patients were included (more swift treatment because of public awareness, different inotropic agents and increased use of corticosteroids).(33) Because mortality from meningococcal sepsis has decreased with improved treatment possibilities, this may have influenced the results of our study with regard to survival. Results with regard to presence of shock or DIC, or correlations with the markers of severity are not likely influenced, as these were scored on admission.

TAFI-AP levels were positively correlated with markers for severity of disease such as the PRISM score, the predicted death rate calculated from the Rotterdam score, lactate, procalcitonin, cytokine levels and ACTH, while a negative correlation was found with CRP and cortisol. Low CRP levels on admission are associated with a rapid onset of disease and are associated with a poor prognosis.(21)

In our study, different results have been obtained for TAFI, AP and TAFIa(i) with regard to outcome and correlation with other disease severity markers. It should be noted that all other studies published so far only described either intact TAFI levels or TAFIa levels upon full activation of intact TAFI and thus indirectly measuring intact TAFI levels.(10, 11, 34) The difference in levels of TAFI, TAFIa(i) and TAFI-AP and the ratio of individual markers may partially result from differences in the half-life or proteolytic degradation of the various fragments. Moreover, TAFIa converts spontaneously to TAFIai which is prone to further cleavage into two degradation products that are not detectable in the TAFIa(i) ELISA.(25) Our results stress the importance of studying both TAFI levels and TAFI activation markers at the same time.

Genetic polymorphisms in *TAFI* were previously associated with TAFI levels and its activation. Previous studies showed that the 325 Ile variant showed an extended half-life of TAFIa *in vitro* to 15 min at 37°C, while the activation had not changed. Moreover, the antifibrinolytic activity of the 325 Ile variant was increased compared to the 325 Thr variant, and it was therefore thought to be a more potent enzyme than the 325 Thr variant.(35, 36) The TAFI 325Ile/Ile genotype was overrepresented in patients with

DIC. This genotype was also reported to be overrepresented in parents of patients who died of meningococcal disease, while in survivors of meningococcal disease the genotype frequency was decreased compared to the general population.(37)
Given the association of TAFI-AP with mortality and DIC during the course of meningococcal sepsis, and the fact that the increase of TAFI-AP is not limited to the first couple of hours, as in cytokine expression, this molecule might serve as a prognostic marker. However, as increased TAFI-AP levels are indicative for an increased activation of TAFI, the current data also suggest that prevention of TAFI activation or pharmacological interaction with TAFIa might be a useful therapeutic intervention.(38)
In conclusion, activation markers of TAFI are associated with mortality and presence of DIC in meningococcal sepsis patients. In addition, an association with severity scores and laboratory parameters reflecting severity was observed. Determination of TAFI, TAFI-AP and TAFIa(i) is required to enable coherent interpretation of the role of TAFI in disease, while the role of genetic polymorphisms and possibilities for therapeutic intervention require further investigation.

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Chapter 2.6

Reduced ADAMTS13 in children with severe meningococcal sepsis is associated with severity and outcome

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Abstract

Objective. Multiple organ failure is a common feature of paediatric meningococcal sepsis and is associated with an imbalance of coagulation and fibrinolysis. This is partly due to an increased secretion of prothrombotic Ultra Large Von Willebrand Factor (VWF) as the result of vascular endothelial damage. Another factor that may contribute is ADAMTS13, which converts VWF into smaller, less active, VWF multimers and thus influences VWF activity in plasma. We investigated the role of ADAMTS13 and VWF in the severity and outcome of sepsis.
Patients and methods. In 71 children with severe meningococcal sepsis we measured ADAMTS13 activity and antigen,Von Willebrand Factor collagen binding activity (VWF:CB) and antigen levels (VWF:Ag),VWF propeptide and Factor VIII at different time points during their stay in the paediatric intensive care unit.
Results. In the acute phase, both ADAMTS13 activity and antigen were decreased (median 23.4% and 33.7% of normal, resp.) and VWF:CB and VWF:Ag levels were strongly increased (325% and 348%, resp.) ADAMTS13 antigen (23.9% vs 34.6%; p=0.06) and VWF:CB (240% and 340%, p<0.001) were lower in non-survivors than in survivors. ADAMTS13 activity and VWF:CB were both correlated with the severity of the disease, as indicated by the Paediatric Risk of Mortality score (R_s= -0.38 and R_s= -0.50, respectively, p<0.001).
Conclusions. In the acute phase of severe sepsis decreased levels of ADAMTS13 and increased levels of VWF are observed, and the changes are related to severity of disease and outcome. This may contribute to the formation of microthrombi and the severity of thrombotic sequelae of sepsis.

Introduction

Meningococcal sepsis is characterized by a systemic inflammatory response to an infection with Neiseria meningitidis and is associated with multiple organ failure and an imbalance between coagulation and fibrinolysis. Although improved treatment possibilities have resulted in a decreased mortality, to date approximately 2% of the meningococcal infections in developed countries are lethal.(1, 2)
In severe sepsis there is often excessive and sustained generalized activation of the endothelium. Prolonged endothelial activation results in pro-inflammatory stimulation and a procoagulant state. Anticoagulants, such as antithrombin, activated protein C and Tissue Factor Pathway Inhibitor are depleted during sepsis and at the same time, fibrinolysis is inhibited, which further potentiates the formation of microthrombi. (3) A well-known marker of endothelial activation is Von Willebrand Factor (VWF). Upon endothelial cell stimulation or damage Ultra Large VWF (ULVWF) is actively secreted from the Weibel-Palade bodies in endothelial cells. VWF mediates platelet-platelet and platelet-subendothelial interactions and has therefore an important role in primary haemostasis. Furthermore VWF is also a carrier protein of factor VIII.

When ULVWF enters the circulation it is rapidly cleaved by ADAMTS13 at the 1605-1606 bond in the A2 domain of VWF into smaller less active forms.
VWF levels are elevated in patients with arterial thrombosis such as acute myocardial infarction, stroke and peripheral vascular diseases.(4-7) Also in sepsis elevated levels of VWF have been reported indicating a possible contribution to microthrombi formation.(8) Limited information is available on the role of ADAMTS13 in sepsis and only one small study (n = 21) reported on decreased ADAMTS13 levels in children with sepsis.(9-12) We hypothesized that reduced ADAMTS13 activity and increased VWF levels can contribute to the severity of the disease in children with severe meningococcal sepsis. We therefore studied the levels of VWF and ADAMTS13 in children with severe sepsis in the acute phase and the convalescent phase of the disease, in relation to inflammatory parameters, severity of disease and outcome.

Methods

Participants. Patients admitted to the paediatric intensive care unit (PICU) of the Erasmus MC Sophia who previously participated in Rotterdam based meningococcal sepsis studies, were eligible for the current study.(13, 14) These studies were approved by the medical ethical committee. Children were enrolled after obtaining informed consent of the parents or guardians. In total 71 patients from whom citrate blood was available, were included in this study.
Inclusion criteria were presentation with tachycardia, tachypnea, rectal temperature < 36ºC or > 38.5ºC, and petechiae. Data on all patients were collected at various time-points in the course of the disease (t = 0, t = 0.5 day, t = 1 day and t = 90 days). The paediatric risk of mortality (PRISM) score, predicted death rate based on the Rotterdam score, and the presence of disseminated intravascular coagulation (DIC) were collected as markers of severity of disease on admission.(15, 16) DIC was defined as having a score of 5 or more on the DIC score, which included platelets, FDP or D-dimers levels, PT and fibrinogen.(17)
Within 4 h after admission to the PICU a baseline plasma sample was collected (t = 0, n = 58). Additionally blood was drawn at t = 1 day, (n = 50). In an unselected patient group blood was also drawn 0.5 day after admission (n = 24) and 90 days after admission (n = 6). Differences in number of patients at different time-points are the result of the fact that patients, with similar baseline characteristics, were included in several studies that did not all include a blood sample at t = 0.5 day. Furthermore no samples were drawn when an arterial line was no longer available, when a patient was transported back to the referral hospital or when the patient died. Blood was drawn after 90 days to study the levels in the convalescent phase, but could only be obtained from 6 cooperative children.
Plasma measurements. ADAMTS13 activity and antigen were measured using the Technozym ADAMTS13 ELISA and antigen kit (Technoclone, Vienna, Austria) performed as described by the manufacturer. Briefly, diluted plasma samples were incubated on micro-titer plates coated with a monoclonal antibody against

ADAMTS13. After two hours the plates were washed and an activity substrate was added. The fluorescence was measured for 15 minutes (1 measurement per minute) at 30ºC. The plate was then washed and incubated with a conjugate substrate. Antigen substrate was added and after an incubation of 15 min at 37ºC the reaction was stopped and the fluorescence was measured at 360/460 nm (Biotek reader FLX800, Vienna, Austria). Normal Human Pooled plasma provided with the kit was used as calibrator. The intra-assay variation was 14.9% for antigen and 9% for the activity. For the validation a pool of 40 healthy men were used. Values were expressed in percentage of normal pooled plasma.

Von Willebrand Factor antigen (VWF:Ag) was determined with an in-house ELISA assay using polyclonal rabbit antihuman VWF antibodies (DakoCytomation, Glostrop, Denmark) for catching and tagging. VWF collagen binding activity (VWF:CB) was measured by an in-house ELISA using type I collagen for catching (Sigma, St Louis, USA) and polyclonal rabbit antihuman VWF antibodies (DakoCytomation, Glostrop, Denmark) for tagging. Factor VIII:C was measured by means of an clotting-based assay (Automatic Coagulation Laboratory (ACL), Instrumental Laboratory, IJsselstein, The Netherlands) using FVIII deficient plasma (Ortho Diagnostic Systems, Beerse, Belgium). The intra-assay variation of VWF:Ag, VWF:CB and Factor VIII:C was 14.1% ,12.4% and 5.1% respectively. For the ratio VWF:CB/ADAMTS13 activity we expressed VWF in percentages, where 1 U/mL equals 100% of normal pooled plasma. VWF propeptide levels were determined by an ELISA using a polyclonal antibody against recombinant VWF propeptide as capture and detection antibody, as described previously.(18)

The concentrations of C-reactive protein, Interleukin (IL-6), thrombin-antithrombin complex (TAT) and plasminogen activator inhibitor I (PAI-I) were determined as described previously.(19-21)

Statistical analysis. Continuous parameters were analyzed using the Mann-Whitney *U* test. Binomial variables were analyzed using Pearson's χ^2 test or Fisher's exact test when appropriate. Spearman correlation coefficient was determined to assess the correlation between ADAMTS13 levels and severity scores or other laboratory parameters. All ADAMTS13 levels were expressed as a percentage relative to normal pooled plasma. VWF levels were expressed in percentages of normal pooled plasma. The statistical analysis was performed using SPSS version 11.0. *P* values less than or equal to 0.05 were considered to be statistical significant.

Results

Patient characteristics. In total 71 patients with meningococcal sepsis were included in this study. The median age in this population was 5.5 years. The mortality rate was 12.6%. The baseline patient characteristics are presented in Table 1.

The median (min-max) ADAMTS13 activity (23.4% (8.3-49.4)) median (min-max) and antigen levels (33.7% (13.1-83.9)) at admission were significantly decreased in children with sepsis compared to normal pooled plasma and levels increased at

three months to 49.5% and 47.9%, respectively (Figure 1). The specific activity of ADAMTS13 (activity over antigen ratio) was 0.7 (0.3-1.2) on admission and 1.2 (1.1-1.8) after three months (n.s). VWF CB (325% (149-637)) and VWF:Ag (348% (200-660))

Table 1. Patient characteristics in survivors and non-survivors.

Characteristics	Total group (n = 71)	Non-survivor (n = 9)	Survivor (n = 62)	p-value[1]
Male gender	44 (62%)	7 (78%)	37 (60%)	0.47
Age in years (min-max)	5.5 (0.1-16.1)	0.9 (0.5-9.4)	4.8 (0.1-16.1)	0.002
Presence of shock	65 (93%)	9 (100%)	56 (92%)	1.0
Mechanical ventilation	45 (64%)	9 (100%)	36 (59%)	0.02
Presence of DIC on admission	41 (58%)	9 (100%)	32 (52%)	0.008
DIC in first 24 h	51 (72%)	9 (100%)	42 (68%)	0.05
PRISM score (first 6 h)	21 (4-43)	33 (23-43)	19 (4-37)	0.001
Predicted death rate based on Rotterdam score	9 (0-99)	94 (55-100)	6 (0-96)	<0.0001

[1]Fisher's Exact test or Mann-Whitney *U* test were performed when appropriate.
Data are presented as n (%) and median (min-max).

levels were strongly increased on admission and after t = 12 and t = 24 h and were (96% (54-120)) for VWF:CB and (76% (60-116)) for VWF:Ag at three months (Figure 1).

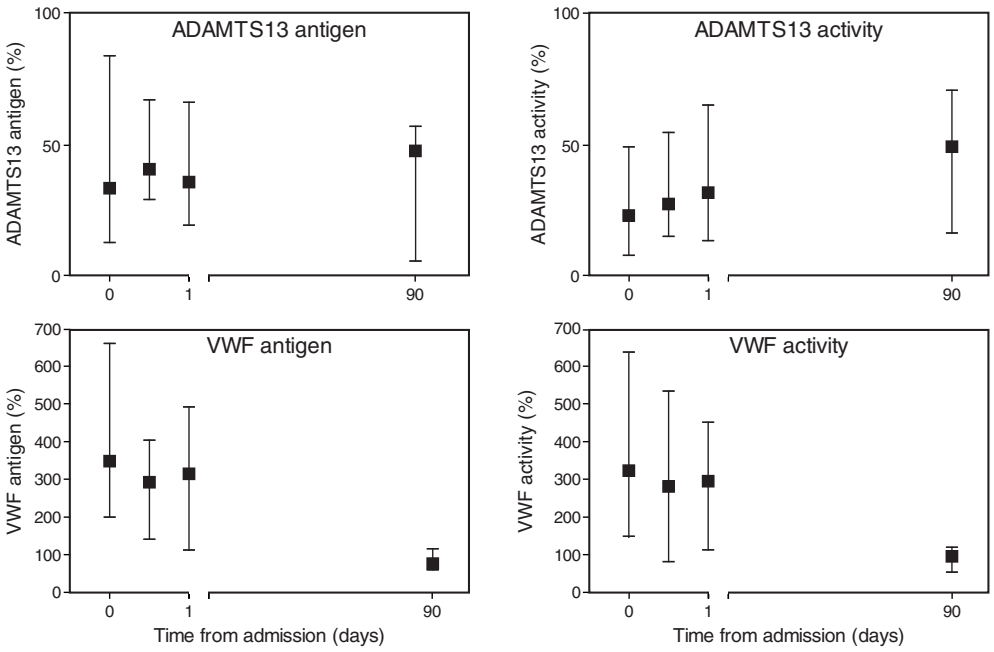

Figure 1. ADAMTS13 and VWF levels in the total study population.
Median ADAMTS13 and VWF levels (% relative to normal pooled plasma for ADAMTS13 and for VWF) on admission, after 0.5, 1 and 90 days are listed. The bars depict the ranges.

The median (min-max) level of FVIII:C on admission was 67% (4-303). The ratio VWF:FVIII:C on admission was 4.6 (1.5-6.0) and 1.7 (1.4-8.0) after three months p=0.003. The ratio VWF:CB/ADAMTS13 activity was calculated at all time points and was 9 (4-27) at t=0 and normalized to 1 (1-5) at three months (Table 2). The median (min-max) VWF propeptide levels upon admission were increased to 27.8 nM (8.0-47) and normalized after three months (4.5 nM (2.9-6.5), p=0.14).

Table 2. VWF:CB activity (%) / ADAMTS13 activity (%) ratio in survivors and non-survivors.

Time point	Total group	Survivors	Non-survivors	p-value
t = 0 day	13 (6-40)	15 (7-40)	12 (6-28)	0.33
t = 0.5 day	10 (2-22)	10 (2-22)	4 (3-4)	0.04
t = 1 day	9 (3-24)	9 (3-24)	4 (4-6)	0.07
t = 90 days	1 (1-7)	1 (1-7)		

Values depicted are median (min-max), VWF:CB 1 U/mL=100%.
P values are Mann-Whitney *U* test, 2 tailed (survivors versus non-survivors).

Plasma levels in survivors and non-survivors. On admission ADAMTS13 antigen was slightly higher (34.6% (14.5-83.9)) in survivors on admission, than in non-survivors (23.9% (13.2-41.8)), (p=0.06) (Table 3). ADAMTS13 activity in survivors was 24.3% (11.5-49.4) vs. 19.4% (8.3-27.6) in non-survivors (p=0.19). In survivors the levels of VWF:Ag were higher than in non-survivors (370% (240-660)) vs. (240% (200-330), p<0.001). Also the VWF:CB was higher in survivors than in non-survivors, (340% (160-640)) vs. (240% (150-320), p<0.001) (Table 3). In survivors the levels of FVIII:C (0.20 U/ml (0-0.6)) were significantly lower than in non-survivors (0.8 U/ml (0-3.0)).

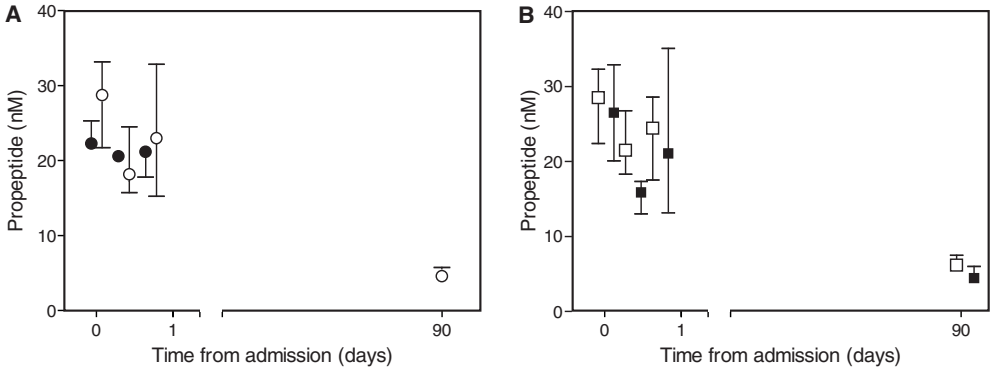

Figure 2a. VWF propeptide levels in survivors and non-survivors.
Median levels of VWF propeptide levels in survivors and non-survivors are plotted. The closed circles represent the survivors and the open circles represent the non-survivors. The bar represents the range.
Figure 2b. VWF propetide levels in patients with DIC versus without DIC.
Median levels of VWF propeptide levels in patients with DIC versus without DIC are plotted. The bar represents the range. The closed squares represent the patients without DIC and the open squares represent the patients without DIC.

Table 3. ADAMTS13 and VWF levels in survivors and non-survivors.

Time point	ADAMTS13 Antigen		p	ADAMTS13 activity		p	VWF antigen		p	VWF activity		p
	Survivors	Non-survivors		Survivors	Non-survivors		Survivors	Non-survivors		Survivors	Non-survivors	
t = 0	34.5 (14.5-83.8)	24.0 (13.2-41.8)	0.06	24.3 (11.5-49.4)	19.4 (8.3-27.6)	0.19	370 (240-660)	240 (200-330)	0.00	340 (160-640)	240 (150-320)	0.00
t = 0.5 day	35.6 (29.5-67)	46.2 (45.8-46.6)	0.10	26.7 (15.5-54.9)	35.3 (27.7-43.1)	0.38	310 (140-400)	240 (230-240)	0.21	280 (80-530)	130 (110-120)	0.06
t = 1 day	36.0 (19.7-66.2)	41.7 (25.7-57.9)	0.88	33.2 (13.9-65.3)	24.5 (20.1-28.9)	0.24	320 (110-490)	210 (180-240)	0.04	300 (120-450)	120 (110-120)	0.02
t = 3 months	47.9 (6.0-57.2)			49.5 (16.8-70.9)			80 (60-130)			100 (50-120)		

All values are presented in percentages of normal pooled plasma.

The ratio VWF/FVIII:C on admission was 4.3 (1.5-46.2) in survivors and 9.7 (3.4-60) in non-survivors (p=0.003). The ratio VWF:CB/ ADAMTS13 activity was higher in survivors than in non-survivors after 0.5 day (Table 2). The propeptide levels were not significantly different in survivors compared to non-survivors: 28.7 nM (8.0-47.0) versus 22.2 nM (21.9-34.6), respectively (Figure 2A). At 90 days the VWF propeptide levels were normal in the survivors.

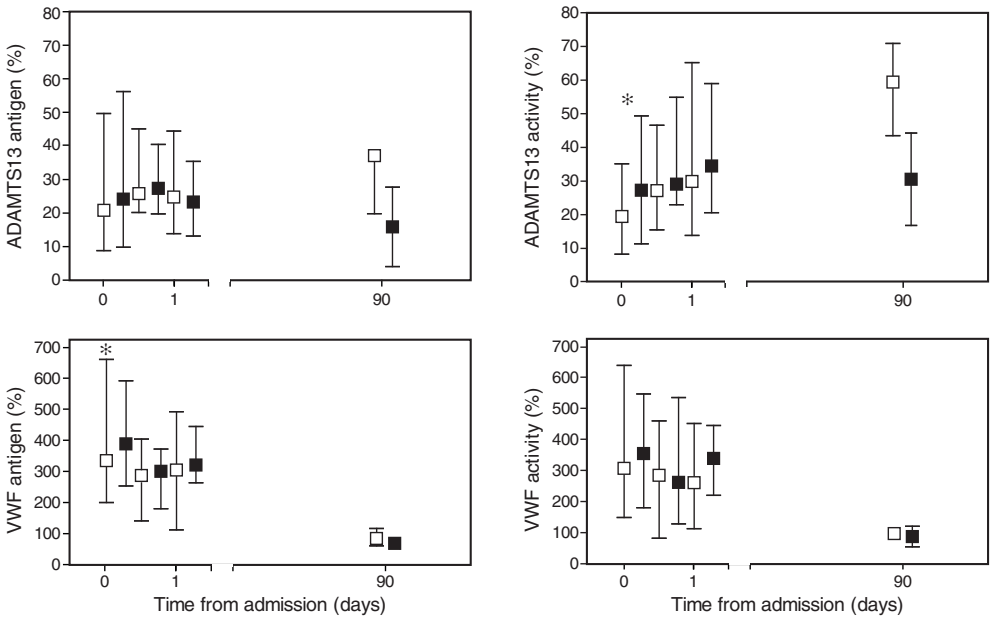

Figure 3. ADAMTS13 and VWF antigen levels in patients with and without DIC.
Median levels of ADAMTS13 and VWF in patients with and without DIC are plotted. The closed squares represent the patients without DIC, the open squares represent the patients with DIC. The bar represents the range.

Plasma levels in patients with DIC and without DIC. For ADAMTS13 antigen no statistical difference between patients with DIC and without DIC was observed (Figure 3). However, ADAMTS13 activity (min-max) on admission was lower in patients with DIC (19.4% (8.3-35.2)) than in individuals without DIC (27.3% (11.5-49.4), p=0.01). On admission VWF:Ag was higher in the patients without DIC (390% (250-590)) than in patients with DIC (330% (200-660), p=0.03) (Figure 3). For VWF:CB there was no difference in levels at the various time points. FVIII:C levels on admission were significantly higher in patients without DIC (1.3 U/ml (0.5-3.0)) than in patients with DIC (0.4 U/ml (0.0-1.45), p<0.001). The ratio VWF/FVIII:C on admission was 2.7 (1.5-5.6) in patient without DIC and 8.3 (3.4-60) in patients with DIC (p=0.001). There was no difference in the ratio VWF:CB/ADAMTS13 activity in patients with DIC or without DIC.

The VWF propeptide levels were similar in patients with DIC (28.5 nM (8.0-43.0)) compared to patients without DIC (26.5 nM (9.0-47.0), p=0.57) (Figure 2B).
Correlations with severity of disease. Both ADAMTS13 and VWF were negatively correlated with the PRISM score and the predicted death rate based on the Rotterdam score (Table 4). There was no correlation between ADAMTS13 antigen or activity and DIC parameters, such as thrombin-antithrombin (TAT) complexes and plasminogen activator inhibitor I (PAI-1). A positive significant correlation was seen for VWF with the levels of CRP. Another inflammatory marker, IL-6, was negatively correlated with VWF activity (Table 4).

Table 4. Correlations of ADAMTS13 and VWF on admission.

Laboratory parameter	n	ADAMTS13 antigen (p)	ADAMTS13 activity (p)	n	VWFag (p)	VWFact (p)
Clinical parameters						
Age in years	58	0.22 (0.10)	0.08 (0.58)	58	0.40 (0.00)	0.28 (0.03)
PRISM score (6 h)	58	-0.31 (0.02)	-0.38 (0.01)	58	-0.46 (0.00)	-0.50 (0.00)
Predicted death rate (Rotterdam score)	58	-0.26 (0.05)	-0.33 (0.02)	58	-0.56 (0.00)	-0.58 (0.00)
IL-6 (ng/mL)	27	-0.21 (0.28)	0.02 (0.94)	27	-0.37 (0.05)	-0.52 (0.01)
CRP (mg/L)	58	0.29 (0.03)	0.05 (0.72)	58	0.39 (0.00)	0.29 (0.03)
Haemostasis						
Platelet count (*10^9/L)	58	0.17 (0.20)	0.34 (0.01)	58	0.51 (0.00)	0.39 (0.00)
TAT (ng/mL)	27	-0.25 (0.21)	-0.03 (0.87)	27	-0.35 (0.07)	-0.48 (0.01)
PAI-1(ng/mL)	27	-0.26 (0.19)	-0.05 (0.81)	27	-0.41 (0.03)	-0.38 (0.05)
Factor VIII:C (U/mL)	58	0.21 (0.11)	0.48 (0.00)	58	0.43 (0.00)	0.59 (0.00)

Discussion

In the present study, we showed that the plasma levels of ADAMTS13 and VWF on admission were associated with the outcome of meningococcal sepsis in children. Furthermore, a strong decrease of ADAMTS13 and increase of VWF were seen in the first 24 hours of the disease and both correlated with disease severity. We observed a strong correlation between ADAMTS13 and markers for disease severity, such as the PRISM score, and the predicted death rate based on the Rotterdam score.
Since ADAMTS13 cleaves ultra large VWF multimers into smaller, less thrombogenic multimers, it is expected that low levels of ADAMTS13 will result in more ULVWF in plasma and is therefore expected to result in a more thrombogenic state. Recently Bockmeyer et al. indeed showed that inflammation-induced ADAMTS13 deficiency is associated with the appearance of ULVWF in plasma.(22) In sepsis patients in whom VWF levels are strongly increased this pro-thrombotic state may further worsen the outcome in these patients.
In our study in a large population of children with meningococcal sepsis, we have shown a reduction in the levels of ADAMTS13 activity and antigen in the acute phase of the disease. These results are in concordance with previous studies in sepsis patients that reported that the levels of ADAMTS13 are decreased and levels of

VWF are increased.(9-12) Most of these studies were in adult patients with sepsis-induced disseminated intravascular coagulation, and it was not yet known whether the contribution of ADAMTS13 to sepsis in children is similar to that in adults. Only one small study had been performed so far in children which is in agreement with our study.(12) In our study we showed for the first time that there is a relationship between ADAMTS13 and DIC and with outcome and severity of the disease in children. In addition, lower levels of ADAMTS13 were inversely related to outcome scores (Rotterdam score and PRISM score), which indicates that low ADAMTS13 levels are associated with poor prognosis. Levels of ADAMTS13 antigen on admission were indeed lower in the non-survivors than in survivors. ADAMTS13 was not correlated with age, but VWF was. Age may play a role, since an immature state of clotting system may cause a more severe coagulation state in young children.(23)

Interestingly, higher levels of VWF were observed in the survivors compared to the non-survivors. One explanation might be that this is caused by a decreased release of VWF in the non-survivors, but it is expected that a more severe disease results in increased endothelial damage and higher VWF levels. An alternative hypothesis is that differences in VWF levels result from an increased consumption of VWF in the non-survivors. To discriminate between these possible mechanisms, we measured the VWF propeptide, which is a marker for the secretion of VWF. VWF propeptide was significantly increased in the first 24 h and was normal after three months. No difference was seen for survivors versus non-survivors for the propeptide levels. This indicates that a decreased release of VWF is not the cause of the lower VWF levels in the non-survivors. The second mechanism, more consumption of VWF, is more likely since patients with DIC had significantly lower VWF levels than patients without DIC. This is further substantiated by the negative correlation between VWF with TAT, a marker of coagulation activation.

A limitation of the study was that we compared the levels of ADAMTS13 in septic children with the normal levels of ADAMTS13 in adults. Nguyen et al used as a control group children that were critically ill but did not have sepsis, and showed that in their control group of children ADAMTS13 levels were around 85% of adult reference values.(12)

Some patients with severe sepsis are treated with coagulation factor concentrates, such as activated protein C, to reduce coagulation activation, which may improve the prognosis.(24) The results of our study may indicate that ADAMTS13, of which a recombinant preparation has recently become available, might also be a therapeutic thrombotic agent in severe sepsis.(25) Theoretically, infusion of ADAMTS13 in these patients may result in increased cleavage of ULVWF and may therefore reduce the thrombogenicity in these patients.(26) However, additional studies on ADAMTS13 levels in both children and adults with severe sepsis should be performed to confirm our findings before this approach is explored.

In conclusion, our study shows that levels of ADAMTS13 are strongly reduced and levels of VWF are strongly increased in children with meningococcal sepsis and that these levels are associated with severity and outcome of the disease, likely by promoting the formation of microthrombi in these affected children.

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Chapter 2.7

Association between high levels of blood macrophage migration inhibitory factor, inappropriate adrenal response, and early death in patients with severe sepsis

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Abstract

Objective. Identification of new therapeutic targets remains an imperative goal to improve the morbidity and mortality of severe sepsis and septic shock. Macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine and counterregulator of glucocorticoids, has recently emerged as a critical mediator of innate immunity and experimental sepsis, and is an attractive new target for treatment of sepsis.
Patients and Methods. Circulating concentrations of MIF were measured in two clinical trial cohorts of 145 paediatric and adult patients with severe sepsis or septic shock caused predominantly by infection with *Neisseria meningitidis* or other Gram-negative bacteria, to study the kinetics of MIF during sepsis, to analyze the interplay between MIF and other mediators of sepsis or stress hormones (adrenocorticotropic hormone and cortisol), and to determine whether MIF is associated with patient outcome.
Results. Circulating concentrations of MIF were markedly elevated in 96% of children and adults who had severe sepsis or septic shock, and they remained elevated for several days. MIF levels were correlated with sepsis severity scores, presence of shock, disseminated intravascular coagulation, urine output, blood pH and lactate and cytokines levels. High levels of MIF were associated with a rapidly fatal outcome. Moreover, in meningococcal sepsis, concentrations of MIF were positively correlated with adrenocorticotropic hormone levels and negatively correlated with cortisol levels and the cortisol:adrenocorticotropic hormone ratio, suggesting an inappropriate adrenal response to sepsis.
Conclusions. MIF is markedly and persistently up-regulated in children and adults with Gram-negative sepsis and is associated with parameters of disease severity, with dysregulated pituitary-adrenal function in meningococcal sepsis and with early death.

Introduction

Numerous adjunctive therapies for patients with severe sepsis and septic shock have been tested in clinical trials. Until recently, most anti-sepsis therapies yielded disappointing results. However, the use of drotrecogin alpha activated, corticosteroid therapy, and early goal-directed therapy has recently given encouraging results in adult patients.(1-3) However, identification of new therapeutic targets to further improve the morbidity and mortality associated with severe sepsis and septic shock remains an imperative goal.
Discovered 40 years ago as a T cell cytokine that inhibited macrophage motility, macrophage migration inhibitory factor (MIF) remained an enigmatic molecule until its rediscovery in the early 1990s as a neuroendocrine peptide and an effector molecule of innate immunity.(4-6) Constitutively expressed by endocrine and immune cells, MIF is released in an hormone-like fashion by the anterior pituitary gland and the adrenal

cortex after exposure to endotoxin (lipopolysaccharide (LPS)), corticotropin-releasing hormone or physiological stress.(6-9) MIF is also released by innate immune cells that are exposed to pro-inflammatory mediators and microbial products, and it acts to promote inflammatory and immune responses.(10, 11)

Interestingly, MIF and corticosteroids together function as a homeostatic counter-regulatory dyad modulating inflammatory and immune responses.(7) Low doses of corticosteroids were found to induce MIF release, which, in turn, counterbalanced the immunosuppressive and anti-inflammatory effects of glucocorticoids. Cytoplasmic phospholipase A2 and mitogen-activated protein kinase phosphatase 1 have been identified as key molecular targets of MIF-glucocorticoid crosstalk.(12, 13) The expression of Toll-like receptor 4, the signal transducing molecule of the LPS receptor complex, is up-regulated by MIF thereby facilitating the detection of Gram-negative bacteria.(14) Moreover, MIF has been shown to play an important role in experimental sepsis, acute respiratory distress syndrome and several inflammatory and auto-immune diseases.(6, 7, 11, 15-17) Thus, many lines of evidence indicate that MIF is an interesting candidate target for therapeutic intervention in patients who have severe sepsis.

In the present study, we measured the circulating concentrations of MIF in two clinical trial cohorts of patients with severe sepsis or septic shock comprising children with meningococcal sepsis and adults with Gram-negative sepsis. We studied the kinetics of MIF release in the systemic circulation during sepsis; the interplay between MIF, mediators of sepsis and stress hormones (adrenocorticotropic hormone (ACTH) and cortisol) and whether MIF was associated with patient's outcome, and if so, whether it might help to identify patients who may benefit from anti-MIF treatment strategies.

Methods

Adult patients. The adult study population consisted of 68 patients with sepsis, severe sepsis or septic shock who were part of a prospective, double-blind study that investigated the efficacy of two IgG preparations for the treatment of Gram-negative severe sepsis and septic shock.(18) Severe sepsis and septic shock were defined as previously reported.(18, 19) Clinical and laboratory parameters were recorded at study entry (i.e. near the onset of severe sepsis or septic shock) and during the course of disease. The evaluation of the patient outcome was made prospectively at the time of the study.(18, 19) Patients were classified as having survived ("survivors"); as having died of fulminant, irreversible septic shock ("early death"); or as having died of an indirect consequence or of a relapse of shock after transient reversal of shock, defined as a normalization of blood pressure and discontinuation of supportive vasopressor therapy ("late death"). The severity of underlying diseases was reported according to the classification proposed by McCabe and Jackson.(20) Concentrations of MIF were also measured in 196 healthy subjects. The study was approved by the ethics committees of the participating centres and informed consent was obtained from patients or from their relatives.(19)

Paediatric patients. The paediatric study population consisted of 77 patients admitted to the paediatric intensive care unit of the Erasmus MC Sophia (Rotterdam, the Netherlands) who had been enrolled in meningococcal sepsis studies.(21-25) All studies were approved by the ethical committee of the Erasmus Medical Center and informed consent was obtained from parents or guardians of patients. Inclusion criteria (tachycardia, tachypnea, rectal body temperature < 36°C or > 38.5°C, and petechiae) were in accordance with recommendations of the International Paediatric Sepsis Consensus Conference.(26) Clinical data, severity scores, and laboratory parameters were collected at study entry and during the course of disease, as has been previously defined.(27, 28) *Neisseria meningitidis* serogroups were determined at the Netherlands Reference Laboratory for Bacterial Meningitis (Academic Medical Center Amsterdam, Amsterdam, the Netherlands).

Blood sampling and cytokine, coagulation factors and stress hormones measurements. In adult patients serum was collected at study entry (i.e. before the infusion of immunoglobulins), 2 h thereafter (for 61 patients), at day 1 (for 57 patients) and at day 10 (for 41 patients). In children, citrate plasma was obtained at baseline (i.e. within 4 h of PICU admission, for 71 patients and at later time-points for 6 patients) and 12 h (for 23 patients) and 24 h (for 62 patients) thereafter. Convalescent blood samples, obtained 3 months after admission, were obtained from 13 patients. Serum and plasma samples were stored at -80^{o}C and underwent, at most, two freeze and thaw cycles before being assayed blindly. Concentrations of MIF were measured using ELISA, as described elsewhere.(29) The analytic sensitivity of the human MIF ELISA was 39 pg/ml. Intra-run and inter-run coefficients of variation were 6% and 12%, respectively. Concentrations of cholesterol, ACTH, cortisol, C-reactive protein, procalcitonin TNF, IL-1β, IL-1ra, IL-6 , IL-8, IL-10, MIP-1β, activated protein C, thrombin-antithrombin III complexes and plasminogen activator inhibitor-1 were determined as previously described.(19, 22-25, 30, 31)

Statistical analysis. Comparison between groups was assessed using the Kruskall-Wallis test for continuous variables and Pearson's χ^2 or Fisher's exact test for categorical variables as appropriate. Spearman's correlation coefficient was used to evaluate the correlation between concentrations of MIF and other laboratory parameters. The interday differences for cytokine levels were evaluated using the Wilcoxon signed-rank test. The risk of death associated with blood MIF levels was assessed using logistic regression analyses. Two-sided *P* values of less than 0.05 were considered to indicate statistical significance. Analyses were performed using SPSS software, version 14.0 (SPSS) and STATA software, version 9.0 (Statacorp).

Results

Adult patients. Among the 68 adult patients, 15 (22%) presented with severe sepsis and 53 (78%) with septic shock. Thirty-two patients died (47%). Non-survivors had been prospectively subdivided into 2 groups: 20 patients (29%) who rapidly died of fulminant, irreversible septic shock (early death; median time to death, 2.5 days) and 12 patients (18%) who experienced late death (median time to death, 14.5 days).

Table 1 lists the demographic, clinical, and laboratory characteristics of the survivor group, the early death group, and the late death group.

Table 1. Characteristics of adult patients in the study population, by outcome.

	Survivors n = 36	Early death n = 20	Late death n = 12
Age (years)	52 (7-78)	58.5 (21-74)	67 (34-76)
Male sex, no. (%)	18 (50)	17 (85)	9 (75)
Severity of underlying			
Diseases, no. of patients			
Rapidly fatal	0	2	3
Ultimately fatal	7	5	5
Non-fatal	29	13	4
Corticosteroid use, no.(%) of patients			
None	27 (75)	13 (65)	6 (50)
Chronic use	2 (6)	4 (20)	1 (8)
Acute use	7 (19)	3 (15)	5 (42)
Site of infection, no. of patients			
Intra-abdominal	11	9	6
Respiratory tract	9	6	2
Genito-urinary tract	6	2	0
Skin and soft tissue	1	2	1
Central nervous system	3	1	0
Other site	0	0	1
No site identified	6	0	2
Pathogen, no. of patients[1]			
Gram-negative bacteria	19	13	5
Gram-positive bacteria	3	0	1
Fungi	3	0	0
Mixed infections	6	7	5
Not documented	5	0	1
Temperature, ^{o}C	38.2 (35.0-40.4)	37.3 (35.6-40.4)	38.1 (35.9-39.4)
Mean arterial pressure, mmHg	63 (17-115)	60 (23-87)	62.5 (47-85)
Duration of hypotension, h	12 (2-36)	11.5 (0-96)	10 (3-144)
Urine output, mL/min	30 (0-200)	0 (0-170)	27.5 (0-60)
Arterial pH	7.39 (7.27-7.57)	7.30 (7.17-7.47)	7.38 (7.26-7.52)
Leukocyte count, G/L	10.25 (0.2-42.0)	6.2 (0.1-100)	6.95 (0.8-13.7)
Thrombocyte count, G/L	89.5 (17-516)	73 (22-352)	109 (12-400)

Data are median (range), unless otherwise indicated. Adult patients were classified as having survived (survivors); as having died of fulminant, irreversible septic shock (early death group); or as having died of an indirect consequence or of a relapse of shock after transient reversal of shock, defined as a normalization of blood pressure and discontinuation of supportive vasopressor therapy (late death group). The three groups of patients differed in sex ratio (p=0.02), severity of the underlying disease (p=0.01), age (p=0.09), urine output (p=0.0001), arterial pH (p=0.02), and time to death (p=0.0002).
[1]Some patients had mixed infections.

Sepsis was caused by Gram-negative bacteria in 54 patients (80%), by Gram-positive bacteria in 4 patients (6%) and by *Candida albicans* in 3 patients (4%). In 7 patients (10%), all sample cultures remained had negative results. Blood cultures had positive results in 43 patients (63%) (Table 2). Polymicrobial bacteraemia occurred in 8 patients (12%).
MIF was detected in the serum samples of all patients who were enrolled in the study. MIF levels were much higher among patients with sepsis (median level at study entry, 103.7 ng/mL; range, 1.4-3200 ng/mL) than among healthy subjects (median level at study entry, 5.2 ng/mL; range, 2.8-15.5 ng/mL; $p<10^{-6}$). Serum concentrations of MIF continued to increase within 2 h after study entry (median, 131.5 ng/mL; range, 2.5-3200 ng/mL) and progressively decreased thereafter (median level at day 1, 73.7 ng/mL; range, 5.2-1772 ng/mL; median at day 10, 50.3 ng/mL; range, 0-388 ng/mL). In

71% of the patients, peak levels of MIF were reached within 2 h of enrolment. Only 3 patients (4%) had peak MIF levels (1.4, 5.9, and 6.5 ng/mL) that were within the range for healthy subjects, probably because of chemotherapy-induced neutropenia (defined as an absolute neutrophil count < 100 cells/mm^3) in one patient and because of treatment with high doses of hydrocortisone or methylprednisolone in the other two patients. Twenty-two patients (32%) were receiving corticosteroid therapy at study entry, which had a significant impact on the circulating MIF levels measured at study entry (p=0.001). Interestingly, patients who had been receiving long-term prednisone therapy (n = 7) had higher MIF levels (median level, 350.3 ng/mL; range, 52.5-525 ng/mL) than those who had not received corticosteroids (median level, 109.1 ng/mL; range, 4.2-1585.5 ng/mL; p=0.07), whereas patients who were treated shortly before enrollment with high doses of hydrocortisone, methyprednisolone or dexamethasone (n = 15) for the treatment of sepsis had much lower MIF levels (median level, 37.7 ng/mL; range, 1.4-3200 ng/mL; p=0.003).

Table 2. Micro-organisms isolated from blood samples obtained from adult study patients.

Microorganisms	Number of isolates
Gram-negative bacteria	
Escherichia coli	14
Pseudomonas species	7
Klebsiella species	4
Enterobacter species	3
Neisseria meningitidis	3
Serratia species	2
Citrobacter species	1
Proteus species	1
Bacteroides species	1
Other	2
Gram-positive bacteria	
Staphylococcus aureus	1
Enterococcus faecalis	1
Coagulase-negative staphylococci	1
Fungi	
Candida albicans	2

Peak MIF serum concentrations (defined as the highest MIF level within the first 2 h of study entry) were inversely correlated with urine output (R= -0.37; p=0.002) and, to a lesser extent, with arterial pH (R= -0.22; p=0.07). There was a trend toward a positive correlation between MIF levels and leucocyte counts (R=0.21; p=0.09), a finding that was in agreement with the observation of low MIF levels in a neutropenic patient. Serum concentrations of MIF were found to correlate with those of macrophage inflammatory protein-1β (p=0.001), IL-1β (p=0.05) and, to a lesser extent, with those of plasminogen activator inhibitor-1 (p=0.06), IL-8 (p=0.09) and IL-6 (p=0.10), but not with those of TNF (p=0.25)

Paediatric patients with meningococcal sepsis. The demographic, clinical, and microbiological characteristics of the 77 paediatric patients are shown in Table 3. Seventy-one patients (92%) presented with septic shock, and 6 (8%) presented

with severe sepsis and 10 patients (13%) died. *N. meningitidis* was isolated from blood samples by culture in 65 patients and by PCR in 2. The other 10 patients had possible meningococcal infections on the basis of clinical criteria.(27) At study entry, concentrations of MIF were found to be negatively correlated with age ($p<0.001$) and positively correlated with paediatric risk of mortality score ($p<0.001$) and predicted mortality ($p<0.001$) on the basis of Rotterdam score.

Table 3. Characteristics of paediatric patients with meningococcal sepsis, by outcome.

	Survivors n = 67	Non-survivors n = 10
Age, median years (range)	4.38 (0.12-16.11)	1.10 (0.46-9.43)
Male sex	41 (61)	8 (80)
Shock	61 (92)	10 (100)
Receipt of mechanical ventilation	41 (62)	10 (100)
Disseminated intravascular coagulation	34 (51%)	10 (100%)
PRISM score within first 6 h, median score (range)	20.0 (4-37)	31.5 (23-43)
Predicted mortality based on the Rotterdam score, % (range)	5.9 (0.0-96.3)	95.9 (55.1-99.6)
Documentation of *Neisseria meningitidis* infection		
Blood culture	55 (82)	10 (100)
PCR	2 (3)	0
Neisseria meningitidis serogroup		
B	39 (75)	7 (87.5)
C	13 (25)	1 (12.5)

Data are no. (%) unless otherwise indicated. The two groups of patients differed in age ($p=0.002$), need for mechanical ventilation ($p=0.03$), presence of Disseminated intravascular coagulation ($p=0.004$), PRISM score ($p=0.001$), and predicted mortality ($p<0.001$). PRISM, paediatric risk of mortality.

MIF levels also were significantly higher in patients with disseminated intravascular coagulation (median level, 74 ng/mL; range, 22-560 ng/mL)than in those who did (median level, 36 ng/ML; range, 8-235 ng/mL; $p<0.001$) and higher in patients with shock (median level, 57 ng/mL; range, 18-560 ng/mL) than in those not experience shock (median level, 20 ng/mL; range, 2-35 ng/mL; $p<0.001$). Moreover, MIF levels were positively correlated with lactate and procalcitonin levels ($p=0.006$ and $p=0.016$, respectively), whereas they were negatively correlated with levels of C-reactive protein ($p=0.02$) and cholesterol ($p=0.001$). Serum concentrations of MIF were positively correlated with levels of IL-1β ($p<0.001$), IL-1ra ($p=0.002$), IL-6 ($p=0.001$), IL-8 ($p=0.001$), and soluble TNF receptor ($p=0.01$) and, to a lesser extent, with levels of TNF ($p=0.08$) and IL-10 ($p=0.08$). In addition, MIF was correlated with thrombin-antithrombin III complexes ($p=0.003$), plasminogen activator inhibitor-1 ($p=0.006$) and activated protein C ($p=0.02$).

MIF and the hypothalamo-pituitary-adrenal (HPA) function. The relationship between circulating levels of MIF and stress hormones (ACTH and cortisol) was examined in children with meningococcal sepsis. Interestingly, circulating concentrations of MIF were found to be positively correlated with ACTH levels ($p=10^{-6}$) (Figure 1A) and negatively correlated with cortisol levels ($p=0.005$) (Figure 1B) and with cortisol:ACTH ratio ($p=10^{-6}$) (Figure 1C). Although ACTH stimulation tests were not performed, the stress hormones profile showing an inverse correlation between ACTH and cortisol levels ($p=0.0003$) was very suggestive of an inappropriate adrenal response to ACTH. This was particularly obvious in non-survivors in whom blood profiles exhibited a typical pattern of high MIF levels and low cortisol:ACTH ratio

pathognomonic of a dysregulated MIF-glucocorticoid balance with an exuberant pro-inflammatory response (Figure 1C).

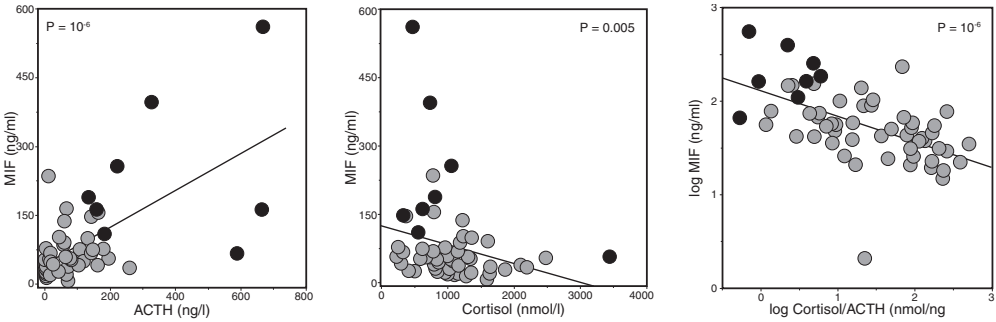

Figure 1. Correlations between levels of macrophage migration inhibitory factor (MIF) and adrenocorticotrophic hormone (ACTH; A), MIF and cortisol levels (B), and MIF and cortisol:ACTH ratio (C), measured at admission to the hospital, in children with meningococcal severe sepsis and septic shock. Black circles, non-survivors; gray circles, survivors. Spearman's correlation coefficients: R=0.597 (A), R=-0.355 (B) and R=-0.627 (C).

MIF and survival. Peak serum levels of MIF differed markedly between the 3 groups of adult patients (p=0.0002) (Figure 2A). Levels were markedly higher in the 20

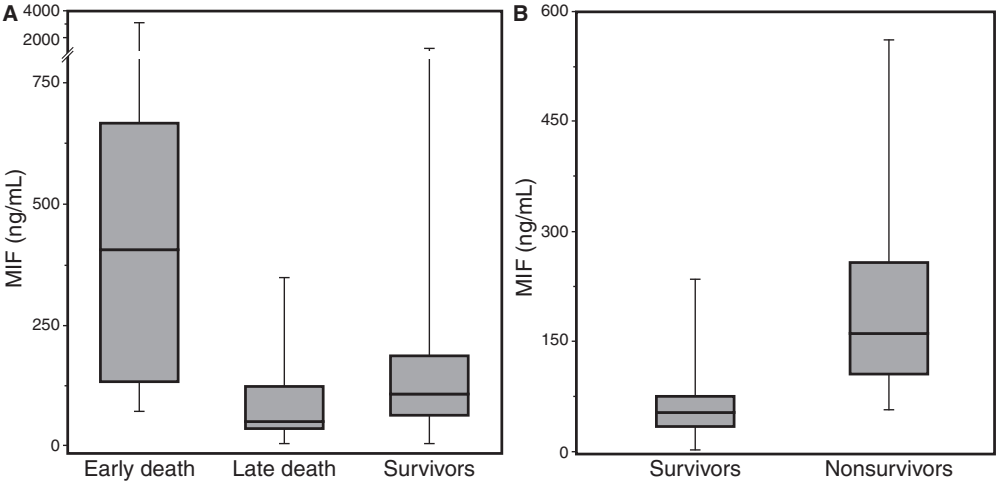

Figure 2. Box plots of macrophage migration inhibitory factor (MIF) levels in adults (A) and in children (B). The bottom, median and top lines of the box mark the 25th, 50th and 75th percentiles, respectively. The vertical line with whiskers shows the range of values. A, Survivors and non-survivors subdivided into those who experienced late death and those who experienced early death. Global *P* value, p=0.0002; those who experienced early death versus survivors, p=0.001, and those who experienced early death versus late death p=0.0001; survivors versus those who experienced late death, p=0.23. B, survivors and non-survivors, p=0.0001.

patients in the early death group (median level, 425.9 ng/mL; range, 73.6- 3200 ng/mL) than in the 36 survivors (median level, 108.2 ng/mL; range 5.9-1236.8 ng/mL; p=0.001) or in the 12 patients in the late death group (median level, 78.0 ng/mL; range, 2.5-350.3 ng/mL; p=0.0001) (p=0.23 for survivors vs. late death group). At study entry, each incremental elevation of 100 ng/mL of MIF increased the risk of early death by a factor of 1.67 (95% CI, 1.17-2.41; p=0.005). This finding remained significant (OR 1.49; 95 % CI 1.02-2.19; p=0.03) after adjustments for sex and urine output, two variables that were significantly associated with MIF levels and the risk of death. Table 4 shows the kinetics of MIF levels in survivors and non-survivors over a 10-day period.

Circulating concentrations of MIF were also associated with outcome in children with meningococcal sepsis. MIF levels were significantly higher in non-survivors than in survivors (at study entry, p=0.0001; at 12 h, p=0.005; and at 24 h, p=0.01) (Figure 2B and Table 4). Similar to the observation made in adults, each incremental elevation of 100 ng/mL of MIF increased the risk of death by a factor of 7.4 (95% CI, 2.1-26; p=0.002). Levels of MIF measured in convalescent children three months after admission were significantly lower than those measured at admission (p=0.009).

Table 4. Kinetics of blood macrophage migration factor (MIF) concentrations in adult and paediatric study patients, by outcome.

MIF concentration	Survivors	Non-survivors	Early death	Late death
Adult Patients				
At study entry	71 (4-717)		165 (124-369)	51 (14-350)
After 2 h	75 (3-1131)		447 (92-743)	106 (27-299)
On day 1	108 (5-862)		670 (110-1772)	87 (23-317)
On day 10	36 (0-273)		231 (118-388)	55 (4-191)
Paediatric patients				
At study entry	53 (2-235)	161 (56-561)		
After 12 h	43 (18-155)	207 (202-735)		
After 24 h	32 (2-386)	347 (213-481)		

Values are median (range). Adult patients were classified as having survived (survivors); as having died of fulminant, irreversible septic shock (early death group); or as having died of an indirect consequence or of a relapse of shock after transient reversal of shock, defined as a normalization of blood pressure and discontinuation of supportive vasopressor therapy (late death group). Paediatric patients were classified as having survived (survivors) or as having died (non-survivors).

Discussion

Analyses of the kinetics of blood MIF in two cohorts of 145 patients with Gram-negative severe sepsis and septic shock caused by *N. meningitidis* infection in children and predominantly by Enterobacteriaceae and *Pseudomonas* species infection in adults revealed that 96% of the paediatric and adult patients with severe sepsis or septic shock had elevated MIF levels that were 10 times above the normal range of MIF concentrations in healthy subjects. These results confirm and extend previous findings of four series of patients who had either systemic inflammatory response syndrome, severe sepsis, or septic shock.(32-35) MIF levels remained elevated for at least 10 days after the onset of severe sepsis and septic shock. This rather unique kinetic profile offers a wide window for therapeutic interventions likely

to be a major advantage for the design of future clinical trials with anti-MIF therapies that are currently under development.

MIF levels were correlated with sepsis severity scores (paediatric risk of mortality score and predicted mortality rates in children with meningococcal sepsis), morbidity (presence of shock, disseminated intravascular coagulation, low urine output and arterial pH, and high lactate levels), and, importantly, with mortality. Indeed, very high MIF levels at the onset of sepsis were associated with fulminant and rapidly fatal disease. In contrast, adults in the late death group of patients who initially recovered from shock but who died later from complications of shock or from a relapse of sepsis had MIF levels that were in the range of those of survivors. Therefore, measurement of blood MIF may help to identify patients with poor prognosis and in whom anti-MIF treatment strategies might improve survival.

Consistent with its pro-inflammatory activities, MIF level was found to be correlated with markers of inflammation or sepsis (C-reactive protein and procalcitonin levels in children) and with pro-inflammatory cytokine levels. However, some discrepancies were observed between children and adults with respect to correlations between MIF and pro-inflammatory cytokine (TNF, IL-6 and IL-8) levels. Several factors may account for these discrepancies, such as the timing of blood sampling; the patient's age, which might influence MIF responses (a hypothesis that deserves further investigation); the type of infections (community-acquired infections in children vs. a mixture of community-acquired and health-care related infections in adults); the underlying risk factors (e.g., meningococcal sepsis typically occurs in young, otherwise healthy subjects with low bactericidal antibody titers, whereas Gram-negative sepsis in adults occurs in the context of co-morbidities likely to affect the magnitude of cytokine responses); and, of course, the aetiology of sepsis (*N. meningitidis* infection in children *vs.* Enterobacteriaceae and *Pseudomonas* species infection in adults). Indeed, in contrast to other types of Gram-negative sepsis, meningococcal sepsis usually follows a fulminant course characterized by a rapid invasion of the bloodstream, very high concentrations of endotoxin in the systemic circulation, and the induction of a vigorous cytokine response and powerful activation of the complement and coagulation systems.(36) Consistent with these observations and previous findings of an association between increased plasminogen activator inhibitor-1 levels and increased mortality associated with meningococcal sepsis or Gram-negative septic shock, as well as with findings regarding the impact of activated protein C on LPS-induced MIF release, MIF levels were positively correlated with disseminated intravascular coagulation and with levels of plasminogen activator inhibitor-1, thrombin-antithrombin III complexes and activated protein C.(30, 37)

Activation of the HPA is an essential feature of the systemic stress response to infection, resulting in the release of glucocorticoids, which play an essential role in the regulation of the host inflammatory and immune responses.(38) One of the intriguing features of MIF has been its abundant expression in the pituitary and adrenal glands and its circulation in the bloodstream with a circadian rhythm synchronous with that of glucocorticoids.(6, 8, 9, 39) Moreover, low doses of glucocorticoids have previously been shown to induce MIF release, while high doses were found to suppress MIF expression.(7, 40, 41) In turn, MIF overrides the immunosuppressive

and anti-inflammatory effects of glucocorticoids, leading to the concept that MIF and glucocorticoids function as a physiological counter-regulatory dyad that modulates inflammatory and immune responses.(7) In line with these observations, treatment with long-term corticosteroids was associated with a 3.5-fold up-regulation of MIF levels at study entry, whereas treatment with short-term high-dose corticosteroids caused a 3.0-fold down-regulation of MIF levels. The positive correlation noted between MIF and ACTH levels measured at admission in children with meningococcal septic shock are well in agreement with the fact that corticotropin-releasing hormone and LPS are powerful MIF secretagogues in the pituitary gland.(8) In contrast to what is observed in adults with sepsis, MIF levels were inversely correlated with cortisol in septic children and, thus, with cortisol:ACTH ratio, which is consistent with previous observations that high doses of corticosteroids inhibit MIF production.(7, 35, 40, 41) Cholesterol, the starting compound of cortisol synthesis, is a major lipid constituent of high-density lipoproteins, which are very potent inhibitors of LPS activity *in vivo* and a prognostic factor of susceptibility to and outcome of sepsis.(24, 42) When forming complexes with high-density lipoproteins and LPS, cholesterol may no longer be used for cortisol synthesis, providing a possible explanation for low cortisol levels in patients with meningococcal sepsis. The observation of high ACTH but low cortisol and low glucose levels was highly suggestive of relative adrenal insufficiency, commonly observed in severe meningococcal sepsis.(21, 25) Taken together with the observation of very high circulating concentrations of MIF in non-survivors, the stress hormone blood profile indicated that the immunoregulatory balancing act played by MIF and glucocorticoids was clearly leaning towards an overwhelming pro-inflammatory response likely to contribute to multiple-organ dysfunction and death.

In summary, the present data showed that MIF was markedly and persistently up-regulated in children and adults who had Gram-negative sepsis. High MIF levels were associated with disease severity scores, pro-inflammatory markers of sepsis and dysregulated pituitary-adrenal function and early death, highlighting the presence of a marked imbalance between pro-inflammatory (MIF) and anti-inflammatory (glucocorticoids) regulatory systems in Gram-negative sepsis. Given the important position of MIF in innate immune responses to microbial pathogens, these results provide a strong rationale for the development of anti-MIF treatment strategies for the treatment of patients with severe sepsis and septic shock.

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Chapter 2.8

Gene expression profiling in children with meningococcal sepsis reveals dynamic changes in NK-cell and cytotoxic molecules

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Submitted for publication

Abstract

Objective. Meningococcal sepsis remains an important cause of childhood morbidity and mortality. Largely due to logistic complexities of research in young children with acute life-threatening disease, very little is known regarding differential expression kinetics and molecular regulation of immune response genes in leukocyte subsets.
Materials and methods. In this prospective case-control study, six children with meningococcal sepsis were included. Blood was drawn at four time points (t = 0, t = 8, t = 24 and t = 72 h after admission to the paediatric intensive care unit). Blood was also collected from matched controls. Detailed immunophenotyping of leukocytes was performed; RNA isolated from whole blood, lymphocytes, monocytes, and granulocytes was used to perform Affymetrix micro-array gene expression analysis.
Results and conclusion. There were no differences in total leukocyte count between patients and controls. In contrast to previous *in vitro* studies we observed an unexpected decrease in NK cell numbers, as well as downregulation of NK cell specific and cytotoxic T-cell related gene expression in patients with meningococcal septic shock. By contrast, expression of genes, involved in innate immunity and several other pathways, differed between the different leukocyte subpopulations in a dynamic fashion. Compared to previously reported gene expression profiles, it was possible to define a meningococcal sepsis specific expression profile.

Introduction

Meningococcal disease is caused by *Neisseria meningitidis*, an intracellular Gram-negative diplococcus, and is known for its variable disease presentation.(1-3) The spectrum varies from mild disease to fulminant septic shock and even death. Especially in children, meningococcal sepsis and meningococcal septic shock are very rapidly progressive disease entities, which may lead to death within 24 h.(3) Mortality in children remains high despite intense clinical and research efforts, and varies from 4% (in meningitis) to 40% (in case of fulminant septic shock).(2, 4) Infection with *Neisseria meningitidis* results in a complex host response including activation of multiple pathways such as the complement system, secretion of pro- and anti-inflammatory mediators and coagulation and fibrinolysis. Several mediators such as complement factors, IL-10, IL-8, TNF-alpha, IL-6 and MIF have previously been identified to play a role, but many factors remain unknown.(5-8) A leading current concept on sepsis proposes that the initial immune and inflammatory hyper-responsiveness is followed by a hyporesponsive period.(9) This concept is appealing but in urgent need of further experimental support from the true clinical situation. Although it is clear that NK-cells, T- and B-cells, granulocytes are all recruited in defence to meningococcal infection, the differential contribution of these leukocyte subsets to disease activity and sepsis are poorly known.(10-13) Furthermore, kinetics of differential immune response gene expression are essential to elucidate how the

reversal of immune hyper- to hyporesponsiveness comes about, and investigating these may lead to the identification of prognostic factors or disease progression markers to identify children with high chance of adverse outcome who may warrant prompt therapy intensification.
One way to further examine the role of inflammatory mediators is to obtain transcription profiles using RNA micro-arrays.(14-17) In this report we present a prospective case-control pilot study which included six patients between the age of 1 month and 18 years who were admitted to the PICU of Erasmus MC-Sophia Children's Hospital with (suspected) meningococcal sepsis. Using RNA obtained from whole blood, as well as from monocytes and lymphocytes separately, RNA expression in the course of meningococcal sepsis was studied. In addition, the RNA expression profiles at admission were compared to those of healthy controls matched for age, gender and ethnic background.

Methods

Participants. A prospective case-control study was conducted between January 2005 and March 2006 at Erasmus MC-Sophia Children's Hospital. Six patients with (suspected) meningococcal sepsis and six healthy age and sex-matched controls were included.
Patients subsequently admitted to the Paediatric Intensive Care Unit (PICU) of Erasmus MC-Sophia Children's Hospital with (suspected) meningococcal sepsis were included if they met the following criteria: a) age between 1 month and 18 years of age, b) tachycardia, c) tachypnea, d) rectal body temperature < 36°C or > 38.5°C, e) petechiae and/or purpura, and f) need of an arterial line. Exclusion criteria were; a) patients with known liver- or kidney malfunction, b) trauma, c) burns, d) organ transplantation, e) cardiogenic shock, f) uncontrollable bleeding, g) malignancies, h) absence of arterial line, i) recent systemic corticosteroid use, and j) pregnancy. The clinical and epidemiological parameters of these 6 patients were similar to those of 287 patients admitted to our hospital with petechia and purpura between August 1988 and June 2006.(18)
Controls children admitted to the hospital for elective minor non-infectious surgery or MRI, and matched for age, gender and ethnic origin.(19) Additional inclusion criteria for controls were a good physical condition and a signed informed consent. Exclusion criteria were a) presence of fever, known immunodeficiencies, heart disease or coagulation disorders, b) current use of antibiotics, or anti-inflammatory medication, including inhalers, d) burns, e) liver- or kidney malfunction and f) organ transplant.
The study was approved by the medical ethics committee of Erasmus MC, and informed consent was obtained from the parents of the patients and controls.
Blood sampling. In meningococcal sepsis patients 12 ml of blood (sodium heparin vacutainers) was drawn from an arterial line and immediately processed at t = 0, 8, 24 and 72 h. T = 0 was defined as the moment of the first blood sampling (max. 6 h after admission to the PICU). Sampling was terminated after 72 h, or prior to that time

point when the arterial line was removed or patients were transferred to the referral hospital.

In controls 12 mL of blood (sodium heparin vacutainers) was drawn once before start of the surgical procedure or MRI. Blood samples were taken with informed parental consent and only after approval of the, independent, treating physician.

Immunophenotyping of leukocytes. Immunophenotyping was performed with standard flowcytometry on 0.5 ml whole blood using the following three 4-color marker combinations: 1) CD4, CD8, CD3, HLA-DR; 2) CD15, CD64, CD45, CD14; 3) CD3, CD16.56, CD45 and CD19.

RNA isolation from leukocyte subsets. One ml of whole blood was used for immediate RNA isolation (internal validation step) using QIAamp RNA Blood Kit (Qiagen, Hilden, Germany). The remaining 10 ml whole blood was used to perform a ficoll density separation using Lymphoprep (Nycomed Pharma, Oslo, Norway). Granulocytes and peripheral blood mononuclear cells (PBMCs) were isolated and immediately frozen at -80°C. From the PBMCs, CD14-positive cells (here referred to as monocytes) and CD14-negative cells, hence enriched for B- and T-cells (and therefore here referred to as lymphocytes) were separated using autoMACS (Miltenyi Biotec). RNA was isolated using RNeasy columns according to the standard protocol provided by the manufacturer (Qiagen, Hilden, Germany). The integrity of the RNA was assessed using the RNA 6000 Nano assay kit on a Bioanalyzer (Agilent Technologies).

Micro-array experiments. A micro-array analysis was performed using the one-cycle target labelling and control reagents as described before (Affymetrix product number 900493).(20)

The fragmented cRNA was hybridized to HG U133 2.0+ microarrays (Affymetrix) and after washing and staining, the arrays were scanned in an HP/Affymetrix scanner at 570 nm. In comparison experiments care was taken that the scaling factor, noise and presence calls were comparable.

Statistical analysis. Continuous variables in cases and controls were analyzed using the Mann-Whitney *U* test in SPSS 11.5.

Micro-array analysis. Background was removed using robust multichip analysis (RMA) and probe intensity levels were quantile normalized.(21, 22) Array groups were compared based on the perfect match (PM) probe intensity levels only, by performing a per-probeset two-way analysis of variance (ANOVA, with factors “probe” and “group”).(22) This results in average expression levels for each probeset in each group, as well as raw *P* values for the significance of the difference between the groups. The latter were adjusted for multiple testing using Šidák step-up adjustment to control the family-wise error rate (FWER), i.e. the probability of incorrectly calling at least one probeset differentially expressed.(23) All differences with adjusted *P* values < 0.05 were considered significant. The Ingenuity program was used to analyse what pathophysiological pathways were differentially expressed between patients at t=0 and controls (Ingenuity Pathway Analysis; 5.5.1-1002).The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE11755 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE11755).(24)

Results

Between January 2005 and March 2006 six patients with suspected or confirmed meningococcal sepsis were admitted to Erasmus MC-Sophia Children's Hospital and included in the study (Table 1). They were boys who all survived the disease.

Table 1. Patient characteristics.

	Patient					
	1	**2**	**3**	**4**	**5**	**6**
Age (years)	5.06	1.37	1.79	2.09	1.51	8.04
Duration of PICU stay (days)	4	5	1	2	60	4
PRISM score[1]	26	23	21	25	28	14
Predicted death rate from Rotterdam score (%)[1]	14.0	72.3	0.3	1.3	92.6	56.3
PIM score[1]	30.0	16.0	8.7	12.5	100.0	48.4
***N. meningitidis* serotype**	B	B		[2]	B	B
DIC	Y	Y	N	Y	Y	N
Mechanical ventilation	Y	Y	Y	Y	Y	Y

[1]On admission; PRISM, Paediatric Risk of Mortality; DIC, disseminated intravascular coagulation; PIM, Paediatric index of mortality.
[2]Culture and PCR negative.

The median age was 1.94 years, ranging from 1.37-8.04 years. All had septic shock and needed mechanical ventilation, while 4 of them had disseminated intravascular coagulation (DIC). The mean Paediatric Risk of Mortality (PRISM) score in the study population was 24 (14-28). Laboratory parameters on admission are listed in Table 2. The six controls were children admitted for minor non-inflammatory surgery (n = 5) or MRI (n = 1).

Table 2. Laboratory parameters on admission.

	Patient					
	1	**2**	**3**	**4**	**5**	**6**
Leukocyte count (*10^9/L)	16.1	5.1	35.5	18.1	8.3	9.9
Platelet count (*10^9/L)	111	26	184	91	116	124
CRP[1] (mmol/L)	51	78	176	258	105	31
Glucose (mmol/L)	10.3	6.5	8.4	9.7	10.4	5.9
Lactate (mmol/L	9.0	5.7	1.7	1.8	9.5	2.2
Base excess (mmol/L)	-9	-11	-7	-6	-12	-9
Creatinin (μmol/L)	85	92	25	55	73	53
Ureum (mmol/L)	7.0	7.4	4.4	11.4	7.6	6.0
Potassium (mmol/L)	3.0	3.7	3.2	3.4	3.2	3.4
Calcium (ionized)	1.04	0.94	1.21	0.77	0.85	1.11
Bilirubin (μmol/L)	6	9	3	3	12	8
Cortisol (nmol/L)	1047	244	415	1309	240	262
ACTH (ng/L)	13.6	179.0	4.3	3.9	303.0	4.9

[1]CRP, C-reactive protein; ACTH, adrenocorticotropic hormone.

Leukocyte subsets in patients and controls. We aimed for as detailed as possible immunophenotyping of leukocytes in all patients at the different time points and in controls, with the purpose of obtaining a broad overall picture of the dynamics of various subsets of immune cells during the course of infection. Patients 3 and 4 had left the PICU and in patient 6 the arterial line was removed before t = 72 h. Therefore,

no samples were available for these patients at this time point. In general a large variability was observed for the leukocyte (subset) cell counts. Total leukocyte counts did not differ significantly between patients and controls (Table 3). However, the

Table 3. Leukocyte subsets in patients on admission and in controls.

Blood cells	Patients median (min-max)	Controls median (min-max)	p[1]
Leukocytes (*10^9/L)	13.0 (5.1-35.5)	8.1 (4.9-9.4)	0.17
% lymphocytes	7.5 (2.6-41.0)	51.3 (26.0-74.3)	0.01
% monocytes	3.8 (1.0-7.0)	5.7 (3.6-14.0)	0.18
% granulocytes	86.5 (58.0-92.4)	37.5 (20.2-65.0)	0.01
CD64 expression granulocytes	30.0 (18.3-47.0)	24.5 (10.0-60.0)	0.94
NK-cells (*10^9/L)	0.06 (0.02-0.11)	0.22 (0.20-0.41)	0.01
B-cells (*10^9/L)	0.54 (0.16-1.23)	0.70 (0.48-1.36)	0.48
T-cells (*10^9/L)	0.79 (0.30-1.05)	2.57 (1.67-5.00)	<0.01
T helper (%)	53.9 (37.3-63.0)	60.0 (46.3-65.6)	0.31
T cytotoxic (%)	39.6 (31.0-54.7)	33.1 (26.9-40.2)	0.18
CD4/CD8 ratio	1.4 (0.7-2.0)	1.8 (1.3-2.4)	0.18
Double negative (%)	6.9 (4.0-8.1)	5.3 (5.1-17.2)	0.82
Activated Th-cells (%)	1.2 (0.6-5.0)	2.9 (0.9-4.8)	0.24
Activated Tc-cells (%)	1.3 (0.5-6.2)	2.6 (1.0-8.4)	0.49
Activated T-cells (%)	2.4 (1.1-10.8)	5.1 (2.5-13.2)	0.31

[1]One-tailed Mann-Whitney *U* test.

percentage of lymphocytes and absolute NK-cell numbers were 6.8 and 3.7 times decreased in patients compared to controls, respectively. The decrease in lymphocyte counts was mainly attributable to a decrease in T-cell counts, while the distribution of $CD4^+$ and $CD8^+$ T-cell was similar for patients and controls. The percentage of granulocytes in the leukocyte population was 2.3 times increased in patients compared to controls.

The absolute number of leukocytes at t = 0 was less than $20*10^9$/L for all patients except patient 3 (Figure 1A). As expected from previous studies this patient had the lowest Paediatric Index of Mortality (PIM) score and predicted death rate based on the Rotterdam score. Only the PRISM score was similar to those of the other patients. In all patients a rise in the leukocyte counts was observed followed by a decline in four patients (1, 3, 5, and 6). The initial percentage of lymphocytes was highest in the two youngest patients (2 and 5), who also had the highest predicted death rate (Figure 1B), and this was followed by a decline. The percentage of granulocytes was markedly declined in these two patients (Figure 1D). The percentage of monocytes (CD14 +) increased over time in all but one patient (patient 4, Figure 1C). In all patients a rise in the number of B-lymphocytes was observed. Remarkably, after an initial substantial rise, a rapid decline in B-cell counts was observed in the most severely ill patient 5 (Figure 2A).

This patient showed the most pronounced rise in T-cells. Whereas others exhibited a gradual increase of the T-cell population over time, in this patient the initial rise was followed by a rapid decline at t = 24 h. It must be noted that T-cell counts at t = 24 h were similar for all patients (Figure 2B). NK-cell counts were low in all patients, when compared to the controls; patients 2 and 5 showed a subsequent temporarily increase in NK-cells (Figure 2C).

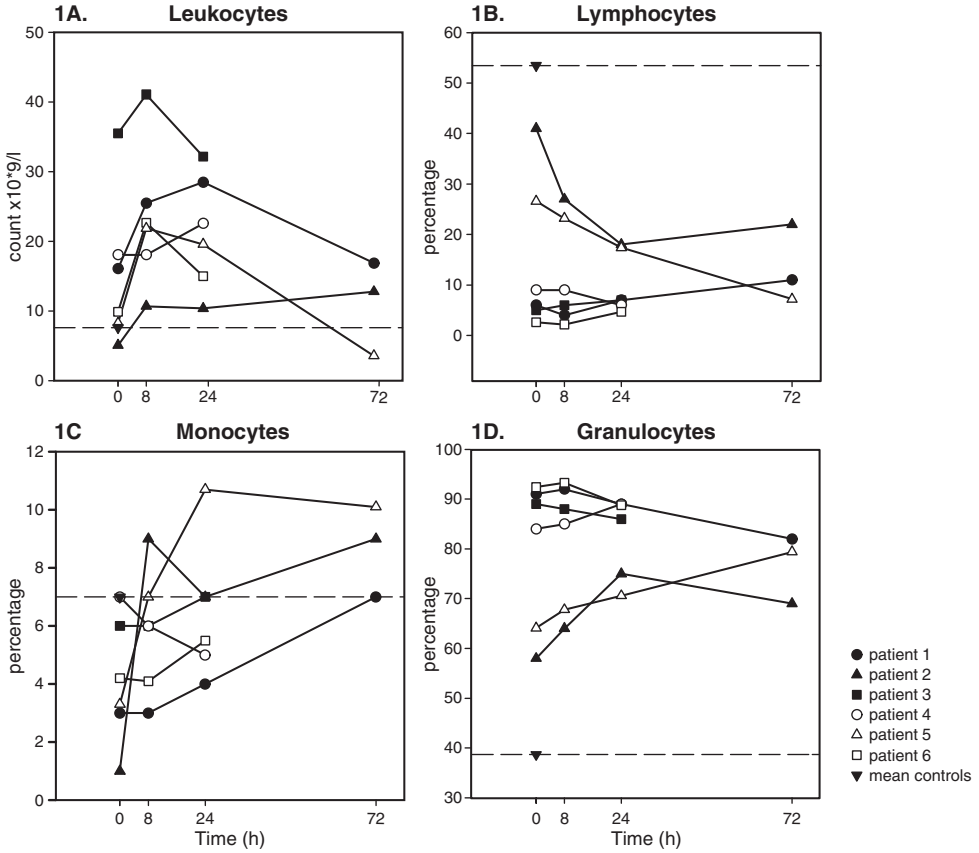

Figure 1. Absolute numbers of leukocytes and subsets.
Total leukocyte counts (1A), percentage of lymphocytes (1B), monocytes (1C) and granulocytes (1D) for individual patients at the different time points, and the means for controls (dashed line).

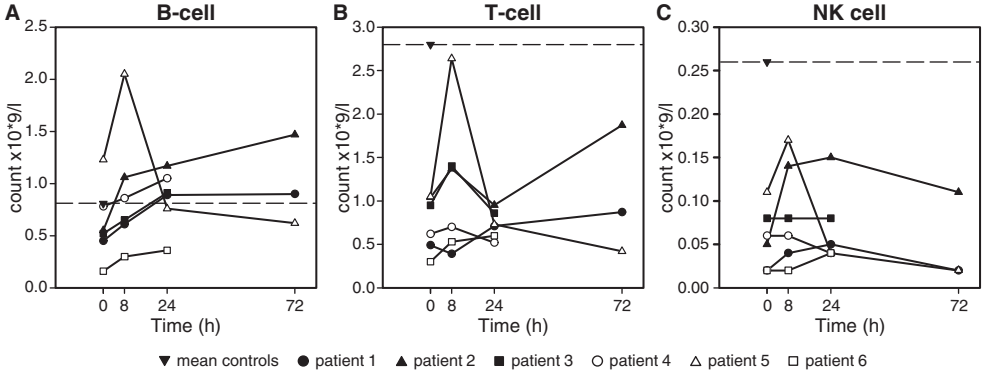

Figure 2. Lymphocyte subsets.
Absolute number of B-lymphocytes (2A), T-lymphocytes (2B) and NK-cells (2C) for individual patients at the different time points, and the means for controls (dashed line).

mRNA expression profiling. Since the volume of blood that could be drawn from young and severely ill children was limited, only a minimum amount of material was available for all the analyses. The quantity and quality of RNA isolated was not always sufficient to allow micro-array analysis. Since no such problems occurred when optimizing the experiments using similar quantities of blood from healthy controls it appears likely that the extreme activation of inflammatory systems in patients with meningococcal sepsis results in loss of good quality RNA. We encountered this problem mainly in the isolation of granulocytes and the RNA from these cells, suggesting that myeloid protease activity is at least in part responsible for RNA degradation. For this reason RNA from granulocytes was not included for micro-array analyses. Table 4 summarizes the number of samples available for each leukocyte subset, and the results of the between-subject analyses at each time point.

Table 4. Results of between-subject analyses.

Source	Time point (h)	Arrays patients at t=0[1]	Arrays controls/ patients at t=T [1,2]	#SDE, ANOVA	#SDE, ANOVA[3]
Blood	0	2 3	a b c	2416	2336
Blood	8	2 3	2	240	235
Blood	24	2 3	1 2 3	1177	1157
Blood	72	2 3	1 2	896	878
Lymphocytes	0	2 3	a b c d	2294	2223
Lymphocytes	8	2 3	1 3 4	1461	1393
Lymphocytes	24	2 3	1 2 3 4 6	1585	1492
Lymphocytes	72	2 3	1 2	571	561
Monocytes	0	1 3 4 6	a b d	2482	2034
Monocytes	8	1 3 4 6	1 2 3	172	170
Monocytes	24	1 3 4 6	1 2 3	630	609
Monocytes	72	1 3 4 6	1	451	441

SDE, significantly differentially expressed probeset ($p<0.05$).
[1]Numbers represent the patient identification numbers.
[2]Characters represent the individual controls.
[3]Number of SDE, when only molecules with mean expression > 100 in patients at t = 0 are included.

Only high quality samples, according to Affymetrix standards, were included in statistical analyses. In addition, samples that would require an additional amplification step (all samples from patient 5) were excluded from the current analysis. Using RMA based statistics and including molecules for which the mean expression level was higher than 100 for at least one time point (a measure roughly corresponding to the Affymetrix presence vs. absence call in our setting), a total of 21219, 20914 and 17073 of the initial 54676 probesets were significantly expressed in whole blood, lymphocytes and monocytes respectively.

Known inflammatory mediators in sepsis. In Figure 3 the expression patterns in whole blood and leukocyte subtypes for inflammatory mediators IL-8, TNF, IL-1B, IL-10, MIF, IL-6, and PFC (properdin), which are all molecules involved in meningococcal infection, are depicted.

Expression of IL8 in patients was increased in whole blood on admission, followed by a marked decrease at t = 24 h. This corresponds to the previously observed serum concentrations of IL-8 in patients with meningococcal sepsis.(25) Notably, IL-8 expression was increased from t = 8 h onwards in lymphocytes. Absolute expression

levels were however markedly lower than in monocytes, in which IL-8 expression was initially decreased and increased only at t = 72 h, suggesting important differences between the adaptive and immune system in the regulated secretion of this cytokine. Ex vivo IL-8 expression in peripheral blood mononuclear cells (PBMC) retrieved from meningococcal sepsis patients was increased in response to stimulation with meningococcal outer membrane complexes (OMCs) compared to PBMCs derived from healthy controls.(26)

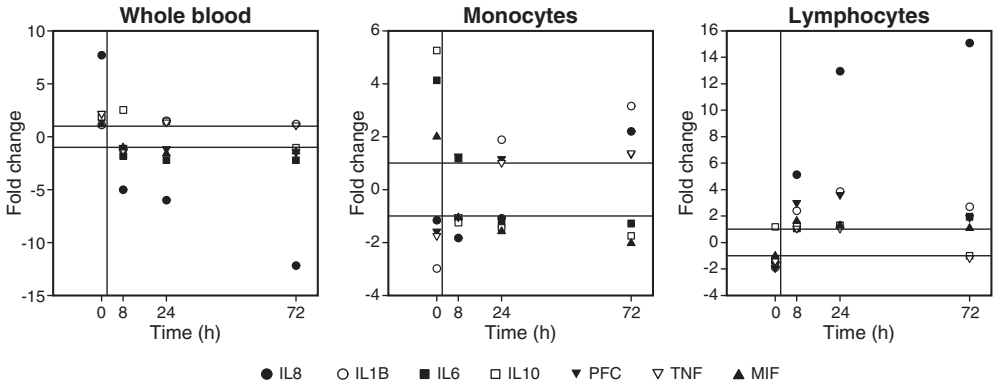

Figure 3. Expression patterns of well studied molecules in meningococcal disease.
Fold change expression of molecules in whole blood (3A), lymphocytes (3B) and monocytes (3C). At t = 0 the fold change expression of probesets in patients on admission is expressed relative to controls. At t = 8, 24 and 72 h, the fold change expression of probesets in patients at these time points relative to the expression in patients at t = 0 is depicted. Horizontal lines at -1 and 1 are inserted to indicate no change (fold change = 1). (IL, interleukin; TNF, tumor necrosis factor; PFC, properdin; MIF, macrophage migration inhibitory factor). Significant differences: A) in whole blood, IL8 at t = 0, 24 and 72; PFC at t = 0; MIF at t = 24 h. B) in lymphocytes IL8 at t = 8, 24 and 72 h; IL1B at t = 24 h; PFC at t = 0 and 24 h; MIF at t = 8 h. C) in monocytes IL8 at t = 8 and 72 h; IL1B at t = 0 and 72 h; IL6, IL10 and PFC at t = 0; MIF at t = 0, 24 and 72 h.

No significant alteration of expression levels was observed for TNF. Increased TNF expression is associated with increased mortality in meningococcal sepsis. In our study all patients survived. In addition, from animal studies it is known that TNF levels reach their maximum already at 1 h after infusion of endotoxin, followed by a decrease.(27) The median (min-max) duration of petechiae in the patients in our study was 9 (5.8-14) h, while the first symptoms occurred with a median (min-max) of 24 (19-27) h before admission to the PICU, which may explain the fact that no differential TNF expression is observed.
IL-1B expression in monocytes was decreased initially while no change in expression levels was observed in whole blood or lymphocytes. Similar to TNF it is suspected that an increased IL-1B expression occurs early in the preclinical phase of disease. (28) IL-10 expression was increased in patient monocytes on admission. This was not reflected by an increased expression in whole blood, stressing the importance of analyzing purified subsets in profiling studies. Plasma IL-10 was previously shown to be increased in meningococcal septic shock, although a wide range was observed. (29) Plasma MIF levels are persistently increased in children with meningococcal

sepsis.(8) RNA expression profiles of MIF showed an increased expression in monocytes on admission followed by a decline, while no differential expression was observed in whole blood. It must be noted that no expression profiles are available for macrophages, which, in addition to the pituitary gland, are one of the major sources of MIF production.(30, 31) IL-6 expression was markedly increased in monocytes of patients at t = 0 when compared to controls. IL-6 was previously found to be a mediator of cardial depression in meningococcal disease and is an important inflammatory mediator and involved in B-cell activation.(6)

Table 5. Top 25 SDE molecules in whole blood, monocytes and lymphocytes in patients when compared to controls.

Function	Whole blood	Monocytes	Lymphocytes
Immune response	**IL1R2, CEACAM1, HGF, C190RF59,** MME, HLA-DQA1	**IL1R2, CXCR7, CD163, C1QB, MRC1,** HLA-DQ1A, ERAP2, ALDH1, HLA-DRB2, HLA-DMB	**HLA-DRB4, PPBP,** FCGR3B, TGFBI, CTSW, KLRF1, c-hluPGFS, BY55, CX3CR1, TCRA, FCER1A, HLA-DQ1A, HLA-DR1B
Coagulation/fibrinolysis	**HGF**	**TIMP4**	**PPBP**
Signalling	**ANKRD22**	**ENPP2, MS4A4, CXCR7,** HM74, OLIG1	**SPRY4,** MYBL1, LRRN3, TGFBI, CX3CR1, EDG8
LPS/ bacterial neutralization	**LCN2, BPI**	**MRC1**	**PPBP**
Apoptosis		LGALS2	GZMB, TGFBI, IGFBP7
Anti-apoptosis	**GW112, HGF**	**CXCR7**	
Wound repair	**MMP8, HGF**	**TMP4?**	TGFBI
Cell adhesion	**GW112, CEACAM1, HGF**	**CXCR7?**	
Insulin resistance	**FIZZ3**	**FIZZ3**	**SPRY4?,** IGFBP7
Iron binding	**MMP8, LTF, HP, HPR**		
Metabolism	**PFKFB2, OLAH**	**ENPP2, OLAH, SLC21A12, METTL7B,** ALDH1	**PPBP**
Mast cell degranulation		FCERA1	FCER1A
Metal transport		**SLC39A8**	
Unknown	**PRV1, NMES1, ASM3A, ORF1-FL49, FLJ38815**	**NKG7,** IFI44L, FAM20A	PSPHL, CCDC146

Genes depicted in bold showed increased expression in patients when compared to controls. '?' indicates possible function for this molecule.

Top 25 fold change differential expression in leukocyte subtypes. In whole blood, lymphocytes and monocytes differential expression was observed for 2336, 2223, and 2034 molecules, respectively, between controls and patients at t = 0 (Table 4). In whole blood predominantly an increased expression was observed (1367 increased versus 969 probesets decreased expression), while in lymphocytes and monocytes

little over half the probesets differentially expressed showed decreased expression at t = 0 when compared to controls (1206 versus 1017 probesets in lymphocytes and 1192 versus 842 probesets in monocytes, respectively). The significantly differentially ($p<0.05$) expressed probesets with the 25 highest fold changes in whole blood, lymphocytes and monocytes between patients at t = 0 and controls are listed in Tables 5, S1, S2, and S3, respectively.

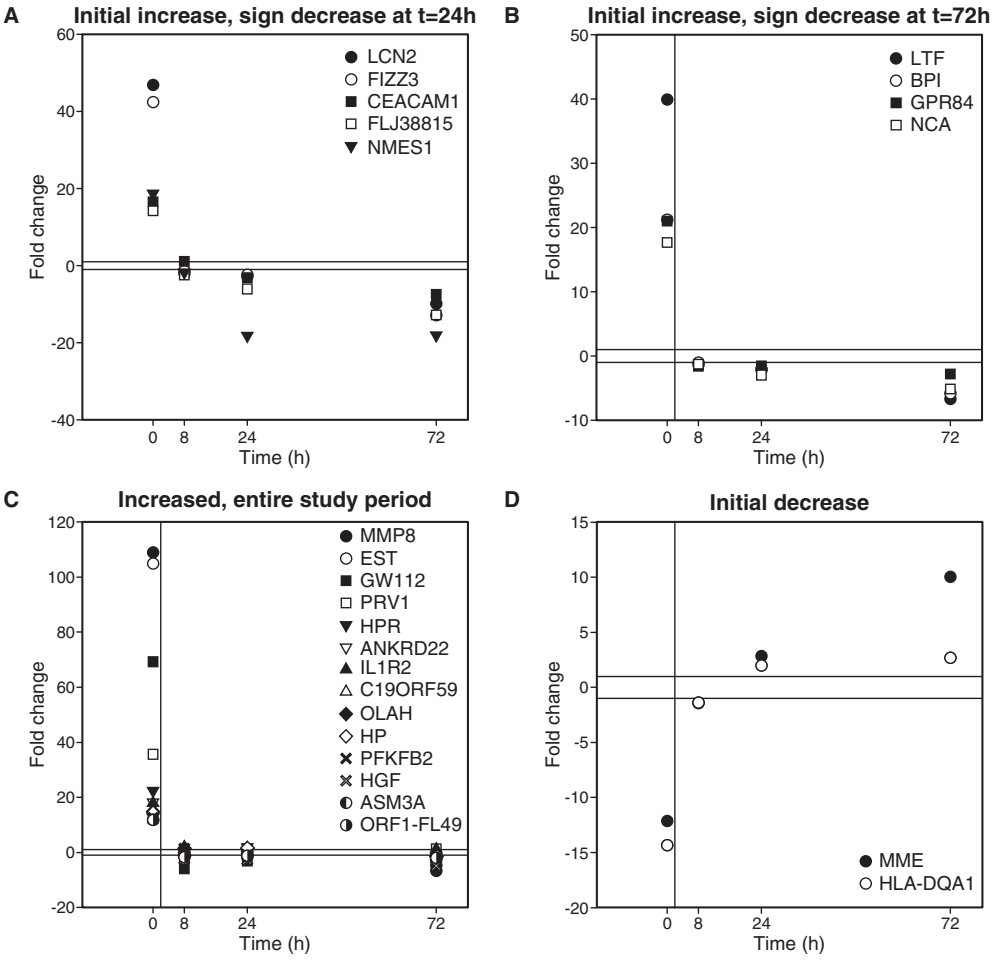

Figure 4. Expression pattern of molecules in whole blood.
At t = 0 the fold change in expression of probesets in patients on admission is expressed relative to controls. At t = 8, 24 and 72 h, the fold change expression of probesets in patients at these time points relative to the expression in patients at t = 0 is depicted. Horizontal lines at -1 and 1 are inserted to indicate no change (fold change = 1). **4A.** Probesets with increased expression at t = 0, when compared to controls, followed by a significant decrease at t = 24 h when compared to t = 0 in patients. **4B.** Probesets with increased expression at t = 0, when compared to controls, followed by a decrease at t = 72 h when compared to t = 0 in patients. **4C.** Probesets with increased expression at t = 0, when compared to controls, that show persistently increased expression. **4D.** Probesets with decreased expression at t = 0, when compared to controls.

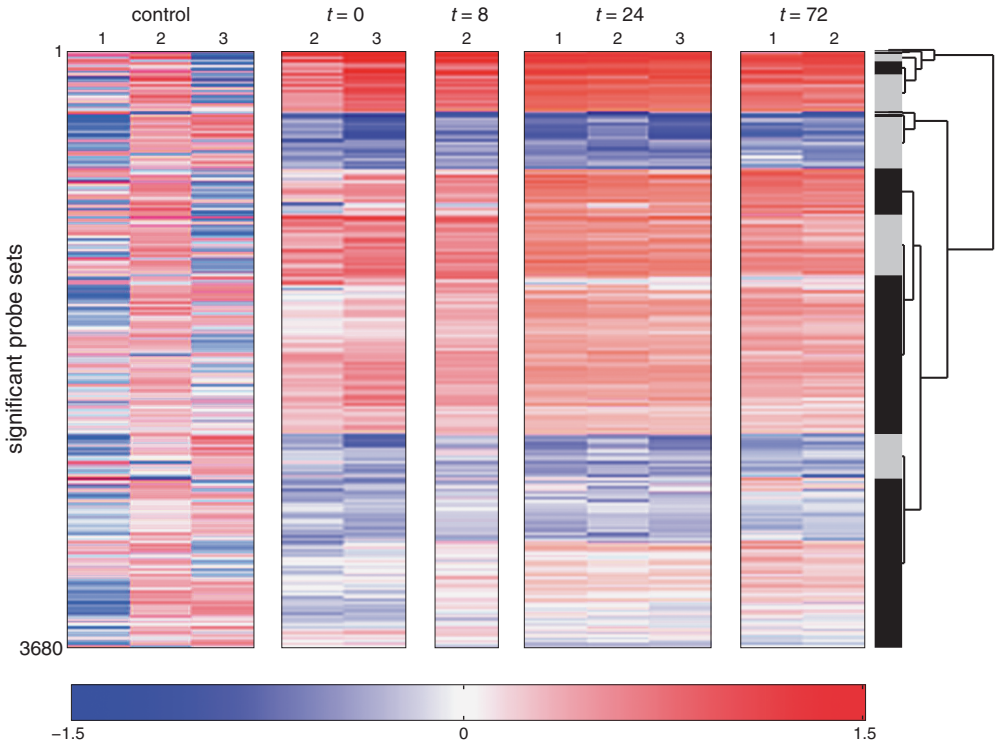

Figure 4S. Expression pattern of molecules differentially expressed in whole blood in patients and controls.
Horizontally: arrays in blocks per time point, the numbers above indicate the patient id number. Vertically: probesets differentially expressed between controls and patients at t = 0, t = 8 and t = 0, t = 24 h and t = 0 and/or t = 72 h and t = 0, and an expression level of at least 100 on at least one array. Values are expressed as log2-ratios between the array and the average of the controls.

In whole blood the top 25 list includes several molecules expressed by granulocytes (MMP8, GW112, PRV1 and HGF), molecules involved in innate immune responses and LPS or bacterial neutralization (IL1R2, CEACAM1, HGF, C19ORF59, MME, LCN2, BPI and GPR84), iron binding (MMP8, LTF, HP and HPR), insulin resistance (FIZZ3), metabolism and signalling (PFKFB2, OLAH, and ANKRD22), and coagulation and fibrinolysis (HGF). Five molecules with unknown function were included in this list (PRV1, NMES1, ASM3A, ORF1-FL49 and FLJ 38815). The expression patterns of these molecules during the study period are depicted in Figure 4, and Figure 4S (including all differentially expressed molecules in whole blood). In contrast to previously, most probesets showed an increased expression even after 24 h (Figure 4C).
Markedly, twenty probesets differentially expressed in lymphocytes present in the top 25 list showed a decreased expression in patients when compared to controls. Five out of these are specific for NK-cells. It is unclear whether this reflects the decreased number of NK cells or a true inhibition of NK cells, since multiple probesets involved

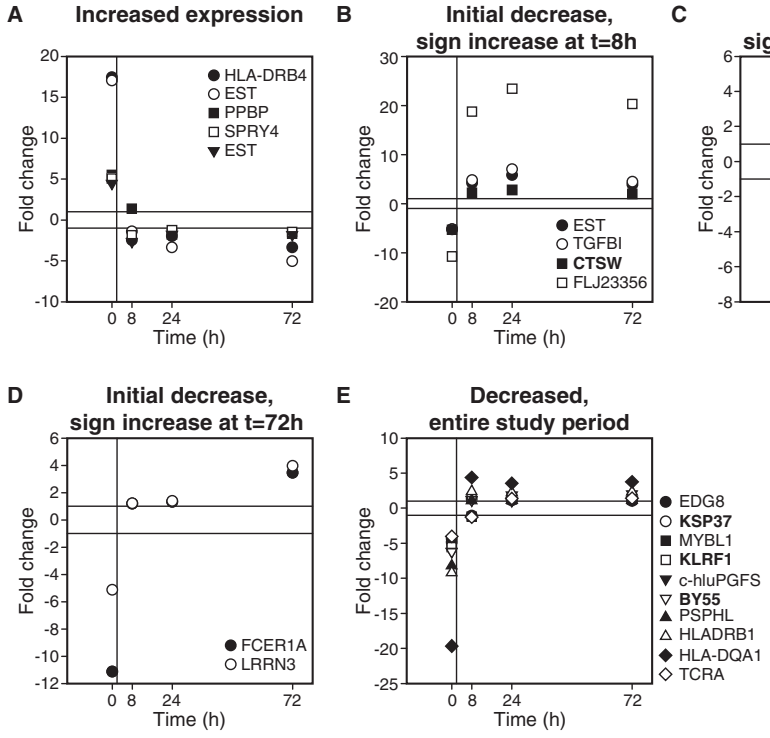

Figure 5. Expression pattern of molecules in lymphocytes.
At t = 0 the fold change expression of probesets in patients on admission is expressed relative to controls. At t = 8, 24 and 72 h, the fold change expression of probesets in patients at these time points relative to the expression in patients at t = 0 is depicted. Horizontal lines at -1 and 1 are inserted to indicate no change (fold change = 1). In **bold** the NK-cell specific probesets are depicted. **5A.** Probesets with increased expression in patients throughout the study period. **5B.** Probesets with decreased expression at t = 0, when compared to controls, that show significantly increased expression at t = 8 h. **5C.** Probesets with decreased expression at t = 0, when compared to controls, that show significantly increased expression at t = 24 h. **5D.** Probesets with decreased expression at t = 0, when compared to controls, that show significantly increased expression at t = 72 h. **5E.** Probesets with decreased expression in patients throughout the study period.

in immune response and signalling pathways showed decreased expression in the lymphocyte population. Expression of half of the depressed probesets remained decreased throughout the study period (Figures 5 and 5S). Two of the probesets in the top 25 have an unknown function (PSPHL and CCDC146).

Most differentially expressed probesets in monocytes represented molecules involved in innate immune responses (IL1R2, CXCR7, CD163, C1QB, MRC1, ERAP2, ALDH1, and HLA-DRB2), metabolism (ENPP2, OLAH, SLC21A12, METTL7B, and ALDH1) or signalling pathways (ENPP2, MS4A4, CXCR7, HM74, and OLIG1). Three probesets of unknown function were in the top 25 (NKG7, IFI44L, and FAM20A). Most of the probesets that exhibited an increased expression at t = 0 showed a significant decline after the first 24 h (Figures 6 and 6S).

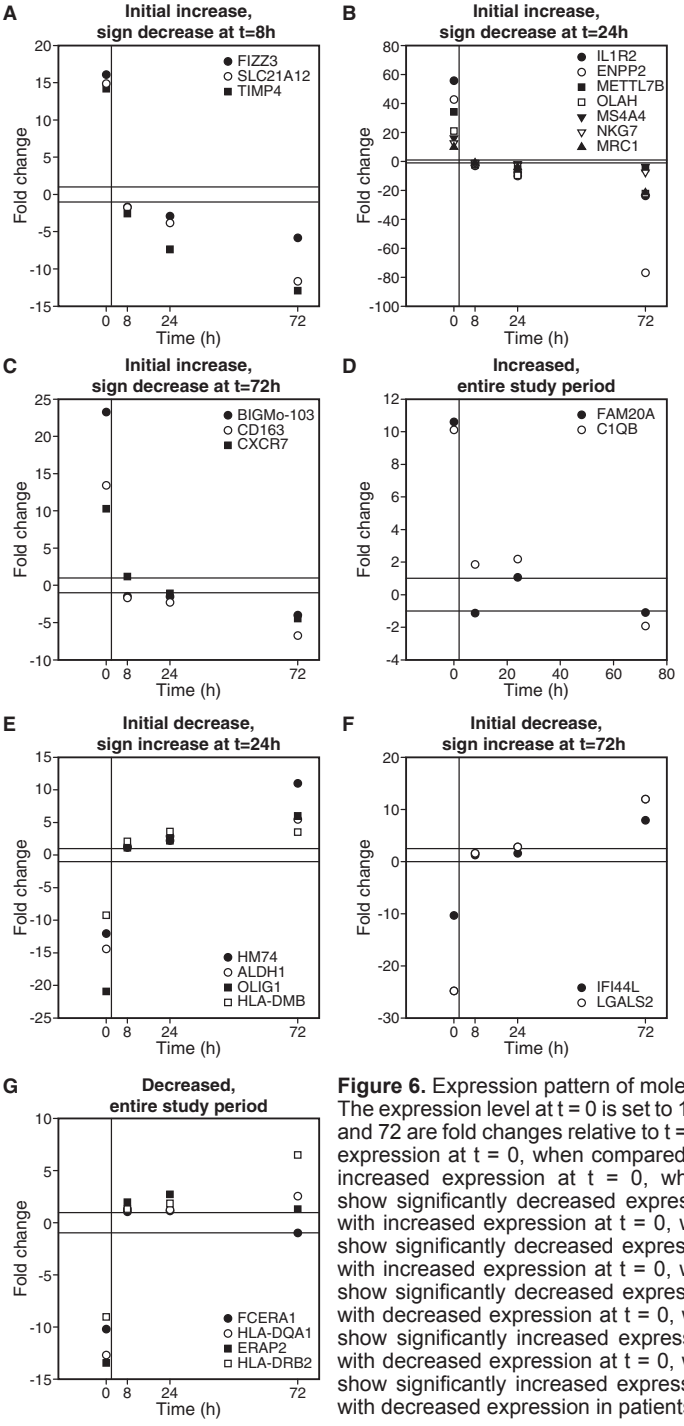

Figure 6. Expression pattern of molecules in monocytes.
The expression level at t = 0 is set to 1. All expression levels at t = 8, 24 and 72 are fold changes relative to t = 0. **6A.** Probesets with increased expression at t = 0, when compared to controls. **6B.** Probesets with increased expression at t = 0, when compared to controls, that show significantly decreased expression at t = 8 h. **6C.** Probesets with increased expression at t = 0, when compared to controls, that show significantly decreased expression at t = 24 h. **6D.** Probesets with increased expression at t = 0, when compared to controls, that show significantly decreased expression at t = 72 h. **6E.** Probesets with decreased expression at t = 0, when compared to controls, that show significantly increased expression at t = 24 h. **6F.** Probesets with decreased expression at t = 0, when compared to controls, that show significantly increased expression at t = 72 h. **6G.** Probesets with decreased expression in patients throughout the study period.

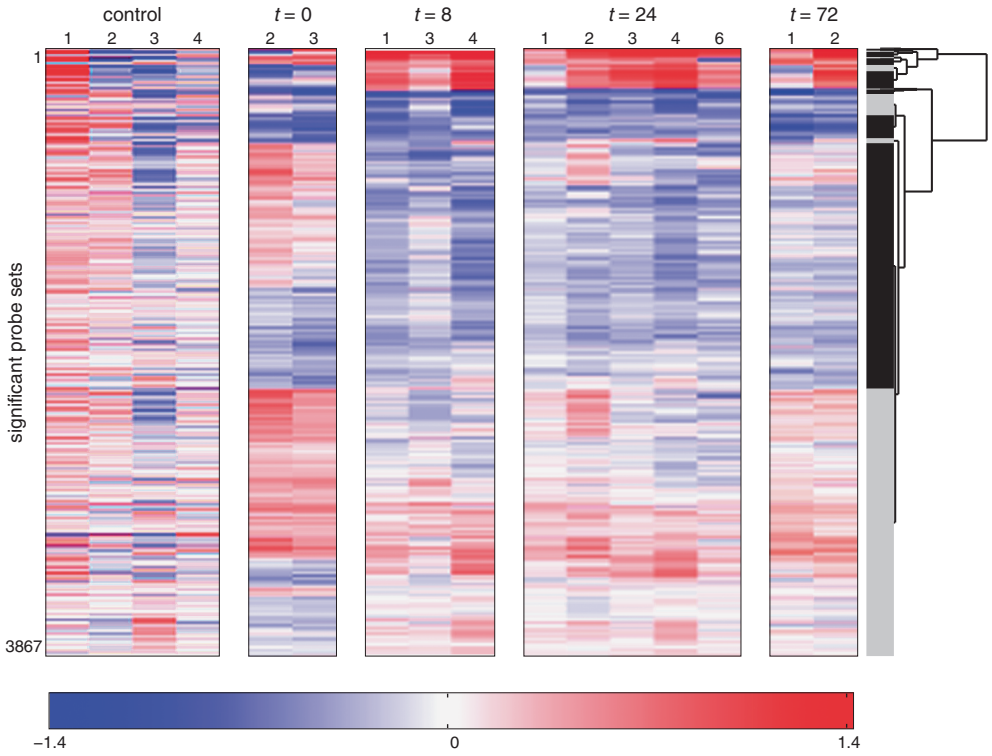

Figure 5S. Expression pattern of molecules differentially expressed in lymphocytes in patients and controls. Horizontally: arrays in blocks per time point, the numbers above indicate the patient id number. Vertically: probe sets differentially expressed between controls and patients at t = 0, t = 8 and t = 0, t = 24 h, and t = 0 and/or t = 72 h, and t = 0, and an expression level of at least 100 on at least one array. Values are expressed as log2-ratios between the array and the average of the controls.

Markedly some B-cell specific markers showed differential expression in the monocytes. This most likely reflects B-cell contamination in the monocyte population, resulting from the autoMACS method used to isolate the monocytes. Most results, however, can be considered true reflections of expression in monocytes, as they relate to moncyte-specific genes.

Most importantly, the differentially expressed probesets identified in whole blood, lymphocytes and monocytes in this study can serve as a disease-specific signature for meningococcal sepsis.

Pathway analysis. Using Ingenuity Pathway Analysis (5.5.1-1002) we analyzed the pathways corresponding to genes differentially expressed between patients and controls (Top 10 in Tables 6, 7, and 8, and complete overview in supplemental Tables S4, S5, and S6).

At admission, an overall increase in both pro-inflammatory and anti-inflammatory pathways was observed in whole blood and monocytes (Tables 6 and 8). In contrast, expression profiles were mainly down regulated in lymphocytes (Table 7).

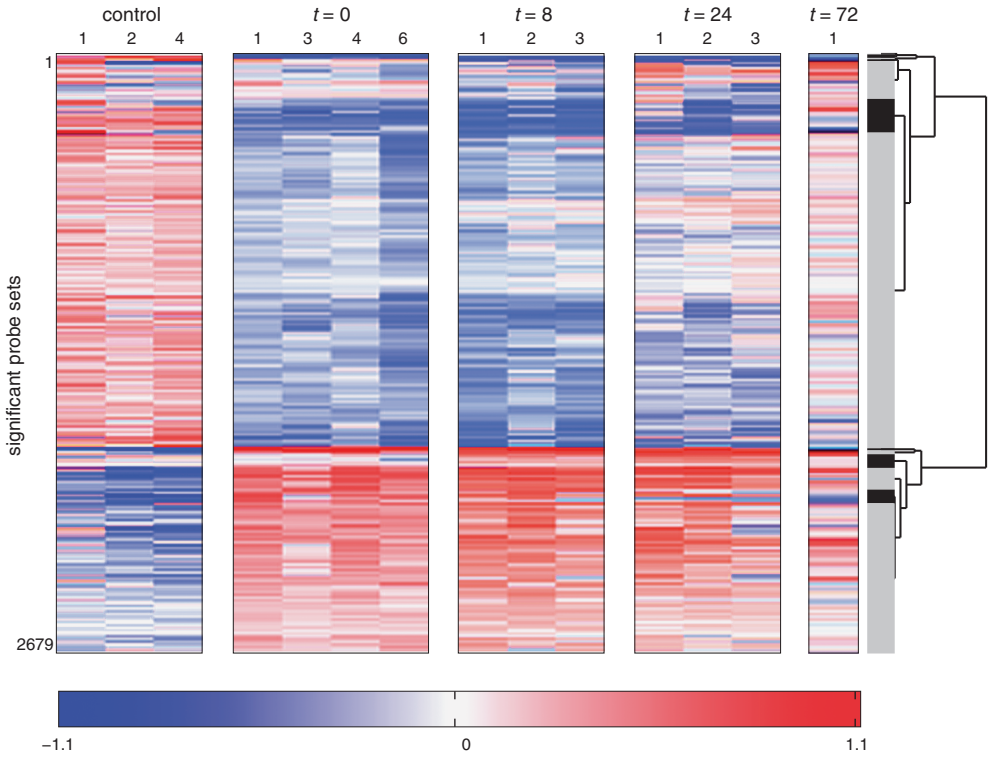

Figure 6S. Expression pattern of molecules differentially expressed in monocytes in patients and controls. Horizontally: arrays in blocks per time point, the numbers above indicate the patient id number. Vertically: probesets differentially expressed between controls and patients at t = 0, t = 8 and t = 0, t = 24 h and t = 0 and/or t = 72 h and t = 0, and an expression level of at least 100 on at least one array. Values are expressed as log2-ratios between the array and the average of the controls.

Table 6. Pathways differentially expressed at t = 0 in whole blood.

		In patients compared to controls		
Pathway	**-Log(p-value)**	**Ratio**	**Upregulated**	**Downregulated**
Protein ubiquitination pathway	*2.09E+01*	*8.03E-01*	*95/203 (47%)*	*78/203 (38%)*
B cell receptor signalling	*1.92E+01*	*8.58E-01*	*80/148 (54%)*	*47/148 (32%)*
Glucocorticoid receptor signalling	*1.42E+01*	*7.06E-01*	*117/265 (44%)*	*72/265 (27%)*
SAPK/JNK signalling	*1.31E+01*	*8.23E-01*	*75/147 (51%)*	*46/147 (31%)*
Oxidative phosphorylation	*1.30E+01*	*6.90E-01*	*54/158 (34%)*	*57/158 (36%)*
Huntington's disease signalling	*1.26E+01*	*7.20E-01*	*112/232 (48%)*	*57/232 (25%)*
Apoptosis signalling	*1.22E+01*	*8.31E-01*	*69/136 (51%)*	*44/136 (32%)*
PI3K/AKT signalling	*1.22E+01*	*7.50E-01*	*83/176 (47%)*	*49/176 (28%)*
Mitochondrial dysfunction	*1.14E+01*	*6.06E-01*	*51/165 (31%)*	*49/165 (30%)*
T cell receptor signalling	*1.09E+01*	*8.04E-01*	*43/102 (42%)*	*39/102 (38%)*

The molecules not represented in the dataset account for the missing numbers and will make up to 100%.

Table 7. Pathways differentially expressed at t = 0 in lymphocytes.

		In patients compared to controls		
Pathway	**-Log(p-value)**	**Ratio**	**Upregulated**	**Downregulated**
Protein ubiquitination pathway	*2.81E+01*	*8.28E-01*	*85/203 (42%)*	*93/203 (46%)*
B cell receptor signalling	*2.02E+01*	*8.51E-01*	*46/148 (31%)*	*80/148 (54%)*
Glucocorticoid receptor signalling	*1.49E+01*	*6.94E-01*	*70/265 (26%)*	*116/265 (44%)*
Huntington's disease signalling	*1.47E+01*	*7.20E-01*	*62/232 (27%)*	*107/232 (46%)*
PI3K/AKT signalling	*1.40E+01*	*7.50E-01*	*52/176 (30%)*	*81/176 (46%)*
Apoptosis signalling	*1.38E+01*	*8.31E-01*	*44/136 (32%)*	*69/136 (51%)*
SAPK/JNK signalling	*1.34E+01*	*8.10E-01*	*47/147 (32%)*	*72/147 (49%)*
Natural killer cell signalling	*1.34E+01*	*8.27E-01*	*15/110 (14%)*	*76/110 (69%)*
Mitochondrial dysfunction	*1.28E+01*	*6.06E-01*	*28/165 (17%)*	*72/165 (44%)*
Oxidative phosphorylation	*1.24E+01*	*6.71E-01*	*25/158 (16%)*	*83/158 (53%)*

The molecules not represented in the dataset account for the missing numbers and will make up to 100%.

Table 8. Top 10 pathways differentially expressed at t = 0 in monocytes.

		In patients compared to controles		
Pathway	**-Log(p-value)**	**Ratio**	**Upregulated**	**Downregulated**
Protein ubiquitination pathway	*2.40E+01*	*7.64E-01*	*72/203 (35%)*	*86/203 (42%)*
B cell receptor signalling	*2.05E+01*	*8.11E-01*	*36/148 (24%)*	*84/148 (57%)*
Huntington's disease signalling	*1.76E+01*	*6.94E-01*	*64/232 (28%)*	*98/232 (42%)*
Oxidative phosphorylation	*1.71E+01*	*6.71E-01*	*67/158 (42%)*	*40/158 (25%)*
Mitochondrial dysfunction	*1.65E+01*	*6.00E-01*	*55/165 (33%)*	*44/165 (27%)*
Glucocorticoid receptor signalling	*1.63E+01*	*6.57E-01*	*56/265 (21%)*	*119/265 (45%)*
Apoptosis signalling	*1.57E+01*	*8.01E-01*	*38/136 (28%)*	*71/136 (52%)*
PI3K/AKT signalling	*1.54E+01*	*7.16E-01*	*42/176 (24%)*	*84/176 (48%)*
NRF2-mediated oxidative stress response	*1.47E+01*	*7.33E-01*	*60/180 (33%)*	*72/180 (40%)*
SAPK/JNK signalling	*1.36E+01*	*7.62E-01*	*35/147 (24%)*	*77/147 (52%)*

The molecules not represented in the dataset account for the missing numbers and will make up to 100%.

Depending on the leukocyte subset analyzed, the interpretation may differ as reflected for instance by an overall increase in the number of genes involved in B-cell receptor signalling and glucocorticoid receptor signalling in whole blood, while in lymphocytes and monocytes a decrease in many of these genes is noted. However, as both positive and negative regulators of each pathway are scored, these results show that both B-cell signalling and glucocorticoid receptor signalling are activated. Consistent with analysis of the individual probesets, most NK-cell related genes are downregulated in expression (70%). In addition, for the oxidative phosphorylation pathway, approximately equal numbers of up- and down-regulated probesets were observed in whole blood, while in lymphocytes a general down-regulation and in monocytes a general up-regulation was observed.

Discussion

Our study is the first to report global gene expression profiling on leukocyte subsets isolated from children with severe meningococcal sepsis over the first 72 h after admission to the PICU. Previous reports were limited to *ex vivo* or *in vitro* studies using cell cultures. Although *in vitro* cell culture studies allow for the exact timing of sampling, they do not allow interaction between multiple different cell types and can only minimally mirror the complexities occurring between bacterium and host in clinical settings.

The design and implementation were elaborate, in terms of ethical acceptability, study logistics, as well as sample acquisition, volume and sample processing. Furthermore, meningococcal sepsis has an exceedingly complex and very poorly understood mechanistic basis, requiring repeated sampling over a short time span. To avoid inter researcher variation, we involved as few people as possible in the actual clinical sampling. Subsequent experimentation and interpretation of the results was performed by an interdisciplinary team including paediatricians, immunologists, intensive care specialists and bioinformaticians. We were able to identify numerous differentially expressed probesets. In the discussion below, we outline our findings and provide several recommendations for further research.

We have hereby defined a disease-specific candidate molecular signature for meningococcal sepsis. Seminal work by Ramilo et al. has identified mRNA expression signatures discriminating *E. coli* versus *S. aureus* infections, or patients with either influenza A, *E. coli*, or *S. pneumoniae* infection.(32) We have overlap of a few genes, as can be expected as common immune response genes likely are involved in any type of bacterial infection. The only 16 overlapping probesets between that and our study are ZNF6, PFDN5, ACTG1, GAPD, RGS2, AF1Q, LRRN3, RASA1, SMAD2, IGHG3, ALOX5AP, P2RX1, SCA1, MAP1LC3B, FLJ10262, and RAC2, which were differentially expressed in lymphocytes between patients and controls. As a caveat in this comparison, several factors should be mentioned. First, we only included patients early in infection in the PICU, while Ramilo et al. included patients with microbiologically confirmed infection. In addition, Ramilo et al. analyzed expression profiles in complete PBMC while we purified monocytes and lymphocytes, allowing assessment of expression profiles in lymphocytes separately. Finally, we studied the kinetics of the expression profiles during the stay in the PICU.

Patient parameters and leukocyte counts. All patients included were in need of mechanical ventilation and four out of six had DIC. Despite the high disease severity, none of the patients in our study died. Five out of six patients had relatively very severe disease, which may result from the fact that these patients were 5 years or younger. (18, 33-36) Although total leukocyte counts did not differ significantly between patients and controls, one could argue that a relative leukopenia was observed in patients 2, 5 and 6, considering the disease severity. This has been observed previously, and ranges in meningococcal infection vary from absolute leukopenia to leukocytosis.

Results of immunophenotyping of leukocyte subsets showed high granulocyte and low lymphocyte percentages, as expected in bacterial infection. The decreased

percentage of lymphocytes was attributable to decreased numbers of T-cells and NK-cells. The relative contribution of T-cell subsets ($CD4^+$ and $CD8^+$) was similar in patients and controls. The increase in granulocyte number was not accompanied by a significantly increased expression of CD64, an Fc receptor which is upregulated during inflammation, indicating that the increased activity mainly results from the increased number of cells rather than the activation of individual cells.

Four patients already showed high granulocyte percentages at t = 0, whereas in the two youngest patients a gradual increase over 72 h was observed. These were also the patients that showed the highest lymphocyte percentage and the lowest total leukocyte count on admission. This may reflect admission to the PICU at distinct time points of disease course. Another explanation could be the lower age of the latter two patients.(37) Together with patient 3, these two patients showed an increase in T-cell counts at t = 8, which was not observed in the older patients. In addition B- en NK-cells counts were increased. This may indicate a general activation of the immune system in this rapid onset severe infectious disease, rather than a more directed activation.

The two youngest patients (1.37 and 1.51 years) showed an initial increase in leukocyte counts after admission, followed by a gradual decrease. Most likely this reflects consumption of leukocytes/granulocytes in the immune response against meningococci. However, after the initial proinflammatory response, an anti-inflammatory response is elicited. This is essential in controlling potential secondary damage of pro-inflammatory mediators.(38) The pro-inflammatory state results from the initial release of pro-inflammatory mediators elicited by LPS. The short pro-inflammatory peak is then followed by an increase in the counterregulatory cytokines (e.g. IL-10), thus resulting in the counterinflammatory response as observed in the decrease of total leukocytes, lymphocytes, monocytes and T-cells. A more detailed analysis of cell counts is not feasible due to the small number of patients included.

Kinetics of global gene expression in early sepsis. Previous studies in adults using expression profiling showed that infection with Gram-positive or Gram-negative microbes results in distinct responses.(39) While infection with Gram-negative organisms resulted in increased expression of molecules involved in inflammation and innate immunity, Gram-positive pathogens elicit expression of genes related to protein synthesis, ribosomal proteins and cell-cycling.(39) Infection with the Gram-negative *N. meningitidis* has a special position, since it is characterized by a very rapid onset of disease in previously healthy individuals.

As expected, analyses of the RNA expression profiles showed an increase of both inflammatory pathways, such as IL-6 and NFκB, and anti-inflammatory pathways, such as IL-10. In addition, Toll like receptor and B-cell receptor signalling pathways showed increased expression. Remarkably, in lymphocytes a downregulation of most pathways was observed, and in addition decreased lymphocyte percentages were observed. Unfortunately, in granulocytes, the contribution of which to the total leukocyte counts was increased in patients when compared to controls, RNA isolation was not successful in patients. Possibly an immense increase of pro-inflammatory mediators resulted in increased degradation of RNA *in vivo* in meningococcal disease,

since no problems were encountered using the same techniques for granulocytes from patients with viral infections or healthy controls. In addition, the samples of the most severely ill patients had to be excluded from the RNA expression analysis due to technical problems that prohibited a reliable comparison of results.

Since it is likely that expression profiles in granulocytes provide important information, future efforts will have to be focused on solving the technical difficulties in RNA isolation from activated granulocytes. Fortunately however, since the granulocytes contributed most to the total leukocyte count, several granulocyte specific probesets were identified in whole blood to show increased expression (MMP8, GW112, PRV1 and HGF). Apparently whole blood analysis allows a significant number of granulocytes to remain extractable for RNA isolation.

When assessing the lymphocyte population, a decreased expression of five probesets specific for NK-cells was observed in the top 25-fold change list. This was also observed when assessing differentially expressed pathways in lymphocytes in which 70% of molecules in the NK-cell signalling pathway were down regulated. This may in part reflect the relatively decreased number of NK cells in patients. In contrast, in meningococcal carriage, NK cells are responsible for the increased IFN-γ levels.(40) *In vitro* NK cells, but not B or T cells are responsible for antibody dependent cellular cytotoxicity (ADCC).(11, 41) For acute meningococcal infection, only few reports regarding the (innate) cellular immune response exist, and none include NK cell involvement. Possibly the contribution of leukocyte subsets differs for meningococcal colonization and acute infection.

Our data on monocytes *ex vivo* can be compared to those of Suzuki et al. who assessed RNA expression using SAGE, in *ex vivo* monocytes from healthy adult donors that were stimulated with LPS for three hours. In line with Suzuki's study, we found increased expression of IL-6 and Cystatin B and decreased expression of CASP, Aldehyde dehydrogenase 2, ATP synthase subunit C, transketolase, lysozyme, CD37 and Jun D.(42) For other molecules, their observations were not confirmed by our results or even in contrast with our results (Gelsolin, NFKB, IL1B, IL7R, IFN stimulated protein 15 kD, RANTES, COX2, IFN inducible protein 78 kD, MARCKS, and interferon-inducible gene I-8D). In mice, differences have been observed in expression profiles in macrophages derived from young and old mice upon *ex vivo* stimulation with LPS.(43) Immune response and signal transduction genes were specifically decreased in aged mouse macrophages. Moreover, *in vivo* interaction with other cell types is likely to alter gene expression profiles when compared to the *ex-vivo* or *in vitro* systems. In addition, both in the study by Suzuki et al. and our study, groups were small (8 donors and 4 patients and 3 controls, respectively).

Differences in expression profiles upon stimulation of PBMCs derived from different donors have previously been described.(44) These differences are even more pronounced when different cell lines are used to study LPS response.(44)

Limitations and recommendations. Our study is limited in the number of patients due to complexities in studying severely ill children. In order to validate our findings, a larger, prospective, case-control study should be performed. Despite the differences in expression profiles observed in studies with regard to the use of different cell types and

diverse pathogens, there seems to be a common host-transcriptional-response.(45) Our study is the first to investigate RNA expression profiles in paediatric meningococcal sepsis patients. Most studies focused on cell culture or animal models using LPS as a stimulus.(44, 46, 47) In the study by Prucha et al. in adult sepsis patients, a list of 50 differentially expressed genes was compiled indicating a homogeneous expression profile using inflammation specific arrays.(48) Despite the heterogeneous group of patients, sepsis patients could be distinguished from patients undergoing spine surgery for which no inflammatory symptoms were observed. Prucha et al. used only whole blood and no leukocyte subset analyses were performed.(48)

In our study, expression analysis was performed on both whole blood and leukocyte subsets. For logistic reasons and diagnostic efficiency the possibility to study whole blood expression patterns is favourable. However, we observed that expression patterns differ between leukocyte subsets, and moreover, the relative contribution of the leukocyte subsets to the whole leukocyte counts differs between patients. Using only whole blood therefore leads to biased and limited results. Previous studies showed that the concordance of expression patterns was higher within one healthy individuals' samples than between different healthy individuals, and that the concordance was higher in healthy individuals when compared to trauma patients. Moreover, concordance in leukocyte subsets was higher than in total leukocyte expression patterns, while the expression patterns between the different subsets differed markedly.(49) For these reasons we would argue to analyze leukocyte subsets instead of whole blood. As in our study we realize that this is not always possible due to the limited amounts of blood that can be drawn, which is especially true in severely ill (paediatric) patients. The development of novel methods that require low amounts of RNA to perform gene expression profiling will increase the possibility of further subset testing. To improve the quality and avoid finding differential expression resulting from contamination with other leukocyte subtypes, purity of isolated leukocyte subset should be optimized using stringent selection criteria.

Biological, immunological and clinical interpretation of the results requires the involvement of specialists in the field of immunology, paediatric intensive care, paediatric infectious diseases, and bioinformatics.

In conclusion, while leukocyte counts showed no overall difference between patients and controls, the percentage of granulocytes was increased in patients, while the contribution of lymphocytes, and markedly, NK-cells, was decreased.

Overall, as expected, RNA expression profiles were upregulated for diverse pathways, both pro- and anti-inflammatory, indicative for a complex pathogenesis. In lymphocytes, RNA expression was more often down regulated. Markedly a number of NK-cell and cytotoxic T-cell function specific probesets showed decreased expression, reflecting a possibly less important role for this leukocyte subset in meningococcal infection. Compared to haematological malignancies, gene expression profiling of infectious diseases, especially in paediatric settings is still in its infancy. However, similar to leukemias, strict guidelines on handling of samples, purification of leukocyte subsets and data interpretation are required.(50) Efforts formulating such guidelines and inclusion of larger cohorts of patients are currently being undertaken.

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Supplemental tables

Table S1. Top 25 SDE molecules in whole blood in patients when compared to controls.

Gene	Description	Fold Change[1]	accession no
MMP8	*matrix metalloproteinase 8 preproprotein*	*108.95*	*gb:NM_002424.1*
	ESTs	*104.83*	*gb:AW337833*
GW112	*differentially expressed in hematopoietic lineages*	*69.24*	*gb:AL390736*
LCN2	*lipocalin 2 (oncogene 24p3)*	*47.97*	*gb:NM_005564.1*
FIZZ3	*resistin*	*42.43*	*gb:NM_020415.2*
LTF	*lactotransferrin*	*39.87*	*gb:NM_002343.1*
PRV1	*polycythemia rubra vera 1; cell surface receptor*	*35.66*	*gb:NM_020406.1*
HPR	*haptoglobin-related protein*	*22.42*	*gb:NM_020995.1*
BPI	*bactericidal/permeability-increasing protein precursor*	*21.17*	*gb:NM_001725.1*
GPR84	*orphan G protein-coupled receptor 84*	*20.96*	*gb:AF237762.1*
NMES1	*normal mucosa of oesophagus specific 1*	*18.72*	*gb:AF228422.1*
ANKRD22	*ankyrin repeat domain 22, transcription factor*	*18.16*	*gb:AI925518*
IL1R2	*soluble type II interleukin-1 receptor*	*17.97*	*gb:U64094.1*
NCA	*non-specific cross reacting antigen*	*17.66*	*gb:M33326.1*
CEACAM1	*TM1-CEA preprotein, transmembrane carcinoembryonic antigen BGPa (biliary glycoprotein)*	*16.54*	*gb:X16354.1*
C19ORF59	*ESTs, possibly involved in immune response*	*15.18*	*gb:BF433657*
OLAH	*oleoyl-ACP hydrolase*	*14.95*	*gb:NM_018324.1*
HP	*haptoglobin*	*14.48*	*gb:NM_005143.1*
FLJ38815	*Homo sapiens cDNA FLJ38815 fis, clone LIVER2007548*	*14.23*	*gb:AK096134.1*
PFKFB2	*6-phosphofructo-2-kinase heart isoform*	*12.56*	*gb:AB044805.1*
HGF	*competitive HGF antagonist*	*12.18*	*gb:M77227.1*
ASM3A	*acid sphingomyelinase-like phosphodiesterase*	*11.86*	*gb:AA873600*
ORF1-FL49	*Homo sapiens mRNA for putative nuclear protein (ORF1-FL49)*	*11.75*	*gb:AL522667*
MME	*membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10)*	*-12.15*	*gb:AI433463*
HLA-DQA1	*DC class II histocompatibility antigen alpha-chain*	*-14.33*	*gb:X00452.1*

[1]Fold change between expression in patients at t = 0 and controls; a positive fold change indicates that expression in patients is increased compared to controls. EST; expressed sequence tag.

Table S2. Top 25 SDE molecules in lymphocytes in patients when compared to controls.

Gene	Description	Fold change[1]	accession no
HLA-DRB4	*Major histocompatibility complex, class II, DR beta 4*	*17.44*	*gb:BC005312.1*
	ESTs	*17.05*	*gb:AW262311*
PPBP	*pro-platelet basic protein*	*5.50*	*gb:R64130*
SPRY4	*sprouty (Drosophila) homolog 4*	*5.08*	*gb:AI355441*
	ESTs	*4.47*	*gb:AA740831*
TCRA	*T-cell receptor alpha*	*-4.01*	*gb:X72501.1*
FCGR3B	*Fc fragment of IgG, low affinity IIIb, receptor for (CD16)*	*-4.18*	*gb:NM_000570.1*
	ESTs	*-4.35*	*gb:AI814092*
GZMB	*granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)*	*-4.43*	*gb:M36118.1*
KSP37	*Ksp37, Killer specific secretory protein, 37-KD*	*-4.85*	*gb:AB021123.1*
MYBL1	*v-myb avian myeloblastosis viral oncogene homolog-like 1*	*-5.09*	*gb:AW592266*
FLJ11129	*LRRN3 similar to murine leucine-rich repeat protein*	*-5.12*	*gb:AI221950*
CCDC146	*Coiled coil domain containing 146*	*-5.24*	*gb:AW135176*
TGFBI	*transforming growth factor, beta-induced, 68kD*	*-5.26*	*gb:NM_000358.1*
CTSW	*cathepsin W (lymphopain)*	*-5.29*	*gb:NM_001335.1*
KLRF1	*killer cell lectin-like receptor F1*	*-5.56*	*gb:NM_016523.1*
IGFBP7	*insulin-like growth factor binding protein 7*	*-5.68*	*gb:NM_001553.1*
c-hluPGFS	*Prostaglandin F synthase*	*-6.15*	*gb:AB018580.1*
BY55	*natural killer cell receptor, immunoglobulin superfamily member*	*-6.22*	*gb:NM_007053.1*
CX3CR1	*V28*	*-6.81*	*gb:U20350.1*
PSPHL	*L-3-phosphoserine phosphatase homolog*	*-8.21*	*gb:NM_003832.1*
	ESTs, possible tissue factor precursor	*-9.21*	*gb:AI377755*
FLJ23356	*Homo sapiens cDNA: FLJ23356 fis, clone HEP14919*	*-10.77*	*gb:AV711904*
FCER1A	*Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide*	*-11.13*	*gb:BC005912.1*
HLA-DQA1	*DC class II histocompatibility antigen alpha-chain*	*-19.66*	*gb:X00452.1*

[1]Fold change between expression in patients at t = 0 and controls; a positive fold change indicates that expression in patients is increased compared to controls. EST; expressed sequence tag.

Table S3. Top 25 SDE in monocytes blood in patients when compared to controls.

Gene	Description	Fold change[1]	accession no
IL1R2	*interleukin 1 receptor, type II*	*55.62*	*gb:NM_004633.1*
ENPP2	*autotaxin, phosphodiesterase I alpha*	*42.65*	*gb:L35594.1*
METTL7B	*methyltransferase like 7B*	*34.18*	*gb:AI827972*
BIGMo-103	*BCG induced integral membrane protein BIGMo-103*	*23.26*	*gb:AB040120.1*
OLAH	*oleoyl ACP hydrolase*	*20.98*	*gb:NM_018324.1*
MS4A4	*membrane-spanning 4-domains, subfamily A, member 4*	*16.09*	*gb:NM_024021.1*
FIZZ3	*resistin*	*16.08*	*gb:NM_020415.2*
SLC21A12	*organic anion transporter OATP-E*	*14.86*	*gb:NM_016354.1*
TIMP4	*tissue inhibitor of metalloproteinase 4 precursor*	*14.20*	*gb:NM_003256.1*
CD163	*M130 antigen cytoplasmic variant 2*	*13.41*	*gb:Z22970.1*
NKG7	*natural killer cell group 7 sequence*	*12.62*	*gb:NM_005601.1*
FAM20A	*family with sequence similarity 20, member A*	*10.59*	*gb:AI860568*
CXCR7	*RDC1, G protein-coupled receptor*	*10.28*	*gb:AI817041*
C1QB	*complement component 1, q subcomponent, beta polypeptide precursor*	*10.11*	*gb:NM_000491.2*
MRC1	*mannose receptor, C type 1*	*9.88*	*gb:NM_002438.1*
HLA-DRB2	*major histocompatibility complex, class II, DQ beta 1*	*-9.04*	*gb:M16276.1*
HLA-DMB	*major histocompatibility complex, class II, DM beta*	*-9.25*	*gb:NM_002118.1*
FCERA1	*receptor for Fc fragment of IgE, high affinity I; alpha polypeptide*	*-10.22*	*gb:BC005912.1*
IFI44L	*interferon-induced protein 44-like*	*-10.36*	*gb:NM_006820.1*
HM74	*putative chemokine receptor; GTP-binding protein*	*-12.04*	*gb:NM_006018.1*
HLA-DQA1	*DC classII histocompatibility antigen alpha-chain*	*-12.70*	*gb:X00452.1*
ERAP2	*endoplasmic reticulum endopeptidase 2*	*-13.45*	*gb:NM_022350.1*
ALDH1	*aldehyde dehydrogenase 1, soluble*	*-14.44*	*gb:NM_000689.1*
OLIG1	*oligodendrocyte transcription factor 1*	*-20.91*	*gb:AL355743.1*
LGALS2	*lectin, galactoside-binding, soluble, 2 (galectin 2)*	*-24.83*	*gb:NM_006498.1*

[1]Fold change between expression in patients at t = 0 and controls; a positive fold change indicates that expression in patients is increased compared to controls.

Table S4. Pathways differentially expressed at t = 0 in whole blood.

		In patients compared to controls		
Pathway	**-Log(p-value)**	**Ratio**	**Upregulated**	**Downregulated**
Protein ubiquitination pathway	2.09E+01	8.03E-01	95/203 (47%)	78/203 (38%)
B cell receptor signalling	1.92E+01	8.58E-01	80/148 (54%)	47/148 (32%)
Glucocorticoid receptor signalling	1.42E+01	7.06E-01	117/265 (44%)	72/265 (27%)
SAPK/JNK signalling	1.31E+01	8.23E-01	75/147 (51%)	46/147 (31%)
Oxidative phosphorylation	1.30E+01	6.90E-01	54/158 (34%)	57/158 (36%)
Huntington's disease signalling	1.26E+01	7.20E-01	112/232 (48%)	57/232 (25%)
Apoptosis signalling	1.22E+01	8.31E-01	69/136 (51%)	44/136 (32%)
PI3K/AKT signalling	1.22E+01	7.50E-01	83/176 (47%)	49/176 (28%)
Mitochondrial dysfunction	1.14E+01	6.06E-01	51/165 (31%)	49/165 (30%)
T cell receptor signalling	1.09E+01	8.04E-01	43/102 (42%)	39/102 (38%)
NRF2-mediated oxidative stress response	1.06E+01	7.61E-01	81/180 (45%)	56/180 (31%)
Hypoxia signalling in the cardiovascular system	8.92E+00	8.31E-01	41/71 (58%)	18/71 (25%)
Integrin signalling	8.85E+00	7.60E-01	100/192 (52%)	46/192 (24%)
Inositol phosphate metabolism	8.62E+00	6.07E-01	65/173 (38%)	41/173 (24%)
Natural killer cell signalling	8.43E+00	7.82E-01	45/110 (41%)	41/110 (37%)
ERK/MAPK signalling	8.24E+00	7.39E-01	110/226 (49%)	57/226 (25%)
GM-CSF signalling	7.91E+00	8.71E-01	36/62 (58%)	18/62 (29%)
Toll-like receptor signalling	7.91E+00	8.43E-01	32/51 (63%)	11/51 (22%)
Insulin receptor signalling	7.75E+00	7.29E-01	61/133 (46%)	36/133 (27%)
JAK/Stat signalling	7.71E+00	8.98E-01	36/59 (61%)	17/59 (29%)
Death receptor signalling	7.31E+00	8.36E-01	28/61 (46%)	23/61 (38%)
Estrogen receptor signalling	7.27E+00	7.54E-01	61/118 (52%)	29/118 (25%)
IL-2 signalling	6.98E+00	8.68E-01	33/53 (62%)	13/53 (25%)
Ubiquinone biosynthesis	6.98E+00	4.38E-01	18/105 (17%)	29/105 (28%)
NF-κB signalling	6.73E+00	7.27E-01	67/143 (47%)	37/143 (26%)
VEGF signalling	6.72E+00	7.39E-01	39/92 (42%)	29/92 (32%)
PDGF signalling	6.57E+00	7.97E-01	41/74 (55%)	18/74 (24%)
EGF signalling	6.46E+00	8.51E-01	25/47 (53%)	15/47 (32%)
IGF-1 signalling	6.21E+00	7.61E-01	48/92 (52%)	22/92 (24%)
IL-10 signalling	6.14E+00	7.50E-01	36/68 (53%)	15/68 (22%)
PPARα/RXRα activation	6.06E+00	6.36E-01	72/176 (41%)	40/176 (23%)
IL-4 signalling	5.99E+00	7.79E-01	29/68 (43%)	24/68 (35%)
Pyrimidine metabolism	5.93E+00	4.91E-01	53/226 (23%)	62/226 (27%)
IL-6 signalling	5.82E+00	7.69E-01	55/91 (60%)	15/91 (16%)
p53 signalling	5.61E+00	8.16E-01	39/87 (45%)	32/87 (37%)
Antigen presentation pathway	5.52E+00	7.18E-01	7/39 (18%)	21/39 (54%)
Purine metabolism	5.40E+00	4.69E-01	93/416 (22%)	106/416 (25%)
Actin cytoskeleton signalling	5.24E+00	6.55E-01	120/267 (45%)	55/267 (21%)
Ephrin receptor signalling	5.09E+00	6.59E-01	105/232 (45%)	48/232 (21%)
PPAR signalling	4.91E+00	6.74E-01	45/95 (47%)	19/95 (20%)
PTEN signalling	4.82E+00	7.39E-01	44/92 (48%)	24/92 (26%)
Citrate cycle	4.49E+00	4.58E-01	13/59 (22%)	15/59 (25%)
Fc epsilon RI signalling	4.17E+00	7.20E-01	43/100 (43%)	29/100 (29%)
Leukocyte extravasation signalling	3.90E+00	6.70E-01	85/188 (45%)	42/188 (22%)

Aminoacyl-tRNA biosynthesis	3.78E+00	4.12E-01	16/85 (19%)	21/85 (25%)
Cell cycle: G1/S checkpoint regulation	3.65E+00	7.33E-01	23/60 (38%)	21/60 (35%)
Amyloid processing	3.55E+00	7.88E-01	29/52 (56%)	12/52 (23%)
Neuregulin signalling	3.43E+00	6.81E-01	34/91 (37%)	28/91 (31%)
N-Glycan biosynthesis	3.39E+00	4.60E-01	27/87 (31%)	13/87 (15%)
Aryl hydrocarbon receptor signalling	3.39E+00	6.12E-01	54/152 (36%)	39/152 (26%)
Nucleotide excision repair pathway	3.37E+00	8.57E-01	14/35 (40%)	17/35 (49%)
Fructose and mannose metabolism	3.36E+00	2.50E-01	22/140 (16%)	13/140 (9%)
chemokine signalling	3.33E+00	7.33E-01	35/75 (47%)	20/75 (27%)

The molecules not represented in the dataset account for the missing numbers and will make up to a 100%.

Table S5. Pathways differentially expressed at t = 0 in lymphocytes.

		In patients compared to controls		
Pathway	**-Log(p-value)**	**Ratio**	**Upregulated**	**Downregulated**
Protein ubiquitination pathway	2.81E+01	8.28E-01	85/203 (42%)	93/203 (46%)
B cell receptor signalling	2.02E+01	8.51E-01	46/148 (31%)	80/148 (54%)
Glucocorticoid receptor signalling	1.49E+01	6.94E-01	70/265 (26%)	116/265 (44%)
Huntington's disease signalling	1.47E+01	7.20E-01	62/232 (27%)	107/232 (46%)
PI3K/AKT signalling	1.40E+01	7.50E-01	52/176 (30%)	81/176 (46%)
Apoptosis signalling	1.38E+01	8.31E-01	44/136 (32%)	69/136 (51%)
SAPK/JNK signalling	1.34E+01	8.10E-01	47/147 (32%)	72/147 (49%)
Natural killer cell signalling	1.34E+01	8.27E-01	15/110 (14%)	76/110 (69%)
Mitochondrial dysfunction	1.28E+01	6.06E-01	28/165 (17%)	72/165 (44%)
Oxidative phosphorylation	1.24E+01	6.71E-01	25/158 (16%)	83/158 (53%)
T cell receptor signalling	1.21E+01	8.04E-01	23/102 (23%)	59/102 (58%)
NRF2-mediated oxidative stress response	1.02E+01	7.39E-01	52/180 (29%)	81/180 (45%)
Pyrimidine metabolism	1.01E+01	5.18E-01	65/226 (29%)	57/226 (25%)
JAK/Stat signalling	9.48E+00	9.15E-01	19/59 (32%)	35/59 (59%)
IL-4 signalling	9.19E+00	8.24E-01	21/68 (31%)	35/68 (51%)
Death receptor signalling	9.03E+00	8.52E-01	17/61 (28%)	35/61 (57%)
p53 signalling	8.98E+00	8.62E-01	33/87 (38%)	42/87 (48%)
Hypoxia signalling in the cardiovascular system	8.94E+00	8.17E-01	26/71 (37%)	33/71 (46%)
Ubiquinone biosynthesis	8.69E+00	4.48E-01	12/105 (11%)	35/105 (33%)
Purine metabolism	8.67E+00	4.81E-01	97/416 (23%)	108/416 (26%)
Insulin receptor signalling	8.44E+00	7.22E-01	35/133 (26%)	61/133 (46%)
Estrogen receptor signalling	8.41E+00	7.54E-01	37/118 (31%)	53/118 (45%)
Inositol Phosphate Metabolism	8.34E+00	5.90E-01	46/173 (27%)	58/173 (34%)
GM-CSF signalling	7.88E+00	8.55E-01	15/62 (24%)	38/62 (61%)
IL-2 signalling	7.70E+00	8.68E-01	16/53 (30%)	30/53 (57%)
ERK/MAPK signalling	7.69E+00	7.12E-01	49/226 (22%)	113/226 (50%)
Aminoacyl-tRNA biosynthesis	7.68E+00	4.59E-01	29/85 (34%)	12/85 (14%)
Integrin signalling	7.52E+00	7.24E-01	40/192 (21%)	99/192 (52%)
EGF signalling	7.10E+00	8.51E-01	18/47 (38%)	22/47 (47%)
VEGF signalling	7.02E+00	7.28E-01	25/92 (27%)	42/92 (46%)
Cell cycle: G1/S checkpoint regulation	6.85E+00	8.00E-01	19/60 (32%)	29/60 (48%)
IGF-1 signalling	6.55E+00	7.50E-01	25/92 (27%)	44/92 (48%)
Nucleotide excision repair pathway	6.29E+00	9.43E-01	12/35 (34%)	22/35 (63%)
Ephrin receptor signalling	6.16E+00	6.55E-01	57/232 (25%)	95/232 (41%)
Antigen presentation pathway	5.99E+00	7.18E-01	7/39 (18%)	23/39 (59%)
PPARα/RXRα activation	5.68E+00	6.14E-01	44/176 (25%)	64/176 (36%)
PDGF signalling	5.49E+00	7.57E-01	24/74 (32%)	32/74 (43%)
Amyloid processing	5.32E+00	8.27E-01	16/52 (31%)	27/52 (52%)
PTEN signalling	5.15E+00	7.28E-01	22/92 (24%)	45/92 (49%)
Citrate cycle	4.92E+00	4.58E-01	8/59 (14%)	20/59 (34%)
NF-κB signalling	4.62E+00	6.71E-01	33/143 (23%)	63/143 (44%)
IL-10 signalling	4.34E+00	6.91E-01	16/68 (24%)	31/68 (46%)
Valine, Leucine and Isoleucine degradation	4.34E+00	4.39E-01	25/107 (23%)	23/107 (21%)

IL-6 signalling	4.25E+00	7.14E-01	26/91 (29%)	39/91 (43%)
Neuregulin signalling	4.10E+00	6.81E-01	22/91 (24%)	40/91 (44%)
Actin cytoskeleton signalling	4.05E+00	6.18E-01	51/267 (19%)	114/267 (43%)
Aryl hydrocarbon receptor signalling	3.92E+00	6.05E-01	37/152 (24%)	55/152 (36%)
Neurotrophin/TRK signalling	3.81E+00	6.58E-01	17/73 (23%)	31/73 (42%)
Toll-like Receptor signalling	3.77E+00	7.25E-01	14/51 (27%)	23/51 (45%)
Fc Epsilon RI signalling	3.76E+00	6.90E-01	24/100 (24%)	45/100 (45%)
Cell Cycle: G2/M DNA damage checkpoint regulation	3.71E+00	7.44E-01	13/43 (30%)	19/43 (44%)
Interferon signalling	3.63E+00	8.28E-01	14/29 (48%)	10/29 (34%)
Pentose phosphate pathway	3.41E+00	3.15E-01	11/89 (12%)	18/89 (20%)
N-Glycan biosynthesis	3.38E+00	4.48E-01	21/87 (24%)	19/87 (22%)

The molecules not represented in the dataset account for the missing numbers and will make up to a 100%.

Table S6. Pathways differentially expressed at t = 0 in monocytes.

		In patients compared to controls		
Pathway	**-Log(P-value)**	**Ratio**	**Upregulated**	**Downregulated**
Protein ubiquitination pathway	2.40E+01	7.64E-01	72/203 (35%)	86/203 (42%)
B cell receptor signalling	2.05E+01	8.11E-01	36/148 (24%)	84/148 (57%)
Huntington's disease signalling	1.76E+01	6.94E-01	64/232 (28%)	98/232 (42%)
Oxidative phosphorylation	1.71E+01	6.71E-01	67/158 (42%)	40/158 (25%)
Mitochondrial dysfunction	1.65E+01	6.00E-01	55/165 (33%)	44/165 (27%)
Glucocorticoid receptor signalling	1.63E+01	6.57E-01	56/265 (21%)	119/265 (45%)
Apoptosis signalling	1.57E+01	8.01E-01	38/136 (28%)	71/136 (52%)
PI3K/AKT signalling	1.54E+01	7.16E-01	42/176 (24%)	84/176 (48%)
NRF2-mediated oxidative stress response	1.47E+01	7.33E-01	60/180 (33%)	72/180 (40%)
SAPK/JNK signalling	1.36E+01	7.62E-01	35/147 (24%)	77/147 (52%)
ERK/MAPK signalling	1.30E+01	7.12E-01	56/226 (25%)	105/226 (46%)
Hypoxia signalling in the cardiovascular system	1.27E+01	8.31E-01	24/71 (34%)	36/71 (51%)
Estrogen receptor signalling	1.01E+01	7.29E-01	34/118 (29%)	52/118 (44%)
Inositol phosphate metabolism	9.97E+00	5.66E-01	28/173 (16%)	70/173 (40%)
Insulin receptor signalling	9.83E+00	6.92E-01	28/133 (21%)	64/133 (48%)
T Cell receptor signalling	9.58E+00	7.25E-01	21/102 (21%)	53/102 (52%)
GM-CSF Signalling	9.44E+00	8.39E-01	17/62 (27%)	35/62 (56%)
PDGF signalling	9.22E+00	7.84E-01	14/74 (19%)	44/74 (59%)
VEGF signalling	9.13E+00	7.17E-01	22/92 (24%)	44/92 (48%)
EGF signalling	9.04E+00	8.51E-01	9/47 (19%)	31/47 (66%)
Ubiquinone biosynthesis	8.92E+00	4.29E-01	27/105 (26%)	19/105 (18%)
PPARα/RXRα activation	8.81E+00	6.08E-01	37/176 (21%)	70/176 (40%)
Purine metabolism	8.80E+00	4.42E-01	99/416 (24%)	89/416 (21%)
IGF-1 signalling	8.69E+00	7.39E-01	23/92 (25%)	45/92 (49%)
Death receptor signalling	8.68E+00	8.03E-01	19/61 (31%)	30/61 (49%)
Integrin signalling	8.65E+00	6.82E-01	45/192 (23%)	86/192 (45%)
Pyrimidine metabolism	8.18E+00	4.65E-01	56/226 (25%)	53/226 (23%)
p53 signalling	8.10E+00	7.93E-01	19/87 (22%)	50/87 (57%)
IL-2 signalling	8.02E+00	8.30E-01	11/53 (21%)	33/53 (62%)
Antigen presentation pathway	7.41E+00	7.18E-01	5/39 (13%)	23/39 (59%)
JAK/Stat signalling	6.87E+00	8.14E-01	13/59 (22%)	35/59 (59%)
IL-4 signalling	6.87E+00	7.35E-01	11/68 (16%)	39/68 (57%)
NF-κB signalling	6.84E+00	6.57E-01	33/143 (23%)	61/143 (43%)
Ephrin receptor signalling	6.71E+00	6.08E-01	51/232 (22%)	90/232 (39%)
PTEN Signalling	6.57E+00	7.07E-01	20/92 (22%)	45/92 (49%)
IL-10 signalling	6.21E+00	6.91E-01	20/68 (29%)	27/68 (40%)
Toll-like receptor signalling	5.99E+00	7.45E-01	15/51 (29%)	23/51 (45%)
Nucleotide excision repair pathway	5.92E+00	8.86E-01	14/35 (40%)	17/35 (49%)
Aminoacyl-tRNA biosynthesis	5.78E+00	4.12E-01	22/85 (26%)	18/85 (21%)
Citrate cycle	5.25E+00	4.41E-01	13/59 (22%)	16/59 (27%)
IL-6 signalling	5.10E+00	6.81E-01	24/91 (26%)	38/91 (42%)
PPAR signalling	4.95E+00	6.11E-01	22/95 (23%)	36/95 (38%)
Actin cytoskeleton signalling	4.53E+00	5.69E-01	56/267 (21%)	97/267 (36%)
Natural killer cell signalling	4.43E+00	6.36E-01	21/110 (19%)	49/110 (45%)

Neuregulin signalling	4.41E+00	6.37E-01	22/91 (24%)	36/91 (40%)
Fc epsilon RI signalling	4.33E+00	6.50E-01	19/100 (19%)	46/100 (46%)
N-Glycan biosynthesis	4.31E+00	4.37E-01	19/87 (22%)	20/87 (23%)
Neurotrophin/TRK signalling	4.05E+00	6.16E-01	13/73 (18%)	32/73 (44%)
Chemokine signalling	4.01E+00	6.80E-01	21/75 (28%)	30/75 (40%)
Propanoate metabolism	3.98E+00	3.41E-01	24/126 (19%)	20/126 (16%)
Amyloid processing	3.97E+00	7.31E-01	13/52 (25%)	25/52 (48%)
Aryl hydrocarbon receptor signalling	3.84E+00	5.53E-01	35/152 (23%)	49/152 (32%)
p38 MAPK Signalling	3.74E+00	6.74E-01	25/95 (26%)	39/95 (41%)
Nicotinate and nicotinamide metabolism	3.57E+00	4.65E-01	19/129 (15%)	41/129 (32%)
Fructose and mannose metabolism	3.46E+00	2.29E-01	15/140 (11%)	18/140 (13%)
Lysine degradation	3.43E+00	3.19E-01	22/144 (15%)	25/144 (17%)
Xenobiotic metabolism signalling	3.24E+00	5.32E-01	54/250 (22%)	80/250 (32%)
Interferon signalling	3.22E+00	7.59E-01	6/29 (21%)	16/29 (55%)
Valine, Leucine and Isoleucine degradation	3.10E+00	3.83E-01	23/107 (21%)	18/107 (17%)
Fatty acid biosynthesis	3.07E+00	2.04E-01	6/49 (12%)	4/49 (8%)
Leukocyte extravasation signalling	3.06E+00	5.74E-01	29/188 (15%)	79/188 (42%)

The molecules not represented in the dataset account for the missing numbers and will make up to a 100%.

Chapter 2.9

Plasminogen activator inhibitor-1 4G/4G genotype is associated with sepsis in severely injured trauma patients

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In preparation

Abstract

Objective. While the association of *PAI1* 4G/5G polymorphism with sepsis and outcome was repeatedly shown in meningococcal disease, no confirmatory results have been presented regarding its association with the clinical course after severe trauma. Our objective was to study the *PAI1* 4G/5G polymorphism in an independent cohort of multiple trauma patients.
Patients and methods. The *PAI1* 4G/5G polymorphism was genotyped in 136 patients with multiple trauma admitted to the ICU. Injury Severity Score (ISS) and Apache II scores were assessed on admittance and at onset of sepsis with or without multiple-organ dysfunction. Additionally, the *PAI1* polymorphism was genotyped in 463 healthy controls.
Results. Sepsis was observed in 28 (93%) of fatal cases, compared to 38 (36%) of survivors. Patients with the *PAI1* 4G/4G genotype had an increased risk for sepsis compared with those homozygous for the 5G variant. However, no significant difference in *PAI1* genotype distributions was observed between survivors and non-survivors.
Conclusions. The *PAI1* 4G/4G genotype, previously shown to be associated with increased PAI-1 serum levels, was confirmed to be associated with increased risk of sepsis in an independent cohort of severe trauma patients. Both less severe disease and improved treatment regimes of the current study population are considered to have contributed to improved survival, and consequently, to the lack of previously observed association with mortality.

Introduction

The 4G/5G plasminogen-activator-inhibitor-1 (*PAI1*) promoter polymorphism was previously associated with altered expression levels. Both inhibitory and stimulatory transcription factors can bind the 5G variant promoter region, while in the 4G variant only a transcription activator can bind, resulting in increased transcription upon stimulation.(1, 2) The *PAI1* 4G/4G genotype has repeatedly been associated with severity and mortality in meningococcal disease.(3-6) In patients with dengue infection, however, no association was observed between *PAI1* polymorphism and outcome or PAI-1 plasma concentrations, even though PAI-1 levels were highest in non-survivors.(7)
Upon severe trauma, an adequate stress response including activation of the immune system and coagulation and fibrinolytic pathways are required. Previously, an increase of PAI-1 concentrations has been observed in trauma patients. Additionally, the *PAI1* 4G/4G promoter genotype, which results in increased PAI-1 transcription upon activation was associated with increased mortality.(8)
In the current study we further investigated the role of *PAI1* 4G/5G polymorphism in the development of sepsis and outcome in an independent multiple trauma patient

cohort. We confirmed the association of the *PAI1* 4G/4G genotype with increased disease severity, but not with survival.

Methods

***Participants*.** Between January 1999 and December 2002 136 multiple trauma patients consecutively admitted to the intensive care unit (ICU) participated in our study. Patients were enrolled in the study after obtaining approval of the Ethic Study Board of the University of Giessen. Informed consent was obtained from the legal custodian at baseline and from all patients upon recovery of their mental status. The clinical criteria for inclusion were: severe injuries of at least two body regions or three major fractures, and admission to the ICU within 12 h after the accident. The exclusion criteria were: less severe injuries, pregnancy, pre-existing coagulation abnormalities, and malignant disease. Apache II and ISS scores were determined. (9-11) The patients were categorized as having or not having sepsis and/or multiple organ dysfunction syndrome (MODS) by the intensivist on duty who was blinded to the genotype and the aim of the study.(12) Patients received standard treatment according to hospital protocols. Additionally, healthy Caucasian controls (n = 463), were derived from the Dutch Blood bank Sanquin South West region.

***Experimental procedures*.** DNA was extracted from peripheral blood leukocytes using standard protocols (DNA minikit; Qiagen, Hilden, Germany). The *PAI1* 4G/5G genotype (rs1799889) was determined using Bi-allelic discrimination with Taqman analysis (ABIPrism 7700, Applied Biosystems, Darmstadt, Germany). Forward and reverse primers were PAI-675-675F 5'GCCAGACAAGGTTGTTGACACA3' and PAI-675-675R 5'GCCGCCTCCGATGATACA3', respectively. The probe to detect the 4G allele was FAM-CTGACTCCCCACGTGT, and the VIC-CTGGACCCCCCACGTG probe was used to detect the 5G variant.

***Statistical analyses*.** Statistical analyses were performed using SPSS 11.0. For univariate analyses Fisher's Exact test or Mann-Whitney *U* test were performed when appropriate. Verification of Hardy-Weinberg equilibrium (HWE) of genotypes was performed using the χ^2 test (1df). A comparison of genotype frequencies was made between survivors and non-survivors and between patients with and without sepsis, using binary logistic regression adjusting for age (log transformed to obtain a normal distribution). Probability (*P*) values ≤ 0.05 were considered to be statistically significant. Apache II and ISS scores were compared between the different genotypes using non-parametric analyses.

Results

In total 136 multiple trauma patients were included in our study. Thirty (22%) of them died. Males represented 85 (62.5%) of patients. Gender was not significantly different between survivors and non-survivors (Table 1). Non-survivors were older

than survivors and had increased severity scores compared to survivors. Twenty-eight (93.3%) of the non-survivors developed sepsis, while this was only 35.8% in survivors. The two non-survivors, who had no signs of sepsis, died of cerebral stroke and fulminant thromboembolism of the lung, respectively. Sepsis patients, who died had more severe disease compared to patients who survived sepsis. Despite increased incubation time for blood cultures and denaturing HLPC testing of the samples, no microbial pathogens were detected in any of the patients with sepsis (13). It must be noted that all patients received antibiotic treatment from the start of therapy on admission to the hospital.

Table 1. Patient characteristics.

	Survivors n = 106	Non-survivors n = 30	p-value
Male gender (%)	67 (63.2)	18 (60)	0.83
Age (years)	33 (18-74)	44.5 (21-79)	0.001
Weight (kg)	79 (50-130)	75.5 (60-100)	0.28
Scores at admission to ICU:			
Injury severity score (ISS)	18 (5-63)	34 (10-58)	<0.001
APACHE II	7.5 (1-26)	13 (2-26)	<0.001
Sepsis (% of total)	**38 (35.8)**	**28 (93.3)**	
Male gender (% of sepsis patients)	25 (65.8)	16 (57.1)	0.61
Age (years)	32 (18-74)	44.5 (21-79)	<0.01
Weight (kg)	80 (50-120)	75.5 (50-100)	0.05
Sepsis patients:			
Injury severity score (ISS)	14.5 (7-63)	34.5 (10-58)	0.04
APACHE II at admission	8 (1-26)	13 (2-26)	0.001
APACHE II at onset sepsis	11.5 (5-24)	21 (7-28)	<0.001

The *PAI1* 4G/5G genotype distribution (%) for the 5G/5G, 5G/4G and 4G/4G genotypes in controls was 99 (21.4%), 241 (52.1%), and 123 (26.6%), respectively. The corresponding frequencies in patients were 31 (22.8%), 60 (44.1%) and 45 (33.1%), respectively. Genotype distribution in both groups reached HWE (p=0.35 and 0.21, respectively). The 4G/4G genotype was over-represented in patients with

Table 2. Plasminogen activator inhibitor-1 genotype distribution in trauma patients with and without sepsis.

	Genotype distribution		Logistic regression analyis	
	No sepsis	Sepsis	OR (p) crude	OR (p) adjusted [1]
PAI1	n = 70	n = 66		
4G/4G	19 (27.1)	26 (39.4)	2.87 (0.03)	2.72 (0.046)
4G/5G	30 (42.9)	30 (45.5)	2.10 (0.11)	2.04 (0.13)
5G/5G	21 (30.0)	10 (15.2)	1	1

[1]Adjusted analysis included log(age).

sepsis compared to patients who did not develop sepsis (crude OR 2.87, p=0.03; adjusted OR 2.72, p=0.046) (Table 2).
Since only 11 patients met the MODS criteria, genotype distributions were not compared between patients with and without MODS. Assessing the effect of *PAI1* genotypes on severity using Apache II and ISS scores, no significant difference was observed for the latter (p=0.54). Individuals having the 4G/4G genotype displayed slightly increased median Apache II scores (min-max) on admission compared to those with the 4G/5G and 5G/5G genotype (10 (3-26), 7 (2-26) and 8 (1-24), respectively; p=0.035).
In contrast to previous observations, no association was observed between the *PAI1* genotype and survival (Table 3). Similar results were obtained when the two patients, who died of causes other than sepsis, were excluded from the analysis.

Table 3. Plasminogen activator inhibitor-1 genotype distribution in trauma patients.

	Survivors		Non-survivors	Survivors vs. non-survivors	
	No sepsis	Sepsis		OR (p) crude	OR (p) adjusted [1]
PAI1	n = 68	n = 38	n = 30		
4G/4G	17 (25.0)	17 (44.7)	11 (36.7)	1.11 (0.85)	0.71 (0.55)
4G/5G	30 (44.1)	18 (47.4)	12 (40.0)	0.86 (0.77)	0.59 (0.37)
5G/5G	21 (30.9)	3 (7.9)	7 (23.3)	1	1

[1]Adjusted analysis included log(age).

Discussion

In the current study we demonstrated that the 4G homozygous genotype was associated with increased occurrence of sepsis in multiple trauma patients. However, in contrast with our previous study no association of the *PAI1* 4G allele with fatal outcome of multiple trauma was observed.(8) The discrepancy between the two studies could have several causes. While mortality was 34% (21/61) in the previous study, only 22% of the patients died in the current study. This may be related to the less severe disease observed, according to the decreased ISS scores, in the current patient cohort. Additionally, in recent years substantial progress has been made in the supportive treatment of multiple trauma and sepsis patients resulting in decreased mortality. Tight glycaemic control, intensivated insulin therapy, hydrocortisone administration in septic shock and improved detoxification have been implemented in the ICU in the last trimester of 2001, and a substantial part of the patients (n=40) was eligible for this therapy.(14-16) PAI-1 gene expression in vascular endothelial cells is suggested to be induced by hyperglycaemia, and control of blood glucose levels may have had a major impact on PAI-1 plasma levels and on mortality.(17) Since outcome of severe multiple trauma is dependent on multiple factors, improved treatment options in the current study may have resulted in decreased mortality even despite increased PAI-1 levels.
In addition, PAI-1 transcription is induced by IL-1β and TNF-α.(1, 18) Differences

in expression of these cytokines are therefore expected to result in altered PAI-1 expression. A correlation between TNF-α and PAI-1 levels has indeed been observed in meningococcal sepsis.(19) Additionally, meningococcal sepsis non-survivors display increased PAI-1 levels compared to survivors with similar TNFα concentrations. These differences in PAI-1 concentrations are associated with the *PAI1* 4G/5G polymorphism. This indicates that the stress response induced by infection or inflammation is complex and involves interaction of immune response, fibrinolysis and coagulation, and endocrinological pathways. The same holds true for multiple trauma patients. Notably, no causative micro-organisms were detected despite laborious efforts. While this may result from the fact that all patients received antibiotics from the start of treatment before blood cultures were drawn, this may also imply that the sepsis syndrome observed in multiple trauma patients has a different pathogenesis than microbial infection. The combination of variations in the individual pathways involved is expected to co-determine the observed phenotype. In the current study population, factors other than PAI-1, such as improved treatment options may have had a higher impact on survival. Consequently, this may have resulted in the finding that no longer an association was observed with mortality although *PAI1* 4G homozygosity was associated with the occurrence of sepsis. Another explanation for the differences observed between the prior and the current study might be that either the first study showed a false positive association or the current study a false negative with regard to mortality. First publications regarding polymorphisms and their association with disease are usually positive and therefore more likely to be false positive results.(20) In addition, association studies in small populations have an increased risk of false positive results since a small shift in patient numbers for different outcomes results in large percentage changes.(21) The first study comprised the data of only 61 multiple trauma patients, while in the current study 136 patients were included. The observation that the *PAI1* 4G homozygous genotype is confirmed to be associated with increased risk of sepsis strengthens the assumption that PAI-1 is an important risk factor for more severe disease course after multiple trauma.
In conclusion, the *PAI1* 4G/5G polymorphism was confirmed to be associated with severity but not with mortality. Less severe disease and improved treatment options are likely involved in co-determining occurrence of sepsis and outcome.

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Chapter 3

Immune response genes and their effects on Guillain Barré syndrome, rheumatoid arthritis, Barrett oesophagus and hepatitis C virus infection

Chapter 3.1

Genetic polymorphisms of macrophage-mediators in Guillain-Barré syndrome

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Abstract

Objective. Macrophages infiltrate peripheral nerves and may contribute to neural damage in the Guillain-Barré syndrome (GBS).
Patients and methods. We determined whether single nucleotide polymorphisms (SNP) in genes encoding macrophage-mediators are related to the susceptibility and severity of GBS.
Results and conclusion. The frequencies of SNPs in the *TNFA*, *MMP9*, *IL10,* and *NOS2a* genes did not differ between 263 GBS patients and 210 healthy subjects. The *MMP9* C(-1562)T and *TNFA* C(-863)A SNP were associated with severe weakness and poor outcome, indicating that these SNPs may be one of the factors predisposing to a severe form of GBS.

Introduction

Macrophages seem to play a crucial role in the pathogenesis of the various variants of the Guillain-Barré syndrome (GBS). These cells phagocytose myelin in patients with acute inflammatory demyelinating neuropathy (AIDP) and axons in patients with acute motor axonal neuropathy (AMAN).(1) Macrophages usually exert their functions by the release of cytokines and other inflammatory mediators. Tumour necrosis factor-alpha (TNF-α), matrix metalloproteinase 9 (MMP-9) and inducible nitric oxide synthase (iNOS) are involved in leukocyte attraction, extracellular matrix degradation, demyelination and axonal degeneration. IL-10, another mediator released by macrophages, may exert pro-inflammatory as well as anti-inflammatory effects, since it promotes both antibody production as well as the termination of inflammatory response and tissue destruction.(2) These mediators of inflammation probably play a decisive role in the actions of macrophages at the peripheral nerves of patients with GBS.
Single nucleotide polymorphisms (SNP) in genes encoding these mediators affect the levels or functions of these mediators and may control the extent of the macrophage-related nerve damage in GBS. Previous studies showed that elevated levels of TNF-α and MMP-9 are associated with disease severity and electrophysiological changes in GBS patients.(3-6) Another report showed that a SNP in the promoter region of *IL10* associated with high IL-10 production was a susceptibility factor for the onset of GBS.(7)
In this study we determined whether SNP in the promoter region of *TNFA*, *MMP9*, *IL10*, and in the coding region of *NOS2a* are susceptibility factors or disease modifying factors in a cohort of clinically well-characterized GBS patients (n = 263).

Methods

Study population. In this study were included 263 Caucasian patients (median age at disease onset 45.0 years, range 7-82 years, male/female ratio = 1.10), who fulfilled the diagnostic criteria for GBS. Caucasian healthy blood bank donors (n = 210, median age 35 years, range 19-60 years, male/female-ratio = 0.64) served as controls. Of the patients, 149 had participated in one of the Dutch randomized clinical trials and were clinically and serologically documented in detail.(8) Twenty-five patients participated in a Dutch prospective study, 19 in a trial investigating the effect of amantidin in patients with fatigue as one of the major residual deficits, and six patients in a pilot study studying the effect of intravenous immunoglobulin, methylprednisolon combined with mycophenolate mofetil.(9-11) The other patients visited the outpatient's clinic. Severe (versus mild) GBS was defined as a nadir with (1) an MRC-sumscore of less than 40 out of 60 (ranging from 60 (normal strength) to 0 (tetraparalysis)) or (2) the inability to walk independently (GBS disability score of 3, 4 or 5). Poor outcome was defined as the inability to walk independently after six months of follow-up.

DNA isolation. Isolation of genomic DNA from EDTA anti-coagulated peripheral blood samples was performed with the use of the Invisorb® MaxiBlood kit (Invitek, Berlin, Germany) according to the manufacturer's instruction.

SNP detection. The C(-1562)T polymorphism in *MMP9* was genotyped with the restriction fragment length polymorphism (RFLP)-technique using the restriction enzyme *Sph1.* Genotyping of the SNPs in the promoter region of *TNFA* (C(-863)T, G(-308)A, G(-238)A), two SNPs in the promoter region of *IL10* (G(-1082)A and C(-819)T), and one SNP in the coding region of *NOS2a* (S608L) were performed by using multiplex single base extension reactions. Primers and conditions for the conventional PCR-reactions and multiplex SBE reactions are listed in Tables 1 and 2.

Table 1. PCR primer sequences.

Gene	Upper 5'→ 3'	Lower 5' → 3'	SNPnumber	Change
NOS2a	GCAGGGCTAGGAGTAGGA	AGCCCCATATGTAAACCAA	rs2297518	608 S/L
IL10	TCCCCTTACCTTCTACACAC	GACCCCTACCGTCTCTATTT	rs1800896	G-1082A
			rs3021097	C-819T
TNFa.2	GGAGAATGTCCAGGGCTATG	AAAATCAGGGACCCCAGAGT		
			rs1800630	A-863C
TNFa.1	CCCCTCCCAGTTCTAGTT	GGGACACACAAGCATCA	rs361525	G-238A
			rs1800629	G-308A
MMP-9	AAATGGCAGAGCCGGGAT	ACCAGCAGCCTCCCTCACT	rs3918242	C-1562T

Table 2. Primer sequences for SBE reactions.

	Primer sequence	Primer length
	5' → 3'	
PrNOS2aU	TTTTGCTCTTTCAGCATGAAGAGC	24
PrTNFa_2863U	AGTCGAGTATGTGGACCCCC	20
PrIL10_1082L	TTTTTTTTTTTTACCTATCCCTACTTCCCC	30
PrTNFa1238U	TTTTTTTTTTTTTTTTTTTTTTAGAAGACCCCCCTCGGAATC	42
PrTNFa_1308L	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTAGAGGCTGAACCCCGTCC	54
PrIL10_819U	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCCCTTGTACAGGTGATGTAA	82

Statistical analysis. Verification of Hardy-Weinberg equilibrium and comparison of genotype and allele frequencies were performed using Pearson's χ^2 test or Fisher's exact test when appropriate. An expectation-maximization algorithm was used to test for linkage disequilibrium and to compare the estimated haplotype distributions.(12) *P* values < 0.05 were considered to be statistically significant.

Results

In this study all SNPs were in Hardy-Weinberg equilibrium and the genotype distributions and allele frequencies did not differ between GBS patients and healthy controls (Table 3). No associations were found between the SNP in *TNFA*, *MMP9*, *NOS2a* and *IL10* and the parameters age, sex, antecedent infection and the presence of anti-ganglioside antibodies. The three SNP in the promoter region of *TNFA* and the two SNP in the promoter region of *IL10* were in linkage disequilibrium (p<0.001).

Table 3. Genotype distribution in Guillain-Barré patients versus healthy controls

SNP	Guillain-Barré patients n = 263 %	Healthy controls n = 210 %
***MMP9* C (-1562)T**		
CC	78.3	78.1
CT	21.3	21.9
TT	0.4	0
***TNFA* C(-863)A**		
CC	70.7	70.9
CA	25.9	26.7
AA	3.4	2.4
***TNFA* G(-308)A**		
GG	73.4	65.2
AG	22.1	32.9
AA	4.5	1.9
***TNFA* G(-238)A**		
GG	87.4	86.7
AG	11.8	13.3
AA	0.8	0
***IL-10* G(-1082)A**		
GG	27.0	27.1
AG	46.4	45.2
AA	26.6	27.6
***IL-10* C(-819)T**		
CC	61.6	63.3
CT	32.7	30.0
TT	5.7	6.7
***NOS2*a S608L**		
GG	63.9	61.0
AG	32.3	33.3
AA	3.8	5.7

MMP9, gene encoding matrix metalloproteinase 9; *TNF*, gene encoding tumour necrosis factor alpha; *IL10*, gene encoding interleukin 10; *NOS2a*, gene encoding inducible nitric oxide synthase. No significant differences were found.

Therefore, the haplotype distributions between GBS patients and controls could be compared, and these were also not significantly different between patients and controls.
Considering the role of the macrophage in nerve damage and the clinical heterogeneity of GBS we determined whether the SNPs were associated with disease severity and outcome. The SNP at position -1562 of the *MMP9* gene and at position -863 of the *TNFA* gene were significantly associated with disease severity in patients with GBS (Table 4). In addition, the variant allele of the SNP at position -1562 of the *MMP9* gene was more frequent in patients with severe GBS (MRC-sumscore < 40) than in patients with mild GBS (13.6% vs. 5.7%, OR 2.6, 95% CI 1.1 to 6.0, p=0.02).

Table 4. Genotype distributions in severely versus mildly affected GBS patients.

	Severely affected (n = 70) %	Mildly affected (n = 79) %	OR (95% CI)	p-value
MMP9				
C/C	72.9	88.6		0.01
C/T	17.1	11.4	2.9 (1.2-7.0)	
***TNFA* -863**				
C/C	58.6	77.2		0.049
C/A	35.7	20.3	2.3 (1.1-4.9)	
A/A	5.7	2.5	2.9 (0.5-17.4)	

Severity of disease was based on the Medical Research Council-sumscore at nadir ranging from tetraparalysis (score 0) to normal muscle strength (score 60). Patients with a MRC-sumscore at nadir of < 40 were defined as severely affected. GBS, Guillain-Barré syndrome; OR, odds ratio; CI, confidence interval; TNFA, gene encoding tumour necrosis factor-alpha; *MMP9*, gene encoding matrix metalloproteinase 9.

A similar difference between severe and mild cases was found for the variant allele of the SNP at position -863 of *TNFA* (23.6% vs. 12.7%, OR 2.1, 95% CI 1.1 to 3.9, p=0.01). The haplotype distribution of the three SNPs in the promoter region of *TNFA* did not significantly differ between mildly and severely affected patients.
The genotype distribution of the SNP at position -863 of *TNFA* also differs between the subgroup of patients with poor prognosis and the patients with good prognosis (Table 5). The variant allele of this SNP was also more frequently found in the subgroup with unfavourable outcome (36.7% vs. 15.7%, OR 3.1, 95% CI 1.4 to 7.0, p=0.004).

Table 5. Genotype distribution of *TNFA* C(-863)A polymorphism in GBS patients with poor or good outcome.

	Poor outcome (n = 15) %	Good outcome (n = 134) %	OR (95% CI)	p-value
***TNFA* -863**				
C/C	40.0	71.4		0.02
C/A	46.7	25.6	3.3 (0.3-10.7)	
A/A	13.3	3.0	7.9 (1.2-53.9)	

The GBS functional disability score at six months was used to define the clinical outcome. Patients who were not able to walk independently after six months of follow-up were defined as having a poor outcome. GBS, Guillain-Barré syndrome; OR, odds ratio; CI, confidence interval; *TNFA*, gene encoding tumour necrosis factor-alpha.

Discussion

The variant alleles of two SNPs in the promoter region of the *MMP9* gene and *TNFA* gene were associated with more severe weakness in patients with GBS. Moreover, the variant allele of *TNFA* was also associated with poor outcome at six months of follow-up. These SNP may therefore predispose to the development of severe forms of GBS. A previous study showed that the A-allele at position -863 of *TNFA* was associated with higher TNF-α concentration due to an inadequate down regulation of its production.(13) In the case of the SNP at position -1562 of *MMP9*, the T-allele is associated with an increased promoter activity that might result in higher MMP-9 levels.(14) Previous reports also showed that high serum levels of MMP-9 as well as TNF-α were associated with severity of disease.(3-6, 15) MMPs play an important role in the recruitment of macrophages and penetration of the blood-nerve barrier and macrophage-derived TNF-α propagates inflammation within the peripheral nervous system.(2) Therefore, these genetic polymorphisms may partly determine the patient's outcome by influencing the levels of pro-inflammatory cytokines.

In this study we did not confirm the previously reported associations between the SNP at position -819 in the promoter region of *IL10* and disease susceptibility (n = 87), and between a *TNFA* microsatellite marker and *Campylobacter jejuni*-positive GBS patients (n = 81).(7, 16) This discrepancy could be caused by chance, but also by the higher sample size of our study (n = 263), or the inclusion of a different population of GBS patients. The expected effects of SNPs in disease development and severity are usually small, underlining the importance of using large cohorts of well-defined patients in genetic association studies. Also our results need further confirmation in new studies with larger cohorts and evaluation in meta-analysis. Larger cohorts are also required to study the effect of the haplotype in the promoter region of *TNFA*.

In principle, SNPs may both influence the susceptibility and course of disease. Thus far, genetic studies in our cohort of GBS patients have identified polymorphisms that predominantly influence the severity of disease, including SNPs in the mannose-binding lectin and Fcgamma-receptorIIIB genes.(17, 18) Accordingly, the current study identified SNPs in the MMP9 and TNFA genes as other disease-modifying factors. In the future, these factors may be used as biological markers to delineate the clinical subgroups in GBS and help to identify patients with poor outcome who need additional forms of treatment.

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Chapter 3.2

Polymorphisms in genes controlling inflammation and tissue repair are associated with rheumatoid arthritis

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Abstract

Objective. Various cytokines and inflammatory mediators are known to be involved in the pathogenesis of rheumatoid arthritis (RA). We hypothesized that polymorphisms in selected inflammatory response and tissue repair genes contribute to the susceptibility to and severity of RA.
Patients and Methods. Polymorphisms in *TNFA*, *IL1B*, *IL4*, *IL6*, *IL8, IL10*, *PAI1*, *NOS2a*, *C1INH*, *PARP*, *TLR2* and *TLR4* were genotyped in 376 Caucasian RA patients and 463 healthy Caucasian controls using single base extension. Genotype distributions in patients were compared with those in controls. In addition, the association of polymorphisms with the need for anti-TNF-α treatment as a marker of RA severity was assessed.
Results. The *IL6* -174 G/G, *IL8* 781 C/C and *PAI1* 5G/5G genotypes were independently associated with RA susceptibility when adjusted for age and gender. In addition, we showed that in patients diagnosed with RA, the *TNFA* -308 G/G and -238 A/A genotypes were associated with RA severity.
Conclusions. We here for the first time report an association between *IL8* and *PAI1* polymorphisms and RA susceptibility. In addition, carriage of the *IL6* -174 C allele was associated with susceptibility to RA, while two *TNFA* polymorphisms were associated with RA severity. Our findings support the role for variations in these genes involved in the immune response and in tissue repair in RA pathogenesis.

Introduction

Rheumatoid arthritis (RA) is a severely disabling chronic inflammatory disease that affects millions of people worldwide. Like in other autoimmune diseases females are affected more often than males. The disease course differs widely between patients. While some have low grade disease which is easily controlled by therapy with one disease-modifying anti-rheumatic drug (DMARD), others suffer from rapidly progressive disease with erosions that is therapy resistant, resulting in the use of many different DMARDs or even the need for therapy with the new biologicals directed against TNF-α. Cytokines and other proteins involved in the inflammatory response play a role in RA. Pro-inflammatory cytokines such as TNF-α and IL-1β are regarded as key players in the pathogenesis of RA.(1, 2) This is further underlined by the development of several anti-cytokine drugs in the treatment of RA, with anti-TNF monoclonal antibodies and soluble TNF-α receptor being the most effective. TNF-α is an important pro-inflammatory cytokine. IL-1β in its turn, stimulates expression of IL-6 and IL-8.(2) Antibodies directed against the IL-6 receptor are effective in the treatment of RA.(3) IL-4 and IL-10 on the other hand, have been suggested to ameliorate arthritis.(4, 5) Deficiency of IL-4 is associated with increased severity of arthritis in a mouse model.(6) In addition, the V50 variant in the IL-4 receptor causing unresponsiveness to IL-4, is associated with rapidly erosive RA.(7)

In addition to inflammatory tissue destruction, factors involved in tissue repair and necrosis or apoptosis are likely to be involved in erosive disease and may also codetermine disease susceptibility. Plasminogen activator inhibitor 1 (PAI-1) inhibits fibrinolysis and tissue repair.(8, 9) Plasminogen deficiency is associated with decreased susceptibility to and severity of arthritis in mice models for RA.(10)
Previously the influence of genetic polymorphisms in inflammatory diseases such as RA has become evident. The association with the HLA DRB1 locus is the best characterized and replicated.(11, 12) Linkage studies in RA families and genome wide screening in case control studies have identified multiple additional genetic loci associated with RA.(11, 13) However, many studies only focus on a single polymorphism and/or only include few patients thereby lacking sufficient power. Polymorphisms of interest include those in genes known or expected to be involved in RA, specifically those with known or proposed effect on transcription or function such as in *IL4*, *IL6*, *PAI1*, *PARP*, *IL10* and *IL1B*.(14-20) In addition, polymorphisms, such as in *TNFA* and *IL8*, previously associated with RA or other inflammatory diseases were considered relevant as well.(21-24)
Therefore, in this study including 376 RA patients and 463 controls, we investigated the possible association of polymorphisms in immune response genes (*TNFA*, *IL1B*, *IL4*, *IL6*, *IL8*, *IL10*, *C1INH*, *TLR2*, *TLR4*) and genes involved in tissue repair and apoptosis (*PAI1*, *NOS2a*, *PARP*) in relation to RA susceptibility, and with the use of anti-TNF-α therapy as selected marker of disease severity.

Methods

Participants. Non-related Caucasian patients who met the 1987 ACR criteria for RA and visited the rheumatology outpatient clinic of the Erasmus MC university hospital (n = 76), the Medical Centre Rijnmond Zuid (n = 195) or the St. Franciscus Gasthuis Hospital (n = 105), were included in the study. Additionally, the patients had to have at least either a positive rheumatoid factor test, or a positive anti-CCP test, or joint erosions. The study was approved by the medical ethical boards of the participating hospitals. Patients were included after informed consent. Gender, age at inclusion in the study, age at diagnosis, clinical parameters and current and prior medication were derived from the clinical charts. The need for therapy with biologicals directed towards TNF was used as a marker of disease severity, since in the Netherlands the prescription of anti-TNF is strictly regulated and the costs for anti-TNF treatment are only reimbursed by the health insurance companies for RA-patients with therapy-resistant disease. This latter has been defined as failure on at least two DMARDs, including methotrexate and still active disease (defined as DAS28 > 3.2) despite therapy with methotrexate 25 mg weekly or with methotrexate at the maximum tolerated dosis. Blood was drawn for DNA isolation and routine laboratory analysis. Since the anti-CCP test has only recently been introduced into routine patient care, only for a minority of patients (n = 125) anti CCP status was known. Caucasian controls representing the general adult population (n = 463) were derived from the Sanquin

Blood Bank South West Region.

DNA isolation. For RA patients DNA isolation from heparinized whole blood was performed as described previously.(25) DNA from controls was derived from whole blood with column methods using standard protocols (Qiagen, Leusden, the Netherlands).

Genotyping. Single base extension (SBE) analysis was used to determine genotypes of inducible nitric oxide synthase (*NOS2A*) S608L (rs2297518), poly (ADP-ribose) polymerase (*PARP*) V762A (rs1136410), complement component inhibitor-1 (*C1INH*) V480M (rs4926), *PAI1* -675 4G/5G (rs1799889), *IL4* C-524T (rs2243250), *IL10* G-1082A (rs1800896), *IL10* C-819T (rs3021097), *IL1B* C-31T (rs1143627), *TNFA* A-863C (rs1800630), *TNFA* T-857C (rs1799724), *TNFA* G-376A (rs3093659), *TNFA* G-308A (rs1800629), *TNFA* G-238A (rs361525), *IL6* G-174C (rs1800795), *IL8* C781T intron (rs2227306), *TLR4* D299G (rs4986790) and *TLR4* T399I (rs4986791) (http://www.ncbi.nlm.nih.gov/SNP/, Applied Biosystems, SNaPShot, Nieuwerkerk aan den IJssel, the Netherlands). The genomic region of interest was amplified by PCR. After purification, a single base extension was performed using a primer ending one nucleotide prior to the single nucleotide polymorphism (SNP) location. Both forward and reverse strands were tested. In a multiplex assay one of the primers was used to genotype the SNP of interest. Up to seven SNPs were analysed in one assay. A poly-T-tail attached to the primer combined with the use of a Liz size marker served to distinguish SNPs in the multiplex analysis. A subset of PCR samples was sequenced to confirm genotypes. Primer and probe sequences are available on request. All genotypes were annotated independently by two investigators who were blinded for the clinical data.

Statistical analysis. Statistical analysis was performed using SPSS 11.5. Haplotype analysis was carried out with Thesias version 2.(26, 27) Genotype frequencies were compared between patients with RA and controls using the Cochrane-Armitage trend test for additive effects. In addition, genotypes were compared after adjusting for age and gender, using binary logistic regression.(28) The median number of different drugs used per year since the moment of diagnosis was assessed. Additionally, genotype distributions between patients who received anti-TNF-α medication were compared with those who did not. These analyses were adjusted for age using binary logistic regression. Subsequently, all SNPs that were considered relevant in univariate analyses were assessed together in one multivariate logistic regression model with regard to outcome, to assess their individual contributions in relation to the other SNPs. Binomial variables were analysed using Pearson's χ^2 test (2df) or Fisher's exact test when appropriate. For continuous variables Student's *t* test or Mann-Whitney *U* test were used when appropriate. Verification of Hardy Weinberg equilibrium (HWE) of genotypes was performed using χ^2 test (1df). Probability (*P*) values < 0.05 were considered to be statistically significant. Since no international consensus exists on what method to use best in genetic studies no correction was made for multiple testing. Power calculations using Quanto revealed a power of 0.8 to detect an OR of 1.5 comparing patients and controls given an allele frequency of 0.1 and α of 0.05.(29)

Results

Patient characteristics. 376 patients, mean age (sd) 59.3 years (13.7) and 463 controls mean age (sd) 39.1 years (8.5), were included in the study (p<0.001). In the patient and control cohort 277 (73.7%) and 230 (49.7%) individuals were female, respectively (p<0.001). All patients were included when comparing RA patients with healthy controls. For 6 patients records regarding medication use were incomplete due to (multiple) transfers of patients from other hospitals. For this reason the 370 patients for whom medication data was complete were included in the analysis regarding disease severity (Table 1).

Table 1. Patient characteristics.

Mean age (sd)	**RA patients n = 370**
Mean age (sd)	59.1 (13.7)
Female gender (%)	272 (73.5)
Median nr years with RA (min-max)	8.5 (0-54)
Anti-CCP positive (%)[1]	104/125 (83.2)
Rheumatoid factor positive (%)	337/369 (91.3)
Erosion (%)	291/357 (81.5)
Median number of DMARDs (min-max)	3 (1-13)
Median number of DMARDs per disease year (min-max)	0.35 (0.03-18)
Number of patients with past or present used DMARDs (%)	
Methotrexate	331 (89.5)
Salazopyrine	262 (70.8)
Antimalaria	176 (47.6)
Leflunomide	33 (8.9)
Intramuscular gold	55 (14.9)
Infliximab	35 (9.5)
Etanercept	61 (16.5)
Adalilumab	60 (16.2)
Anakinra	14 (3.8)
Oral corticosteroids	164 (44.3)
Azathioprine	33 (8.9)
Others n = 1	32 (8.6)
n = 2	5 (1.4)

[1]Anti-CCP was not routinely analyzed.

Genotypes and disease susceptibility. SNPs not mentioned in the results section showed no significant associations and only significant associations will be discussed further. In controls, genotype distribution of all SNPs except the *IL10* G-819A polymorphism reached Hardy Weinberg Equilibrium (HWE). To rule out a technical problem, an initial set of individuals was typed using both the reverse and forward primer in the single base extension reaction. Results for both strands were identical. Sequencing of a subset of 13 random controls showed identical genotypes excluding technical errors (data not shown). Allele frequencies of polymorphisms in controls and RA patients are listed in Table 2.

An association was observed for the *IL6* -174 G/C polymorphism. The *IL6* -174 G/G genotype was significantly overrepresented in RA patients when compared to controls even when corrected for age and gender (crude OR (95% CI) 1.66 (1.24-2.22), p=0.001, adjusted OR (95% CI) 1.90 (1.22-2.96), p=0.004) (Table 3).

Table 2. Allele frequencies in RA patients and controls.

SNP	Allele	Controls n (%)	RA Patients n (%)	p[1]
***TNFA* -863**	**C**	775 (84)	601 (81)	0.16
	A	147 (16)	137 (19)	
***TNFA* -857**	**C**	838 (92)	676 (93)	0.29
	T	76 (8)	50 (7)	
***TNFA* -376**	**G**	907 (98)	746 (99)	0.04
	A	15 (2)	4 (1)	
***TNFA* -308**	**G**	747 (81)	615 (82)	0.60
	A	175 (19)	135 (18)	
***TNFA* -238**	**G**	876 (95)	734 (98)	0.002
	A	46 (5)	16 (2)	
***IL1B* -31**	**C**	328 (36)	261 (35)	0.62
	T	584 (64)	489 (65)	
***IL4* -524**	**C**	790 (86)	625 (84)	0.30
	T	130 (14)	119 (16)	
***IL6* -174**	**C**	395 (43)	245 (37)	0.01
	G	523 (57)	425 (63)	
***IL8* 781**	**C**	538 (58)	453 (60)	0.40
	T	386 (42)	299 (40)	
***IL10* -1082**	**G**	465 (51)	348 (49)	0.49
	A	453 (49)	364 (51)	
***IL10* -819**	**C**	711 (77)	566 (77)	[2]
	T	209 (23)	166 (23)	
***PAI1* -675**	**5G**	438 (47)	374 (51)	0.12
	4G	486 (53)	356 (49)	
***NOS2a* 608**	**S**	742 (80)	612 (83)	0.22
	L	182 (20)	128 (17)	
***PARP* 762**	**V**	738 (82)	618 (84)	0.40
	A	160 (18)	120 (16)	
***C1INH* 480**	**V**	686 (75)	551 (76)	0.47
	M	234 (25)	173 (24)	
***TLR4* 299**	**D**	804 (93)	684 (92)	0.62
	G	60 (7)	56 (8)	
***TLR4* 399**	**T**	810 (93)	683 (92)	0.42
	I	58 (7)	57 (8)	

[1]*P* value represents analysis not corrected for multiple testing. When Bonferroni correction is applied only the *TNFA* -238 polymorphism remain significant (p=0.02).
[2]The *IL10* -819 C/T genotype distribution did not reach Hardy Weinberg equilibrium in controls. For this reason no p value is given. The genotype distribution did not differ significantly between patients and controls.

For other polymorphisms, associations were only observed in either logistic regression models without correction for age and gender (*TNFA* -863, *TNFA* -376, *TNFA* -238) or with correction (*PAI1* -675 4G/5G, *IL8* 781 C/T) for age and gender. Analysis of *TNFA* haplotypes yielded similar results as for the individual SNPs (Table 4).

When all significant polymorphisms were included in a logistic regression model comparing all RA patients with healthy controls without correction for age and gender, only *IL6* genotypes were differentially distributed (OR (95% CI) *IL6* -174 G/G vs. G/C and C/C 1.62 (1.20-2.18), p=0.002). A trend was observed for the *TNFA* -238 A allele carriers (OR (95% CI) 0.54 (0.27-1.07), p=0.08). When adjusting for age and gender the *IL6* -174 G/G, the *IL8* 781 C/C and the *PAI1* 5G/5G genotypes were independently associated with increased risk for RA (Table 3).

Since RA patients were significantly older than healthy controls and controls are by definition still 'at risk' to develop RA at a later age, the difference observed may also

Table 3. Genotype frequencies in RA patients and controls.[1]

SNP	Genotype frequency		Patients vs.		Log regression	
	Controls	RA patients	controls	OR crude	OR adjusted[2]	OR adjusted[3]
	n (%)	n (%)	p[4]		(95% CI)	(95% CI)
***IL6* -174**					G/G vs. C/G and C/C	
C/C	82 (17.9)	56 (16.7)	0.01	1.66 (1.24-2.22)	1.90 (1.22-2.96)	1.75 (1.11-2.78)
C/G	231 (50.3)	133 (39.7)				
G/G	146 (31.8)	146 (43.6)				
***IL8* 781**						
C/C	152 (32.9)	141 (37.5)	0.40	1.10 (0.74-1.65)	2.14 (1.15-3.99)	2.04 (1.05-3.94)
C/T	234 (50.6)	171 (45.5)		0.87 (0.59-1.28)	1.00 (0.55-1.82)	0.84 (0.45-1.58)
T/T	76 (16.5)	64 (17.0)		1	1	1
***PAI1* 4/5G**						
5G/5G	99 (21.4)	101 (27.7)	0.12	1.36 (0.93-2.01)	2.12 (1.17-3.87)	1.96 (1.03-3.74)
5G/4G	240 (51.9)	172 (47.1)		0.96 (0.69-1.34)	1.29 (0.78-2.12)	1.24 (0.72-2.14)
4G/4G	123 (26.6)	92 (25.2)		1	1	1
***TNFA* -863**					C/C vs. C/A and A/A	
C/C	329 (71.4)	239 (64.8)	0.16	0.74 (0.55-0.99)	0.68 (0.44-1.05)	0.66 (0.41-1.06)
C/A	117 (25.4)	123 (33.3)				
A/A	15 (3.3)	7 (1.9)				
***TNFA* -857**					C/C vs. C/T and T/T	
C/C	386 (84.5)	314 (86.5)	0.29	1.18 (0.80-1.75)	1.02 (0.56-1.89)	
C/T	66 (14.4)	48 (13.2)				
T/T	5 (1.1)	1 (0.3)				
***TNFA* -376**						
G/G	446 (96.7)	371 (98.9)	0.04			
G/A	15 (3.3)	4 (1.1)		0.32 (0.11-0.98)	0.71 (0.18-2.81)	0.96 (0.14-6.80)
A/A						
***TNFA* -308**					G/G vs. G/A and A/A	
G/G	300 (65.1)	248 (66.1)	0.60	1.05 (0.79-1.40)	1.24 (0.81-1.90)	
G/A	147 (31.9)	119 (31.7)				
A/A	14 (3.0)	8 (2.1)				
***TNFA* -238**						
G/G	415 (90.0)	359 (95.7)	0.002			
G/A	46 (10.0)	16 (4.3)		0.40 (0.22-0.72)	0.55 (0.24-1.25)	0.50 (0.17-1.43)
A/A						

[1]Polymorphisms for which no significant association was observed are not shown; genotype data are available on request.
[2]Adjusted OR for age and gender.
[3]Adjusted OR for age and gender including the following SNPs in the model: *IL6* -174, *IL8* 781, *PAI1* 4G/5G, *TNFA* -863, *TNFA* -376 and *TNFA* -238.
[4]Cochrane-Armitage trend test for additive effects.

result from a difference in susceptibility to develop RA at a younger age. For this reason the relation of all SNPs with the age at diagnosis was analysed. Indeed, the *TNFA* -376 A allele carriers were diagnosed at a significantly younger age than those with the G/G genotype (mean age (SD) at diagnosis 30.3 (14) and 48.4 (14) years, respectively, p=0.01). The *IL8* 781 C/C genotype was associated with younger age

at RA diagnosis compared to both the *IL8* 781 C/T and T/T genotype (mean age (SD): *IL8* 781 T/T 52.2 (14) years, T/C 50.0 (14) years and C/C 44.2 (13) years, respectively, p<0.001). No associations with age at diagnosis were observed for the *PAI1* -675, IL6 -174 and the *TNFA* -863 and *TNFA* -238 polymorphisms.

Table 4. TNFA haplotypes in RA patients and controls.

TNFA haplotype[1]	Controls n = 463 (%)	RA patients n = 376 (%)	OR crude (95% CI)	OR adjusted (95% CI)[2]
CCGG	51.9	54.3	1	1
CCGA	5.0	2.1	0.40 (0.22-0.72)	0.53 (0.23-1.20)
CCAG	19.0	18.0	0.90 (0.69-1.18)	0.79 (0.51-1.22)
CTGG	8.1	6.9	0.82 (0.54-1.24)	0.85 (0.46-1.58)
ACGG	15.8	18.6	1.12 (0.85-1.49)	1.13 (0.74-1.72)
ATGG	0.2	0.1	-	-

[1]Haplotype: *TNFA* -863 C/A, -857 C/T, -308 G/A, -238 G/A.
[2]Adjusted analyses include sex and age. Haplotype analyses were performed using Thesias.

Genetic polymorphisms and severity of disease. The use of TNF-α modifying therapy was considered to be an indicator of severe disease. Anti-TNF-α drugs were administered to 120 of 370 patients. The mean age (sd) of patients who used anti-TNF-α was 55.7 (12.8) years compared with 60.8 (13.9) years in the group that did not receive anti-TNF-α (p=0.001). The *TNFA* -308 G/G genotype was consistently associated with severe disease even after adjusting for age (p=0.05; Table 5).
Carriage of the *TNFA* -238 A allele was also associated with severe disease (p=0.05), however, this was no longer significant after correction for age (=0.09). A trend was observed for *IL6* -174 C allele carriage and severe disease when only the *IL6* -174 genotype was included in the statistical model (p=0.10). A trend for severe disease was also observed for the *PARP* 762 C allele carriers (p=0.08).
Including the *PARP* 762 T/C, *IL6* C/G and the *TNFA* -308 G/A and -238 G/A polymorphisms in one multivariate logistic regression model, the *TNFA* -308 G/G genotype was significantly (p=0.04) associated with severe disease after adjusting for age (Table 5).

Discussion

In this study we observed that polymorphisms in *IL6*, *PAI1*, *IL8* and *TNFA* were associated with RA susceptibility. Two promoter polymorphisms in *TNFA*, at positions -308 and -238, were associated with severity of RA, while a trend toward RA severity was observed for polymorphisms in *PARP* and *IL6*.
The most evident association was observed for the *IL6* -174 G/G genotype that was overrepresented in RA patients when compared to healthy controls. The association observed in our study remained significant even after adjusting for age, gender and the effect of other polymorphisms that showed a significant association in univariate analysis. Recently, the importance of IL-6 in the pathogenesis of RA was shown by Maini et al. who reported a substantial improvement of RA upon treatment with

Table 5. Comparison of genotype frequencies between RA patients with or without anti-TNF therapy, as marker of disease severity.[1]

SNP	Genotype frequency			Log regression			
	No anti-TNF 250 n (%)	Anti-TNF 120 n (%)	P[2]	OR crude[3] (95% CI)	OR adjusted[4] (95% CI)	OR adjusted[5] (95% CI)	OR adjusted[6] (95% CI)
***PARP* 762**					T/T vs. T/C and C/C		
T/T	180 (73.2)	75 (64.1)	0.37	0.66 (0.41-1.05)	0.64 (0.39-1.03)	0.63 (0.37-1.03)	0.62 (0.37-1.03)
T/C	55 (22.4)	41 (35.0)					
C/C	11 (4.5)	1 (0.9)					
***TNFA* -308**					G/G vs. G/A and A/A		
G/G	156 (62.4)	89 (74.2)	0.13	1.73 (1.07-2.80)	1.65 (1.01-2.68)	1.81 (1.07-3.06)	1.71 (1.00-2.91)
G/A	91 (36.4)	26 (21.7)					
A/A	3 (1.2)	5 (4.2)					
***TNFA* -238**							
G/G	243 (97.2)	111 (92.5)	0.04	0.36 (0.13-0.98)	0.41 (0.15-1.15)	0.28 (0.09-0.87)	0.31 (0.10-0.97)
G/A	7 (2.8)	9 (7.5)		1	1	1	1
A/A							
***IL6* -174**							
C/C	33 (15.1)	22 (19.8)	0.06	1.72 (0.90-3.29)	1.76 (0.91-3.42)	1.82 (0.92-3.61)	1.85 (0.93-3.68)
C/G	83 (37.9)	49 (44.1)		1.52 (0.92-2.52)	1.62 (0.97-2.73)	1.62 (0.96-2.75)	1.71 (1.00-2.92)
G/G	103 (47.0)	40 (36.0)		1	1	1	1

Only patients for whom data regarding medication were complete (n = 370) were included.
[1]Polymorphisms for which no significant association was observed are not shown; genotype data are available on request.
[2]Cochrane-Armitage trend test for additive effects.
[3]Univariate analysis.
[4]As in 3, adjusted for age.
[5]This multivariate model includes the *PARP* 762 T/C, the *TNFA* -308 G/A and -238 G/A and the *IL6* C/G polymorphism simultaneously.
[6]As in 5, adjusted for age.

anti-IL-6 receptor antibody.(3) The *IL6* G/G genotype is associated with increased IL-6 levels compared to the C/C genotype, contributing to the complex regulation of IL-6 production.(15, 30-32) IL-6 expression in synovial tissue is higher in end-stage RA than in chronic active RA.(33) Furthermore, IL-6 expression is influenced by TNF-α, and interaction of polymorphisms in these and other genes may co-determine the disease phenotype. Indeed, although the *TNFA* -308 polymorphism was not differentially distributed between our RA patients and controls, the *TNFA* -238A allele was underrepresented in RA-patients. However, this was no longer significant after correction for age and gender. The findings for the *TNFA* -238 polymorphism match those reported for both a Mexican and a Colombian cohort.(23, 24) Additionally, in the Mexican cohort, the -308 A allele was associated with more severe disease, while it is also reported to be associated with erosion in RA patients.(23, 34) Moreover, carriage of the *TNFA* -308A allele, related to higher TNF-α production, was previously reported to be associated with non-response to anti-TNF-α in patients with different autoimmune diseases among which RA.(35-37) In the past numerous studies have been performed to investigate the association of *TNFA* promoter polymorphisms and TNF-α levels in different inflammatory and infectious diseases, reporting contradictory

results.(38) TNF-α expression is probably not determined by one but by a combination of polymorphisms in *TNFA* and -associated genes. In our study, and in contrast to those described above, the *TNFA* -308 G/G genotype and the *TNFA* -238A allele were associated with the severe disease (Table 5). It must however be noted that the allele frequency is very low and a slight difference in genotype distribution would markedly alter the results.

Two other polymorphisms, the *PAI1* 4G/5G and the *IL8* 781 C/T polymorphisms, were associated with susceptibility to RA. The *PAI1* 4G homozygous variant was previously shown to be associated with increased expression of PAI-1 compared to the *PAI1* 5G/5G genotype.(16, 21, 39, 40) The *PAI1* 5G homozygous variant, related to low PAI-1 levels and therefore decreased inhibition of plasminogen activator was associated with RA when compared with controls. No link was observed with severity of disease. Plasminogen deficiency has been associated with decreased susceptibility to and or severity of arthritis in mice models.(10) PAI-1 deficiency, however, has also been associated with decreased severity in murine antigen-induced arthritis.(41)

The *IL8* 781 C/C genotype was overrepresented in RA patients when compared with normal controls. Increased IL-8 expression was associated with RA when compared with osteoarthritis.(42) No data are available on the effect of the intronic *IL8* 781C/T polymorphism on IL-8 expression. It must be noted that the effect of this polymorphism appears to be age dependent. *IL8* 781 C/C genotype was associated with a younger age at RA diagnosis.

With regard to RA severity, a trend was observed for carriers of the *PARP* 762 C allele, who were overrepresented in RA patients receiving anti-TNF-α therapy compared to those who did not. PARP plays a role in inflammation and apoptosis and is involved in maintaining genomic stability of cells upon oxidative stress.(43) The C allele represents the valine to alanine amino acid substitution at position 762 in the regulatory domain, which is associated with reduced activity after H_2O_2 exposure.(17) This might result in increased tissue damage, increased disease severity, and thus the need for more extensive anti-inflammatory medication. Previously the *PARP* promoter haplotype B (410C-$[A]_{11}$-$[CA]_{13\text{-}20}$-1362T) was reported to be overrepresented in an RA patient group compared to healthy controls in a Spanish population.(44) Like in other studies we observed no association between RA and polymorphisms in the gene encoding inducible nitric oxide synthase, which is also involved in apoptosis.(45)

No association was observed for the *IL10* polymorphisms with RA susceptibility or severity. Previously, contrasting results were reported for the role of *IL10* polymorphisms in RA.(46, 47)

Although a polymorphism in *TLR4* has previously been associated with rapid response to treatment in RA no association with RA was observed for the *TLR4* polymorphisms in our study.(48)

Many would argue that functional studies are preferred over genetic association studies to elucidate the pathogenesis of RA. However, interpretation of RNA expression analyses is difficult due to the intra and inter-patient variation.(49) Careful sampling may overcome this problem and reduce the finding of false positive differentially expressed genes. With the exception of those studies assessing cytokine expression

only in patients without DMARDs at diagnosis, results are likely to be influenced by the use of these DMARDs and disease progression. For this reason comparison of the results of different studies is limited.
One can question whether therapy with anti-TNF is an appropriate marker for disease severity. In the modern era of aggressive treatment of RA medication is intensified till the goal of low disease activity or even remission is achieved. That disease activity was low is underscored by the low geometric mean ESR level in our cohort of RA-patients (16.2; 95% CI 14.7-17.7 mm/h), which was not different (p=0.17) between patients on conventional DMARDs (15.4; 95% CI 13.8-17.3 mm/h) or those on biologicals (17.6; 95% CI 15.1-20.5 mm/h). It was therefore decided not to use disease activity as marker of disease severity, but whether low disease activity could be achieved by conventional DMARDs or by use of biologicals directed against TNF. That therapy with a biological against TNF could be used as a marker of disease severity in this study is also due to the fact that its use is strictly regulated in the Netherlands; therapy with biologicals against TNF is available for all RA-patients that failed on two DMARDs and still have active disease (DAS28 > 3.2) despite therapy with methotrexate 25 mg weekly or at the maximal tolerated dose.
In summary we here for the first time report an association between *IL8* and *PAI1* polymorphisms and RA susceptibility. In addition, we confirm genetic associations or support previous functional findings on polymorphisms in *TNFA* and *PARP*. For other polymorphisms we could not confirm previous findings or found contrasting results such as for *TLR4*. For the *IL6* -174G allele and the *IL8* 781 C/C and *PAI1* 5G/5G genotypes we observed an independent association with RA susceptibility even after adjusting for other polymorphisms and cofactors. This illustrates the need for additional large studies to elucidate the contributing factor of polymorphisms in the susceptibility to and severity of RA. Carefully designed functional studies regarding expression of mRNA and protein levels in combination with genetic analyses are warranted to elucidate the contribution of polymorphisms to disease severity and will contribute to improving our knowledge on RA pathogenesis.

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Chapter 3.3

Polymorphisms in the immune regulatory genes *IL1B* and *IL1RN* and the bacterial sensing genes *CD14* and *TLR4* are associated with Barrett oesophagus

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Abstract

Objective. Patients with Barrett oesophagus are at increased risk for malignancy. Progression to invasive adenocarcinoma follows the metaplasia-dysplasia-carcinoma sequence. The eradication of *H. pylori* influences gastric acid production and reflux disease. Therefore, polymorphisms in genes regulating gastric acid secretion (*IL1*), immune regulation (*IL1* family) and bacterial recognition genes (*CD14* and *TLR4*) may be involved in the susceptibility to develop Barrett oesophagus.
Patients and methods. 210 Dutch Caucasian Barrett patients and 334 ethnically matched controls were typed for *IL1B*-511, *IL1RN* +2018, *CD14* -260 and *TLR4* +896 single nucleotide polymorphisms.
Results. The *IL1RN**C allele at position +2018 was significantly increased in Barrett's patients compared to controls (p=0.033; OR 1.5, 95% CI 1.1-2.1). *CD14* -260 T/T homozygotes were significantly decreased in Barrett's patients compared to controls (p=0.040; OR 0.6, 95% CI 1.0-2.7) and carriers of *CD14* -260 T/T with *TLR4* +896 A/A were also significantly decreased in patients (p=0.007; OR 0.5, 95% CI 0.33-0.83). Combined carriage of specific combinations of these four genes confers protection from Barrett oesophagus. Multivariate logistic regression analysis showed a strong correlation between *IL1RN* +2018 and development of Barrett oesophagus (p=0.006; OR 2.2, 95% CI 1.3-3.0).
Conclusions. *IL1B, IL1RN, CD14* and *TLR4* gene polymorphisms influence susceptibility to Barrett oesophagus. *IL1RN* correlates very strongly in multivariate analysis with Barrett oesophagus.

Introduction

Patients with Barrett oesophagus are at increased risk for malignancy and progression to invasive adenocarcinoma is reflected histologically by the metaplasia-dysplasia-carcinoma sequence. The incidence of development of high grade dysplasia or adenocarcinoma from Barrett oesophagus is small (estimated annual risk 0.2-2.0%), however the risk is 30-125 times that of an age matched population.(1, 2) The presence of low grade dysplasia and long duration of reflux symptoms are independent risk factors for development of high grade dysplasia and oesophageal carcinoma.(3) The diagnosis of Barrett oesophagus is made endoscopically and confirmed by histological examination of biopsies.
Decreasing rates of *H. pylori* infection are reported to coincide with increased rates of Barrett oesophagus in the Western countries. Other risk factors appear to be age over 40, male gender, Caucasian race and increased body mass. Protective factors may be high fibre diets and successful antireflux surgery.(3-6)
Recent studies have implicated the involvement of the host immune system in the pathogenesis of gastrointestinal tract diseases.(7) Although comparatively little is known about the immunogenetics of upper gastrointestinal tract diseases, recent

studies have shown clear associations between polymorphisms in genes encoding cytokines and the development of gastric cancer.(8, 9) For instance, carriage of polymorphisms in *IL1B*, *IL10*, *TNFA* and *IL1RN* increases the risk for non-cardia gastric cancer as compared to single gene analysis OR 2.8 (single gene) vs. OR 27.3 (multiple genes).(8) Furthermore, twin studies have reported the role of genetic factors in the aetiology of reflux disease.(10, 11)

The interleukin 1 (*IL1*) gene family is involved in a wide variety of physiologic processes, including the regulation of inflammatory, metabolic, haematopoietic and immunologic mechanisms. IL-1β induces lymphokine expression and activates natural killer cells, T cells, and B cells.(12) Furthermore, it is a powerful inhibitor of gastric acid secretion and an up regulator of COX2 expression.(13) The IL-1 receptor antagonist (IL-1ra) is the natural inhibitor of IL-1β. Polymorphisms have been described in both genes. The polymorphism at position -31 in the *IL1B* gene is located in a TATA-box and influences DNA-protein interactions. This polymorphism is associated with decreased IL-1β production.(14) The polymorphism at position +2018 in the *IL1RN* gene is in linkage disequilibrium with the *IL1RN* 86bp tandem repeat, which is associated with increased IL-1ra production.(15)

The Toll Like Receptor (TLR) family is a group of pattern recognition receptors, which recognize several microbial products, including bacterial cell wall components and DNA.(16) CD14 acts as a co-receptor for TLR4 and confers responsiveness to LPS, a component of the cell wall of most Gram-negative bacteria. CD14 forms a complex with LPS and the LPS-binding protein (LBP).(17) Combined with TLR4 this complex induces NF-κB associated immune responses including the release of a broad spectrum of cytokines that include tumour necrosis factor alpha (TNF-α), IL-1, IL-6, and IL-8, to initiate immune responses.(18) The *TLR4* gene contains an A>G substitution at position +896, which is associated with LPS hyporesponsiveness.(19) The promoter region of the *CD14* gene contains a single nucleotide polymorphism (SNP) at position -260. The -260 C>T genetic variation affects the binding of transcription factors and has been associated with increased expression of levels of soluble CD14 and membrane-bound CD14.(20) Since *H. pylori* infection has been described in relation to the pathogenesis of reflux disease and Barrett oesophagus development, recognition of *H. pylori* and the subsequent immune response to its presence might be important.

The aim of this study was to identify whether or not polymorphisms in the *IL1B* and *IL1RN* genes (immune regulation), and in the *TLR4* and *CD14* genes (bacterial recognition) influence the susceptibility to and severity of Barrett oesophagus both in single gene and carrier trait analysis.

Methods

Participants. The study comprised of 210 Dutch Caucasian patients, attending the outpatient departments of Gastroenterology of the VU University Medical Centre (n = 88) and the Academic Medical Centre (n = 122), Amsterdam, the Netherlands. A

total of 334 ethnically matched healthy controls were included. The mean age was 59y (21y-81y) and 43y (19y-92y) in the Barrett and control cohorts respectively, while 80.5% was male in the Barrett cohort as compared to 52.7% in the control cohort. All patients underwent an upper gastrointestinal (GI) endoscopy and biopsies (duodenum, antrum, corpus, and Barrett) were taken at regular intervals. Presence of Barrett epithelium and intestinal metaplasia was confirmed by histology. Patients who underwent multiple GI endoscopies were classified as suffering from Barrett oesophagus if this condition was diagnosed in at least 1 endoscopy and confirmed by histology in the biopsy samples. Data was available on alcohol use and smoking for one-third of the patients for logistic regression (gender distribution was equal to the total population). Patients were defined as non-user, previous user or current user of alcohol and/or tobacco. The patient characteristics are presented in table 1.

Table 1. Patient characteristics.

	Cases n = 210	**Controls n = 334**	**Remarks**
Age (years)	59 (21-81)	43 (19-92)	
Males (%)	80.5	52.7	
Smokers (%)		N/A	Data in 71 patients only
Never	12.7		
Previous	7.0		
Current	80.3		
Alcohol use (%)		N/A	Data in 73 patients only
Never	37.0		
Previous	21.9		
Current	41.1		

N/A, not available.

DNA isolation. Peripheral venous blood was collected in 10ml EDTA-tubes and stored at room temperature until the genomic DNA was extracted from peripheral blood mononuclear cells (PBMC) according to an in-house DNAzol extraction procedure (Invitrogen, the Netherlands).

Genotyping. All samples were genotyped for the *IL1RN* T+2018C (rs419598), *CD14* C-260T (rs2569190), *IL1B* C-511T (rs16944) and *TLR4* A+896G (rs4986790) polymorphisms using PCR-RFLP or TaqMan analyses.

The detection of the *CD14* C-260T and *TLR4* A+896G polymorphisms was performed as described previously by our group.(21, 22) The *IL1B* C-511T was assessed by PCR-RFLP as described previously by Di Giovine et al.(23) The *IL1RN* T+2018C, of which the mutant (C) allele is in linkage disequilibrium with the second allele of the *IL1RN* VNTR (rs2234663), was assessed according standard TaqMan protocol in 96 well plates (Greiner Bio-One), using the primers: forward: 5'- CAA CCA CTC ACC TTC TAA ATT GAC AT -3', and reverse: 5'- CTG AGT CCT TTT CCT TTT CAG AAT CT -3'. The probes used were: AGT ATC CAG CAA CTA GT-FAM for the T allele and CAA GTA TCC GGC AAC TA-VIC for the C allele.

Statistical analyses. All groups were tested for Hardy-Weinberg equilibrium to check for Mendelian inheritance. Statistical analyses were performed using Instat

Graphpad and SPSS version 11. Fisher exact and χ^2 tests were used to test for differences in allele / genotype / carrier frequencies between the (sub)groups and *P* values < 0.05 were considered statistically significant. Logistic regression analysis was used to assess the association of different genotypes with Barrett oesophagus, adjusted for age and gender.

Results

All genotype distributions were in Hardy-Weinberg equilibrium, confirming genotype distributions according to Mendelian inheritance principles.

Single gene analyses. An increased prevalence of the IL1RN*C allele was found in patients with Barrett oesophagus compared to controls (C/C and C/T: 48.1% vs. 38.6%; p=0.033; OR 1.5, 95% CI 1.1 - 2.1).

The *IL1B* -511 alleles were equally distributed among cases and controls. The distribution of genotypes and allele frequencies in cases and controls is shown in tables 2 - 5.

Table 2. *TLR4* genotype and allele frequencies in cases and controls.

TLR4	Cases n = 191 n (%)	Controls n = 334 n (%)
Genotype		
A/A	161 (84.3)	289 (86.5)
A/G	30 (15.7)	44 (13.2)
G/G	0 (0)	1 (0.3)
Allele		
A	352 (92.1)	622 (93.1)
G	30 (7.9)	46 (6.9)

Table 3. *CD14* genotype and allele frequencies in cases and controls.

CD14	Cases n = 191 n (%)	Controls n = 334 n (%)
Genotype		
C/C	65 (34.0)	86 (25.7)
C/T	85 (44.5)	154 (46.1)
T/T	41 (21.5)	94 (28.1)
Allele		
C	215 (56.3)	326 (48.8)
T	167 (43.7)	342 (51.2)

Table 4. *IL1RN* genotype and allele frequencies in cases and controls.

IL1RN	Cases n = 191 n (%)	Controls n = 334 n (%)
Genotype[1]		
T/T	99 (51.8)	205 (61.4)
T/C	70 (36.6)	107 (32.0)
C/C	22 (11.5)	22 (6.6)
Allele		
T	268 (70.2)	517 (77.4)
C	112 (29.8)	151 (22.6)

[1]C/C and C/T: cases vs. controls: 48.1% vs. 38.6%; p=0.033; OR 1.5, 95% CI 1.1 - 2.1. *P* values calculated using 2 by 2 χ^2 test.

Table 5. *IL1B* genotype and allele frequencies in cases and controls.

IL1B	Cases n = 191 n (%)	Controls n = 334 n (%)
Genotype		
C/C	81 (42.4)	141 (42.2)
C/T	89 (46.6)	151 (45.2)
T/T	21 (11.0)	42 (12.6)
Allele		
C	251 (65.7)	333 (64.8)
T	131 (34.3)	235 (35.2)

Carrier trait analysis. Carriage of the *CD14* -260 T/T genotype together with the *TLR4* A/A genotype is significantly associated with a decreased risk for Barrett oesophagus compared to controls (14.8% vs. 24.6% (controls); p=0.007; OR 0.5, 95% CI 1.2 - 3.0) (Table 6).

Table 6. Multiple gene analysis of *CD14, TLR4, IL1B* and *IL1RN* gene polymorphisms in Dutch Caucasian Barrett's oesophagus patients and controls.

CD14 -260 C>T	*TLR4* +896 A>G	*IL-1B* -511 C>T	*IL-1RN* +2018 T>C	Cases (%)	Controls (%)	Effect	p	Odd's ratio
T/T	A/A			14.8	24.6	Protection	0.007	0.5
T/T	A/A	C/T	T/T	1.8	6.1	Protection	0.030	0.3
T/C	A/G	C/C	T/T	0.6	4.0	Protection	0.013	0.1

Combination of the two carrier traits. Combining the regulatory and bacteria-sensing carrier traits into a larger carrier trait resulted in significant differences between cases and controls. Homozygous wild type carriage of the IL1B and IL1RN genes combined with heterozygous carriage of the CD14 and TLR4 genes is significantly decreased in cases compared to controls (0.6% vs. 4.0%; p=0.013; OR 0.1, 95% CI 0.014 - 0.83) (Table 6).

Carriage of the carrier trait IL1B -511*C/T, IL1RN +2018*T/T, CD14 -260*C/T and TLR4 +896*A/A is significantly decreased in patients with Barrett oesophagus compared to controls (1.8% vs. 6.1%; p=0.030; OR 0.3, 95% CI 0.1 - 0.91). The results are summarized in table 6.

Logistic regression analysis. Logistic regression analysis was used to assess the association of different genotypes with Barrett oesophagus, adjusted for age and gender. Age, gender and carriage of the *IL1RN* +2018 SNP were significantly associated with Barrett oesophagus, with gender and *IL1RN* being risk factors (Table 7). We analyzed the immune regulatory, bacteria sensing, and combined carrier traits in the logistic regression model; however this did not reach statistical significance.

Table 7. Logistic regression analysis of the *IL-1B, IL-1RN, CD14, TLR4* genotypes in patients suffering from Barrett Oesophagus.

	p	OR	95% CI	
			Lower	Upper
Gender	< 0.001	5.830	3.031	11.214
IL1RN[1]	0.006	2.241	1.266	3.969
TLR4[1]	0.119	1.782	0.862	3.687
Age	< 0.001	0.852	0.827	0.877
CD14[1]	0.251	0.718	0.407	1.265
IL1B[1]	0.081	0.601	0.339	1.065

[1]Mutation carriers vs. homozygous wild type.

Discussion

In this study we found on a single gene level that the *IL-1RN+2018**C allele was significantly increased in Barrett patients compared to controls. The carrier trait *CD14*-260 T/T - *TLR4*+896 A/A was significantly decreased in patients. Combined carriage of specific combinations of these four genes confers protection against Barrett oesophagus (table 6). Finally, multivariate logistic regression analysis showed a strong correlation between *IL-1RN*+2018 and development of Barrett oesophagus.

The studied SNP in *IL1RN* was shown to be associated with altered inflammatory responses. Carriage of the *IL1RN**C allele is associated with risk for Barrett oesophagus. These results concur with those reported in the literature, namely, IL-1β increases COX2 production which is known to up-regulate Th2 cytokines when associated with oesophageal carcinogenesis.(24, 25) Moons et al. have demonstrated that a predominantly humoral immune response is characteristic for Barrett oesophagus.(26) Furthermore, an increased inflammatory response and/or chronic inflammation may result in increased tissue damage and carcinogenesis. (27)

In Japanese it was shown that *IL1B* genotypes protect against gastro-oesophageal reflux disease through induction of corpus atrophy.(28) Even though this population is Japanese, while our population consists of Dutch Caucasian patients, these results indicate that the genetic risk factors for the development of Barrett oesophagus could differ between different ethnicities. The increased expression of CD14 in *CD14*-260*T carriers might explain the association with protection against Barrett oesophagus. A

better recognition of an antrum predominant *H. pylori* infection which is associated with increased acid production may result in a stronger inflammatory response.(29) CD14 stimulates both IL-1 and TNF-α production.(18) Both cytokines reduce gastric acid production and thus reduce the effect of gastric acid in the oesophagus and so reduce the risk for development of Barrett oesophagus.(13, 30, 31) The mutant allele of *TLR4* has been associated with a reduced LPS recognition. The mutant *TLR4**G allele affects the extracellular domain of the TLR4 receptor thus blunting the response to LPS.(19) We observed a trend towards risk for Barrett oesophagus in carriers of this mutant allele.

When the regulatory and bacteria-sensing carrier traits were combined in a larger carrier trait, significant differences between cases and controls could be observed. Specific carrier traits of the *IL1B*, *IL1RN*, *CD14*, and *TLR4* genes were significantly decreased in patients with Barrett oesophagus when compared to controls.

Recent literature has provided conflicting evidence for the role of *IL1* polymorphisms and *H. pylori* infections in Barrett oesophagus. For instance, Gough et al. have shown that the *IL1RN* +2018 2.2 genotype was associated with Barrett oesophagus when compared to oesophagitis, while Moons and colleagues failed to confirm this association in a similar population.(32, 33) In another paper, Moons et al. demonstrated a predominantly humoral immune response in patients with Barrett oesophagus, which may explain a lack of association with the Th1 type cytokine IL-1.

Combined carriage of the *CD14* T/T genotype and the *TLR4* A/A genotype confers optimal recognition of *H. pylori* and thus protection in an antrum predominant infection. In a corpus predominant infection which is associated with reduced acid production, this effect may be reversed and this effect of the location of *H. pylori* infection should be researched in a population with clearly defined corpus or antrum gastritis.(34) It should be noted that conflicting reports have been published on the role of TLR4 in *H. pylori* infection. Both positive and negative associations have been reported.(35-38) Different *H. pylori* LPS types and TLR4 expression and subcellular distribution, have been reported as possible explanations for these conflicting results.(39-41)

Equally conflicting reports have been published on the effect of *H. pylori* on development of reflux disease and Barrett oesophagus. However, recent findings may offer an explanation. An antrum predominant gastritis results in increased acid production, while a corpus predominant (atrophic) gastritis results in reduced gastric acid secretion.(29, 34) These differences in infection pattern may explain the differences in reported influences of *H. pylori* on reflux disease, since increased acid production has been associated with reflux disease after eradication of the bacteria. (42, 43) Unfortunately, we currently do not have data on *H. pylori* infection in this cohort, so we can not comment on the influence of *H. pylori* on Barrett oesophagus.

Pei and colleagues have recently described a variety of bacterial species in oesophageal biopsy specimens in patients with oesophageal reflux related disorders. (44) These bacteria might influence development of oesophagitis and Barrett oesophagus. The interaction between these bacterial species and CD14 – TLR4, and the regulation of the immune response against these bacteria by IL-1 may influence pathogenesis of Barrett oesophagus. However, it should be noted that the study of

Pei et al., is only a preliminary report and further studies are required to confirm the results, to elucidate the role of the bacterial species in oesophageal reflux related disorders, and to elucidate the role of the *IL1B*, *IL1RN*, *TLR4*, and *CD14* genes in upper gastrointestinal tract pathogenesis.
Complex mechanisms underlay development of Barrett oesophagus and pathogenic mechanisms in one disease may be protective against another. An overview of variables currently associated with Barrett oesophagus is presented in table 8. Further research is required to elucidate these pathological mechanisms and to gain a better understanding of Barrett oesophagus. The results of this study and those of published studies suggest that a combined analysis of environmental, bacterial and host factors will help to identify patients at high risk for development of Barrett oesophagus and oesophageal cancer.

Table 8. Overview of current known factors associated with Barrett oesophagus.

Variable	OR (95% CI)	Reference
Gender (Male)	2.7 (2.2 – 3.4)	Ford, 2005 (4)
BMI (Obesity)	3.0 (1.6 – 6.7)	Bu, 2006 (45)
	4.0 (1.4 – 11.1)	El-Serag, 2005 (46)
Ethnicity (Caucasian)	6.0 (3.6 – 10)	Ford, 2005 (4)
Diet (vitamin C)	0.44 (0.2 – 0.98)	Veugelers, 2006 (47)
Hiatal hernia	5.4 (3.1 – 9.4)	García Rodríguez, 2006 (48)
Socio-economical status	1.6 (1.2 – 2.2)	Ford, 2005 (4)
Oesophagitis	1.8 (1.6 – 2.0)	Ford, 2005 (4)
Drinking	p=0.001[1]	Conio, 2002 (49)
Medication (NSAIDs)	0.40 (0.19 – 0.81)	Anderson, 2006 (50)

[1]No OR given.

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Chapter 3.4

Polymorphisms in immune response genes affect susceptibility and treatment response in chronic HCV infection

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Abstract

Objective. Treatment of hepatitis C virus (HCV) infection by peginterferon alfa-2a and ribavirin is beneficial only in part of the patients. Since the innate immune response appears to codetermine outcome, we investigated the role of polymorphisms in innate response genes in initial and sustained treatment response.
Patients and methods. Caucasian controls (n = 463) and patients with chronic HCV infection (n = 262) were included. Polymorphisms in *INFAR1*, *IFNAR2*, *STAT2*, *TNFA*, *IL1B*, *IL4*, *IL6*, *IL10*, *IL8*, *NOS2A*, *PARP*, *C1INH*, *PAI1* and *TLR4* were genotyped. Possible associations of genotypes with initial virological response, end of treatment response (ETR) and sustained virological response (SVR) were analysed.
Results. The *IL6* -174G allele was overrepresented in patients when compared to healthy controls. The *IL6* -174 G/C genotype distributions differed significantly between patients and controls, also after correction for multiple testing. Carriage of the *IL10* -819T allele and the *IL10* -1082A;-819T haplotype may be involved in rapid virological response, ETR and SVR. The *PAI1* 5G/5G genotype was overrepresented in patients with SVR.
Conclusions. Polymorphisms in innate immune response genes appear to influence HCV infection susceptibility and treatment response. Viral load, HCV genotype and fibrosis stage, however, are considered to play a more important role in treatment response than the currently assessed genetic polymorphisms.

Introduction

Infection with hepatitis C virus (HCV) is one of the most important causes of liver disease. While some infected individuals are capable of clearing the infection spontaneously, 50-75% suffer from persistent infection.(1) Only 55-65% of patients receiving combination therapy with peginterferon alfa and ribavirin for 48 weeks achieve a sustained virological response (SVR).(2, 3) The reason for this is multifactorial. Age, body weight, but mainly the hepatitis genotype, viral load and presence of fibrosis codetermine outcome.(4) In addition, an early virological response seems to predict SVR.(2) The importance of the immune system in disease progress, measured as inflammation and fibrosis, and treatment response is recognized but its precise role remains unclear. Figure 1 summarizes these factors that provided the rationale for the selection of single nucleotide polymorphisms in genes that are likely to be involved. First, an imbalance between pro-inflammatory and anti-inflammatory cytokines is, together with apoptosis inducing proteins, involved in the development of liver tissue damage and fibrosis. Loss of tissue integrity may facilitate ongoing infection and can possibly result in lack of treatment response. Second, chemokines like IL-8 seem to increase viral replication and may therefore hinder an appropriate treatment response.(5, 6) Third, factors involved in the IFN-α pathway are likely to be involved in treatment response and even susceptibility to ongoing infection.(7) Variations in immune response genes like *IL10* and *IL4* have

been associated with altered cytokine expression levels, and altered function because of amino acid substitutions has been reported for polymorphisms in *TLR4*.(8-10) This implies that polymorphisms in immune response genes might codetermine susceptibility to infection and treatment outcome. Indeed polymorphisms in genes such as *TNFA*, *IL10*, *IL1B* and *TGFB* have been studied before in relation to HCV infection, however, results were contradictory and mostly only small patient groups were evaluated.(11-19)

In this study, we investigated if variations in *INFAR1*, *IFNAR2*, *STAT2*, *TNFA*, *IL1B*, *IL4*, *IL6*, *IL10*, *IL8*, *NOS2A*, *PARP*, *C1INH*, *PAI1* and *TLR4* are involved in the susceptibility to HCV infection or in the early, end of treatment and sustained virological response in chronic HCV infection in a large multi-centre treatment trial.

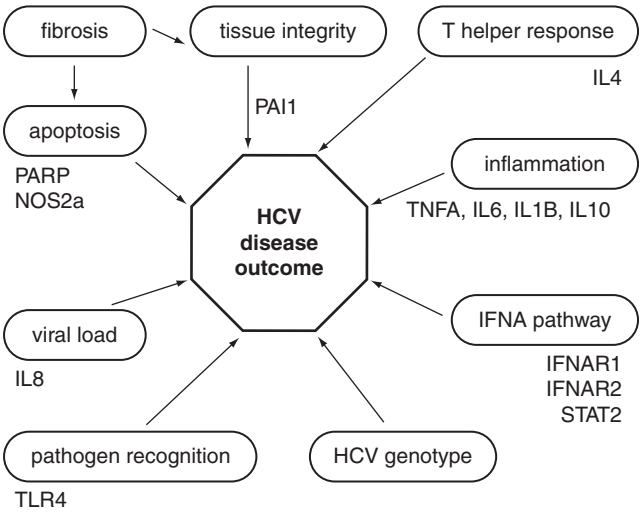

Figure 1. Overview of factors involved in HCV infection and treatment response. This figure depicts major events and pathways likely to be involved in HCV susceptibility, disease course and treatment response (boxed). Candidate genes in which polymorphisms were assessed in this study are indicated below the boxes. HCV, hepatitis C virus; TNFA, tumour necrosis factor alpha; IL, interleukin; IFN, interferon; NOS2A, inducible nitric oxide synthase; PARP, poly (ADP-ribose) polymerase; C1INH, complement component inhibitor-1, PAI1, plasminogen activator inhibitor 1; TLR4 Toll-like receptor 4; IFNAR1/2, interferon alpha receptor 1/2; STAT2, Signal transducer and activator of transcription 2; Th1/2, T helper cell 1/2.

Methods

Participants. Patients included in this study participated in an international, metacentre, randomized, controlled study comparing dynamically individualized versus standard treatment of chronic hepatitis C (n = 270; inclusion period February 2001-November 2003).(20) Only patients who completed at least the randomization phase of the study and of whom DNA derived from blood was available, were included (n = 262 (97%), of whom 250 Caucasian) after obtaining approval of the medical ethical committee of the

Erasmus Medical Centre. All patients provided written informed consent. All patients were previously untreated, had a positive test for anti-HCV antibody and had a pre-treatment liver biopsy consistent with chronic hepatitis C. Patient characteristics were previously described by the DITTO-HCV Study Group.(20) In short, 22 patients (8%) showed no response (NUR) after 6 weeks of treatment while 10 (4%) were defined as flat partial responders (FPR), 62 (24%) as slow partial responders (SPR) and 168 (64%) as rapid virological responders (RVR). In the intention to treat analysis, 201 (77%) patients had a successful response by the end of treatment (ETR). SVR 24 weeks after the end of treatment was observed in 165 (63%) patients. HCV genotype 1 was found in 171 (65%) of patients, while 23 (9%) had genotype 2, 54 (21%) had genotype 3, and 14 (5%) had genotype 4 infection.

Caucasian controls representing the general healthy adult population (n = 463) were derived from the Sanquin Blood Bank (the Netherlands, inclusion period 2002- 2004). All controls were anti-HCV negative. No data are available on anti-HBc status since this was not routinely determined.

***Genotyping*.** Genotyping analysis was performed at the Erasmus MC University Hospital (the Netherlands). Single base extension analysis was used to determine genotypes of inducible nitric oxide synthase (*NOS2A*) S608L (rs2297518), poly (ADP-ribose) polymerase (*PARP*) V762A (rs1136410), complement component inhibitor-1 (*C1INH*) V480M (rs4926), *PAI1* -675 4G/5G (rs1799889), *IL4* C-524T (rs2243250), *IL10* G-1082A (rs1800896), *IL10* C-819T (rs3021097), *IL1B* C-31T (rs1143627), *TNFA* A-863C (rs1800630), *TNFA* T-857C (rs1799724), *TNFA* G-376A (rs3093659), *TNFA* G-308A (rs1800629), *TNFA* G-238A (rs361525), *IL6* G-174C (rs1800795), *IL8* C781T intron (rs2227306), *TLR4* D299G (rs4986790) and *TLR4* T399I (rs4986791) (http://www.ncbi.nlm.nih.gov/SNP/). The genomic region of interest was amplified by PCR. After purification a single base extension was performed using a primer ending one nucleotide prior to the single nucleotide polymorphism (SNP) location. Up to seven SNPs were analysed in one assay. A poly-T-tail attached to the primer combined with the use of a Liz size marker served to distinguish SNPs in the multiplex analysis. A subset of PCR samples was sequenced to confirm genotypes. Genotypes of *IFNAR1* L168V (rs2257167), *IFNAR2* V10F (rs7279064) and *STAT2* I294M (rs2066807) were determined using Taqman analysis. Primer and probe sequences, and PCR specifications are available on request. All genotypes were annotated independently by two investigators who were blinded for the clinical data. Only when identical genotypes were assigned by the investigators was the individual included in the analysis. This resulted in a different number of individuals for the various polymorphisms analysed. No selection was made with regard to outcome when batches were analyzed.

***Statistical analysis*.** Statistical analysis was performed using SPSS 11.0. Haplotype analysis was carried out with Thesias version 2 or Haplostat.(21, 22) Verification of Hardy-Weinberg equilibrium (HWE) of genotypes was performed using χ^2 test (1df). Statistical analyses within the patient cohort represent intention to treat analyses. Binomial variables were analysed using Pearson's χ^2 test (2df) or Fisher's exact test when appropriate. For continuous variables the Student's *t* test or Mann-Whitney

U test were used. A comparison of genotype frequencies was made between the different initial virological response groups at 6 weeks of treatment, and between patients with and without ETR and SVR, 24 weeks after the end of treatment, using logistic regression. A correction was made for possible confounding factors. The effect of genotypes on the initial treatment response was assessed using two different approaches. First a logistic regression analysis comparing the RVR vs. other responses was performed. Second an ordinal regression was performed where initial response was defined as NUR and FPR, SPR or RVR. NUR and FPR were considered to be one group since numbers were small and the clinical consequence similar, i.e. no (adequate) response. Additionally genotype frequencies were compared between patients with chronic hepatitis C and healthy controls using a multiplicative model. (23) Since age and gender distribution differed significantly between patients and controls, this was accounted for in the logistic regression analysis. Non-Caucasian patients, who were evenly distributed among the different response subgroups, were excluded from the analyses comparing patients with healthy Caucasian controls. Probability (*P*) values ≤ 0.05 were considered to be statistically significant. Genetic analyses were performed with and without correction for multiple testing (Bonferroni, times 14 for the number of genes tested).

Results

In controls, genotype distribution of all SNPs except the *IL10* C-819T polymorphism reached HWE. To rule out a technical problem an initial set of individuals was typed using both the reverse and forward primer in the single base extension reaction. Results for both strands were identical. Sequencing of a subset of the controls showed identical genotypes, (data not shown), excluding technical errors. Allele frequencies of all SNPs are depicted in Table 1. Only significant associations will be discussed. SNPs not mentioned in the results section showed no significant associations.

TNFA and IL6 genotype distributions differ between patients and healthy controls. In Caucasian chronic HCV patients and controls 169 of 250 (67.6%) and 233 of 463 (50.3) were males, respectively (p<0.001). The mean age was 41.6 years (SD 10.1) and 39.1 years (SD 8.5), respectively (p<0.001). The *TNFA* -857 T and the *IL6* -174 G allele were overrepresented in chronic HCV patients even after correction for multiple testing (Table 1). The *TNFA* -308 A/A and *IFNAR1* 168 V/V variants were underrepresented in patients compared to controls assuming a multiplicative model (crude OR 0.70 (p=0.02) and OR 0.72 (p=0.04), respectively). In addition, the *TNFA* -857 T/T and the *IL6* -174 G/G genotype were overrepresented in the patients with chronic HCV OR 1.71 (p=0.002, corrected p=0.03) and OR 1.63 (p<0.0001, corrected p<0.001), respectively (Table 2). Only the association with the *IL6* -174 polymorphism remained significant after adjusting for gender and age and correction for multiple testing. When significantly associated polymorphisms were included in the regression model simultaneously, similar results were obtained. The *TNFA* promoter haplotype frequencies are significantly different between patients and

controls (global test p=0.003, corrected p=0.04, 4df) (Table 3). The *TNFA* haplotype -863C, -857T, -376G, -308G and -238G (CTGGG) was overrepresented in patients, while the CCGAG genotype was underrepresented in patients.

Table 1. Allele frequencies in Caucasian patients and controls.

SNP	Allele	Controls n (%)	Patients n (%)	p[1]
***TNFA* -863**	**C**	775 (84)	410 (83)	0.61
	A	147 (16)	84 (17)	
***TNFA* -857**	**C**	838 (92)	428 (86)	0.002
	T	76 (8)	68 (14)	
***TNFA* -376**	**G**	907 (98)	490 (99)	0.55
	A	15 (2)	6 (1)	
***TNFA* -308**	**G**	747 (81)	427 (86)	0.02
	A	175 (19)	71 (14)	
***TNFA* -238**	**G**	876 (95)	480 (96)	0.23
	A	46 (5)	18 (4)	
***IL1B* -31**	**C**	328 (36)	188 (38)	0.42
	T	584 (64)	306 (62)	
***IL4* -524**	**C**	790 (86)	414 (84)	0.39
	T	130 (14)	78 (16)	
***IL6* -174**	**C**	395 (43)	152 (31)	<0.001
	G	523 (57)	332 (69)	
***IL8* 781**	**C**	538 (58)	292 (59)	0.68
	T	386 (42)	200 (41)	
***IL10* -1082**	**G**	465 (51)	230 (47)	0.15
	A	453 (49)	264 (53)	
***IL10* -819**	**C**	711 (77)	355 (75)	[2]
	T	209 (23)	117 (25)	
***PAI1* -675**	**5G**	438 (47)	225 (47)	0.85
	4G	486 (53)	255 (53)	
***NOS2a* 608**	**S**	742 (80)	393 (79)	0.64
	L	182 (20)	103 (21)	
***PARP* 762**	**V**	738 (82)	404 (81)	0.52
	A	160 (18)	96 (19)	
***C1INH* 480**	**V**	686 (75)	364 (74)	0.72
	M	234 (25)	130 (26)	
***TLR4* 299**	**D**	804 (93)	467 (95)	0.17
	G	60 (7)	25 (5)	
***TLR4* 399**	**T**	810 (93)	465 (94)	0.55
	I	58 (7)	29 (6)	
***IFNAR1* L168V**	**L**	107 (12)	74 (16)	0.04
	V	775 (88)	382 (84)	
***IFNAR2* V10F**	**V**	567 (65)	307 (67)	0.49
	F	307 (35)	153 (33)	
***STAT2* I594M**	**M**	817 (94)	386 (97)	0.08
	I	51 (6)	14 (3)	

[1]*P* value represents analysis not corrected for multiple testing. When Bonferroni correction is applied only the *TNFA* -857 and the *IL6* -174 polymorphism remain significant (p=0.03 and p<0.01, respectively).
[2]The *IL10* -819 C/T genotype distribution did not reach Hardy Weinberg equilibrium in controls. For this reason no p value is given. The genotype distribution did not differ significantly between patients and controls.

IL10 polymorphism in rapid initial virological response to treatment. Viral load, HCV genotype (1 or 4 versus other) and fibrosis stage were independently associated with treatment response. Age and gender were not statistically different between the groups. Carriage of the *IL10* -819T allele was significantly associated with RVR

using either ordinal regression or binary logistic regression (Table 4). However, when confounding factors where included in the statistical model or when correction for multiple testing was applied, this association was no longer significant. A similar trend was observed when patients with HCV genotype 1 and 4 were analysed separately (p=0.07).

Table 2. Comparison of genotype frequencies between HCV patients and controls.

SNP	Controls n = 463 n (%)	HCV patients[1] n = 250 n (%)	OR crude (95% CI)	OR adjusted [2] (95% CI)	OR adjusted [3] (95% CI)
***TNFA* -863**					
C/C	329 (71.4)	170 (68.8)	1.08 (0.81-1.44)	1.10 (0.82-1.48)	
C/A	117 (25.4)	70 (28.3)			
A/A	15 (3.3)	7 (2.8)			
***TNFA* -857**					
C/C	386 (84.5)	186 (75.0)	1.71 (1.21-2.40)	1.63 (1.15-2.31)	1.56 (1.06-2.30)
C/T	66 (14.4)	56 (22.6)			
T/T	5 (1.1)	6 (2.4)			
***TNFA* -376**					
G/G	446 (96.7)	243 (98.0)	0.76 (0.30-1.90)	0.74 (0.29-1.87)	
G/A	15 (3.3)	4 (1.6)			
A/A		1 (0.4)			
***TNFA* -308**					
G/G	300 (65.1)	181 (72.7)	0.70 (0.51-0.95)	0.64 (0.47-0.88)	0.67 (0.48-0.94)
G/A	147 (31.9)	65 (26.1)			
A/A	14 (3.0)	3 (1.2)			
***TNFA* -238**					
G/G	415 (90.0)	232 (93.2)	0.71 (0.41-1.25)	0.70 (0.40-1.23)	
G/A	46 (10.0)	16 (6.4)			
A/A		1 (0.4)			
***IL6* -174**					
C/C	82 (17.8)	31 (12.8)	1.63 (1.62-2.66)[4]	1.64 (1.29-2.08)[4]	1.68 (1.31-2.16)[4]
C/G	231 (50.3)	90 (37.2)			
G/G	146 (31.8)	121 (50.0)			
***IFNAR1* L168V**					
L/L	7 (1.6)	7 (3.1)	0.72 (0.52-0.99)	0.72 (0.52-0.99)	0.68 (0.49-0.95)
L/V	93 (21.1)	60 (26.3)			
V/V	341 (77.3)	161 (70.6)			

[1]Only Caucasian patients were included, when initial treatment was completed.
[2]OR adjusted 1; adjusted for gender and age, one SNP per model. OR female gender 0.5 (p<0.001); OR age 1.03 (p<0.01).
[3]OR adjusted 2: model including gender, age and polymorphisms *TNFA* -857 and -308, *IL6* and *IFNAR1*. Only SNPs significant in the adjusted (1) analyses were included.
[4]Only the *IL6* -174 polymorphism was significantly associated after correction for multiple testing (correction factor 14 (Bonferroni)).

Table 3. *TNFA* haplotypes in patients and controls.

Haplotype[1]	Controls n = 463 (%)	Patients[2] n = 250 (%)	Crude OR[3] (p)	Adjusted OR[4] (p)
CCGGG	51.9	51.6	0.95 (0.86)	1
CCGAG	19.0	14.0	0.68 (0.02)	0.41 (0.02)
ACGGG	15.7	17.1	1.09 (0.54)	1.23 (0.58)
CTGGG	8.1	13.7	1.77 (0.001)	2.83 (0.02)
Global test p=0.003, corrected for multiple testing p=0.04				

[1]Haplotype: -863 C/A, -857 C/T, -376 G/A, -308 G/A, -238 G/A.
[2]Non Caucasians were excluded from the analysis.
[3]Haplotypic odds ratio, not adjusted for multiple testing. Only the distribution of the CTGGG haplotype remains significantly different after correction for multiple testing (crude).
[4]Adjusted for age, sex and other haplotypes with frequency above 0.05.

A possible role for IL10 and PARP polymorphisms in virological ETR. Successful ETR was observed in 201 (77%) patients. The *PARP* 762 V/V and *IL10* -1082 A/A genotypes and the *IL10* -819T allele were overrepresented in patients who had undetectable HCV-RNA levels at the end of treatment (p= 0.045, p=0.03 and p=0.02, respectively) (Table 4).

Table 4. Comparison of genotype frequencies between different initial treatment response groups.

SNP	Initial virological treatment response			Logistic regression RVR vs NUR, FPR and SPR	
	NUR and FPR	SPR	RVR	Crude	Adjusted[1]
Total	n (%)	n (%)	n (%)	OR (95% CI)[2]	OR (95% CI)[2]
***IL10* -819**	**31**	**56**	**161**	C/C vs C/T And T/T	
C/C	21 (68)	36 (64)	81 (50)	0.53 (0.31-0.91)	0.62 (0.33-1.17)
C/T	7 (23)	16 (29)	70 (44)		
T/T	3 (10)	4 (7)	10 (6)		

[1]Adjusted analysis includes infection with HCV genotype 1 or 4 vs non 1 or 4, log (viral load) at the start of treatment and the stage of fibrosis (0-6).
[2]Not corrected for multiple testing.

When viral load at the start of treatment, HCV genotype (1 or 4 vs. others) and fibrosis stage were included in the model or correction for multiple testing was applied, these associations were no longer significant. Since the *IL10* SNPs are in linkage disequilibrium, the effect of the haplotype was assessed (Table 5). Only the 246 patients for who complete genotype data was available were included in the analysis. The *IL10* promoter -1082A;-819T haplotype was associated with end of treatment response in the univariate analysis (crude OR 1.81, p=0.03, adjusted OR 1.63, p=0.11). When correction for multiple testing is applied, the IL10 promotor haplotype this is no longer significant. When patients infected with HCV genotype 1 or 4 were analysed separately, similar results were obtained for the crude OR (data not shown).

A possible role for IL10 and PAI1 polymorphisms in SVR. SVR 24 weeks after treatment was observed in 165 (63%) patients. The *PAI1* high producer 4G/4G

genotype was overrepresented in patients who did not have SVR compared to the 5G/5G genotype (crude OR 2.19, p=0.04, adjusted OR 2.62, p=0.02, respectively) (Table 6). Carriage of the *IL10* -819T allele was associated with SVR. Similar results were obtained for the PAI1 genotype, while a trend was observed for carriage of the IL10 -819 T allele, when only patients infected with the HCV genotype 1 or 4 were analysed (data not shown). The *IL10* promoter AT haplotype was associated with SVR (crude OR 1.81 p=0.01, adjusted OR 1.69, p=0.05) (Table 6). These associations were no longer significant after correction for multiple testing.

Table 5. Genotype frequencies and *IL10* haplotypes in chronic HCV patients with and without ETR.

SNP	End of treatment response		Logistic regression	
	No n (%)	Yes n (%)	Crude OR	Adjusted OR[1]
Total	61	201	(95 % CI[2])	(95 % CI[2])
***PARP* 762**			V/V vs V/A and A/A	
V/V	34 (56)	140 (70)	1.82 (1.01-3.28)	1.85 (0.97-3.53)
V/A	23 (38)	54 (27)		
A/A	4 (7)	7 (4)		
***IL10* -1082**				
G/G	17 (28)	34 (17)	0.39 (0.17-0.92)	0.41 (0.16-1.04)
G/A	31 (52)	104 (52)	0.66 (0.32-1.38)	0.71 (0.32-1.56)
A/A	12 (20)	61 (31)	1	1
***IL10* -819**			C/C vs C/T and T/T	
C/C	40 (69)	98 (52)	0.48 (0.26-0.90)	0.55 (0.27-1.09)
C/T	14 (24)	79 (42)		
T/T	4 (7)	13 (7)		
***IL10* haplotype[3]**	n = 57 (%)	n = 189 (%)		
AT	19.3	27.5	1.81 (1.05-3.12)	1.63 (0.89-2.95)
AC	26.3	29.6	1.43 (0.87-2.35)	1.49 (0.86-2.57)
GC	54.4	42.9	1	1

[1]Adjusted analysis includes infection with HCV genotype 1 or 4 vs non 1 or 4, log (viral load) at the start of treatment and the stage of fibrosis (0-6).
[2]Not corrected for multiple testing.
[3]Haplotype: -1082 A/G, -819 C/T

Discussion

In this study we found that *IL6* -174 and the *TNFA* -857 genotypes were differentially distributed between patients with chronic HCV infection and healthy controls. The high producer variant -174G in the *IL6* promoter was observed more often in chronic HCV infected patients than controls. Barrett et al. found the high producer variant to be more prevalent in persistent HCV infection compared with spontaneous viral clearance.(18) Increased IL-6 expression was observed in patients with chronic hepatitis C compared to healthy controls. Whether this is secondary to infection or reflects increased susceptibility to or reduced clearance of infection with HCV in

Table 6. Genotype frequencies and *IL10* haplotypes in chronic HCV patients with and without SVR.

SNP	Sustained response		Logistic regression	
	No n (%)	Yes n (%)	Crude	Adjusted[1]
Total	97	165	OR (95 % CI[2])	OR (95 % CI[2])
***PAI1* -675 4/5G**				
5G/5G	17 (18)	44 (28)	2.19 (1.06-4.53)	2.62 (1.13-6.05)
5G/4G	45 (47)	74 (47)	1.39 (0.77-2.52)	1.16 (0.58-2.30)
4G/4G	33 (35)	39 (25)	1	1
***IL10* -1082**				
G/G	24 (25)	27 (17)	0.55 (0.26-1.15)	0.57 (0.24-1.32)
G/A	48 (50)	87 (53)	0.89 (0.49-1.62)	0.96 (0.49-1.88)
A/A	24 (25)	49 (30)	1	1
***IL10* -819**			C/C vs C/T And T/T	
C/C	61 (67)	77 (49)	0.47 (0.28-0.81)	0.54 (0.30-0.99)
C/T and T/T	30 (34)	80 (51)	1	1
***IL10* haplotype[3]**	**n = 90 (%)**	**n = 156 (%)**		
AT	19.4	29.2	1.81 (1.13-2.91)	1.69 (0.99-2.88)
AC	29.4	28.5	1.17 (0.76-1.80)	1.25 (0.76-2.04)
GC	51.1	42.3	1	1

[1]Adjusted analysis includes infection with HCV genotype 1 or 4 vs non 1 or 4, log (viral load) at the start of treatment and the stage of fibrosis (0-6).
[2]Not corrected for multiple testing.
[3]Haplotype: -1082 A/G, -819 C/T

high IL-6 producers is not clear.(24) Additionally, the *TNFA* -857T variant was overrepresented in patients versus healthy controls. Other studies investigating this or other *TNFA* promoter polymorphisms did not find a difference between patients and healthy controls or between patients with chronic infection and individuals with spontaneous viral clearance.(12-14, 25, 26) Hohler et al. found the -238A allele to be overrepresented in patients, but no significant difference was observed for the *TNFA* -308 genotype.(27) Control groups were small however, and studies were therefore probably underpowered. *TNFA* polymorphisms have been related to differences in expression levels. Results are, however, contradictory and the contribution of each individual polymorphism remains unclear.(28, 29)

Although we realize that a control cohort for whom exposure followed by spontaneous viral clearance is preferred over healthy controls and interpretation of these findings is limited, our current findings do warrant further research.

In this study we found an association between polymorphisms in immune response genes *IL10, PARP, PAI1* and *TNFA* and HCV treatment response. It must be noted, however, that these associations lost significance after correction for multiple testing. Carriage of the IL10 -819T allele was associated with RVR response at 6 weeks of treatment with peginterferon alfa-2a and ribavirin. The *IL10* promoter low producer -1082A;-819T haplotype was associated with ETR and SVR after 24 weeks. When known treatment response predictors like viral load, HCV genotype and fibrosis were

included in the statistical model simultaneously, only the association of the *IL10* AT haplotype with SVR remained significant. This was attributable to the combination of the confounders. This indicates that, although an effect of *IL10* promoter haplotype is present, its contribution to outcome is limited. This is also reflected by the loss of significance after correction for multiple testing. Treatment response is considered to be determined by multiple factors. The effect of individual genetic polymorphisms is expected to be limited, and multiple SNPs are expected to be involved besides other host and environmental factors. Genetic polymorphisms have been implied to play a role in the progression rate of fibrosis. Results have however been contradictory.(30) Carriage of the *PARP* 762A allele was associated with a lack of ETR. PARP plays a role in inflammation and apoptosis and is involved in maintaining genomic stability of cells upon oxidative stress.(31) The *PARP* 762A variant has recently been found to be associated with reduced activity after H_2O_2 exposure.(32) Although a protective effect of PARP inhibition is reported in inflammatory diseases in the early phase, it may be worsening fibrosis formation in a later stage by increasing collagen synthesis.(33) Another factor involved in fibrosis is the pleiotropic PAI-1.(34) The -675 4G/4G promoter genotype is associated with high PAI-1 expression.(35, 36) Since increased PAI-1 levels inhibit wound healing and induce fibrosis, it is reasonable to assume that this mechanism contributes to non sustained response in PAI1 4G homozygous patients. Additionally, PAI-1 deficiency protects against fibrosis formation in diverse animal disease models.(37) Furthermore, plasminogen deficiency interferes with clearance of debris after acute injury, and results in activation of hepatic stellate cells known to be involved in fibrosis progression.(38, 39)

In contrast to previous studies no associations were observed in treatment response for genetic polymorphisms in the IFN pathway.(40, 41) Matsuyama et al. investigated promoter polymorphisms in *IFNAR1* and *IFNAR2* while we analysed SNPs resulting in an amino acid substitution in the coding regions. For *IFNAR1* the dinucleotide repeat in the promoter in the Japanese patients was associated with treatment response. The *IFNAR1* V168L polymorphism was not associated with treatment response in the European patients in our study. However, a difference was observed between patients and healthy controls that was significant also after adjusting for confounding factors and independent of other polymorphisms, but lost significance after correction for multiple testing. This implies a possible role for *IFNAR1* in the endogenous response to viral infection. Previously the *IFNAR* 168L/L genotype was shown to be associated with susceptibility to multiple sclerosis but not to IFN-β treatment response.(42)

Although previous studies suggested a role for TLR, the complement system, apoptosis and interleukins 1, 4 and 8 in the pathogenesis of HCV infection, we found no associations with treatment response or a difference between patients and controls for selected polymorphisms in genes encoding TLR4, C1INH, IL-1β, IL-4, iNOS, STAT2 and IL-8.(5, 6, 14, 43-49)

Studies regarding variations in immune response genes in HCV infection and treatment response have yielded contradictory results, likely due to several reasons. First, the relatively small sample sizes, since it is known that they predispose to finding both false positive and false negative associations.(50) Second, HCV infection and

treatment response are determined by multiple host and environment factors, and some may have different effects depending on disease stage. Th1 and Th2 responses for instance, might be either beneficial of deleterious. The Th1 response appears to be essential in viral clearance, however, over-stimulation of the inflammatory response may result in increased tissue damage, which might be limited by anti-inflammatory action of IL-10.(47) The balance in these responses therefore appears to determine outcome. In addition to SNPs in host genes, genetic variations in HCV play a role in the early viral clearance by combination therapy with IFN and ribavirin.(51) This needs to be accounted for when effects of SNPs are assessed and may explain the differences observed in our study when treatment response is defined at different time points.

In conclusion, the polymorphisms in innate immune response genes *TNFA* and *IL6* appear to affect susceptibility to HCV infection. In addition, polymorphisms in *IL10*, *PAI1*, *IFNAR1* and *PARP* may play a role in the susceptibility to HCV infection or treatment response. The combination of HCV genotype, viral load and fibrosis stage, however, is considered to play a more important role. Given the multifactorial nature of HCV disease course and the likelihood that various genotypes interact both with each other and numerous environmental and host factors, additional functional and genetic studies are warranted to elucidate their individual contributions to HCV infection.

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Chapter 4

SNPs in immune response genes and their effects in otitis media

Chapter 4.1

The 4G/4G plasminogen activator inhibitor-1 genotype is associated with frequent recurrence of acute otitis media

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Abstract

Objective. Plasminogen activator inhibitor-1 (PAI-1) counterregulates cell migration, adhesion and tissue repair. The *PAI1* 4G/5G promoter polymorphism has an effect on expression levels of *PAI1*. After a first acute otitis media (AOM) episode, children are at increased risk for a next episode. Because the *PAI1* 4G allele is associated with higher PAI-1 production and, hence, decreased tissue repair, we hypothesize that this allele may contribute to increased recurrence of AOM.
Patients and Methods. The *PAI1* 4G/5G polymorphism was genotyped in 348 Dutch children aged 1 to 7 years who were suffering from recurrent AOM and participating in a randomized, controlled trial, and 463 healthy control subjects, representative of the general population.
Results. No significant difference in *PAI1* genotype distribution between the whole AOM group and control subjects (p=0.32) was observed. However, children with the *PAI1* 4G/4G genotype had an increased risk of more frequent AOM episodes compared with those who were homozygous for the 5G variant, also after correction for cofactors (p=0.03, OR 2.31). This finding was attributable to children < 4 years of age (p=0.02, OR 2.97).
Conclusions. Our findings suggest that the *PAI1* 4G/4G genotype is associated with an increased risk for the otitis-prone condition, potentially because of impaired healing after a previous otitis media episode.

Introduction

Acute otitis media (AOM) is the most common infection in children, but the number of AOM episodes varies markedly between patients. Around 10% to15% of all children suffer from ≥ 4 episodes per year and are considered otitis prone.(1-3) One of the most prominent risk factors appears to be a first episode of AOM early in life predisposing to a next event.(4, 5) Other issues affecting the risk of developing recurrent middle ear infections include host factors like gender, age, atopy, and ethnic background. (6) Environmental factors associated with recurrent AOM episodes are microbial exposure, like in day care or from older siblings, or triggers, like lack of breastfeeding or exposure to tobacco smoke. Often there is a family history of otitis media.(4, 6, 7) Twin studies have shown a heritability of 57% for acute ear infections and 72% for chronic ear infections.(8-12) Furthermore, the correlation for recurrent otitis media is twofold higher in monozygotic twins (65-71%) compared to dizygotic twins (25-34%). (13) Genetic factors involved in inflammation and tissue repair may play a role in the development of recurrent AOM episodes and the otitis-prone condition. Delayed downregulation of the inflammatory response to infections in the middle ear cavity may play a role in priming the individual for additional AOM episodes. Plasminogen activator inhibitor (PAI-1) is known to have an important role in fibrinolysis.(14, 15) In addition, PAI-1 is involved in the inflammatory reaction, inhibits cell adhesion and

migration, and delays tissue repair.(16, 17) The 4G/5G insertion deletion polymorphism at position -675 of the *PAI1* gene is known to influence the expression of PAI-1.(18, 19) Individuals homozygous for the 4G allele have higher PAI-1 concentrations in peripheral blood than those with the 4G/5G or 5G/5G genotypes.(20, 21) The *PAI1* 4G allele has been reported previously to be associated with asthma, as well as with increased morbidity and mortality in meningococcal disease.(20, 22-25)
We hypothesized that the *PAI1* 4G/4G genotype, known to result in high PAI-1 concentrations, which is associated with decreased tissue integrity, is associated with recurrence of otitis media. In this study we investigated the association between this *PAI1* polymorphism and recurrent AOM. Our results show that 4G homozygous individuals have an increased risk for an otitis prone condition, especially under the age of 4 years.

Methods

Participants. Patients who initially participated in a randomized, controlled study on prevention of recurrent AOM by pneumococcal vaccinations were included in this study.(26) Parents were informed about the study by primary care physicians, paediatricians, and otolaryngologists from across the Netherlands. Children were enrolled in the study after obtaining approval of the medical ethical committee of the Utrecht University Medical Centre and informed consent from the parents or guardians. DNA was available from 348 Dutch Caucasian children, 1 to 7 years of age, who were suffering from recurrent AOM defined as ≥ 2 AOM episodes in the preceding year. The number of AOM episodes before vaccination was based both on parental report (AOM defined as having one or more of the symptoms: acute earache, new-onset otorrhea, irritability, and fever) and on clinical information of the diagnosis by a physician. In the present study cohort, 122 children suffered from 2 to 3 otitis media episodes in the year preceding inclusion, whereas 226 suffered from ≥ 4 episodes (otitis-prone condition).(3) No children with < 2 AOM episodes were included in the vaccination trial. In addition, children with primary or secondary immunodeficiency, cystic fibrosis, immotile cilia syndrome, chromosomal abnormalities such as Down's syndrome, and craniofacial abnormalities such as cleft palate were excluded from the study. The history of atopy, defined as eczema, hay fever, recurrent wheezing or asthma and the number of AOM episodes in the year before inclusion was assessed by the questionnaire filled out by all of the patients and their parents at inclusion in the study. Otorrhea duration was assessed in 214 children for whom *PAI1* genotype were available. Patient characteristics are described in Table 1. Healthy adult Dutch Caucasian controls (n = 463) were derived from the Dutch Blood bank Sanquin. No records of previous history regarding AOM were available for these control subjects; however, in the general population, a total number of ≤ 3.2 AOM episodes is expected in childhood.(3) This implies that control subjects will have had, on average, much fewer AOM episodes per year than the patients. Blood was sampled for DNA isolation. DNA isolation from donor buffycoats was performed according to standard protocols using a QIAamp DNA mini kit (Qiagen, Valencia, CA).

Table 1. Characteristics of patients with recurrent acute otitis media.

Characteristics	2-3 AOM episodes n=122	≥ 4 AOM episodes n=226	p-value[1]
Male gender, n (%)	84 (69)	128 (57)	0.03
Median age (min-max), y	2.42 (1.0-6.3)	2.10 (1.0-7.0)	0.40
Age group (12-48 months), n (%)	84 (69)	180 (80)	0.03
Geometric mean age at first AOM (SD), months	10.1 (2.0)	7.7 (2.1)	0.001
Breastfeeding ≥ 3 months, n (%)	57 (47)	96 (43)	0.50
Tobacco smoke exposure indoors, n (%)	41 (34)	70 (31)	0.63
Day care			
At age 12-24 months, n/N (%)	22/44 (50)	42/99 (42)	0.47
At age 25-48 months, n/N (%)	70/78 (90)	113/127 (89)	1.00
Median number of siblings (min-max), (mean)	1.0 (0-3) (0.93)	1.0 (0-7) (1.17)	0.02
Family history positive for AOM			
Parents (121/225), n (%)	79 (65)	131 (58)	0.21
Siblings, n/N (%)	48/86 (56)	98/182 (54)	0.79
Atopy, n (%)[2]	54 (44)	117 (52)	0.22

[1]Fisher's Exact test, Mann-Whitney *U* test, and *t* test were performed when appropriate.
[2]Atopy was defined as having eczema, hay fever, or recurrent wheezing or asthma.

Experimental procedures. *PAI1* -675 genotype (rs1799889) was determined using single base extension analysis, a technique commonly used in our laboratory (ABI Prism®, SNaPshot™ Multiplex Kit, Applied Biosystems, Foster City, CA). The genomic region of interest was amplified using polymerase chain reaction (37 cycles; annealing temperature of 60 °C; total volume of 10 µl) with the following primers: forward, 5'-CAGCCAGACAAGGTTGTT-3' and reverse 5'-CCACCCGGTGCTCT-3'. Amplification resulted in a 265-nucleotides polymerase chain reaction product. After purification with Shrimp Alkaline Phosphatase and Exonuclease I (both from Amersham Biosciences, Upsala, Sweden), a single base extension with fluorescently labeled dideoxynucleotide triphosphates was performed using the reverse primer 5'-TTTTTGATACACGGCTGACTCCCC-3'. The poly-T tail allows us to distinguish primers in a multiplex analysis for which this assay was originally designed. The products generated were analyzed on an ABI Prism® 3100 genetic analyzer using GeneScan Analysis Software (Applied Biosystems). When no clear genotype was obtained or no consensus was reached between two independent investigators, genotypes were excluded from the analyses.

Statistical analyses. Statistical analyses were performed using SPSS 11.0 (SPSS Inc., Chicago, IL). A comparison of genotype frequencies was made between patients and blood donor control subjects assuming a multiplicative model.(27) Binomial variables were analyzed using Pearson's χ^2 test or Fisher's exact test when appropriate. Continuous variables were compared for the different genotypes using the Mann-Whitney *U* test (nonparametric) or *t* test (normal distribution). Within the patient sample, only genotype distributions of children suffering from 2 to 3 episodes per year before vaccination were compared with those individuals who had ≥ 4 episodes after correction for age at study entry, gender, number of siblings and the log-transformed

age at the time of the first episode of AOM, using binary logistic regression. The log-transformed age at the time of the first AOM episode was included in the analysis, because age at the first AOM episode itself did not show a normal distribution and an early first infection predisposes to a second AOM episode. A child who has had a first AOM episode at a younger age has had a longer period of time to develop multiple AOM episodes than a child of the same age who suffered from the first infection at a later age. Because the interaction between the age at the first AOM episode and the age of inclusion was significant, this was accounted for in the analyses. Because the pathogenesis of AOM in young children may differ from older children due to an immature immune system and different anatomic features such as the Eustachian tube, we also analyzed separately the frequencies of the number of AOM episodes in the year before inclusion in patients 1 to 4 years of age and patients > 4 years. Presence of atopy did not differ between the otitis-prone children and those with fewer episodes. Because asthma cannot, by definition, be diagnosed in children < 6 years of age, which applies to the main fraction of our patient cohort, recurrent wheezing and asthma as reported by the parents were considered the same in the statistical analyses. In addition to the multiplicative model, the effect of *PAI1* genotype on the number of AOM episodes was also assessed comparing 4G/4G homozygotes with those homozygous for the 5G allele.

Results

***The PAI1 4G/4G genotype is not associated with initial susceptibility to AOM*.** The *PAI1* -675 polymorphism was successfully genotyped in 463 control subjects and 317 children with recurrent AOM. *PAI1* genotype frequencies in the control population were in Hardy-Weinberg equilibrium, and the allele frequencies were similar to results published previously.(24) The distribution of genotypes in control subjects was as follows: 5G/5G 99 (21.4%), 4G/5G 241 (52.1%) and 4G/4G 123 (26.6%). A significant difference in genotype distribution was only found between control subjects and those patients suffering from ≥ 4 episodes of AOM (p=0.02). No difference was observed between patients having 2 to 3 AOM episodes a year and control subjects (p=0.34; Table 2).

***Related epidemiological findings*.** In our patient population log-transformed age at first AOM, gender, and the number of siblings were related to AOM recurrence rates (Table 1). Children who suffered from ≥ 4 episodes in the year before inclusion had their first AOM episode at a significantly younger age compared with the children suffering from 2 to 3 episodes. Although the majority in the total group of children with AOM were boys, girls were overrepresented in the group of children suffering from ≥ 4 AOM episodes when compared with the group of children with 2 to 3 AOM episodes. The number of siblings was higher in the otitis-prone group compared with the children with 2 to 3 episodes. Age at inclusion was not significantly different when comparing the group of children suffering from 2 to 3 AOM episodes with the group with ≥ 4 episodes. However, when children were divided into groups under and over

Table 2. Comparison of PAI1 4G/5G genotype distribution in patients with recurrent otitis media and control subjects.

PAI1 4G/5G	controls	2-3 episodes of AOM	≥ 4 episodes of AOM	Co vs. 2-3 OR[1] (p)	Co vs. ≥ 4 OR[2] (p)	2-3 vs. ≥ 4 OR (p)	2-3 vs. ≥ 4 OR (p)
		All patients		**4G/4G vs. 5G/5G**		**Crude**	**Adjusted[3]**
	n = 463 Number (%)	n = 115 Number (%)	n = 202 Number (%)			1.59[4] (0.01)	1.52[4] (0.03)
5G/5G	99 (21.4)	26 (22.6)	28 (13.9)	1[5]	1[5]	1[5]	1[5]
4G/5G	241 (52.1)	65 (55.6)	109 (54.0)	1.03 (0.92)	1.60 (0.05)	1.56 (0.16)	1.57 (0.18)
4G/4G	123 (26.6)	24 (20.9)	65 (32.2)	0.74 (0.34)	1.87 (0.02)	2.52[6] (0.01)	2.31[6] (0.03)
		Patients < 4 years				1.75[4] (0.01)	1.72[4] (0.02)
5G/5G		18 (23.4)	25 (15.8)			1[5]	1[5]
4G/5G		48 (62.3)	85 (53.8)			1.28 (0.50)	1.32 (0.47)
4G/4G		11 (14.3)	48 (30.4)			3.14[6] (0.01)	2.97[6] (0.02)
		Patients ≥ 4 years				1.53[4] (0.21)	1.17[4] (0.69)
5G/5G		8 (21.1)	3 (6.8)			1[5]	1[5]
4G/5G		17 (44.7)	24 (54.5)			3.77 (0.08)	5.25 (0.06)
4G/4G		13 (34.2)	17 (38.6)			3.49[6] (0.11)	2.50[6] (0.30)

[1]Data for patients with 2-3 episodes of AOM (acute otitis media) versus control subjects: p=0.45 (χ^2).
[2]Data for patients with ≥ 4 episodes of AOM versus control subjects: p=0.05 (χ^2).
[3]Adjusted Odds ratio's depicted were corrected for age at study entry, gender, number of siblings and the log-transformed age at the time of the first episode of AOM.
[4]Odds ratio's represent the effect of the PAI1 4G/5G polymorphism (binary logistic regression analysis, multiplicative model).
[5]The reference OR = 1.
[6]Data are for the 4G homozygous vs. 5G homozygous patients, crude or adjusted, binary logistic regression analysis. In this analysis the PAI1 -675 polymorphism was included in the model as a categorical variable.

the age of four years, the distribution of AOM frequency differed significantly (Figure 1). The occurrence of atopy and the presence of a family history positive for AOM did not differ between children with 2 to 3 vs ≥ 4 episodes of AOM.

The PAI1 4G/4G genotype is associated with the otitis-prone condition in children < 4 years of age. Comparing patients having 2 to 3 AOM episodes to patients with ≥ 4 episodes, the log-odds ratio (OR) of the 4G/4G versus the 5G/5G genotype was about two times the logOR of the 4G/5G versus 5G/5G genotype (OR 4G/4G vs 5G/5G = 2.52, logOR 0.4 and OR 4G/5G versus 5G/5G = 1.52, logOR 0.19, respectively; Table 2). Hence, the 4G allele probably has a multiplicative effect on AOM recurrence rate (27). After correction for confounding factors as described in the "Statistical analysis," we still found that the PAI1 4G/4G genotype was associated with recurrence of disease in patients (p=0.01, crude OR 1.59; p=0.03, adjusted OR 1.52). The PAI1 4G/4G individuals had an increased risk for ≥ 4 AOM episodes compared to patients homozygous for the 5G variant (p=0.01, crude OR 2.52; p=0.03, adjusted OR 2.31). Because the distribution of the number of AOM episodes differed between the groups of children 1 to 4 years of age and > 4 years of age, these groups were also analyzed separately (Figure 1). The association of the PAI1 4G/4G genotype with the otitis-prone condition was only found in the group of children 1 to 4 years of age (p=0.01, crude OR 1.75; p=0.02 adjusted OR 1.72; Table 2). Comparing the children with PAI1 4G/4G genotype with the 5G homozygotes yielded a crude OR of 3.14

(p=0.01; adjusted OR 2.97, p=0.02). The PAI1 4G/4G genotype was not significantly associated with the otitis-prone condition in children > 4 years of age. The number of days with otorrhoea showed a trend toward longer duration in the 4G homozygous children (geometric mean days (SD) for genotypes: 5G/5G 5.3 (3.9); 4G/5G 6.5 (4.0); and 4G/4G 7.2 (4.2)). This was, however, not significant (p=0.66, ANOVA).

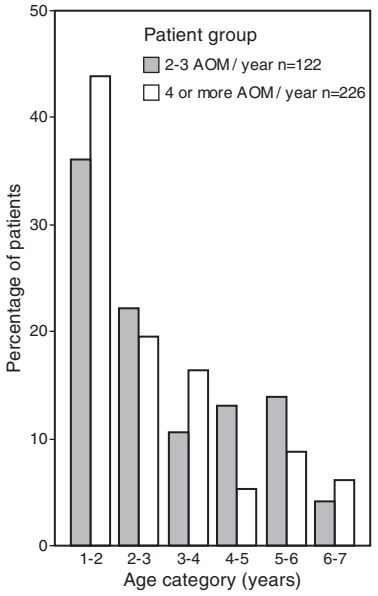

Figure 1. Distribution of the number of AOM episodes for different age categories at the time of inclusion. The number of patients (age category in years) for the group of children with 2 to 3 AOM episodes in the year before inclusion is 44 (1-2), 27 (2-3), 13 (3-4), 16 (4-5), 17 (5-6) and 5 (6-7). The number of patients (age category) for the group of children with ≥ 4 AOM episodes in the year before inclusion is 99 (1-2), 44 (2-3), 37 (3-4), 12 (4-5), 20 (5-6) and 14 (6-7). The distribution of 2 to 3 versus ≥ 4 AOM episodes differs between patients < 4 and > 4 years of age (p=0.03).

Discussion

In this study we show that, in the patient group with a history of ≥ 2 AOM episodes, children 1 to 4 years of age, with the *PAI1* 4G/4G genotype, which is related to high PAI-1 levels, have increased risk for more frequent recurrent AOM.(18, 19) The association is significant even after correction for possible confounding factors, with only a minimal change in odds ratios. PAI-1 inhibits cellular migration and invasion, processes essential in tissue repair in inflammatory disease.(16, 17) Hence, higher PAI-1 levels might result in longer persistence of tissue inflammation, or abnormally repaired tissue, prone to bacterial colonization and new infections.

Two possible mechanisms for PAI-1 interaction and inhibition of tissue repair have been proposed. First, PAI-1 may inhibit a proteolytic cascade at the cell surface

that results in matrix destruction, allowing cellular migration and invasion for tissue repair. The proteolytic cascade mechanism involves the activation of surface-bound plasminogen by surface bound urokinase plasminogen activator (uPA). PAI-1 can bind to uPA on the cell surface and inhibit its proteolytic activity and, hence, tissue repair.(15, 28, 29) A second proposed pathway involves competitive binding of PAI-1 and integrin to vitronectin. Both share the same binding site region on vitronectin. Vitronectin/integrin interaction is needed for cell migration and adhesion. The inhibiting effect of PAI-1 is negated when excess nonsurface-bound uPA forms a complex with PAI-1 resulting in a release of PAI-1 from vitronectin, which is then available for interaction with integrin, favouring cell adhesion.(30)

A possible role for high PAI-1 concentrations in the stronger inhibition of healing of inflamed otolaryngeal tissue that may, thus, favour an otitis-prone condition is supported by the results of Hansson et al. that imply a further role for PAI-1 and plasminogen in tissue repair. They have shown that perforated tympanic membranes (TMs) of plasminogen-deficient mice exhibit reduced healing 15 days after myringectomy compared with TM from wild-type mice.(31) Intravenous administration of plasminogen, in plasminogen-deficient mice, resulted in restored TM healing even if plasminogen was administered 30 days after perforation. The distribution of inflammatory cells in the perforated TM differed significantly between plasminogen-deficient and wild-type mice in vivo. Plasminogen-deficient mice with initially normal TMs showed spontaneous development of middle ear effusion. Interestingly, in an otherwise healthy 10 year old boy with a history of chronic otitis media, low levels of serum plasminogen were detected.(32) Interaction of PAI-1 with uPA prohibits catalytic activation of plasminogen to plasmin. High PAI-1 levels might, therefore, result in similar clinical findings as seen in plasminogen deficiency.

Furthermore, in skin wound healing, PAI-1, uPA, and uPA receptor are expressed at the site of regenerative epithelial outgrowths at the edge of the wound.(33, 34) Plasminogen-deficient mice showed impaired wound healing.(35) In addition, Chen et al. reported that uPA expression is increased upon infection with *Streptococcus pneumoniae* in an otitis media model in the rat.(36) *S. pneumoniae* is one of the major causative microbes of otitis media in humans.(37-39) We recognize that these findings do not prove a role for PAI-1 in AOM, but we do feel that they deserve further attention. Additional functional assays in an otitis model are needed to further investigate the precise role of PAI-1 in AOM. These studies would preferably also be directed toward analyzing the therapeutic role of PAI-1 inhibitors and plasminogen in overcoming the effect of high PAI-1 expression and their effects on recurrence of otitis media.

We found that *PAI1* 4G/4G had no effect on the initial susceptibility to AOM as reflected by the lack of significant differences between AOM patients and control subjects. This fits in our concept of tissue repair where only once an episode of AOM has occurred may impaired tissue healing in 4G homozygous individuals facilitate microbial reinfection and the recurrence of otitis media. We realize that the control group was not an age-matched group without any AOM episodes, which may have biased our results. However, occurrence of AOM in the control subjects was < 2

episodes per year based on the prevalence of AOM in childhood. The association between *PAI1* 4G/4G genotype and the otitis-prone condition remained significant even after correction of the effect of the polymorphism on AOM recurrence rates for gender, number of siblings, and both age and age at first AOM for the children < 4 years of age. After 4 years of age we found a difference in the distribution of the number of AOM episodes compared with the younger children. AOM (and recurrence) has its peak in the first years of life. Ongoing recurrence in older children most likely selects a specific group that differs from the larger group of 10% to 15% of all children with recurrent AOM at early age. We possibly selected for the older patients as a result of the nature of the vaccination trial in which the patients participated selecting more otitis-prone children and particularly at older age those who had ongoing recurrent disease.

We only studied one genetic polymorphism in a complex multifactorial disease. As for many risk factors, only a small increase in risk can be found, which may be related to other risk factors as well. These may be both genetic and environmental. The balance of the different risk factors will determine the phenotype observed. Studying multiple factors simultaneously does, however, require large numbers of individuals. It must be mentioned that the association found in this study may reflect the result of linkage disequilibrium, meaning that not the *PAI1* polymorphism itself but a genetic variation linked to this polymorphism is the actual disease associated genetic variant. Additional studies are, therefore, required to confirm our findings. However, our data show that studies of genes involved in the inflammatory responses and tissue repair will be of significance in understanding recurrent AOM.

In conclusion, 4G homozygous individuals show an increased risk for an otitis-prone condition, especially under the age of 4 years. Impaired tissue integrity, because of high PAI-1 levels in *PAI1* 4G homozygous individuals once an episode of AOM has occurred, may possibly facilitate bacterial colonization, and, thereby, the recurrence of otitis media.

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Chapter 4.2

Genetic polymorphisms in immune response genes *TNFA, IL6, IL10* and *TLR4* are associated with recurrent acute otitis media

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Abstract

Objective. Cytokines and other inflammatory mediators are involved in the pathogenesis of otitis media. We hypothesized that polymorphisms in inflammatory response genes contribute to the increased susceptibility to acute otitis media (AOM) in otitis-prone children.

Patients and Methods. DNA samples from 348 children with ≥ 2 AOM episodes participating in a randomized controlled vaccination trial, and from 463 healthy adult controls were included. Polymorphisms in *TNFA*, *IL1B*, *IL4*, *IL6*, *IL10*, *IL8*, *NOS2A*, *C1INH*, *PARP*, *TLR2* and *TLR4* were genotyped. Genotype distributions in children with AOM were compared with those in controls. Within the patient group, the number of AOM episodes before vaccination and the clinical and immunological response to pneumococcal conjugate vaccinations were analyzed.

Results. The *IL6* -174 G/G genotype was overrepresented in children with AOM when compared to controls. In the patient group, *TNFA* promoter genotypes -238 G/G and -376 G/G and the *TLR4* 299 A/A genotype were associated with an otitis-prone condition. Furthermore, lower specific anticapsular antibody production after complete vaccination was observed in patients with the *TNFA* -238 G/G genotype or *TNFA* -863 A allele carriage. Finally, the *IL10* -1082 A/A genotype contributed to protection from recurrence of AOM after pneumococcal vaccination.

Conclusion. Variation in innate immune response genes like *TNFA* -863A, -376G and -238G, *IL10* -1082 A and *IL6* -174G alleles in the promoter sequences may result in altered cytokine production that leads to altered inflammatory responses, and, hence, contributes to an otitis-prone condition.

Introduction

Acute otitis media (AOM) is the most common bacterial infection in children. Overall, 10% to 15% of all children suffer from ≥ 4 AOM episodes per year, which causes a great disease burden.(1) Genetic polymorphisms in immune response genes are known to influence susceptibility to and severity of infectious diseases. For example, allelic variations in *TNFA*, *IL1B* and *IL6* have been associated with meningococcal infection.(2) Although cytokines and other inflammatory mediators are also involved in the pathogenesis of otitis media, the role of polymorphisms in immune response genes in recurrent AOM is relatively unexplored thus far. Increased expression of TNF-α, IL-1β, IL-6 and IL-10 was observed during experimental otitis media in animals. (3, 4) Therefore, in a common disease such as otitis media, genetic variations may lead to altered inflammatory responses and an otitis-prone condition. For instance, bacterial endotoxin is recognized by several Toll-like receptors (TLRs), which in turn stimulate TNF-α production, thus affecting numerous other pathways such as cytokine production, immunoglobulin responses, and mucin production.(5-8) Remarkably, IL-1β, IL-6 and TNF-α levels in nasopharyngeal secretions were found to be lower in

children with recurrent otitis media than in healthy children.(9)
The influence of genetically determined variations on otitis media can be illustrated by twin studies, which have shown a heritability of 57% for acute ear infections and 72% for chronic ear infections.(10-14) Correlation for recurrent otitis media is higher in monozygotic twins (65%-71%) compared to dizygotic twins (25%-34%).(15) *Streptococcus pneumoniae* is an important pathogen in otitis media, involved in at least 20% to 40% of all cases.(16-18) Hence, genetic polymorphisms that influence recurrence of otitis media may also be related to response to pneumococcal antigens.
The effect of polymorphisms may result, for instance, from altered expression levels or altered function due to amino acid substitutions. Variations in immune response genes such as *IL10, IL6* and *IL4* have been associated with altered cytokine expression levels.(19-21) An altered function because of amino acid substitutions has been reported for polymorphisms in *TLR4* and poly (ADP-ribose) polymerase (*PARP*).(22, 23) The *PARP* 762A variant was found recently to be associated with reduced activity after H_2O_2 exposure, which is known to be present in inflammation.(23) Other polymorphisms were selected in this study because of previous associations with infectious or inflammatory diseases such as the *IL8* C781T polymorphism, which was reported recently to be associated with bronchiolitis caused by the respiratory syncytial virus.(24)
In this study, we investigated whether polymorphisms in selected immune response genes may contribute to recurrence of otitis media and to clinical and immunologic response to pneumococcal vaccination.

Methods

Participants. Patients who initially participated in a randomized, controlled study on prevention of recurrent AOM by pneumococcal vaccinations were included.(25) Children were enrolled in the study after obtaining approval of the medical ethical committee of the participating hospitals and informed consent from the parents or guardians. DNA was available from 348 Dutch Caucasian children, 1 to 7 years of age, who suffered from ≥ 2 AOM episodes in the preceding year. The number of AOM episodes prior to vaccination was based both on parental report (AOM defined as having ≥ 1 of these symptoms: acute earache, new-onset otorrhea, irritability, and fever) and on clinical information of the diagnosis by a physician. Children who did not have AOM episodes were not included, because they were not likely to benefit from vaccination. In the present study cohort, 122 children suffered from 2 to 3 otitis media episodes whereas 226 children suffered from ≥ 4 episodes (defined as otitis-prone condition) (Table 1).(26) Children received either pneumococcal vaccinations (n = 168) or control vaccines i.e. hepatitis A (2 years or older) or hepatitis B (younger than 2 years) (n = 180) vaccines. In the pneumococcal vaccine group 1 dosage of 7-valent conjugate vaccine (Prevnar®, Wyeth, Rochester, NY, USA) was administered to children 2 to 7 years of age, whereas 2 dosages were given with a 1-month

Table 1. Characteristics of patients with 2 or more AOM episodes.

Characteristics	2-3 AOM episodes n=122	≥ 4 AOM episodes n=226	p-value[1]
Male gender, n (%)	84 (69)	128 (57)	0.03
Median age (min-max), y	2.42 (1.0-6.3)	2.10 (1.0-7.0)	0.40
Age group (12-48 months), n (%)	84 (69)	180 (80)	0.03
Geometric mean age at first AOM (SD), months	10.1 (2.0)	7.7 (2.1)	0.001
Breastfeeding ≥ 3 months, n (%)	57 (47)	96 (43)	0.50
Tobacco smoke exposure indoors, n (%)	41 (34)	70 (31)	0.63
Day care			
At age 12-24 months, n/N (%)	22/44 (50)	42/99 (42)	0.47
At age 25-48 months, n/N (%)	70/78 (90)	113/127 (89)	1.00
Median number of siblings (min-max), (mean)	1.0 (0-3) (0.93)	1.0 (0-7) (1.17)	0.02
Family history positive for AOM			
Parents (121/225), n (%)	79 (65)	131 (58)	0.21
Siblings, n/N (%)	48/86 (56)	98/182 (54)	0.79
Atopy, n (%)[2]	54 (44)	117 (52)	0.22

[1]Fisher's Exact test, Mann-Whitney *U* test, and *t* test were performed when appropriate.
[2]Atopy was defined as having eczema, hay fever, or recurrent wheezing or asthma.

interval to children of 1 to 2 years. In both groups, this procedure was followed after 6 months by 1 dose of the 23-valent polysaccharide vaccine (Pneumune®, Wyeth). Childrens' progress was followed until 18 months after completion of the vaccine scheme to check for the recurrence of physician-diagnosed AOM.(25)

Dutch Caucasian controls (n = 463) were derived from the Dutch blood bank Sanquin, after informed consent was obtained and represented healthy adult donors. No records of prior history regarding AOM were available for these adult controls; however, in the general population a total number of ≤ 3.2 AOM episodes is expected in childhood. (26) For children aged 0 to 13 years had an estimated number of 120 episodes of physician diagnosed AOM per 1000 person-years in the Netherlands in the period 1995-2003.(27) This infers that controls will have experienced, on average, fewer AOM episodes per year than the patients.

Genotyping. Single base extension analysis was used to genotype inducible nitric oxide synthase (*NOS2A*) S608L (rs2297518), *PARP* V762A (rs1136410), complement component inhibitor-1 (*C1INH*) V480M (rs4926), *IL4* C-524T (rs2243250), *IL10* G-1082A (rs1800896), *IL10* C-819T (rs3021097), *IL1B* C-31T (rs1143627), *TNFA* A-863C (rs1800630), *TNFA* T-857C (rs1799724), *TNFA* G-376A (rs3093659), *TNFA* G-308A (rs1800629), *TNFA* G-238A (rs361525), *IL6* G-174C (rs1800795), *IL8* C781T (rs2227306), *TLR4* D299G (rs4986790) and *TLR4* T399I (rs4986791) (http://www.ncbi.nlm.nih.gov/SNP).

In short, the genomic region of interest was amplified using polymerase chain reaction. After purification, a single base extension was performed using a primer ending 1 nucleotide prior to the single nucleotide polymorphism (SNP) location. Up to seven SNPs were analyzed in one multiplex assay. A poly-T-tail attached to the primer combined with the use of a Liz size marker served to distinguish

SNPs in the multiplex analysis (Tables 2 and 3). The *TLR2* R753Q polymorphism (rs5743708) was determined using Taqman analysis using primers, TLR2-753F CCATTCCCCAGCGCTTCT and TLR2-753R CCAGGTAGGTCTTGGTGTTCATT and probes TLR2-753V1 VIC-AAGCTGCAGAAGAT and TLR2-753M1 FAM-AAGCTGCGGAAGAT. A subset of polymerase chain reaction samples was sequenced to confirm genotypes. All genotypes were annotated independently by 2 investigators who were blinded for the clinical data.

Table 2. Polymerase chain reaction primer sequences.

Pool 1	Upper 5'→ 3'	Lower 5' → 3'	rs No.	Change
NOS2A	GCAGGGCTAGGAGTAGGA	AGCCCCATATGTAAACCAA	rs2297518	608 S/L
PARP	CTGCCCCTGTTCTACCA	ACTGTAGGCCACCTCGAT	rs1136410	762V/A
Pool 2				
C1INH	CCTCCGCCATCTCTGT	GCTCGCCCTAACCTGA	rs4926	480 V/M
IL4	CTTGCCAAGGGCTTCCTTAT	TGGAAACTGTCCTGTCATGG	rs2243250	C-524T
Pool 3				
IL10	TCCCCTTACCTTCTACACAC	GACCCCTACCGTCTCTATTT	rs1800896	G-1082A
IL1B	CTTGCCCTTCCATGAAC	TGCCTCGAAGAGGTTTG	rs1143627	C-31T
TNFA.1	CCCCTCCCAGTTCTAGTT	GGGACACACAAGCATCA	rs361525	G-238A
			rs1800629	G-308A
			rs3093659	G-376A
TNFA.2	GGAGAATGTCCAGGGCTATG	AAAATCAGGGACCCCAGAGT	rs1800630	A-863C
			rs1799724	T-857C
Pool 4				
TLR4	ATGCCCCTACTCAATCTCTCT	GCCAGCCATTTTCAAGACT	rs4986790	299 D/G
			rs4986791	399 T/I
IL6	TTGTCAAGACATGCCAAGTGCT	GCCTCAGAGACATCTCCAGTCC	rs1800795	G-174C
IL8	AGCTTGCCTACTATAAATAACA	CTAGCCCTTGACCTCAG	rs2227306	C781T
Pool 5				
IL10	TCCCCTTACCTTCTACACAC	GACCCCTACCGTCTCTATTT	rs3021097	C-819T

Antibody measurements. In children with ≥ 2 AOM episodes, a blood sample was taken for immunologic assessment before and 1 month after complete vaccination. Prevaccination and postvaccination immunoglobulin G (IgG) levels to the 7 pneumococcal serotypes included in the conjugate vaccine were measured in serum by enzyme-linked immunosorbent assay (ELISA) as described previously.(28, 29)
Statistical analysis. Statistical analysis was performed using SPSS 11.0 (SPSS Inc., Chicago IL) and Stata 8 (Stata Corp, College Station, TX). Verification of Hardy-Weinberg equilibrium of genotypes was performed using the χ^2 test (1df). Binomial variables were analyzed using Pearson's χ^2 test (2df) or Fisher's exact test when appropriate. Continuous variables were compared for the different genotypes using the Mann-Whitney *U* test. When necessary, variables were log-transformed to

Table 3. Primer sequences for single base extension reactions.

	Primer sequence	Primer length (bases)
Pool 1	5' → 3'	
PrNOS2A_U	TTTTGCTCTTTCAGCATGAAGAGC	24
PrPARP_L	TTTTTTTTTTGCAGGTTGTCAAGCATTTCC	30
Pool 2		
PrIL4_U	TAAACTTGGGAGAACATTGT	20
PrC1INH_U	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCAGCAGCCCTTCCTCTTC	66
Pool 3		
PrTNFA_2863U	AGTCGAGTATGTGGACCCCC	20
PrIL10_1082L	TTTTTTTTTTTTACCTATCCCTACTTCCCC	30
PrTNFA1238U	TTTTTTTTTTTTTTTTTTTTTTAGAAGACCCCCCTCGGAATC	42
PrIL1B_L	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTCCCTCGCTGTTTTTAT	48
PrTNFA_1308L	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTAGAGGCTGAACCCCGTCC	54
PrTNFA_1376U	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCCTGCATCCTGTCTGGAA	66
PrTNFA_2857L	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTCTACATGGCCCTGTCTTC	72
Pool 4		
PrIL6_L	AATGTGACGTCCTTTAGCAT	24
PrIL8_L	TTTTTTTTTTCATAACTGACAACATTGAAC	30
PrTLR4_299U	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTTAGACTACTACCTCGATG	60
PrTLR4_399L	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGATCTAAATACTTTAGGCTG	72
Pool 5		
PrIL10_819U	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCCCTTGTACAGGTGATG TAA	82

obtain an approximately normal distribution.

A comparison of genotype frequencies was made between patients and the reference control group. In addition, genotype frequencies in children suffering from 2 to 3 episodes per year were compared with those of patients having ≥ 4 episodes after correction for gender, number of siblings, age, log-transformed age at the time of the first AOM episode, and the interaction term between the latter 2 by using binary logistic regression. Log-transformed age at the first AOM episode was included in the analysis because an early first infection predisposes to a second AOM episode. A child who has had a first AOM episode at a younger age has had a longer period of time to develop multiple AOM episodes than a child of the same age who suffered from the first infection at a later age. Since the interaction between the age at the first AOM episode and the age of inclusion was significant, it was accounted for in the analyses. Log-transformed antibody levels were compared between individuals with different genotypes. Age, number of AOM episodes (2-3 vs. ≥ 4) and the number of conjugate vaccinations (1 or 2), were included in the analyses that assessed the effect of the different genotypes. Only when genotypes were consistently associated with different serotype-specific antibodies was this association considered relevant. To determine the involvement of SNPs on occurrence of AOM after complete vaccination, negative binomial regression was used, because it allows for extra variation (overdispersion). The time of follow-up was measured from one month after

complete vaccination until the end of the study. Effects were corrected for treatment (anti pneumococcal vaccinations or hepatitis vaccinations) and the number of AOM episodes in the year preceding vaccinations (2-3 vs. ≥ 4). *P* values ≤ 0.05 were considered to be statistically significant. No correction was made for multiple testing.

Results

We investigated the association between variations in genes listed in Table 2 and occurrence of ≥ 2 episodes of AOM, the number of AOM episodes before vaccination, specific anti-pneumococcal IgG levels after vaccination and AOM after complete vaccination, respectively. Only significant associations will be discussed. SNPs not mentioned in the results section showed no significant associations.

Genotype distribution for all SNPs but the *IL10* G-819A polymorphism reached Hardy Weinberg equilibrium in controls. To rule out technical problems, 14 individuals were typed using both the reverse and forward primer in the single base extension reaction. Results for both strands were identical. Sequencing of 13 random controls also showed identical genotypes, excluding technical errors (data not shown). Genotype distributions in controls are listed in Table 4.

IL6 -174 G/G genotype is associated with susceptibility to AOM. The number of individuals with the *IL6* -174 C/C, C/G and G/G genotypes in the total cohort of AOM patients were 49 (14.1%), 156 (45%), and 142 (40.9%), respectively. The corresponding frequencies in controls were 82 (17.8%), 232 (50.4%) and 146 (31.7%), respectively. The *IL6* -174 G/G genotype was found more frequently in AOM patients compared with controls than the C/G genotype (OR 1.45, p=0.02) and the C/C genotype (OR 1.64, p=0.02).

TNFA promoter genotypes -238 G/G and -376 G/G and TLR4 299 D/D genotype are associated with an otitis-prone condition. Similar to other otitis studies, in our study, population risk factors for AOM such as a high number of siblings and low age at first AOM are related to an otitis-prone condition (Table 1) (13, 30, 31). Females were overrepresented in the group of children suffering from ≥ 4 AOM episodes when compared with the children who had 2 to 3 AOM episodes. Children under the age of 4 were more likely to have had ≥ 4 AOM episodes in the previous year than older children. The interaction of age and age at the first AOM episode was significantly associated with AOM recurrence rate (p<0.01). Therefore, the effect of the polymorphisms on recurrence rate of AOM was corrected for these factors (Table 5).

The *TNFA* -238 G/G genotype was overrepresented in the otitis-prone children compared with the children with 2 to 3 AOM episodes (crude OR 2.13, p=0.03, adjusted OR 2.29, p=0.03). Because a difference was observed in recurrence-rate distribution in children over and under the age of 4 years, these were also analyzed separately. The association was mainly attributable to children under the age of 4 years. The *TNFA* -376 G/G genotype was associated with the otitis-prone condition

Table 4. Genotype distribution of polymorphisms in Dutch Caucasian controls.

SNP	Genotype frequency Controls n (%)	SNP	Genotype frequency Controls n (%)
***NOS2a* 608**		***IL4* -524**	
G/G	302 (65.2)	C/C	340 (73.8)
G/A	140 (30.2)	C/T	112 (24.3)
A/A	21 (4.5)	T/T	9 (2.0)
***PARP* 762**		***IL1B* -31**	
T/T	300 (66.7)	C/C	57 (12.5)
T/C	140 (31.1)	C/T	215 (47)
C/C	10 (2.2)	T/T	185 (40.5)
***C1INH* 480**		***IL10* -1082**	
G/G	256 (55.5)	G/G	123 (26.7)
G/A	176 (38.2)	G/A	219 (47.6)
A/A	29 (6.3)	A/A	118 (25.7)
***TNFA* -863**		***IL10* -819**	
C/C	330 (71.4)	C/C	284 (61.6)
C/A	117 (25.3)	C/T	145 (31.5)
A/A	15 (3.2)	T/T	32 (6.9)
***TNFA* -857**		***IL6* -174**	
C/C	387 (84.5)	C/C	82 (17.8)
C/T	66 (14.4)	C/G	232 (50.4)
T/T	5 (1.1)	G/G	146 (31.7)
***TNFA* -376**		***IL8* 781**	
G/G	447 (96.8)	C/C	152 (32.8)
G/A	15 (3.2)	C/T	235 (50.8)
A/A		T/T	76 (16.4)
***TNFA* -308**		***TLR4* 299**	
G/G	301 (65.2)	A/A	374 (86.4)
G/A	147 (31.8)	A/G	58 (13.4)
A/A	14 (3.0)	G/G	1 (0.2)
***TNFA* -238**		***TLR4* 399**	
G/G	415 (89.8)	C/C	379 (87.1)
G/A	47 (10.2)	C/T	54 (12.4)
A/A		T/T	2 (0.5)

Table 5. Genotype distribution of *TNFA* and *TLR4* polymorphisms in children with AOM.

SNP	controls	2-3 episodes of AOM	≥ 4 episodes of AOM	2-3 vs ≥ 4 OR (p)	
		All patients		Crude	Adjusted[1]
***TNFA* G-238A**	n = 462 n (%)	n = 120 n (%)	n = 222 n (%)	G/G vs G/A	
G/G	415 (89.8)	101 (84.2)	204 (91.9)	2.13 (0.03)	2.29 (0.03)
G/A	47 (10.2)	19 (15.8)	18 (8.1)		
A/A	0 (0)	0 (0)	0 (0)		
		Patients < 4 years			
G/G		68 (82.9)	162 (92.0)	2.38 (0.03)	3.06 (0.01)
G/A		14 (17.1)	14 (8.0)		
A/A		0 (0)	0 (0)		
		Patients > 4 years			
G/G		33 (86.8)	42 (92.3)	1.59 (0.51)	1.41 (0.66)
G/A		5 (13.2)	4 (8.7)		
A/A		0 (0)	0 (0)		
		All patients			
***TNFA* G-376A**	n = 462 n (%)	n = 120 n (%)	n = 222 n (%)	G/G vs G/A and A/A	
G/G	447 (96.8)	112 (93.3)	217 (97.7)	3.10 (0.05)	3.06 (0.07)
G/A	15 (3.2)	8 (6.7)	4 (1.8)		
A/A	0 (0)	0 (0)	1 (0.5)		
		Patients < 4 years			
G/G		77 (93.9)	172 (97.7)	2.79 (0.13)	3.05 (0.12)
G/A		5 (6.1)	3 (1.7)		
A/A		0 (0)	1 (0.6)		
		Patients > 4 years			
G/G		35 (92.1)	45 (97.8)	3.85 (0.25)	2.74 (0.44)
G/A		3 (7.9)	1 (2.2)		
A/A		0 (0)	0 (0)		
		All patients			
***TLR4* 299 D/G**	n = 433 n (%)	n = 121 n (%)	n = 216 n (%)	D/D vs D/G and G/G	
D/D	374 (86.4)	99 (81.8)	194 (89.8)	1.96 (0.04)	1.66 (0.14)
D/G	58 (13.4)	20 (16.5)	22 (10.2)		
G/G	1 (0.2)	2 (1.7)	0 (0)		
		Patients < 4 years			
D/D		67 (80.7)	157 (91.3)	2.50 (0.02)	2.49 (0.03)
D/G		14 (16.9)	15 (8.7)		
G/G		2 (2.4)	0 (0)		
		Patients > 4 years			
D/D		32 (84.2)	37 (84.1)	0.99 (0.99)	0.65 (0.54)
D/G		6 (15.8)	7 (15.9)		
G/G		0 (0)	0 (0)		

Data are from the crude and adjusted logistic regression analysis.
[1]OR adjusted: effect of SNP adjusted for gender, number of siblings, age, log (age at first AOM episode) and the interaction of the latter two.

in children (crude OR 3.10, p=0.05, adjusted OR 3.06, p=0.07). No significant association was found when older and younger children were analyzed separately. In addition, carriage of the *TLR4* 299 G allele was associated with a lower number of AOM episodes (OR 0.5, p=0.04). This finding, however, remained significant only

after correction for confounding factors when the children under the age of 4 years were analyzed separately (crude OR 2.50, p=0.02, adjusted OR 2.49, p=0.03).

TNFA -863 C/C genotype and carriage of the TNFA -238 A allele are associated with higher specific anti-pneumococcal IgG levels after complete vaccination.

IgG anti-pneumococcal antibody levels were evaluated in 80 children of the pneumococcal vaccine group; 34 aged 12 to 24 months and 46 were aged 25 to 84 months. Four or more AOM episodes in the year preceding inclusion were observed in 36 children, whereas 44 had 2 to 3 episodes of AOM. Before vaccination, all IgG anti-pneumococcal antibody levels were low for patients with 2 to 3 AOM and ≥ 4 AOM episodes (Table 6).

Table 6. Geometric mean (95% Confidence Interval) of specific anti-pneumococcal IgG in children with 2 to 3 and children with ≥4 AOM episodes, before vaccination and one month after complete vaccination.

	Prior to vaccination			One month after complete vaccination		
Total IgG against capsular type	2-3 AOM episodes n = 36	≥ 4 AOM episodes n = 44	p[1]	2-3 AOM episodes n = 36	≥ 4 AOM episodes n = 44	p[1]
4	0.05 (0.04-0.07)	0.05 (0.04-0.06)	0.61	5.91 (4.6-7.6)	3.55 (2.6-4.8)	<0.01
6B	0.04 (0.03-0.05)	0.04 (0.04-0.05)	0.74	1.39 (0.7-2.7)	0.75 (0.4-1.4)	0.20
9V	0.22 (0.16-0.31)	0.18 (0.13-0.25)	0.37	43.48 (31-61)	20.18 (14-30)	<0.01
14	2.20 (1.27-3.82)	1.17 (0.84-1.61)	0.05	117.84 (85-164)	59.94 (47-77)	0.001
18C	0.27 (0.18-2.50)	0.20 (0,14-0.28)	0.29	12.80 (9.9-17)	7.79 (6.0-10)	<0.01
19F	0.43 (0.26-0.71)	0.23 (0.18-0.30)	0.02	25.81 (18-36)	8.16 (5-12)	<0.001
23F	0.53 (0.40-0.71)	0.57 (0.43-0.75)	0.65	4.91 (3.6-6.7)	3.28 (2.3-4.8)	0.12

[1]*P* values were corrected for age, by using regression analysis.

Serum IgG anti-pneumococcal antibody levels against all serotypes were lower in the children with an AOM recurrence rate of ≥ 4 compared with those with only 2 to 3 AOM episodes after complete vaccination. This was significant for all except the anti-serotype 6B and 23F antibody levels. Age, independent from the number of AOM episodes (2-3 or ≥ 4), had a significant effect on specific IgG levels against serotype 14 at baseline, and on anti-serotype 4 antibody levels after vaccination. By using a logistic regression model adjusting for age, number of AOM episodes and number of conjugate vaccinations, a significantly lower anti-pneumococcal serotype 23F antibody level was observed for the carriers of the *TNFA* -863 A allele compared with children homozygous for the C allele (p<0.001). Furthermore, a trend for lower specific IgG levels was observed against 5 of 7 vaccine serotypes (p=0.05-0.10) in the carriers of the -863A allele. The *TNFA* -238 A allele carriers had higher geometric mean specific IgG levels against all serotypes (Table 7). This was, however, only significant for antibodies against pneumococcal serotypes 18C and 19F without correction for confounding factors (p=0.04 and p=0.04, respectively).

No significant differences in specific IgG levels were observed for the *IL6* -174 G/C and the *IL10* -1082 G/A and -819 C/T genotypes (data not shown).

Table 7. Geometric mean (95% Confidence Interval) of specific anti-pneumococcal antibodies 1 month after complete vaccination for different *TNFA* -863 and *TNFA* -238 genotypes.

	IgG against pneumococcal capsular type						
	4	**6B**	**9V**	**14**	**18C**	**19F**	**23F**
***TNFA* -863**							
C/C	5.00	1.27	33.29	90.11	10.12	16.00	5.22
(n = 54)	(3.9-6.4)	(0.8-2.1)	(24-45)	(69-118)	(8.0-13)	(11-23)	(4.0-6.8)
C/A and A/A	3.52	0.60	20.66	65.52	8.99	9.93	2.19
(n = 26)	(2.3-5.3)	(0.3-1.4)	(12-36)	(47-92)	(6.4-13)	(6.0-17)	(1.4-3.5)
p (crude)	0.12	0.10	0.10	0.16	0.80	0.13	0.001
p (adjusted)[1]	0.05	0.10	0.06	0.10	0.42	0.05	<0.001
***TNFA* -238**							
G/G	4.53	0.93	27.36	76.22	8.87	11.86	3.74
(n = 66)	(3.7-5.5)	(0.6-1.5)	(21-36)	(61-95)	(7.4-11)	(8.6-16)	(2.9-4.9)
G/A	4.17	1.89	34.88	125.04	15.54	27.83	5.03
(n = 12)	(1.6-11)	(0.5-6.7)	(9.7-125)	(61-257)	(6.9-35)	(12-67)	(2.1-12)
p (crude)	0.78	0.23	0.54	0.10	0.04	0.04	0.41
p (adjusted)[1]	0.70	0.46	0.77	0.21	0.06	0.07	0.39

Data are from the crude and adjusted logistic regression analysis.
[1]*P* (adjusted): indicates the effect of SNP adjusted for age, AOM rate (2-3 vs ≥ 4) and number of conjugate vaccinations (1 or 2).

IL10 -1082 A/A genotype is associated with protection from AOM recurrence after vaccination. Previously, we have reported that the number of AOM episodes in the per-protocol analysis was higher in the pneumococcal vaccine group than in the control vaccine group, in particular among children who suffered from ≥ 4 AOM episodes in the year preceding inclusion.(25) We corrected for this feature to assess whether the polymorphisms were correlated to the number of AOM episodes after complete vaccination. Patient characteristics did not differ between the pneumococcal and control vaccine group.(25) We observed that the *IL10* -1082 A/A genotype protects patients from AOM recurrence during follow-up after vaccination (IRR 0.63, p=0.01). No significant differences were observed for polymorphisms in *TNFA* or *IL6* (data not shown).

Discussion

In this study, we found an association between *TNFA* promoter polymorphisms and otitis-prone condition and specific IgG production after pneumococcal vaccination. The *TNFA* -238 G/G genotype was associated with the otitis-prone condition and a trend for lower specific anti-pneumococcal antibody levels after vaccinations compared with carriers of the A allele. The *TNFA* -863 A allele was also associated with a trend for lower specific anti-pneumococcal IgG levels compared with children with the -863 C/C genotype. The *TNFA* -376 G/G genotype was also associated with an otitis prone condition. Similar to the study of Joki-Ekkila et al., we found no association between the *TNFA* -308 promoter polymorphism and recurrent AOM.(32) In a recent study, an association was reported between carriage of the rare *TNFA* -308 allele and susceptibility to AOM. The allele frequencies of the *TNFA* -308 polymorphism, however, differed from what is usually reported for this polymorphism.(33) Differences

in the observed associations between the *TNFA* promoter polymorphisms may, indeed, result from differences in allele frequency in the population and the effect of haplotypes. This finding may be specifically true for rare polymorphisms like the *TNFA* -376 G/A polymorphism. For our study, however, the limited number of patients prohibited haplotype analysis.

TNF-α levels in nasopharyngeal secretions are decreased in children with recurrent otitis media compared with healthy children.(9) TNF-α stimulates immunoglobulin and mucin production, and low TNF-α concentrations may compromise these defence mechanisms.(7) The association between various *TNFA* polymorphisms and otitis media parameters found in our study may indicate that, indeed, there is a role for these polymorphisms in TNF-α production in vivo. In the recent past, numerous studies have been performed to investigate the association of *TNFA* promoter polymorphisms and TNF-α levels in different inflammatory and infectious diseases, and contradictory results have been reported.(34) TNF-α expression is probably not determined by one polymorphism, but, rather, by a combination of polymorphisms in *TNFA* and *TNFA*-associated genes. Different pathogens may induce a variety of cytokine responses, and because numerous pathogens, both bacterial and viral, are known to cause otitis media, unravelling the role of polymorphisms on TNF-α production in the human setting is very difficult. Because TNF-α levels are expected to change during the course of infection, the timing of sampling is likely to be a very important determinant of the results.

In addition, the *IL6* -174 G/G promoter genotype was found more frequently in patients with AOM than in healthy adult controls in our study. Our findings support the findings of others (e.g., the study by Nieters et al.) who found homozygosity of the *IL6* -174 C allele to be associated with a lower frequency of reported common colds.(35) Common colds are known to predispose for recurrent otitis media. In addition, Patel et al. reported *IL6* -174 G allele carriage to be increased in otitis media susceptible children.(33) The *IL6* G/G genotype was shown previously to be associated with high IL-6 levels compared with the C/C genotype.(21) This association, however, is not consistent, however, and a more complex regulation of IL-6 production that depends on multiple polymorphisms in the *IL6* promoter region appears to play a role.(36, 37) Furthermore, IL-6 expression is influenced by TNF-α, and interaction of polymorphisms in these and other genes may co-determine the phenotype.

Carriage of the *TLR4* 299 G allele was associated with a lower number of AOM episodes, but was significant only in children younger than 4 years after corrections for confounding factors. Possibly, a low allele frequency in our population (G allele 10% in the group with 2-3 AOM episodes versus 5% in the otitis-prone group) hampered identification of an association. TLRs recognize microbial patterns in a specific way. It is thought that, depending on the micro-organism involved, a combination of TLRs is triggered, which determines the direction of the immune response.(38) Detailed information on the causative pathogens in each disease episode is needed to elucidate the precise role of TLRs and their genetic variation in AOM and other diseases. Unfortunately, these data are not available in the current study.

The *IL10* -1082 A/A genotype was associated with protection from AOM after vaccination. The *IL10* promoter haplotype that includes the -1082 A/A genotype is

associated with low IL-10 production.(19, 39, 40) For the *IL10* -819 C/C genotype, which is in the same haplotype, a similar trend was observed (data not shown). In IL-10 deficient mice immunized with non-virulent unencapsulated *S. pneumoniae* (strain R36A), elevated induction of proinflammatory cytokines was observed, which supports the hypothesis that low IL-10 producers confer better response upon vaccination. Antibody titers against pneumococcal proteins were increased compared with those in wild-type mice.(41) Although no association was observed between the *IL10* polymorphism and specific antibody levels in our population, one might expect a similar effect. Possibly, the concentration of IL-10 in low producers is sufficient to preclude finding differences in antibody titers.

Because otitis media is a multi-factorial disease, the effect of each polymorphism on its own is expected to be limited. In addition, we are aware that most of these associations are expected to lose significance after correction for multiple testing. However, because no consensus has been reached on what method to use to correct for multiple testing in genetic association studies, we felt it most appropriate to provide p-values as they are.

The associations in our study were attributable mainly to children younger than 4 years. Several factors may explain this finding. First, a selection for children suffering from ≥ 2 episodes of otitis media for the vaccination trial may have resulted in a biased group in the older children, including those children with the highest recurrence rates or ongoing infection. Second, the immune system and anatomy, like the Eustachian tube, of young children are both still developing and differ from older children and adults, which likely results in a more prominent role for innate immunity at younger age. In contrast to previous studies, our study only included patients with recurrent AOM and no age-matched controls without otitis were included.(30, 42)

In conclusion, several polymorphic variants in immune response genes (ie, *IL6* -174 G/C, *TNFA* -863 A/C, *TNFA* -376 G/A, *TNFA* -238 G/A, *TLR4* D/G and *IL10* -1082 G/A) are suggested to have a potential influence on middle ear infections. Because the various genotypes are expected to interact with each other and numerous environmental and host factors, additional functional and genetic studies are warranted to elucidate their individual contributions to recurrence of AOM.

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Chapter 5

Genetic variability and nasal carriage of *S. aureus*

Chapter 5.1

Association between nasal carriage of *Staphylococcus aureus* and the human complement cascade activator serine protease C1 inhibitor (C1INH) valine vs. methionine polymorphism at amino acid position 480

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Abstract

Objective. *Staphylococcus aureus* produces compounds that interfere with complement deposition. We hypothesized that humans have developed countermeasures to staphylococcal complement evasion.

Methods. We screened for single nucleotide polymorphisms in the serine protease C1 inhibitor (*C1INH*) gene at amino acid position 480 (valine vs. methionine) and nasal carriage of *S. aureus*.

Results. In our study cohort, 38 individuals were persistently colonized by *S. aureus*, whereas 50 were invariably culture-negative. A trend was observed towards an increased prevalence of the Val/Val genotype in non-carriers compared to persistent carriers (OR 0.50, p=0.07). The Val/Val genotype was significantly overrepresented in non-carriers compared to 463 Caucasian blood donors (OR 0.52, p=0.02).

Conclusion. These findings suggest that susceptibility to *S. aureus* nasal carriage is associated with the *C1INH* V480M polymorphism.

Introduction

Staphylococcus aureus uses various mechanisms that facilitate escape from the human innate immune response.(1-3) This will probably contribute significantly to bacterial survival in the hostile host environment, although it is currently unclear how nasal colonization is affected by for instance anti-complement features. A central molecular regulator of the human complement system is the serine protease C1 inhibitor (C1-Inh), which prevents over-activation of the complement cascade. C1-Inh is a possible candidate that might facilitate host circumvention of the staphylococcal 'anti-complement' activity. C1-Inh harbours a serpine or serine protease domain that has proteolytic capacities and is essential for the inhibitory capacity of C1-Inh.(4) C1-Inh also inhibits contact system proteases, such as kallikrein and blood coagulation factor XII, and is involved in endothelial binding of leukocytes.(5-7) A polymorphism in this protein has not yet been linked to infectious disease susceptibility. However, considering the important role of C1-inh in complement regulation and the observation that about 25% of the general population carries *S. aureus* persistently in the nasal cavity, we hypothesized that genetic variation in the protease domain of *C1INH* might be associated with nasal carriage of *S. aureus.*

Methods

A total number of 111 Caucasian volunteers were included in the current analysis. Volunteers were co-workers from the Department of Medical Microbiology & Infectious Diseases, Erasmus MC, Rotterdam (n = 19), participants of an ongoing epidemiology cohort study in the elderly in Rotterdam (n = 36), and medical students

of the Erasmus MC (n = 56). Seven (36.8%) of the co-workers from the Department of Medical Microbiology & Infectious Diseases were male, and the median age (range) was 29 (20-52) years.

The median age (range) of the elderly participants was 71 (61-101) years; 15 (41.7%) were male. Among the medical students 26 (46.4%) were male, and the median age (range) was 19 (18-22) years. In addition, blood samples from Caucasian controls representing the general Dutch adult population (n = 463) were provided by the Sanquin Blood Bank South West Region. In the control cohort, 233 (50.3%) were male and the mean age was 39.1 years (SD 8.5). No records regarding *S. aureus* carriage were available for the controls. However, previous studies in both children and elderly from the same geographical region revealed that carriage rates for these and other groups are similar.(8, 9) We therefore assume that the groups that we compare in the present study are not significantly different with respect to carriage rates. Both controls and volunteers originated from the South West area of the Netherlands and their *S. aureus* carriage rates are expected to be similar. Numbers of individuals in test and control groups allow significant differences to be detected at 4-10% change in allele frequencies at 80% power and $p<0.05$. All participants gave written informed consent and the study protocol was approved by the Medical Ethics Committee of Erasmus MC.

Serial nasal swab cultures were performed as described previously.(9, 10) Persistent carriers were defined as those having at least 80% of their nasal cultures positive for *S. aureus*. Intermittent carriers are positive in between 20% and 80% of all culture efforts. Non-carriers were defined as those being repeatedly culture-negative for *S. aureus*.

Blood samples were used for DNA extraction and genotyping. DNA was extracted from peripheral blood leukocytes using standard protocols (DNA minikit; Qiagen). The *C1INH* valine to methionine substitution at amino acid position 480 (V480M) (rs4926) genotype was determined using single base extension analysis (ABI PRISM SNaPshot multiplex kit, Applied Biosystems, Warrington, UK). The genomic region of interest was amplified in 10 µl containing 0.2 mM dNTPs, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.1 U of Taq (Integro BV, Leuvenheim, the Netherlands), 2 pmol of each primer (5'-CCTCCGCCATCTCTGT-3' and 5'-GCTCGCCCTAACCTGA-3') and 5 ng of DNA. Cycle conditions were 95°C for 5 min, followed by 36 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 1 min and 1 cycle of 72°C for 5 min. The *C1INH* V480M genotype was determined by single base extension of the primer 5'-$(T)_{47}$GCAGCAGCCCTTCCTCTTC-3', ending one nucleotide prior to the SNP location. To confirm accuracy of the genotyping analysis, 11 controls covering all sequence variants were sequenced. The sequences and genotypes assessed by SNaPshot were in concordance.

Statistical analysis was performed using SPSS 11.0. Verification of Hardy-Weinberg equilibrium of genotypes was performed using the χ^2 test (1 df). A comparison of genotype frequencies was made between persistent carriers or non-carriers and controls using binary logistic regression. Additionally, genotype frequencies were compared between non-carriers and persistent carriers, the latter categories

representing the extremes of the phenotype tested.(9) Probability (*P*) values ≤ 0.05 were considered to be statistically significant. Since no consensus exists on what methods to use in genetic association studies, no correction was made for multiple testing.

Results and discussion

In the test group, 38 individuals were persistently colonized by *S. aureus*, whereas 50 were invariably culture-negative; 23 were intermittent carriers. The *C1INH* Val480Met genotype distributions for the V/V, V/M and M/M genotypes in the total cohort of microbiologically assessed individuals were 72 (64.9%), 33 (29.7%), and 6 (5.4%), respectively. The corresponding frequencies among bloodbank controls were 256 (55.5%), 179 (38.2%) and 29 (6.3%), respectively. Genotype distribution in both groups reached Hardy-Weinberg equilibrium (p=0.40 and 0.86, respectively). A trend was observed towards a higher prevalence of the V/V genotype in the non-carriers compared to persistent carriers (OR 0.50, p=0.07) (Table 1).

Table 1. *C1INH* V480M genotype frequency compared between S. aureus persistent carriage or non-carriage groups and controls.

	n (%)			NC v CO[1]	PC vs CO[2]	NC vs PC
***C1INH* V480M**	**Non-carrier**	**Persistent carrier**	**Controls**	**OR[3] (p) (95% CI)**	**OR (p) (95% CI)**	**OR (p) (95% CI)**
Total (n)	50	38	461			
V/V	36 (72.0)	21 (55.3)	256 (55.5)	0.52 (0.02)	1.05 (0.86)	0.50 (0.07)
V/M	13 (26.0)	14 (36.8)	176 (38.2)	(0.29-0.92)	(0.62-1.79)	(0.24-1.07)
M/M	1 (2.0)	3 (7.9)	29 (6.3)			

[1]NC, non-carrier; CO, controls.
[2]PC, persistent carrier.
[3]OR, Odds ratio.
Comparing non-carriers to the controls, the log(OR) of the *C1INH* 480 V/V versus M/M genotype was two times the log(OR) of the V/M versus M/M genotype (0.6 and 0.3, respectively). Hence, the relation between the G allele and *S. aureus* carriage is probably additive and the *C1INH* genotype was therefore included in the binary logistic regression analysis as a linear factor (SPSS 11.0).

The V/V genotype was overrepresented among non-carriers when compared to the general population (72.0 vs. 55.5%, respectively, OR 0.52, p=0.02). The fact that the association between *C1INH* polymorphism and nasal carriage of *S. aureus* is not absolute is considered likely because the interaction between bacteria in general and man is thought to be complex and dependent on a large variety of both human and bacterial factors.(11)
As the functional relevance of the V480M substitution remains to be determined, the role of this polymorphism in staphylococcal colonization is unknown.(12) Previously, no differences between the V480 C1-Inh and the M480 C1-Inh were observed with regard to structure, protein stability, serum concentration or inhibition of C1.(13) It is

therefore unlikely that there is a difference in the direct interaction between *S. aureus* and C1-Inh.(14) It might very well be that the polymorphism has an effect on one of the other functions of C1-Inh.(6, 13) Future opsonophagocytosis experiments or characterization of complement deposition on bacterial cells using serum samples from donors with different *C1INH* genotypes may help to elucidate the molecular basis of *S. aureus* - C1-Inh interactions. To perform such experiments, serum is needed from individuals with the different *C1INH* genotypes, multiple *S. aureus* strains need to be tested and the influence of antibodies against *S. aureus* needs to be assessed. (15, 16) We here demonstrate that a genetic polymorphism in a complement gene seems to be associated with staphylococcal colonization of the nose. This observation suggests that human complement gene variability is relevant in counterbalancing the complement-interfering abilities of *S. aureus*, although nothing is as yet known on the mechanistic aspects of this host-pathogen interaction.

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Chapter 5.2

The role of human innate immune factors in nasal colonization by *Staphylococcus aureus*

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Abstract

Objective. *Staphylococcus aureus* colonization of the human nares predisposes to sometimes severe auto-infection. We investigated whether genetic polymorphisms affect the *S. aureus* carriage status.
Methods. Sequence variation in α-defensin and β-defensin, and mannose-binding lectin (MBL) genes were determined for a group of volunteers (n = 109) with known *S. aureus* nasal carriage status. *DEFA1/3* expression was measured in a subset of the volunteers (n = 32).
Results. None of the single nucleotide polymorphisms studied could clearly distinguish the (non) carriage groups. *S. aureus* carriers differed from non-carriers in baseline level of HNP1-3 peptide production (median: 218 vs. 89 µg/ml, p=0.016). No association between HNP1-3 levels and the individual sequence polymorphisms was documented. The combined copy numbers of *DEFA1*/*A3* genes ranged from 5 to 23 per diploid genome. A linear correlation between combined copy numbers and HNP1-3 peptide concentrations in nasal secretions of non-carriers was noted (r^2 =0.8991). *DEFA3* gene was absent in 25% of the individuals. *MBL* haplotype A was overrepresented in persistent *S. aureus* carriers (87% vs 67%; p=0.038).
Conclusion. Defensin gene polymorphism, both in sequence and in gene copy numbers, does not seem to be involved in *S. aureus* carriage predisposition. However, *MBL* haplotypes do so significantly. Baseline HNP1-3 production is more the consequence of *S. aureus* colonization than a reason for the (non) carrier status.

Introduction

Despite of a variety of antimicrobial substances secreted by the human nasal mucosae, *Staphylococcus aureus* is still capable of persistently colonizing the vestibulum nasi in approximately 1 out of 4 individuals.(1) Nasal carriage of *S. aureus* provides an important source for blood-borne, surgical site and other nosocomial infections.(1, 2) Conversely, persistent nasal *S. aureus* carriage seems to protect against a more severe course of staphylococcal disease especially bacteraemia.(1) Consequently, it is important to elucidate the immunological mechanisms prohibiting or leading to *S. aureus* nasal carriage. It has been suggested that persistent *S. aureus* nasal carriers may suffer from dysregulation of innate humoral factors present in their nasal secretions.(3-5) In addition, polymorphisms in innate immune-response genes are known to be associated with susceptibility to other infectious diseases.(6, 7) We hypothesized that single nucleotide polymorphisms (SNPs) previously reported in defensin- and mannose-binding lectin (MBL) genes, encoding for proteins produced at the nasal mucous lining, might play a role in *S. aureus* carriage.(7, 8) It has been demonstrated that defensin gene transcription can be strongly enhanced upon stimulation with bacterial cell wall associated lipopolysaccharide (LPS).(9)
We here present a comparative sequence and SNP analysis of the *DEFA1/3*,

DEFA4, *DEFB1* and *MBL* genes for a group of volunteers with known *S. aureus* nasal carriage status. A variety of SNPs was identified and the frequency of those SNPs within a validation cohort of persistent carriers and non-carriers of *S. aureus* was determined. Furthermore, we measured the expression of DEFA1/3 in nasal secretions of individuals with different *S. aureus* carriage phenotypes, before and after mupirocin treatment and after active *in vivo* re-colonization with *S. aureus*. We also performed quantitative PCR analysis to determine both the gene copy number variability and presence of the *DEFA1* and *DEFA3* genes.

Methods

Study population. A total number of 109 volunteers were included in the current analysis: 37 were demonstrated to be persistently colonized by *S. aureus*, 22 were colonized intermittently, whereas 50 were continuously culture-negative. Volunteers were co-workers from the Department of Medical Microbiology & Infectious Diseases, Erasmus MC, Rotterdam (n = 19), elderly people taking part in an ongoing clinical-epidemiology cohort study in Rotterdam (n = 35), and medical students of the Erasmus MC (n = 55). Blood samples were used for DNA extraction prior to genotyping. *S. aureus* nasal carriage status was determined by longitudinal culturing of nasal swabs of each individual as described previously.(10, 11) Persistent carriage was defined as having at least 80% of the cultures testing positive for *S. aureus*. Non-carriers were those individuals having no positive cultures, whereas intermittent carriers had at least one but less than 80% of positive cultures.(10, 11) All participants provided written informed consent and the study protocol was approved by the Medical Ethics Committee of the Erasmus MC.

Collection of nasal secretions. Nasal secretions were collected from a subset (n = 32) of the abovementioned volunteer group, participating in another study. (11) Nasal secretions were collected from 11 non-carriers, 13 intermittent carriers and 8 persistent carriers. These volunteers were also treated with mupirocin nasal ointment to eliminate *S. aureus* from the nose. Ten weeks later, with nasal swab cultures being still negative, both nostrils of all participants were inoculated with a 10^9 CFU/ml combination of *S. aureus* strains: the laboratory *S. aureus* 502a strain, 2 strains obtained from persistent *S. aureus* nasal carriers and 1 strain originated from an intermittent carrier of *S. aureus* in the nose. Moreover, the intermittent- and persistent carriers in this study population were also reinoculated with their own strain. At base line, before and after inoculation, nasal secretions were obtained from the volunteers. The materials were processed as described before.(3) The nasal secretions were collected by vacuum-aided suction, without chemical stimulation. The secretion volume varied from 0.4 to 2 ml per donor per occasion. The secretions were sonicated briefly (3 times 10 sec bursts at low power) to disrupt the mucoprotein aggregates and to facilitate reproducible handling. The secretions were stored in aliquots at -20^{o}C until use.

DEFA 1/3 quantitative dot-blotting. Sonicated whole nasal fluid was diluted 1:10 in 10% acetic acid, vortexed for 20 min, and centrifuged at 13,000 rpm for 10 min, all at 4°C. The supernatant was collected and kept on ice. The pellet was re-extracted with 400 µl of 10% acetic acid for another 20 min. Following centrifugation, both supernatants containing acid-soluble proteins were pooled and vacuum dried using a ThermoSavant SPD1010 SpeedVac system. Protein pellets were resuspended to the original fluid volume in 1% acetic acid and stored at -20°C. The immuno-dotblot technique was performed as described previously.(12) In short, DEFA1/3 standards and extracted nasal fluid samples were dotted onto Immobilon-P PVDF membranes that were pre-wet with Tris-buffered saline (500 mM NaCl, 20 mM Tris-HCl pH 7.4, TBS). After dotting, the proteins were fixed to the membranes by TBS (150mM NaCl and 10mM Tris-HCl, pH 7.5) containing 0.05% glutaraldehyde. Blots were blocked using Superblock (Pierce, Rockford, USA) containing 0.05% Tween-20 for 30 min at 37°C in an environmental shaker at 30 rpm. Primary antibody incubation was carried out overnight at room temperature using 1:3000 diluted rabbit anti-*DEFA1/3* antisera. The secondary antibody, goat anti rabbit IgG complexed to horse radish peroxidase, was diluted 1:20,000 and incubated for 1 h at room temperature. Blots were blocked again and developed using Immun-Star reagent (BioRad) according to the manufacturer's protocol. Blots were visualized using a ChemiDoc XRS Imaging System (BioRad, Hercules, USA) and quantitative analysis was performed by fitting unknown values to the standard curve using Quantity One Software (BioRad).

Sequence analysis of DEFA1/3 genes. Human genomic DNA was extracted from peripheral blood leukocytes with the Magna-Pure Large Volume protocol (Roche, Almere, the Netherlands). In a pilot experiment, using genomic DNA from 2 persistent carriers, 2 non-carriers and 1 intermittent carrier, the complete human α-1/3 (*DEFA1/3*) and β-defensin-1 (*DEFB1*) genes (Genebank accession numbers L12690 and U50930/U50931, respectively) were amplified using overlapping amplicons, synthesized while employing the oligonucleotides described in Table 1. It has to be emphasized that these are all general primers, incapable of distinguishing between the various *DEFA1/3* (pseudo)genes. Amplification of the genes was achieved with a straightforward thermocycling program (Table 1). Double stranded DNA sequencing using the PCR primers, dideoxy terminator technology and capillary ABI sequencing machines were performed at BaseClear (Leiden, the Netherlands). Sequences were aligned using MEGALIGN (DNA Star Software, Michigan, USA) and homo- and heterozygous mutations were verified by visual examination of the electropherograms. Based on the sequence results, an adapted sequence protocol for *DEFA1/3* intron 2 was designed to determine the G1623T and the C1748T SNPs within the *DEFA1/3* genes (GenBank Accession No. L12690) for the whole group of volunteers (n = 109).

Gene copy number assessment by quantitative PCR. The cumulative copy numbers of the *DEFA1/3* genes were determined for a subset of volunteers (n = 32, see nasal secretion) as described by Linzmeier and Ganz.(13) In short, fivefold serial dilutions were made to generate initial DNA concentrations of 50, 10 and 2 ng for target and reference amplifications. For accurate control measurement, the genes for TATA box-binding protein (TBP) and myeloperoxidase were selected as references.

Both genes do not have pseudogenes and are present on the haploid genome as a single copy. Quantitative real-time PCR was performed on a MiniOpticon instrument (BioRad, Veenendaal, the Netherlands) in a 48-well plate format.

Table 1. Sequences for the primers used for the amplification of the α- and β-defensin and mannose-binding lectin genes.

Amplified region	Oligo name	Nucleotide sequence (5' to 3')	Annealing temperature (°C)	Reference[1]
***DEFA1/3* 5'UTR**	963 (F)	gaa.ttc.cct.gta.agc.cct.gt	57	This study
	964 (R)	aca.att.gtc.tag.gat.cat.ct		
***DEFA1/3* 5'UTR**	963 (F)	gaa.ttc.cct.gta.agc.cct.gt	58	This study
	966 (R)	ggt.gag.gtt.aaa.gta.gaa.ct		
***DEFA1/3* exon-1**	967 (F)	gac.caa.ggt.aga.tga.gag.gt	59	This study
	968 (R)	aga.cca.agg.acc.taa.ata.gg		
***DEFA1/3* intron-1**	969 (F)	act.atc.aca.ggt.ctt.tgg.aa	57	This study
	970 (R)	gtg.aca.cag.agt.ggt.tta.ag		
***DEFA1/3* intron-1/exon-2**	971 (F)	gtc.taa.gta.gag.gtg.gaa.at	57	This study
	972 (R)	cag.tga.gag.agg.agg.tgt.gca.t		
***DEFA1/3* exon-2, 3**	HHNP (F)	aga.cat.taa.agg.tac.aga.tgt	56	This study
	HHNP (R)	ttg.gta.gct.gta.tcc.caa.gtg		
***DEFA4* 3'UTR**	U2 (F)	ccc.agc.atg.aca.ttc.tgg.a	50	This study
	U2 (R)	gtg.ttg.gga.act.gcc.tca.ttg		
	U2 VIC probe	agc.aga.a**c**g.tta.atc		
	U2 FAM probe	cag.cac.aa**t**.gtt.aat		
***DEFB-1* promoter**	P44 (F)	cga.ctg.gca.ggc.aac.ac	50	(29)
	P44 (R)	tgt.cag.ctc.agc.ctc.caa.ag		
	P44 VIC probe	cca.gc**g**.tct.ccc.cag		
	P44 FAM probe	cca.gc**c**.tct.ccc.cag		
***MBL* codon 54/57**	1731 (F)	gta.gga.cag.agg.gca.tgc.tc	58	(29, 30)
	1732 (R)	cag.gca.gtt.tcc.tct.gga.agg		
***MBL* promoter -221**	1729 (F)	gtt.tcc.act.cat.tct.cat.tcc.cta.ag	60	(29, 30)
	1720 (R)	gaa.aac.tca.ggg.aag.gtt.aat.ctc.ag		

[1]The DEFA1/3 and DEFA4 primers are homologous to GenBank accession L12960 and U18745, respectively. The DEFB-1 primers correspond to U50930.

For every DNA sample, target and both reference genes were amplified simultaneously in separate wells. PCR was performed independently in triplicate. The average of the results obtained from the three different DNA concentrations formed the given gene copy number.
The copy numbers (CN) of the target genes were calculated as follows:

$$\Delta C_t = C_{t\ reference} - C_{t\ target}$$

$$CN_{target} = 2^{\Delta Ct} \times CN_{reference}$$

$CN_{reference}$ is known to be 2 per diploid genome and C_t, the threshold cycle, is the PCR cycle number at which the fluorescent signal emitted by the PCR products significantly exceeds the background fluorescence level.
DEFA1 and DEFA3 allele discrimination by real-time PCR. The protocol was described before.(13) In short, two sets of PCR primers were designed to detect the presence and absence of the *DEFA1* and *DEFA3* genes, based on the detection of the C3400A SNP. Each allele-specific PCR was performed in duplicate. PCRs using allele-specific primer sets were performed separately. Real-time PCR was carried

out according to the protocol used for gene copy number assessment, except for the primer annealing temperatures. Those were adapted to 70°C and 68°C for primer sets 1 and 2, respectively.

Taqman assay for DEFA and DEFB-1. The allele frequency of SNP rs736227 (NCBI, SNP database) in the 3' UTR region of *DEFA4* and of rs1800972 in the promoter region of *DEFB-1* were examined with real time tests using a 7900 HT thermocycler (Applera, Nieuwerkerk a/d IJssel, the Netherlands). Primers and FAM- and VIC-dye probes were designed by ABI (Table 1) in such a way that pseudogenes were not co-amplified.

PCR-RFLP analysis of MBL. SNPs were detected for the *MBL* codons 54 and 57 in exon1, and for promotor position -221 using PCR restriction fragment length polymorphism (RFLP) analysis with restriction endonucleases *Ban*I, *Mbo*II and *BsaJ*I, respectively (Table 1).(7) *Ban*I reveals a restriction site in codon 54 of the *MBL* exon1, that results in discrimination between homozygous wild-type (GGC/GGC), wild-type/mutant GGC/GAC and homozygote mutant (GAC/GAC) genotypes. *Mbo*II restriction analysis detects polymorphism in codon 57 of *MBL* exon 1 and distinguishes between wild-type (GGA) and mutant (GAA) alleles. *BsaJ*I discriminates the wild-type (G) and mutant (C) alleles in the promoter sequence position -221. The *MBL* haplotypes are defined as described previously.(7) Haplotype A combines wild-type alleles in both codon 54 and 57 (W54, W57), haplotype B (m54, W57) and haplotype C (W54, m57).

Statistical analysis. Association between *S. aureus* nasal carriage status and the presence of a polymorphism in the various genes was assessed by the Fisher's Exact test. The persistent *S. aureus* carriage group was compared to the combined non-carrier and intermittent *S. aureus* carrier group. The latter two groups were combined because only persistent *S. aureus* carriage, characterized by a high bacterial load, is associated with an increased risk for infections.(1, 14) The odds ratio (OR) was defined as the cross-product ratio of the numbers shown in the 2 x 2 table. Means were compared using paired and unpaired tests. *P* values less than 0.05 were considered significant.

Results

Analysis of polymorphisms in the defensin genes. Defensin genes have been mapped to chromosome 8p22/p23 and *DEFA1* has been noted to display copy number polymorphism with two to five copies per haploid genome. In a pilot experiment, *DEFA1/3* genes of five volunteers (known carrier status) were sequenced in full. This was done to determine the presence of relevant SNPs in the defensin genefamily with the ultimate intention to distinguish *S. aureus* nasal carriers from non-carriers. The PCR primers of the overlapping amplicons (Table 1) appeared to be adequately selective and the amplified products all had the expected size. Sequencing revealed near identity with GeneBank Accession reference sequence number L12960. The results of our sequence analysis revealed two SNPs in the *DEFA1/3* genes.

Table 2a. Association of allele frequencies in *DEFA1, DEFA3, DEFA4, DEFB1* or *MBL* genes and *S. aureus* carriage status.

SNP	Allele 1/2	Allele frequency						Fisher's Exact Test *P* value
		Non-carrier (n = 50)		Intermittent carrier (n = 22)		Persistent carrier (n = 37)		
		Allele 1 (%)	Allele 2 (%)	Allele 1 (%)	Allele 2 (%)	Allele 1 (%)	Allele 2 (%)	NC+IC vs. PC[1]
***DEFA1/3* 1632**	G/T	33 (33.0)	67 (67.0)	21 (47.7)	23 (52.3)	34 (45.9)	40 (54.1)	0.246
***DEFA1/3* 1748**	C/T	47 (47.0)	53 (53.0)	17 (38.6)	27 (61.4)	35 (47.3)	39 (52.7)	0.774
***DEFA4*-3' UTR**	A/G	64 (64.0)	36 (36.0)	30 (68.2)	14 (31.8)	51 (68.9)	23 (31.1)	0.651
***DEFB1*-5' UTR**	G/C	78 (78.0)	22 (22.0)	41 (93.2)	3 (6.8)	59 (79.7)	15 (20.3)	0.586
***MBL* codon 54 exon 1**	GGC/GAC	84 (84.0)	16 (16.0)	39 (88.6)	5 (11.4)	69 (93.2)	5 (6.8)	0.122
***MBL* codon 57 exon 1**	GGA/GAA	96 (96.0)	4 (4.0)	43 (97.7)	1 (2.3)	73 (98.6)	1 (1.4)	0.666
***MBL* promoter -221**	G/C	50 (50.0)	50 (50.0)	28 (63.6)	16 (36.4)	36 (48.6)	38 (51.4)	0.476

[1]NC, non-carrier; IC, intermittent carrier; PC, persistent carrier.

Table 2b. Association of genotype frequencies in *DEFA4, DEFB1*, or *MBL* genes and *S. aureus* carriage status.

SNP	Allele 1/2	Genotype frequency									Fisher's Exact Test *P* value
		Non-carrier (n = 50)			Intermittent carrier (n = 22)			Persistent carrier (n = 37)			
		W/W (%)[1]	W/m (%)	m/m (%)	W/W (%)	W/m (%)	m/m (%)	W/W (%)	W/m (%)	m/m (%)	NC+IC vs. PC[1]
***DEFA4*-3' UTR**	A/G	19 (38.0)	26 (52.0)	5 (10.0)	10 (45.5)	10 (45.5)	2 (9.0)	17 (45.9)	17 (45.9)	3 (8.1)	NS
***DEFB1*-5' UTR**	G/C	31 (62.0)	16 (32.0)	3 (6.0)	19 (86.4)	3 (13.6)	0	24 (64.9)	11 (29.7)	2 (5.4)	NS
***MBL* codon 54 exon 1**	GGC/GAC	36 (72.0)	12 (24.0)	2 (4.0)	17 (77.3)	5 (22.7)	0	33 (89.2)	3 (8.1)	1 (2.7)	NS
***MBL* codon 57 exon 1**	GGA/GAA	46 (92.0)	4 (8.0)	0	21 (95.5)	1 (4.5)	0	36 (97.3)	1 (2.7)	0	NS
***MBL* promoter -221**	G/C	17 (34.0)	17 (34.0)	16 (32.0)	8 (36.4)	2 (9.1)	12 (54.5)	12 (32.4)	13 (35.1)	12 (32.4)	NS

[1]W/W, homozygous wild-type sequence; W/m, heterozygous wild-type/mutant sequence; m/m, homozygous mutant sequence; NC, non-carrier; IC, intermittent carrier; PC, persistent carrier; NS, not significant.

Notably, these polymorphisms are located in non-coding regions of the genes only.
Based on the sequence data obtained during the pilot experiment, the amplified intron 2 region of *DEFA1/3* was sequenced for the entire volunteer group (n = 109) to detect the different allelic profiles of the various *DEFA1/3* (pseudo)genes. Table 2a summarizes the comparison of the allelic frequencies among the *S. aureus* carrier and the non-carrier group. The latter group includes the intermittent *S. aureus* carriers. Variability in allelic frequencies in which the SNPs occurred could not distinguish between the *S. aureus* carriage phenotypes.
The *DEFA1/3* gene copy numbers ranged from 7 to 22 for the non-carrier group (average of 13 copies) and from 5 to 23 for the carrier group (average of 13 copies). Again, different *S. aureus* carriage profiles could not be distinguished on the basis of these quantitative data.
The presence of *DEFA1* and *DEFA3* genes was achieved by robust amplification compared to non amplification. The *DEFA1* gene was detected in every individual, while the *DEFA3* gene is absent in 10% of the non carrier group and in 15% of the carrier group. The absence of *DEFA3* was unrelated to the *S. aureus* carriage phenotype (p=1.000). The TaqMan tests did not reveal polymorphisms in the promoter region of *DEFB1* and in the 3'- UTR domain of *DEFA4* that could discriminate effectively between both carriage groups (Tables 2a and 2b).
The frequency of the different restriction patterns of the codon 54 and 57 of exon 1 resulting in classification of 3 *MBL* alleles A (wild-type 54 and 57), B (mutant 54 and wild-type 57), and C (wild-type 54, mutant 57), did not show significant differences between the allelic profiles of the *MBL* gene between the carrier groups. However, analysis of the genotypes documented a slight haplotype A overrepresentation in the persistent carrier group (p=0.038, Table 2c). The genotypes were proportionally distributed according to the Hardy-Weinberg Equilibrium analysis (codon 54, HWE p=0.59 and codon 57, HWE p=0.77).

Table 2c. Association of *MBL* haplotypes and *S. aureus* carriage status.

Haplotype	Non-carrier (%)	Intermittent carrier (%)	Persistent carrier (%)	Fisher's exact Test p value NC+IC vs. PC[1]
MBL2 A	32 (64.0)	16 (72.8)	32 (86.5)	0.038
MBL2 B	14 (28.0)	5 (22.7)	4 (10.8)	0.080
MBL2 C	4 (8.0)	1 (4.5)	1 (2.7)	0.662

[1]NC, non-carrier; IC, intermittent carrier; PC, persistent carrier.

HNP1-3 level in nasal secretions. The overall results are presented in Figure 1 and show an apparent increase in the HNP1-3 levels (i.e. *DEFA1/3* expression) in nasal secretions during the elimination-recolonization studies. Starting at baseline values, going through decolonization and re-colonization, the HNP1-3 values demonstrated a decreased level after *S. aureus* elimination which increased again to a higher level after re-colonization of the nares with *S. aureus*. This differential expression did not reach statistical significance. It is interesting to note that large variations in the local production of HNP1-3 were documented, ranging from borderline positive (50µg/mL) to values of more than 2 mg/mL.

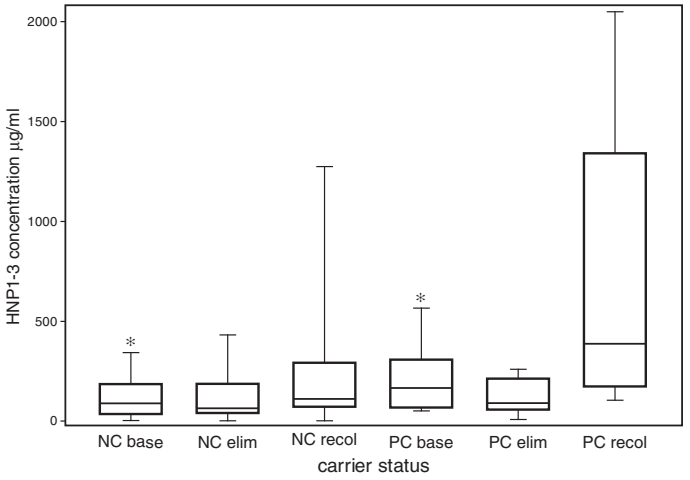

Figure 1. Quantitative box-plot analysis (median, minimum-, maximum value, 25% and 75% percentile) of DEFA1/3 production in various groups of defined S. aureus (non)carriers in various states of colonization. The carrier status is shown on the X-axis; NC, non-carrier plus intermittent carrier group (n = 23); PC, persistent carrier group (n = 7). The abbreviations base, elim and recol indicate the state of colonization: base line level, after elimination of S. aureus from the nasal cavity with mupirocin and after recolonization with 109 CFU/mL S. aureus, respectively.
* indicates the significant difference in HNP 1-3 level in both groups (p=0.016).

Only for those samples that were positive for *S. aureus* as determined by culture at baseline, higher median HNP1-3 values were scored than for culture negative samples (218 versus 89 µg/mL; p=0.016, data not shown). No significant differences were found in the baseline expression levels of HNP1-3 peptides in nasal secretions between the two different genotypes (G-1623-T). Good correlation was found between the *DEFA1/3* copy number in non-*S. aureus* nasal carriers and the HNP1-3 baseline expression level (r^2 = 0.8991, Table 3).

Discussion

Our data show that genetic polymorphism in the defensin genes are not associated with nasal carriage of *S. aureus*. This lack of association contradicts an apparent difference in the expression of *DEFA1/3* at the protein level, irrespective of the fact that colonized individuals show elevated *DEFA1/3* levels in nasal secretions. Also, there is no apparent difference in the physiological response upon mupirocin-mediated elimination of *S. aureus*. Interestingly, an increase in the *DEFA1/3* levels is found after recolonization of the nose with *S. aureus*. This suggests an ineffective local inflammatory response to the presence of *S. aureus.*
Presence of SNPs in the 3'UTR region and promotor regions may lead to instability of the transcript, possibly resulting in variability of translation. We have not tested this hypothesis. We observed no association between *S. aureus* nasal carrier status and

Table 3. Association of the *DEFA1/3* copy numbers and the *DEFA1/3* expression (HNP1-3) at baseline level in nasal secretions of 32 volunteers.

***S. aureus* carriage status**					
Non-carrier (n = 11)		**Intermittent carrier (n = 13)**		**Persistent carrier (n = 8)**	
HNP[1]	**CN[2]**	**HNP**	**CN**	**HNP**	**CN**
19	10	223	9	81	15
240	18	188	15	51	23
246	22	79	13	321	14
36	11	241	12	566	13
145	15	182	18	218	14
7	9	3	5	52	12
89	13	189	11	113	11
89	12	181	10	295	9
66	14	343	22		
3	7	28	5		
89	14	118	14		
		128	9		
		32	7		
r^2	0.8991		0.5626		0.1115

Correlation coefficient (r^2) displays the linear correlation between HNP1-3 peptide concentration in nasal secretions and the number of *DEFA1-3* gene copies of the individuals within each *S. aureus* carriage group.
[1]HNP; HNP1-3 concentration (μg/mL) in nasal secrete.
[2]CN; copy number of DEFA1/3 genes.

allelic polymorphism of *DEFA4* and *DEFB1* genes.

Transcription of some members of the α and β defensin gene families is strongly enhanced upon tissue damage or stimulation with bacterial lipopolysaccharide, although the latter is still controversial.(9, 15, 16) The question whether defensin insufficiency predisposes towards nasal colonization by *S. aureus* is an obvious one. It has been shown that the colonized state is characterized by increased local concentrations of defensins, which is in agreement to our findings.(4) Nasal secretions display antimicrobial activity and this effect was found to vary strongly among donors, suggesting differential effects on the expression regulation of genes encoding for the secreted peptides by staphylococcal products, environmental factors or genetic host factors. Bactericidal activity of nasal secretions from persistent nasal carriers of *S. aureus* was very low in relation to those of non-carriers.(3, 4) Genetic variation but also individual differences between post-translational modification of the defensin propeptides within neutrophils or regulation of defensin gene expression by cytokines could be important mediators.(17) Besides host factors, variation in pathogenicity between different *S. aureus* strains is likely to play a role. Resistance to cationic antimicrobial peptides of *S. aureus* strains colonizing the nose has been noted before.(18, 19) Also, diverse pathogenic factors, such as (lipo)teichoic acid or staphylokinase have an effect on the bactericidal activity of defensins and are probably prerequisites for colonization by *S. aureus* strains.(18, 19) Our current protein data confirm that *S. aureus* nasal carriage is a low grade infectious process triggering variable innate host responses.(20, 21) This implies that colonization by *S. aureus* may result in tissue damage.(22) Therefore, it is important to study the role of major staphylococcal macromolecules, such as peptidoglycan or lipoteichoic acid, in the process of neutrophil recruitment.(23, 24)

Defensin gene copy number is related to effective expression. In a recent study, the HBD-2 gene copy number predisposes to colonic Crohn Disease, perhaps leading to a decreased beta-defensin 2 production. Linzmeier and Ganz showed that the HNP1-3 peptide levels in human neutrophils are proportional to the copy number of the *DEFA1* and *DEFA3* genes.(13) The authors hypothesize that the variation in defensin gene copy number could underlie variability in individual resistance to infection. Large numbers of gene copies would generate increased amounts of the antimicrobial peptides HNP1-3 and may have an influence on the *S. aureus* colonization status in the human nose. However, in our study, no significant variation in defensin gene copy number between *S. aureus* non carrier- and carrier groups could be detected. A good correlation was found between *DEFA1/3* gene copy number and baseline level of HNP1-3 expression in nasal secretions of non-carriers. It seems that the HNP1-3 concentration in the nasal mucous is a combined effect of the number of defensin gene replicates and the presence of *S. aureus* in the nose. The number of *DEFA1/3* gene copies per diploid genome in our study group seems higher than in Linzmeier's study population.(13) The frequent absence of the *DEFA3* gene, which encodes for the HNP3 peptide, is likely to have an effect on effectiveness of innate immunity. Again, in our study no significant difference in the *DEFA3* absence was measured for the *S. aureus* carrier- and non-carrier groups. The percentage *DEFA3* loss in our study population was similar to the observation of Linzmeier and Ganz.(13)

The *MBL* haplotype A was overrepresented in the *S. aureus* persistent carrier group. This *MBL* type is associated with normal levels and good opsonisation activity of the MBL protein.(25) Genetic polymorphism in the *MBL* gene is known to be associated with infectious disease susceptibility. The lectin-dependent complement activation pathway contributes significantly to the opsonophagocytosis of *S. aureus*.(26) Genetic polymorphism in *MBL* is associated with increased numbers of infections during childhood.(27) A recent study provides evidence that MBL plays a key role in restricting the complications associated with *S. aureus* infection in mice and raises the hypothesis that the *MBL* gene may act as a disease susceptibility gene in relation to staphylococcal infections in humans.(28) We here present data that suggest that *MBL* haplotypes are also associated with *S. aureus* nasal colonization.

Although the sample size used in our study is limited, the results are obvious. Our current data show that genetic predisposition towards staphylococcal carriage exists. It has been shown recently that genotype-dependent variability in the sensitivity to glucocorticoids is associated with tolerance towards staphylococcal nasal colonization.(5) Although we could not find a difference in gene expression, a possible role of this polymorphism in the translational or post-translational maturation of the α-defensins could not be excluded and needs to be investigated further**.** The results obtained from this study suggest that genetic polymorphism in genes encoding defensins does not predispose to *S. aureus* carriage. Our future research will be focussed on genome wide SNP analysis, involving all innate immunity factors. Secondly, expression profiles of those genes and (post-)translational proteomic screening (e.g., 2D electrophoresis) should be performed to measure the relevance of innate immunity factors in the *S. aureus* nasal carriage status.

The current working hypothesis that staphylococcal colonization of the vestibulum nasi is a complex, multi-factorial process, depending on both host and microbe capacities still holds and deserves additional research.

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Chapter 5.3

Host polymorphisms in interleukin 4, complement factor H and C-reactive protein associated with nasal carriage of *Staphylococcus aureus* and occurrence of boils

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Abstract

Objective. *Staphylococcus aureus* is capable of persistently colonizing the vestibulum nasi. We hypothesized that polymorphisms in host inflammatory response genes and genetic variation of *S. aureus* contribute to the susceptibility to *S. aureus* carriage and infection.

Methods. The prevalence of persistent *S. aureus* nasal carriage in 3851 participants aged 61-101 years was 18% (678 of 3851 participants), whereas 73% of volunteers (2804 of 3851) were not colonized. A total of 1270 individuals had boils. Polymorphisms in *TNFA* (C-863T*)*, *IL4* (C-542T), *CFH* (Tyr402His), and *CRP* (C1184T, C2042T and C2911G) were determined. Genetic similarity among 428 *S. aureus* strains was determined by use of amplified fragment length polymorphism analysis (AFLP)-mediated genotyping.

Results*.* The *IL4* -524 C/C host genotype was associated with an increased risk of persistent *S. aureus* carriage, irrespective of *S. aureus* AFLP genotype. The *CRP* haplotype 1184C; 2042C; 2911C was overrepresented in individuals who were not colonized. In individuals with boils, carriers of the *CFH* Tyr402 variant and the *CRP* 2911 C/C genotype were overrepresented.

Conclusion. Persistent carriage of *S. aureus* is influenced by genetic variation in host inflammatory response genes. As would be expected in multifactorial host-microbe interactions these effects are limited. Interestingly, host genotype was associated with the carriage of certain *S. aureus* genotypes. Apparently, a close interaction between host and bacterial determinants are prerequisites to long term colonization.

Introduction

The nasal mucous membrane secretes inflammatory mediators such as complement and cytokines.(1) Together with anti-microbial agents such as defensins, lysozyme, lactoferrin, and mucin these seem to protect the host from microbial infections.(2) Despite these local defence mechanisms, *Staphylococcus aureus* is capable of persistently colonizing the vestibulum nasi in approximately one out of four to five individuals.(3-5) Because nasal carriage of *S. aureus* is associated with an increased risk of infection, particularly in patients who have undergone surgery or who are immunocompromised, identification of the determinants of *S. aureus* colonization is important.(4) The prevalence of nasal carriage of *S. aureus* is increased in individuals who produce nasal secretions that are deficient in anti-microbial activity. (6, 7) Genetic polymorphisms in immune response genes are known to influence individuals' susceptibility to infectious diseases and the severity of those diseases. Likewise, polymorphisms may be involved in determining *S. aureus* nasal carriage and *S. aureus* infections such as boils.(8, 9)

Major pathways in *S. aureus* colonization are the activation of cytokines and interaction with the complement system upon interaction with *S. aureus.*(10-12) Lipoteichoic acid and protein A of *S. aureus* induce TNF-α, IL-6, IL-8 and IL-1β, which indicates

that these inflammatory mediators play a role in the defence against staphylococcal colonization and infection.(10) TNF-α, IL-4 and IL-1β are known to stimulate the production of mucin, IL-6 and IL-8.(1, 13) Several variants in the *TNFA* promoter region have been associated with differences in concentrations of its protein.(14) In addition, genetic polymorphisms in *TNFA, IL1B* and *IL6* have previously been associated with meningococcal infection, while *IL4* polymorphisms have been associated with atopy, asthma and severe respiratory infections.(15-17) The *IL4* -524 T allele has previously been associated with atopy and asthma, possibly due to an increased immunoglobulin class switch to the IgE type.(18) The *IL4* -524 C allele was found to be associated with decreased transcriptional activity in reporter gene studies, and carriage of the C allele is associated with a significantly lower serum concentration of IL-4.(18, 19) IL-4 skews the immune response towards the Th2 response and was shown to stimulate mucin production.(20) Although IL-4 deficiency is associated with aggravated *S. aureus* infection in a mouse model, no data are available on its role in carriage of *S. aureus*.(21)

C-reactive protein (CRP) is another major acute phase protein that influences complement activation through opsonisation, which increases the clearance of microbes.(22, 23) Recently, it has been shown that CRP can bind staphylococcal protein A and it may, therefore, be expected that differences in basal CRP expression levels might influence colonization with *S. aureus*.(24) Genetic polymorphisms in the CRP gene are known to influence basal CRP levels.(25-27) In addition to cytokines and CRP, factors of the complement system itself are important in the first line defence against microbes. An important regulator of the complement system is complement factor H (CFH), which inhibits excess activation of C3 and metabolizes activated C3.(28) We therefore hypothesize that a polymorphism resulting in an amino acid substitution in the CRP- binding region (Tyr402His) of CFH may affect the innate immune response upon colonization with *S. aureus*. Recently, it has been shown that *S. aureus* can evade the immune response by inhibiting C3 convertases, which indicates the importance of direct host and pathogen interaction.(12) The possibility of *S. aureus* counteracting host immune responses likely depends on the capability of *S. aureus* to express specific proteins in variable quantities. This implies that genetic variation in both the host and the microbe may contribute to the *S. aureus* carriage phenotype. Recently, amplified fragment length polymorphism (AFLP) analysis, used to assess genetic variability in *S. aureus* strains, revealed that certain genetic clones are more virulent than others.(29)

In this study we investigated if genetic polymorphisms in *IL4*, *TNFA*, *CFH* and *CRP* are associated with nasal carriage of *S. aureus* and the occurrence of boils in a large, well-characterized cohort of 3851 men and women aged 61 years and over from the Netherlands.(30) In addition, we studied the effect of genetic variation of *S. aureus* itself on the likelihood of colonization in a subset of 428 subjects of this cohort.

Methods

Study cohort. This analysis was conducted as part of a prospective, population-based study on the occurrence and determinants of disease in elderly individuals, known as the Rotterdam Study. This longitudinal study commenced in 1990 and was approved by the Medical Ethics Committee of the Erasmus University.(30) Informed consent was obtained from all participants. The second follow up -between April 1997 and December 1999- involved 4797 participants that remained of the initial 7983 individuals. Of these 4797, there were 3882 (81%) participants who were able to visit the study centre. A complete bacteriological assessment was obtained from 3851 participants. An incomplete set of nasal swab cultures was obtained for 31 persons, hence these individuals were excluded. The median age in this study population was 71 years (range, 61-101 years), and 58% were female. Medical information (including age, sex, smoking behaviour, diabetes, and history of eczema or boils, defined as an acute folliculitis with a diameter of more than 1 cm) was obtained from questionnaires and medical records at baseline and during the two follow-up visits that were performed thus far. In addition, laboratory assessments, including fasting glucose level and serum CRP level, were obtained at baseline. Serum CRP levels at baseline were determined as described previously.(27)

Definition of S. aureus carriage status. *S. aureus* carriage status was assessed for all participants by two quantitative nasal swab cultures separated by a one-week interval, as described previously.(31) Persistent carriers (n = 678) were defined as individuals for whom both cultures were positive for *S. aureus*. Non-carriers were defined as those who had no positive culture results (n = 2804), whereas intermittent carriers were those who had only one culture positive for *S. aureus* (n = 369).(31)

Genotyping. The *TNFA* A-863C (reference single nucleotide polymorphism accession number rs1800630) genotype was determined by use of single base extension analysis (SNaPshot, Applied Biosystems) (Table 1).

Table 1. Primers and probes for *TNFA* A-863C and *IL4* C-542T.

Gene	SNP[1]	rs number[1]	Method	Primer or probe
TNFA	A-863C	rs1800630	SBE[1]	F5'GGAGAATGTCCAGGGCTATG3' R5'ACTCTGGGGTCCCTGATTTT3' SBE 5'TTTTTTGAAGTCGAGTATGTGGACCCCC3'
IL4	C-524T	rs2243250	Taqman	F5'GGCAGAATAACAGGCAGACTCT3' R5'GACCTGTCCTTCTCAAAACACCTAA3'
	C			VIC-CATTGTCCCCCAGTGCT
	T			FAM-CATTGTTCCCCAGTGCT

[1]SNP, single nucleotide polymorphisms; rs number, reference SNP accession number; SBE, single base extension.

Bi-allelic discrimination with Taqman analysis was used to determine the *IL4* C-524T (rs2243250) genotype. Primers were ordered from the Applied Biosystems Assay-by-Design service (www.appliedbiosystems.com). The *CFH* C402T (rs1061170), *CRP* C1184T (rs1130864), *CRP* C2042T (rs1205) and *CRP* C2911G (rs3093068) genotypes were determined using Taqman analysis (Applied Biosystems), as described previously.(27, 32)

***Molecular typing of S. aureus*.** Using AFLP analysis, the presence or absence of 147 markers was determined for a subset of *S. aureus* isolates recovered from 428 (41%) of 1047 culture positive subjects, as described elsewhere.(29) In short, by use of the predictive software package Recomb, optimal enzyme and primer combinations were selected. Bacterial DNA was digested with the enzymes *Mbo*I and *Csp*6I and the linker oligonucleotide pair for *Mbo*I (5'-CTCGTAGACTGCGTACC-3' and 5'-GATCGGTACGCAGTCTAC-3') and for *Csp*6I (5'-GACGATGAGTCCTGAC-3' and 5'-TAGTCAGGACTCAT-3') was ligated. Subsequently, a non-selective pre-amplification was performed using the *Mbo*I primer (5'-GTAGACTGCGTACCGATC-3' and *Csp*6I primer: 5'-GACGATGAGTCCTGACTAC-3'). In the final amplification, a ^{33}P-labeled *Mbo*I primer containing one selective nucleotide (either +C or +G) and a *Csp*6I primer containing two selective nucleotides (+TA) were used. Amplified material was analyzed using standard polyacrylamide slab gels and autoradiography. Marker fragments were scored and a binary table that scored marker fragment absence (0) or presence (1) was constructed.

The 428 *S. aureus* isolates were obtained from 103 individuals with intermittent nasal carriage and 325 individuals with persistent nasal carriage who were randomly selected from our cohort. Of these 428 carriers, 225 (53%) were female. A total of five phylogenetic clusters (I, II, III, IVa, and IVb) were defined combining these markers. (29)

***Statistical analysis*.** Statistical analysis was performed using SPSS (version 11.0, SPSS). The verification of Hardy-Weinberg equilibrium of genotypes was performed using Pearson's χ^2 test. Binomial variables were analyzed using Pearson's χ^2 test or Fisher's exact test, as appropriate. Continuous variables were analyzed using the students' *t*-test or the Mann-Whitney *U* test, as appropriate. Initially, a comparison of host genotype frequencies was made between the non-*S. aureus* carriers and the group with persistent carriage. Individuals with intermittent carriage were excluded from this host genetics analyses because they represented a group that was considered less likely to enable discrimination between the features of individuals with persistent carriage and those with no carriage.(31, 33) Because age, sex, active smoking, eczema and fasting glucose were previously reported to be associated with *S. aureus* carriage, risk estimates were adjusted for these parameters in the logistic regression analyses to determine their influence.(9) Genotype distributions were compared between individuals who reported having had boils at least once in their life and individuals who had not had boils. Because the occurrence of boils was associated with age, sex and smoking, these factors were included in the logistic regression analyses to determine their influence. CRP haplotypes were determined using Haplo Stats (CRAN).(34)

In a subset of 428 individuals (41% of 1047) with persistent and intermittent nasal carriage of *S. aureus* the effect of genetic variation in *S. aureus* strains on carriage phenotype (intermittent or persistent) was assessed using Pearson's χ^2 test and logistic regression analysis. Both *S. aureus* phylogenetic clusters and individual AFLP markers were analyzed. In a logistic regression model the effect of host polymorphisms on carriage phenotype (intermittent or persistent) was corrected for

eczema and for AFLP markers found to contribute significantly to carriage phenotype in a univariate analysis. In this last analysis, comparison is only possible between intermittent and persistent carriers, because *S. aureus* genotypes were included in the model, which, of course, is only possible when an isolate is cultured. Probability (*P*) values ≤ 0.05 were considered to be statistically significant. No correction was performed for multiple testing.

Results

S. aureus nasal carriage. Persistent carriage of *S. aureus* was observed in 678 (18%) of 3851 participants. Median age, gender, smoking behaviour, presence of eczema, and median fasting glucose level were independently significantly different between *S. aureus* non-carriers and persistent carriers (Table 2).

Table 2. Characteristics of the study population.

			***S. aureus* carriage status n = 3482**			**Presence of boils n = 3815**		
Characteristic			**None**	**Persistent**	**p**	**No**	**Yes**	**p**
Total			2804	678		2545	1270	
Median age (range) years			72 (61 -101)	71 (61-95)	0.11[1]	72 (61-101)	70 (61-95)	<0.001
Male sex			1121 (40)	333 (49)	<0.001	868 (34)	744 (59)	<0.001
Smoking history								
Non smoker			945 (34)	230 (34)	0.046	979 (39)	309 (24)	<0.001
Past smoker			1326 (48)	348 (52)	0.007	1147 (45)	712 (56)	0.66
Current smoker			515 (19)	96 (14)	1	418 (16)	249 (20)	1
Eczema			231 (8)	100 (15)	<0.001	233 (9)	130 (10)	0.29
Fasting glucose level median (range), mmol/L			5.6 (1.6-18.9)	5.6 (4.0-19.8)	0.001	5.6 (1.6-19.8)	5.6 (3.0-20.5)	0.31
C-reactive protein, mg/L (95% CI)			1.53 (1.47-1.58)	1.64 (1.51-1.78)	0.09	1.54 (1.48-1.60)	1.60 (1.51-1.69)	0.47
Gene	**SNP**	**allele**						
IL4[2]	**-524**	**C**	1478 (85)	366 (91)	0.008	1360 (87)	674 (85)	0.28
		T	252 (15)	38 (9)		204 (13)	116 (15)	
TNFA[2]	**-863**	**C**	1450 (83)	336 (83)	0.78	1298 (83)	672 ((84)	0.26
		A	290 (17)	70 (17)		274 (17)	124 (16)	
CFH	**402**	**T**	3272 (64)	784 (63)	0.46	2913 (63)	1524 (65)	0.22
		C	1812 (36)	456 (37		1675 (37)	820 (35)	
CRP	**1184**	**C**	3406 (69)	823 (68)	0.86	3087 (69)	1551 (68)	0.34
		T	1550 (31)	379 (32)		1387 (31)	735 (32)	
CRP	**2042**	**C**	3414 (68)	798 (66)	0.10	3055 (67)	1565 (68)	0.66
		T	1610 (32)	420 (34)		1489 (33)	745 (32)	
CRP	**2911**	**C**	4698 (94)	1129 (93)	0.30	4225 (94)	2168 (95)	0.11
		G	294 (6)	81 (7)		285 (6)	122 (5)	

Data are no. (%) of participants, unless otherwise indicated. CI, confidence interval; CRP, C-reactive protein; SNP, single nucleotide polymorphism. Data on intermittent carriers were omitted from *S. aureus* carriage analysis, and data were missing for 36 individuals for the analysis regarding boils.

[1]In multivariate analysis, after correction for sex, smoking history, eczema, serum glucose and serum CRP level, the median age was significantly different (p=0.02).

[2]*IL4* and *TNFA* polymorphisms were determined in a subset of ~ 34% of the total population for whom *S. aureus* carriage information was available (1186 and 1192 participants, respectively; intermittent *S aureus* nasal carriers (n = 119) included).

Table 3. Frequency of *S. aureus* carriage, according to host genotype.

SNP	P for HWE[1]	Persistent *S. aureus* carrier/ (non and persistent carrier) (%)	OR for persistent carriage of *S. aureus* (95% CI)[2]
***IL4* -524**	0.15		C/T and T/T vs C/C
C/C		167/802 (20.8)	0.58 (0.4-0.8)
C/T		32/240 (13.3)	
T/T		3/25 (12.0)	
***TNFA* -863**	0.90		C/C vs C/A and A/A
C/C		138/744 (18.5)	0.93 (0.7-1.3)
C/A		60/298 (20.1)	
A/A		5/31 (16.1)	
***CFH* 402**	0.22		
T/T		246/1324 (18.6)	0.97 (0.7-1.3)
T/C		292/1408 (20.7)	1.11 (0.8-1.5)
C/C		82/430 (19.1)	1
***CRP* 1184**	0.85		
C/C		279/1447 (19.3)	1.01 (0.7-1.4)
C/T		265/1335 (19.9)	1.04 (0.8-1.4)
T/T		57/297 (19.2)	1
***CRP* 2042**	0.26		
C/C		250/1405 (17.8)	0.90 (0.7-1.2)
C/T		298/1402 (21.3)	1.12 (0.8-1.5)
T/T		61/314 (19.4)	1
***CRP* 2911**	0.04		C/G and G/G vs C/C
C/C		530/2744 (19.3)	1.11 (0.8-1.5)
C/G		69/339 (20.4)	
G/G		6/18 (33.3)	
***CRP* Haplotype**	**Haplotype frequency, %**		
Haplo 1 CTC	32.7		
0 copy		243/1354 (17.9)	1
1 copy		287/1363 (21.1)	1.22 (1.0-1.5)
2 copies		60/307 (19.5)	1.11 (0.8-1.5)
Haplo 2 CCC	29.8		
0 copy		309/1483 (20.8)	1
1 copy		243/1278 (19.0)	0.89 (0.7-1.1)
2 copies		38/263 (14.4)	0.64 (0.4-0.9)
Haplo 3 CCG	6		
0 copy		516/2677 (19.3)	1
1 copy		68/331 (20.5)	1.08 (0.8-1.4)
2 copies		6/16 (37.5)	2.51 (0.9-6.9)
Haplo 4 TCC	31.4		
0 copy		273/1419 (19.2)	1
1 copy		260/1311 (19.8)	1.04 (0.9-1.3)
2 copies		57/294 (19.4)	1.01 (0.7-1.4)

Haplotypes of CRP single nucleotide polymorphisms (SNPs) could be determined for only 3024 individuals. Differences in denominators reflect differences in the number of individuals for whom the SNP was successfully genotyped. CI, confidence interval; OR, odds ratio.
[1]Hardy-Weinberg equilibrium, for all individuals for whom *S. aureus* carriage was determined.
[2]Univariate analysis, binary logistic regression; OR, Odds ratio.

Genotype distribution for all single nucleotide polymorphisms (SNPs) followed Hardy-Weinberg equilibrium (HWE), but was borderline significantly deviant for *CRP* C2911G (p=0.04).

Individuals who had the *IL4* -524 T allele were less frequently had persistent *S. aureus* carriage compared to those who did not have this allele (Tables 2 and 3). Although individual SNPs in *CRP* were not associated with *S. aureus* carriage, we observed a 1.4-1.5 times overrepresentation of the *CRP* (1184-2042-2911) C-C-C haplotype 2 (one or two copies vs. no copies) in *S. aureus* non-carriers, whereas an opposite effect was observed for the C-C-G haplotype 3 (Table 3). The T-C-G haplotype was too rare (0.1% of participants) for meaningful analyses. It seemed that the polymorphism at position 2911 was driving the association, but only for a particular haplotype background (haplotype 2 versus 3). Serum CRP levels were slightly but not significantly increased in persistent *S. aureus* carriers compared to non-carriers (Table 2). Similar results were obtained in a multivariate analysis adjusted for for age, sex, smoking behavior, eczema and serum glucose level (data not shown).

***Skin infections*.** A total of 1270 (33%) of 3815 participants reported having had boils had at least once (data were missing for 36 of 3851 individuals, Table 2). Participants with a history of boils were on average 1.5 years younger and 2.7 times more likely to be male than those without a history for boils (Table 2). Non-smoker status was associated with protection from the occurrence of boils, compared with current smokers (odds ratio (OR) 0.53 (95% CI 0.43-0.65), p<0.001). Geometric mean CRP levels were similar in individuals with and without boils. Eczema and fasting glucose levels were not associated with occurrence of boils.

The *TNFA* -863 polymorphism was not associated with nasal carriage of *S. aureus* or the occurrence of boils (Tables 3 and 4). Homozygosity for the *CFH* 402 C (His) allele and carriage of the CRP 2911 G allele were associated with decreased occurrence of boils. The occurrence of boils was decreased in carriers of the *CRP* C-C-G haplotype 3 (n = 109 participants (29%)), compared to individuals who did not have the C-C-G haplotype (n = 1008 participants (34%) (OR crude 0.78 (95% CI 0.6-1.0); p=0.03). If nasal carriage was introduced into the logistic regression analysis, identical results were obtained. Other *CRP* haplotypes were not associated with occurrence of boils (data not shown).

***S. aureus phylogenetic clusters and S. aureus nasal carriage*.** Subsequently, in a random subgroup of 428 (41%) of 1047 participants with cultures positive for *S. aureus*, we assessed the effect of genetic variation in *S. aureus* strains on *S. aureus* carriage phenotype. We had previously found that analysis of the AFLP markers revealed the presence of five *S. aureus* phylogenetic clusters.(29) The frequency of the clusters among isolates recovered from 103 individuals with intermittent carriage and 325 individuals with persistent carriage strains are depicted in Table 5. Individual clusters were not differentially distributed between *S. aureus* carriage phenotypes (data not shown). We assessed the incidence of *S. aureus* carriage status (intermittent or persistent) for each AFLP cluster by genotype for each human SNP. Individuals with the *IL4* -524 C/C genotype from whom an *S. aureus* isolate belonging to cluster I was recovered, were 3.2 times (95% CI 1.0-10.1 times) more

Table 4. Distribution of genotype in individuals with and without a history of boils.

SNP	Genotype frequency, particpants with a history of boils/all particpants (%)	Crude OR for presence of boils (95% CI)[1]
***IL4* -524**		C/C vs C/T and T/T
C/C	290/884 (23.6)	0.87 (0.7-1.2)
C/T	94/266 (35.3)	
T/T	11/27 (40.7)	
***TNFA* -863**		C/C vs C/A and A/A
C/C	138/673 (20.5)	1.18 (0.9-1.5)
C/A	102/330 (30.9)	
A/A	11/34 (32.4)	
***CFH* 402**		C/C vs T/T and T/C
T/T	490/1436 (34.1)	0.83 (0.67-1.0)
T/C	544/1565 (34.8)	
C/C	138/465 (29.7)	
***CRP* 1184**		
C/C	528/1593 (33.1)	0.89 (0.7-1.1)
C/T	495/1452 (34.1)	0.93 (0.7-1.2)
T/T	120/335 (35.8)	1
***CRP* 2042**		
C/C	528/1543 (34.2)	1.02 (0.8-1.3)
C/T	509/1534 (33.2)	0.98 (0.8-1.2)
T/T	118/350 (33.7)	1
***CRP* 2911**		C/G and G/G vs C/C
C/C	1032/3012 (34.3)	0.79 (0.6-1.0)
C/G	104/369 (28.2)	
G/G	9/19 (47.4)	
***CRP* Haplo 3 CCG**		1 or 2 vs 0 copies
0 copy	1008/2937 (34.3)	0.78 (0.6-1.0)
1 copy	102/361 (28.3)	
2 copies	7/17 (41.2)	

[1]Univariate analysis, binary logistic regression. Similar results were obtained after adjusting for age, gender and smoking history. OR, Odds ratio.

likely to have persistent *S. aureus* carriage, compared with individuals with the C/T and T/T genotypes combined (Table 5). Individuals with the *CRP* 2042 C/C genotype from who a cluster II isolate was recovered were 5.5 times (95% CI 1.1-28.2 times) more likely to have persistent *S. aureus* carriage, compared with participants with the T/T genotype, indicating minor contributions as expected. No significant differences in individual clusters were observed for the SNPs in *TNFA* or *CFH* or for the *CRP* 1184 and 2911 polymorphisms (data not shown).

Individual S. aureus AFLP markers and S. aureus nasal carriage. By use of univariate logistic regression only the presence of eczema was significantly associated with persistent *S. aureus* carriage as compared with intermittent *S. aureus* carriage (OR 1.77, p=0.007). Age, sex, smoking, and fasting glucose levels were not significantly different between intermittent and persistent carriers (data not shown).

No significant difference was observed for the distribution of host genotypes between

Table 5. Host genotype frequencies in *S. aureus* carriers stratified by amplified fragment length polymorphisms analysis clusters.

		Strains from persistent carriers/ all carriers (%)[1]				
	All	**I**	**II**	**III**	**IVa**	**IVb**
***S. aureus* carriage status[2]**						
Intermittent		56/103 (54)	20/103 (19)	22/103 (21)	4/103 (4)	1/103 (1)
Persistent		105/325 (46)	92/325 (28)	68/325 (21)	10/325 (3)	5/325 (2)
By host genotype						
***IL4* -524**						
C/C	84/111 (76)	45/59 (76)	19/24 (79)	14/19 (74)	4/6 (67)	2/3 (67)
C/T and T/T	18/30 (60)	8/16 (50)	6/8 (75)	3/5 (60)	1/1 (100)	0/0
OR C/C vs. CT and T/T		3.21 (1.0-10.1)	1.27 (0.2-8.3)	1.87 (0.2-14.6)	-	-
***CRP* 2042**						
C/C	120/157 (76)	52/75 (69)	33/37 (89)	26/35 (74)	6/7 (86)	3/3 (100)
C/T	151/199 (76)	69/93 (74)	48/59 (81)	29/40 (73)	3/4 (75)	2/3 (67)
T/T	28/38 (74)	11/14 (79)	6/10 (60)	10/12 (83)	1/2 (50)	0/0
OR C/C vs T/T		0.49 (0.2-2.4)	5.50 (1.1-28.2)	0.58 (0.1-3.2)	6 (0.2-196)	-

OR, Odds ratio (95% CI).
[1]Data are isolates recovered from individuals with persistent carriage/isolates recovered from all individuals with carriage (%).
[2]P=0.43.

individuals with intermittent and persistent carriage with or without adjustment for eczema and bacterial genotype clusters.

Because most markers were not 100% specific for one of the clusters (data not shown), which were also not differentially distributed among individuals with intermittent carriage and persistent carriage, we assessed the prevalence of the 147 individual *S. aureus* AFLP markers in the carriage groups. Five of the 147 markers were differentially distributed between isolates from individuals with intermittent carriage and individuals with persistent carriage in a univariate analysis (Table 6). When we analyzed these five markers simultaneously in a logistic regression model or after correction for multiple testing, none of them remained statistically significant. When we analyzed the effect of human genetic polymorphisms, no significant

Table 6. Distribution of *S. aureus* amplified fragment length polymorphisms (AFLP) analysis markers in isolates recovered from participants with intermittent and persistent colonization.

Marker	**Percentage of isolates positive for the marker, by *S. aureus* AFLP cluster**					**Carriage group no. (%) of isolates**		
n strains	**I 206**	**II 112**	**III 90**	**IVa 14**	**IVb 6**	**Intermittent (n = 103)**	**Persistent (n = 325)**	**OR (95% CI)[1]**
F2115.628[2]	87.3 (205)	5.5 (109)	97.5 (80)	92.9	83.3	77/101 (76.2)	204/313 (65.2)	0.58 (0.3-1.0)
F2217.206	1	99	1	50	0	20 (19.4)	101 (31.1)	1.87 (1.1-3.2)
F2340.870	2	100	0	29	100	21 (20.4)	104 (32.0)	1.84 (1.1-3.1)
F2386.357	0	0	8	0	0	5 (4.9)	2 (0.6)	0.12 (0.0-0.6)
F2305.620	0	0	9	0	33	6 (5.8)	4 (1.2)	0.20 (0.1-0.7)

[1]Odds ratio (OR) for persistent versus intermittent carriage. Only those markers differentially distributed in univariate analysis without correction for multiple testing are included in the table.
[2]A total of 414 isolates were successfully typed for the F2115628 AFLP marker: 205 isolates for cluster I, 109 for cluster II, 80 for cluster III, 14 for cluster IVa and, 6 for cluster IVb.

differences were observed between individuals with intermittent and persistent carriage when polymorphisms were individually included in the model or when the effect was adjusted for eczema. A trend was observed for the *IL4* C-524T and *CRP* C1184T polymorphisms (Table 7). When the effect of human polymorphisms was corrected for eczema and the five relevant AFLP markers, a significant association between the *IL4*-524 C/C genotype and persistent carriage was observed (OR 2.52, 95% CI 1.0-6.2), indicating that interaction between *S. aureus* and human genetic variation may codetermine *S. aureus* carriage phenotype. It must be noted however, that after correction for multiple testing, these associations would no longer be significant.

Table 7. *S. aureus* carriage status, according to host genotypes.

			Binary logistic regression		
		Genotype frequency	Crude	Adjusted	Adjusted
SNP	Total	Persistent/all carriers (%)[1]	OR (95% CI)[2]	OR (95% CI)[3]	OR (95% CI)[4]
***IL4* -524**	141		C/C vs C/T and T/T		
C/C		84/111 (75.7)	2.07 (0.9-4.9)	2.06 (0.9-4.8)	2.52 (1.0-6.2)
C/T and T/T		18/30 (60.0)			
***TNFA* -863**	142		C/C vs C/A and A/A		
C/C		64/93 (68.8)	0.57 (0.2-1.3)	0.56 (0.2-1.3)	0.61 (0.3-1.5)
C/A		34/43 (79.1)			
A/A		5/6 (83.3)			
***CFH* 402**	401				
T/T		120/158 (75.9)	0.96 (0.5-2.0)	0.98 (0.5-2.0)	0.84 (0.4-1.8)
T/C		143/187 (76.5)	0.98 (0.5-2.0)	0.97 (0.5-2.0)	0.80 (0.4-1.7)
C/C		43/56 (76.8)	1	1	1
***CRP* 1184**	390				
C/C		132/177 (74.6)	0.43 (0.2-1.2)	0.43 (0.2-1.2)	0.43 (0.2-1.2)
C/T		131/174 (75.3)	0.45 (0.2-1.2)	0.46 (0.2-1.2)	0.43 (0.2-1.2)
T/T		34/39 (87.2)	1	1	1
***CRP* 2042**	394				
C/C		120/157 (76.4)	1.16 (0.5-2.6)	1.12 (0.5-2.5)	1.22 (0.5-2.8)
C/T		151/199 (75.9)	1.12 (0.5-2.5)	1.09 (0.5-2.4)	1.13 (0.5-2.6)
T/T		28/38 (73.7)	1	1	1
***CRP* 2911**	390		C/C vs C/G and G/G		
C/C		263/343 (76.7)	1.13 (0.6-2.3)	1.18 (0.6-2.4)	1.17 (0.6-2.4)
C/G		35/47 (74.5)			
G/G					

[1]Data are individuals with persistent carriage/all individuals with carriage (%). "All individuals with carriage" indicates both those with intermittent and those with persistent carriage.
[2]Univariate analysis, binary logistic regression; OR, Odds ratio.
[3]Adjusted for eczema.
[4]Adjusted for eczema and *S. aureus* AFLP markers F2115.628, F2217.206, F2340.870, F2386.357, and F2305.620.

Discussion

We documented an association between the -524 promoter polymorphism in *IL4* and nasal carriage of *S. aureus*. In elderly participants with the *IL4* -524 C/C genotype persistent *S. aureus* carriage was more often observed than in individuals with the *IL4* -524 T allele, even after adjustment for confounding factors. The C-allele, with lower IL-4 serum concentration and, therefore, lower mucin production, is associated with carriage of *S. aureus*, which suggests that decreased mucociliary clearance might play a role here.(35) This refutes the possibility that a higher mucin production promotes carriage by improving bacterial adherence.(8, 36) Most of the studies on IL-4 have been performed during infection rather than during colonization. The role of IL-4 in systemic infection seems crucial since IL-4 deficient mice are more susceptible to shock induced with staphylococcal enterotoxin B and D-galactosamine than wild type mice.(37) In addition, IL-4, in combination with IL-10, seems to protect mice from aggravation of the pro-inflammatory immune response in *S. aureus* infection.(21) The outcome of *S. aureus* sepsis in IL-4 deficient mice, however, is dependent on the genetic background of the mouse strain in which where IL-10 deficiency leads to an aggravated course of infection, possibly due to impaired bacterial clearance.(38) It must be noted that most sepsis models use staphylococcal superantigens, whereas these are not always present in *S. aureus*, and moreover, these superantigens are accompanied by different antigens, which may elicit either predominant Th1 or Th2 responses.(39-43) Besides IL-4, IL-1β, TNF-α and IL-13 also stimulate mucin production.(1, 44) The *TNFA* C-863A polymorphism was not associated with *S. aureus* nasal carriage. Although TNF-α was shown to be involved in the pathogenesis of staphylococcal sepsis, we now presume that its role in colonization is less pronounced.(10)

When assessing the possible association of host genetic polymorphisms between intermittent and persistent carriers, we observed a significant difference for the *IL4* C-524T polymorphism when *S. aureus* AFLP markers, significantly associated with carriage phenotype, were included in the model. This difference was not significant when *S. aureus* genotype was not taken into account or when *S. aureus* AFLP clusters, instead of individual markers, were included in the model. Not all individual AFLP markers are specific for a single cluster. Remarkably, the two markers associated with increased risk for persistent carriage, were present in the vast majority of cluster II *S. aureus* strains (99-100%). Although cluster II strains were recovered more frequently from individuals with persistent carriage, compared to with isolates recovered from individuals with intermittent carriage, this difference was not significant. It should be noted that if correction for multiple testing was applied when analyzing the individual markers, these would no longer have been significant, and assessing their true effect and functional relevance requires further research.

Individual SNPs in *CRP* were not consistently associated with *S. aureus* carriage, but when the effect of haplotypes was assessed, overrepresentation of the *CRP* C-C-C haplotype 2 was observed in non-carriers. *CRP* haplotypes have been shown to be associated with differences in basal CRP expression levels.(27) However,

the C-C-C (haplo 2) haplotype is associated with intermediate levels whereas the C-T-C (haplo 1) haplotype is associated with a lower CRP level and the T-C-C (haplo 4) haplotype with higher CRP levels.(27) This renders interpretation of the current results difficult. In addition, the CRP level itself was not significantly associated with *S. aureus* colonization. A possible explanation could be that this single time point of CRP measurement may not be relevant, whereas the *CRP* haplotypes reflect lifelong exposure. Alternatively, this haplotype may be linked to another unknown polymorphism which is associated with *S. aureus* colonization. In addition, carriage of one or two copies of the *CRP* C-C-G haplotype 3, previously associated with the highest basal CRP levels, was associated with a decreased occurrence of boils in our study, although CRP levels were not associated with boils.(27) Because CRP levels were measured as a baseline value, irrespective of the presence of boils, no conclusion can be drawn about the possible effect of the haplotype on CRP level upon infection.

Because the complement system is involved in the defence against microbes we expected that the CFH Tyr402His variant might play a role in colonization with *S. aureus*. Although we did not find an association with *S. aureus* carriage in our population, the occurrence of boils was associated with presence of the CFH 402Tyr variant. The functional relevance of the Tyr to His substitution is not known, but since the polymorphism is located in the CRP binding region of CFH, it may affect the innate immune response. Moreover, recent findings underline the importance of the *S. aureus* immune evasion strategy that relies on the inhibition of C3 convertases. (12)

Although the *IL4*, *CFH* and *CRP* variants appear to be involved in susceptibility to nasal carriage of *S. aureus*, the exact role that the individual polymorphisms we studied play in colonization and occurrence of boils remains unclear.

Besides environmental and genetic host factors that can influence carriage, *S. aureus* bacteria themselves are considered to be responsible due to different mechanisms that facilitate escape from the human immune response.(11) Recently, the importance of staphylococcal proteins such as staphylococcal complement inhibitor and staphylokinase, in the evasion of the complement system, and thereby the innate immune system, has become evident.(45)

The present study involved a large number of participants. Although this decreases the risk of finding false-positive or false-negative associations, replication in an independent similar population, in populations involving other age groups, and/or complementing functional studies are still warranted. This is specifically important for the assessment of the effect of AFLP markers, because multiple testing very likely contributed to finding the associations. In addition, the presence of boils was defined as ever having had boils. This information was derived from medical records, but information also came from questionnaires, which may have resulted in a recall bias.

In conclusion, the *CRP* C-C-C haplotype was associated with negative nasal swab cultures for *S. aureus*. Carriage of the *CFH* 402 Tyr allele were overrepresented in individuals with boils, while boils were observed less frequently among carriers

of the *CRP* (1184-2042-2911) C-C-G haplotype. The *IL4* -524 C/C genotype was overrepresented in among individuals with persistent nasal carriage of *S. aureus*, compared with non-carriers. When accounting for the effect of *S. aureus* AFLP markers that were differently distributed between isolates recovered from individuals with intermittent carriage and those with persistent carriage, an independent association between the *IL4* -524 C/C genotype and persistent carriage was observed. Additional genetic and functional studies in large populations are warranted to further clarify the contribution of these individual factors in staphylococcal nasal carriage and infections in humans.

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Chapter 6

Summarizing discussion and future perspectives

Chapter 6.1

Inflammatory diseases: Common pathways

Summarizing discussion

Introduction

This thesis is the result from a collaboration between a series of clinical and preclinical departments at the Erasmus MC, as well as selected other national and international centres that was started because of a mutual interest in the role of genetic variation in inflammatory disease susceptibility and pathogenesis. The diseases of interest were considered to have similarities in at least a part of their pathogenesis. All diseases included had inflammatory components, thought to be elicited by common (innate) immune response pathways. These immune response pathways could be initiated by infection or colonization with bacteria or viruses, or be an effect of autoimmune response. The availability of well described, homogenous patients was a prerequisite for the inclusion of a cohort into the study, to minimize the chance of introducing bias as seen in poorly defined heterogeneous cohorts.
In this chapter the findings in the diseases described in this thesis are summarized. Genetic polymorphisms were analyzed in patient cohorts with inflammatory diseases: meningococcal infection, chronic hepatitis C virus (HCV) infection and recurrent acute otitis media (AOM) being infectious diseases; rheumatoid arthritis (RA) from the group of autoimmune diseases; and Guillain Barré syndrome (GBS) and Barrett oesophagus as intermediates between infectious and autoimmune disease. In addition, we investigated *S. aureus* nasal carriage as a risk factor for infection, and sepsis after severe multiple trauma as an inflammatory condition.
Table 1 summarizes the positive results in the studies. Polymorphisms in *IL4*, *PAI1*, *TNFA*, *IL6*, *IL10* and *C1INH* were associated with more than one of the studied diseases. This shows that our strategy, assessing what could be common denominators in inflammatory diseases based on the literature available at that time appears accurate. It must be noted that some polymorphisms were only studied in one or two, but not all patient cohorts (*CRP*, *CFH*, *MBL*, *CD14*, *IL1RN*, *TAFI*, and *MMP9*). This resulted from a specific interest in these genes by the different collaborators.

IL4 -542 C/T polymorphism in meningococcal infection and nasal colonisation with *S. aureus*

The *IL4* -524 C allele was transmitted more often from parents to patients with meningococcal disease than expected under Mendelian inheritance (Chapter 2.4). In elderly individuals with the *IL4* -524 C/C genotype persistent *S. aureus* carriage was more often observed than in individuals with the *IL4* -524 T allele even after adjustment for confounding factors (Chapter 5.3). Previously, *IL4* high producer genetic variants have been associated with atopy, asthma and severe respiratory infections.(1-3) The *IL4* -524 C/C genotype is associated with lower *IL4* expression, while low IL-4 serum concentrations are associated with low mucin production.(4-6) This indicates that an increased susceptibility to both meningococcal infection and *S. aureus* colonization might result from a decreased mucociliary clearance.

Table 1. Positive gene/SNP-disease associations identified in this thesis.

Disease	Susceptibility	Severity	Treatment response
Meningococcal infection	*DEFB1*[1] G/G > UK *IL4* -542 C >, tdt *C1INH* 480 Val >, tdt	*DEFB1* G/G > DIC, UK[1] *DEFB1* C/C > mortality, UK[1] *IL4* -524 T/T > mortality, Dutch *TAFI* 325Ile/Ile > DIC, Dutch[2]	NA
Sepsis in trauma patients	*PAI1* 4G/4G >	-	NA
Guillain Barré syndrome	-	*MMP9* -1562 T > severe[3] *TNFA* -863 A/A > severe	NA
Rheumatoid arthritis	*IL6* -174 G/G > *IL8* 781C/C > *PAI1* 5G/5G >	*TNFA* -308 G/G > severe *TNFA* -238 A/A > severe	NA
Barrett oesophagus	*IL1RN* 2018C >[4] *CD14* -260 T/T <[4] *CD14* -260 T/T and *TLR4* +896 A/A combined < *IL1B* -511 C/C + *IL1RN* 2081 T/T + CD14 -260 T/C+TLR4 896 A/G combined <	NA	NA
Chronic HCV infection	*IL6* -174 G/G > *TNFA* -857 T >	-	*IL10* -819 T *IL10* -1082A;-819T > viral response *PAI1* 5G/5G SVR *PARP* 762 A < ETR[5]
Recurrent acute otitis media	*IL6* -174 G/G >	*PAI1* 4G/4G > *TNFA* -238 G/G > *TNFA* -376 G/G > *TLR4* 299 A/A >	*TNFA* -238 G/G *TNFA* -863 A lower specific antibody response upon vaccination *IL10* -1082 A/A: protection from more recurrent AOM upon complete vaccination
Staphylococcus aureus nasal colonization	*C1INH* 480 Val > MBL haplotype A >[6] *IL4* -524 C/C > *CRP* haplotype 1184C; 2042C; 2911C <[6]	-	NA
Staphylococcus aureus infection: boils	*CFH* Tyr402 > *CRP* 2911 C/C >		

NA, not addressed thus far.
[1]The *DEFB1* polymorphism was genotyped in meningococcal infection and for *S. aureus* carriage only.
[2]*TAFI* polymorphisms were determined in meningococcal sepsis patients only. DIC, disseminated intravascular coagulation; tdt, transmission disequilibrium test.
[3]*MMP9* was genotyped only in patients with Guillain Barré syndrome and controls.
[4]*IL1RN* and *CD14* polymorphisms were genotyped in Barrett oesophagus patients and controls only.
[5]ETR, end of treatment response.
[6]*MBL, CFH* and *CRP* polymorphisms were determined for *S. aureus* carriage analysis only.

Mainly recent, but not long-lasting colonization results in increased risk for infection. Colonization with microbes will elicit specific antibody response. This however takes time. For recently acquired microbes there has not been enough time for the host to produce specific antibodies, and the lack of these specific antibodies results in an increased risk of infection.(7) The role of IL-4 in systemic infection is crucial since IL-4 deficient mice are more susceptible to shock induced with a combination

of staphylococcal enterotoxin B and D-galactosamine than wild type mice.(8) In addition, IL-4, in combination with IL-10, protects mice from aggravation of the pro-inflammatory immune response in *S. aureus* infection.(9) In adult sepsis patients IL-4 mRNA levels were decreased when compared to patients with bacteraemia or controls.(10)
One might expect that given these results co-colonization of *N. meningitidis* and *S. aureus* is likely to occur in susceptible individuals. Although a positive correlation was observed in children between *N. meningitidis* and *S. pneumoniae* colonization, no correlation exists between *S. aureus* and meningococcal carriage.(11) Possibly only limited interaction between *S. aureus* and meningococci exists allowing co-colonization of these two microbes without favouring or inhibiting colonization of the other in the presence of one. The interaction of the microbes and the host in a given environmental context, for example the nasopharynx, will determine the outcome after exposure of the host to the microbe: asymptomatic colonization, infection, severity of infection, and outcome of infection.(12)
IL-4 is a typical Th2 cytokine.(6) We observed an increased susceptibility for microbial colonization and infection in IL-4 low producers, while high IL-4 production genotypes were associated with asthma and atopy. This underlines the importance of the adequate regulation of both Th1 and Th2 responses, to prevent either phenotype.

PAI1 4G/5G polymorphism important in tissue integrity

PAI-1 inhibits cellular migration and invasion, processes essential in tissue repair in inflammatory disease.(13, 14) Hence, higher PAI-1 levels might result in longer persistence of tissue inflammation, or abnormally repaired tissue, prone to bacterial colonization and new infections. The -675 4G/4G promoter genotype is associated with high PAI-1 expression.(15, 16) The high production genotype is associated with poor outcome in meningococcal disease.(17)
Children with the *PAI1* 4G/4G genotype had an increased risk of more frequent AOM episodes compared with those who were homozygous for the 5G variant, also after correction for cofactors (p=0.03, OR 2.31). This finding was attributable to children < 4 years of age (p=0.02, OR 2.97) (Chapter 4.1). In addition, homozygosity for the *PAI1* 4G allele in patients with HCV infection associated with non-sustained response to therapy (Chapter 3.4), while in multiple trauma patients it associated with susceptibility to sepsis (Chapter 2.9). The *PAI1* 5G homozygous variant was associated with increased RA susceptibility (Chapter 3.2).
Two possible mechanisms for PAI-1 interaction and inhibition of tissue repair have been proposed. First, PAI-1 may inhibit a proteolytic cascade at the cell surface that results in matrix destruction, allowing cellular migration and invasion for tissue repair. The proteolytic cascade mechanism involves the activation of surface-bound plasminogen by surface bound urokinase plasminogen activator (uPA). PAI-1 can bind to uPA on the cell surface and inhibit its proteolytic activity and hence tissue repair.(18-20) A second proposed pathway involves competitive binding of PAI-1 and integrin

to vitronectin. Both share the same binding site region on vitronectin. Vitronectin/integrin interaction is needed for cell migration and adhesion. The inhibiting effect of PAI-1 is negated when excess non-surface bound uPA forms a complex with PAI-1 resulting in a release of PAI-1 from vitronectin, which is then available for interaction with integrin, favouring cell adhesion.(21)

A possible role for high PAI-1 concentrations in the stronger inhibition of healing of inflamed otolaryngeal tissue that may thus favour an otitis prone condition is supported by the results of Hellström and co-workers that imply a further role for PAI-1 and plasminogen in tissue repair. They have shown that perforated tympanic membrane of plasminogen deficient mice exhibits reduced healing 15 days after myringectomy compared with TM from wild type mice.(22) Intravenous administration of plasminogen, in plasminogen deficient mice, resulted in restored TM healing even if plasminogen was administered 30 days after perforation. The distribution of inflammatory cells in the perforated TM differed significantly between plasminogen deficient and wild type mice *in vivo*. Plasminogen deficient mice with initially normal tympanic membranes showed spontaneous development of middle ear effusion. In addition, uPA expression is increased upon infection with *Streptococcus pneumoniae* in an otitis media model in the rat.(23) *S. pneumoniae* is one of the major causative microbes of otitis media in humans.(24-26)

Interaction of PAI-1 with uPA prohibits catalytic activation of plasminogen to plasmin. High PAI-1 levels might therefore result in similar clinical findings as seen in plasminogen deficiency. Furthermore, in skin wound healing, PAI-1, uPA and uPA-Receptor are expressed at the site of regenerative epithelial outgrowths at the edge of the wound.(27, 28) Plasminogen deficient mice showed impaired wound healing. (29)

The pleiotropic PAI-1 molecule is also involved in fibrosis.(30) Additionally, PAI-1 deficiency protects against fibrosis formation in diverse animal disease models. (31) Furthermore, plasminogen deficiency interferes with clearance of debris after acute injury, and results in activation of hepatic stellate cells known to be involved in fibrosis progression.(32, 33) Since increased PAI-1 levels inhibit wound healing and induce fibrosis, it is reasonable to assume that this mechanism contributes to the nonsustained response to treatment with peginterferon alfa-2a and ribavirin in *PAI1* 4G homozygous HCV infected patients (Chapter 3.4).

Whether the role of PAI-1 in pathophysiologic processes proposed in otitis media and HCV infection is similar for susceptibility to sepsis in multiple trauma patients is not yet clear, but plausible. PAI-1 concentrations are increased in multiple trauma patients. (34) This was thought to result in a decreased fibrinolysis and the occurrence of microthrombi in disseminated coagulation as in meningococcal sepsis. Additionally, the high producer *PAI1* 4G/4G promoter genotype was associated with increased mortality.(34) The latter could not be confirmed in our study, possibly due to less severe disease and improved treatment regimes of the current study population.

The role for PAI-1 in rheumatoid arthritis seems to differ from the mechanisms proposed above for otitis media, HCV infection and sepsis in trauma patients. The *PAI1* 5G homozygous variant, related to low PAI-1 levels and therefore decreased

inhibition of plasminogen activator was associated with RA when compared with controls. No link was observed with severity of disease. Plasminogen deficiency is associated with decreased susceptibility to and or severity of arthritis in mice models.(35) PAI-1 deficiency, however, has also been associated with decreased severity in murine antigen-induced arthritis.(36)
In general, the role of factors involved in tissue repair and integrity, such as filaggrin and myosin became more evident in the past few years, as illustrated in celiac disease and atopic dermatitis.(37-39) This underlines the importance of tissue repair and integrity in the pathogenesis of inflammatory diseases.

IL6 and increased susceptibility to inflammatory disease

The *IL6* -174 G/G genotype was overrepresented in rheumatoid arthritis patients (Chapter 3.2), and individuals with hepatitis C infection (Chapter 3.4) when compared to healthy controls. In addition, the *IL6* -174 G/G promoter genotype was found more frequently in AOM patients in our study (Chapter 4.2). This is consistent with the increased expression of IL-6 during experimental otitis media in animals.(40, 41) The *IL6* G/G genotype is associated with increased IL-6 levels compared to the C/C genotype. This SNP therefore contributes to the complex regulation of IL-6 production involving multiple polymorphisms.(42-45) Furthermore, IL-6 expression is influenced by TNF-α, and interaction of polymorphisms in these and other genes may co-determine the disease phenotype.
Barrett et al. found the high producer variant to be more prevalent in persistent HCV infection compared with spontaneous viral clearance.(46) Patients with chronic hepatitis C have increased IL-6 expression when compared to healthy controls. Whether this is secondary to infection or reflects increased susceptibility to or reduced clearance of infection with HCV in high IL-6 producers is not clear.(47)
The substantial improvement of RA upon treatment with anti-IL-6 receptor antibody underscores the importance of this pleiotropic cytokine in the pathogenesis. (48) The exact functional role of the pleiotropic pro-inflammatory IL-6 increasing the susceptibility in these diseases is not yet known. Experimental models assessing early disease in normal and IL-6 deficient animals could provide further insight into the role of IL-6 in susceptibility to RA.

IL10 low producer variants enable enhanced proinflammatory responses

IL-10 is a well-known anti-inflammatory cytokine, which serves with other cytokines, such as IL-4, to counterbalance the pro-inflammatory response elicited in infection. The promoter -1082A;-819T haplotype was previously associated with decreased expression of IL-10.(49-51)
In our study carriage of the *IL10* -819T allele was associated with rapid viral response

(a decrease or disappearing in viral titers) in HCV patients at 6 weeks of treatment with peginterferon alfa-2a and ribavirin (Chapter 3.4). The *IL10* promoter low producer haplotype was associated with end of treatment viral response and sustained viral response 24 weeks after the termination of the treatment.
In addition, the *IL10* -1082 A/A genotype was associated with protection from AOM after vaccination (Chapter 4.2). These observations point towards an improved pro-inflammatory response in low IL-10 producers in patients with recurrent AOM vaccinated against pneumococci and patients treated for HCV infection. The first concept is supported by observations in animal studies. In IL-10 deficient mice immunized with non-virulent unencapsulated *S. pneumoniae* (strain R36A) elevated induction of pro-inflammatory cytokines was observed, supporting the hypothesis that low IL-10 producers confer better response upon vaccination. Moreover, antibody titers against pneumococcal proteins were increased in IL-10 deficient mice compared to wild type mice.(52) Our results indicate that it may be possible to identify individuals who are more likely to fail a proper response to vaccination. These individuals could in the future possibly be selected for adjusted vaccination regimes providing optimized vaccination response for everybody.

C1INH polymorphism in meningococcal infection and nasal colonization with *S. aureus*

The *C1INH* 480 Val variant was associated with increased susceptibility for meningococcal infection and also with nasal carriage of *S. aureus* (Chapters 2.4 and 5.1).
The complement system is important in the defence against bacterial infections evidenced by the increased susceptibility to infection with meningococci in patients with deficiencies of complement factors, and the mechanisms *S. aureus* has developed to evade the complement system.(53, 54) Over-activation of the complement cascade is prevented by the serine protease C1 inhibitor (C1-Inh), which is an important molecular regulator of the human complement system. This multifunctional protein also inhibits activity of contact system proteases, such as kallikrein and blood coagulation factor XII, and is involved in endothelial binding of leukocytes.(55-57)
As the functional relevance of the V480M substitution remains to be determined, the role of this polymorphism in staphylococcal colonization and the pathogenesis of meningococcal infection is unknown.(58) Previously, no differences between the V480 C1-Inh and the M480 C1-Inh were observed with regard to structure, protein stability, serum concentration or inhibition of C1.(59) It is therefore unlikely that there is a difference in the direct interaction between microbes and C1-Inh.(60) It might very well be that the polymorphism has an effect on one of the other functions of C1-Inh. (56, 59) Future opsonophagocytosis experiments or characterization of complement deposition on bacterial cells using serum samples from donors with different *C1INH* genotypes are required to elucidate the molecular basis of (indirect) microbe- C1-Inh interactions.

TNFA expression: too much, too little?

The *TNFA* -863 A variant was associated with increased severity of GBS and a decreased specific antibody response upon anti-pneumococcal vaccination (Chapters 3.1 and 4.2). A previous study showed that the A-allele at position -863 of *TNFA* was associated with higher TNF-α concentration due to an inadequate down regulation of its production.(61) Previous reports also showed that high serum levels of TNF-α were associated with severity of GBS.(62-66) This is possibly mediated by the extent of macrophage activation, resulting in an insufficiently controlled pro-inflammatory response.
In addition, the *TNFA* -238 G/G genotype was associated with more recurrent AOM, while the -238 A/A genotype was associated with more severe RA (Chapters 3.2 and 4.2).
The interpretation of these findings is more complex. Both an excess and insufficient *TNFA* expression may explain more severe disease in AOM. An excess of TNF-α may result in increased tissue damage, prone to recurrent infection while a decreased TNF-α response may prevent adequate clearance or inactivation of the microbes resulting in (ongoing) infection. The effect of the TNFA -238 polymorphism on TNF-α expression is unclear. In the recent past numerous studies have been performed to investigate the association of *TNFA* promoter polymorphisms and TNF-α levels in different inflammatory and infectious diseases, reporting contradictory results.(67) TNF-α expression is probably not determined by one but by a combination of polymorphisms in *TNFA* and -associated genes. This is further complicated by the low minor allele frequency. Small changes in numbers are likely to substantially influence the results, which increases the risk of false positive and false negative associations.
Furthermore, and specifically in AOM, different pathogens may induce a variety of cytokine responses, and since numerous pathogens, both bacterial and viral, are known to cause otitis media, unravelling the role of polymorphisms on TNF-α production in the human setting is very difficult. Moreover, since TNF-α levels are expected to change during the course of infection, timing of sampling is likely to be a very important determinant of the results. Differential expression of TNF-α is very likely to influence disease susceptibility and severity due to its pleiotropic pro-inflammatory actions. TNF-α stimulates, for example, immunoglobulin and mucin production and low TNF-α concentrations compromise these defence mechanisms.(68) TNF-α levels in nasopharyngeal secretions are decreased in children with recurrent otitis media compared with healthy children.(69)
The association between various *TNFA* polymorphisms and AOM, RA, and GBS described in this thesis indicate that indeed there is a role for these polymorphisms in TNF-α production *in vivo*. Analyzing TNF-α expression in our patient cohorts in relation to the *TNFA* haplotypes, could clarify the effect of the polymorphisms tested in these patients.

Conclusion

The above shows that several of the polymorphisms included in this thesis were indeed associated with two or more phenotypes. This phenomenon of pleiotropy supports the hypothesis that similar pathogenic processes are likely to be involved in different diseases. While observations were consistent in some, others like for the SNPs in *TNFA* in the different diseases appeared contradictory. Low minor allele frequency, relatively small patient populations still, and therefore increased risk of finding false positive or false negative findings may have contributed to these findings. This is also reflected by the loss of significance for some of the associations after correction for multiple testing. One must however consider that the effect of genetic polymorphisms is expected to be limited at most, and multiple SNPs are expected to be involved besides other host and environmental factors.

Observational studies but also controlled (*in vitro*/animal model) experiments, assessing protein levels, such as those for TAFI, ADAMTS13, VWF, and MIF in meningococcal disease (Chapters 2.5 to 2.7) are required to elucidate the relevance of SNPs associated with disease. In addition, RNA expression profiling studies as described in chapter 2.8 can support genetic association, and they can also provide new insights and point toward other candidate genes possibly involved in the pathogenesis of inflammatory diseases.

The emerging interest in tissue integrity is further supported by our observations for *PAI1* polymorphism in AOM, HCV infection, and possibly sepsis in multi trauma patients. In addition, the role of *IL4* and *C1INH* in susceptibility to *S. aureus* nasal carriage and susceptibility to meningococcal infection deserves further attention. Functional studies elucidating the contribution of these molecules to the pathogenesis are required.

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Chapter 6.2

Genotyping studies: Future perspectives

A personal view

Introduction

In this chapter I will discuss the strategies that are being used or should in my opinion be implemented when assessing the effect of single nucleotide polymorphisms (SNPs) in multifactorial (complex) traits and diseases. Complex diseases are conditions that are influenced by the actions of multiple genes, their interactions with each other and with the environment.
Genetic variants, including di-, tri- or multiple-nucleotide repeats (microsatellite regions), and copy number variation, although diverse and relevant, fall outside the scope of this overview.

Since the discovery of the double helix structure of DNA by Watson and Crick in 1953 the knowledge about this coding structure has expanded and is still expanding with great speed including the sequencing of the complete genome of Jim Watson. (1-3) Although heritability had been a recognized phenomenon long before that, understanding the DNA structure made it possible to determine the molecular origin of the features observed. The search for mutations in single gene diseases such as Huntingtons disease and cystic fibrosis has been very successful using linkage analysis in large multigenerational families.(4)
The idea that all diseases and traits can be explained by single mutations has however been proven wrong long ago. In single gene diseases it was observed that many different mutations in the same gene result in similar phenotypes. In cystic fibrosis for instance, over 1000 mutations and 200 polymorphisms have been described in the cystic fibrosis transmembrane conductance regulator gene, although their individual functional effects are not yet all known.(5) And, especially for complex traits and diseases, in which not only genetic but also environmental factors can be involved, this simplified view was found to be erroneous. Most likely multiple variations in different genes will co-determine the phenotype in interaction with environmental factors. This implies that most of these common variants are expected to have only a modest effect.
Inflammatory diseases can be included in the category of complex diseases. Inflammation is a process by which the white blood cells and chemicals protect the body from infection, irritation or other injury. Autoimmune diseases in which the inflammation is directed against ‘self’ and causes tissue damage are also part of the inflammatory diseases. Multifactoriality of these complex diseases is illustrated by an increased susceptibility to certain infectious diseases, which will have no immediate consequences, unless one is exposed to the causative micro-organism.

Replication of genetic associations

In the recent past numerous associations have been published between SNPs and complex traits. Some of these have been confirmed in independent (patient)

cohorts.(6) For most however, replication has been proven difficult. Numerous factors are likely to have contributed to the discrepancies observed (Table 1). First of all disease definition, or subgroup classification often differs between studies. Assessing subtle clinical differences on a greyscale rather than looking at black and white differences may result in different findings, especially since the expected contribution of each of the genetic factors is small and the observed phenotype is the result of the combination of these factors. The use of internationally standardized diagnostic criteria helps to deal with this issue. Although such criteria are available for an increasing number of diseases, still usually several similar, but not identical, internationally recognized standards exist and are being used simultaneously. Examples include severity scores used in paediatric meningococcal disease patients, for whom a.o. sequential organ failure assessment (SOFA) score, Rotterdam score, paediatric index of mortality (PIM) score, different paediatric risk of mortality (PRISM) scores, and organ system specific severity scores have been used.(7-12) The use of multiple different scoring systems limits comparison of different study populations.

Table 1. Factors involved in non replication of genetic associations.

Factors involved in non-replication	Explanation
Disease definition	Existence of (unidentified) clinical subgroups
Ethnic heterogeneity	Applicability limited to a single specific population
Winners curse	Initial positive findings are not often replicated
Lack of statistical power	Small sample sizes
Genotyping of other SNPs in the gene of interest	SNPs may or may not be in linkage disequilibrium
Using different genome wide genotyping platforms	There is no 100% overlap of SNPs included in the platforms

Furthermore, with increased knowledge of complex diseases, more clinical subgroups are being identified. Disregarding the existence of subgroups, would bias the results, since the distribution of these subgroups may differ between different study populations examined. Since existence of subgroups is not always acknowledged at the time of the study, it is not surprising that associations now considered to be true, may in the future be determined to be actually false based on increased knowledge of disease definition. Moreover, the choice of the control group also determines what can and cannot be analyzed. When assessing the contribution of a given SNP to disease susceptibility one should - if possible - correct for (confounding) factors that may codetermine disease. For example, when the effect of SNP A on the susceptibility to diabetes mellitus is assessed in an obese patient cohort, one should preferably use a matched control population. Since an increased body mass is a known risk factor for diabetes, this infers including individuals that are of the same ethnic origin, not diabetic, but do have an increased body mass index, which is not significantly different from the diabetes patients. This was demonstrated by the differences observed when analyzing a genetic variant in *FTO* in type 2 diabetes and obesity.(13) When the

FTO variant was compared between patients with diabetes and controls matched for body mass index, no association was observed, while the FTO variant was reported to be associated with diabetes when a lean healthy control cohort was used. Given these differences it was noted that the FTO variant was actually not associated with diabetes, but with obesity, and through that, with diabetes.(14, 15)
Additionally, positive findings are easier to publish than negative results, resulting in publication bias. And since small sample sizes are more prone to obtain false positive associations than large sample sizes, it is not unexpected that initial positive results have not been replicated in subsequent larger studies.(16) This has several implications. First, sample sizes should be large enough to ensure statistical power, and second it should be made easier to publish negative results. While until recently cohort sizes of several hundreds of individuals were considered sufficient, now the call has been placed to include at least several (ten)thousands.(13, 17-19) Single centres usually do not have sufficient numbers of patients to meet this criterion. This implies the need for collaboration and an (international) multicentre approach, and as mentioned above, further standardization of diagnostic criteria and severity scores. Recently, several consortia have been formed like the Wellcome Trust Case Control Consortium (WTCCC GWA) in the United Kingdom that has collected ~2,000 patients per diagnosis (Crohn's disease, diabetes type I and II, bipolar disorder, rheumatoid arthritis, coronary artery disease) and ~3,000 controls, consisting of a birth cohort and healthy blood donors.(17) Yet other more recent initiatives have collected or are still collecting over 100,000 individuals.(17, 18)
Collaboration sounds easy. One must however not forget that this requires another way of thinking in the scientific community. For decades competition has been the key word. And even now, researchers are rewarded for the number of top 10-25% publications in which their name is either first or last in the author list. Even more, financial support is at least partially determined based on these criteria. The collaborative effort needed to enable the collection of thousands of patients per disease or trait and an equal number of controls sometimes requires, however, giving up these prominent authorship positions. While the need for collaboration is clear and progress is being made towards the formation of multiple collaborative efforts, scientists are sometimes still reluctant, given the importance of authorship. Reforming the system toward scientists being rewarded for collaborative efforts would positively contribute to improvement of genetic association studies.

Relevance of biobanks

Large patient sample collections require a structured organization, managing the storage and use of samples and associated information, for instance in the form of a biobank. Besides 'sacrifices' from scientists, sharing collections and giving up first or last authorship for 'the good cause', this implies further thinking about legislation and medical ethical considerations. For example, who owns the samples and, anonimized, clinical information: The individual patient, the researcher, the

consortium, the institutes involved, or the biobank? And how long can samples be stored before they need to be destroyed. Medical ethical committees now ask for destruction of patient materials after a maximum of 15 and sometimes 30 years. Collection of these large numbers of samples, especially for the more rare diseases, however, may require a long period of time. In my view it is ethically not justifiable to have to ask a patient for a new sample when material would still be available if legislation had not required its destruction. Even more, in diseases associated with increased mortality, like meningococcal disease or multiple trauma, it is impossible to obtain new samples. Hence, I hereby plead for an increased storage time that may be even indefinite for some very rare diseases. For the same reasons, while not disregarding the rights of the donors of the material, so only if samples and clinical data are anonimized, I suggest for genetic studies to allow additional genes to be screened without additional explicit informed consent of the donor. For initial recruitment of individuals, of course informed consent to perform genetic association studies is still required. One of the goals of biobank formation is to create large patient cohorts from national and international collaborating research teams. Current legislation, however, differs between countries. Uniformity of (inter)national legislation is therefore required to avoid hindrance of these collaborative efforts.(20)
These biobanks may through registration of studies also serve as a control system to ensure public (online) availability of results, whether positive or negative. This would contribute to a decrease in publication bias.

The power of quantitative trait loci (QTL)

Most genetic association studies focus on binomial outcome variables such as patient or control, complication or no complication, and death or survival. The reason for this is most likely clinical routine, convenience, and relatively easy statistics.

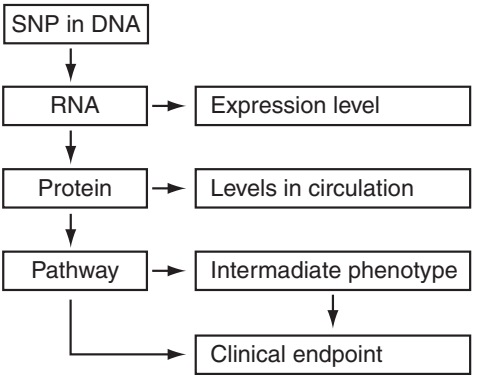

Figure 1. Endophenotypes.
Factors that can be used as quantitative trait loci, increasing the statistical power compared to only analyzing dichotomous clinical endpoints.

While some outcomes are clearly dichotomous, others, like complications can, however, also be subdivided into categories from “no” via “mild” to “severe” complications. Moreover, outcomes are usually determined by multiple factors of which some (if not most) may be quantitative like levels of C-reactive protein, plasminogen activator inhibitor type 1, or viral load. Instead of assessing dichotomous endpoints of phenotypes, one could also focus on quantitative trait loci (QTL). These provide the advantage of increased statistical power compared to dichotomous outcome parameters. In addition, SNPs if having an influence will do so most likely on one of the quantitative traits that codetermine the disease phenotype. Focusing on the more direct link, endophenotypes (Figure 1), instead of the indirect disease phenotype, also eliminates confounding factors that influence clinical traits but not the QTL.

Genome wide approaches

Efficient genotyping of the extended cohorts now required in genetic association studies would not have been feasible without the progress that has been made in the techniques for genotyping. High throughput genotyping platforms (in terms of number of samples) such as Taqman and Sequenom approaches, analyzing up to 384 or even more individuals in one assay simultaneously, and the availability of high density microarray techniques, genotyping up to 1 million SNPs in a single experiment, enable the scientific world to obtain enormous amounts of data in a short time frame. Nearly 12 million unique reference SNPs are known today, of which approximately 6 million have been validated, and this number is still expanding on a daily basis. Approximately 4.6 million of the SNPs are located in genes, of which most (3.8 million) are located in intronic regions, while 80,000 SNPs result in an amino acid substitution. (21, 22) Additionally, other types of genetic variation such as variable number of tandem repeat polymorphisms, restriction fragment length polymorphisms, different numbers of pseudogenes, and large structural variations, potentially influencing gene function, are present in the human genome.(23-25) Only part of the polymorphisms has been genotyped thus far. However, since SNPs located at close proximity are known to form haplotype blocks that are inherited together, there is no need to type all possible SNPs. Since the completion of the human genome much effort is put into documentation of existing haplotypes in diverse populations by the Hapmap project (Table 2).(26) Using the Tagger program developed by de Bakker et al., one can select SNPs that tag one or more other SNPs and only genotype the tagging SNPs.(27) This enables efficient genotyping of SNPs. However, it must be noted that coverage of SNPs throughout the genome using this approach differs for different ethnic populations. Using the first human haplotype map (2005), coverage ranged from 71% in African Americans to 84% in European Americans.(28) This results from the fact that haplotype blocks in African Americans are smaller and have different allelic structure than in European Americans. Using high density oligonucleotide arrays it was estimated that in Chinese approximately 250,000 SNPs are needed to obtain reasonable coverage, while in European Americans and African Americans

these numbers are approximately 300,000 and 550,000, respectively.(26, 28) This difference is illustrated by Nicolae at al. who reported that using the Affymetrix 100k SNP array, coverage in phase I was less in the African HapMap samples than in the European HapMap samples.(29) Taking an r^2 of 0.8 about 60% of SNPs are covered in Europeans using the Affymetrix 500k SNP microarray. Using specific multimarker tests, combining two SNPs to be minimally involved in the haplotype, an additional 20% is covered. With an r^2 of 0.95, 72% of SNPs is covered using the Illumina 300k array. None of the platforms has a 100% coverage. In general coverage is less in regions near telomeres and in GC-rich regions since here recombinations occur more often. Recently the second generation human haplotype map was published covering ~3.1 million SNPs. Currently available genome wide platforms cover the phase II SNPs with an r^2 of up to 0.8 in African and up to 0.95 in non-African populations.(30) Taking an r^2 of 0.8, 1.1 million tag SNPs are needed to capture phase II SNPs in African populations, while ~550,000 and ~520,000 tag SNPs are needed in populations from European descent and Chinese and Japanese populations combined, respectively. (30) Using an r^2 of 1 would require a double number of tag SNPs since most SNPs are already captured using r^2 of 0.8, while the remaining SNPs have lower minor allele frequencies or are even untaggable. The small although substantial increase in coverage is also illustrated by the fact that with the phase II HapMap taking an r^2 of ≥ 0.8, about 68% of SNPs are covered in Europeans using the Affymetrix 500k SNP microarray, while 77% is covered using the Illumina 300k array.(30) Only recently other types of polymorphisms, such as structural variants have been included in the Hapmap project. Moreover, a high-resolution sequencing map of these human structural variants has been published recently.(31)

From the above it is clear that there are differences in genetic variation between ethnic populations. However, 85-90% of the variation is present between individuals within a population, while 1-2% of the variation occurs between populations within a continent and 10-12% between continents.(32, 33) To improve coverage of the HapMap, additional populations are currently being genotyped allowing implementation of the HapMap in genetic association studies regarding populations world wide (Table 2).

Table 2. Populations genotyped for HapMap.

Populations	Number of individual DNA samples
Yoruba in Ibadan, Nigeria	180
Han Chinese in Beijing, China	90
Japanese in Tokyo, Japan	91
CEPH collection (NIGMS human genetic cell repository) (Utah residents with ancestry from northern and western Europe)	90
Additional populations being genotyped	
Maasai in Kinyawa, Kenya	90
Luhya in Webuye	100
Chinese in Metropolitan Denver, CO, USA	100
Gujarati Indians in Houston, TX, USA	100
Tuscany in Italy	100
Mexican ancestry in LA, CA, USA	90
African ancestry in SW, USA	90

CEPH, Centre d'Etude du Polymorphisme Humain; NIGMS, National Institute of General Medical Sciences, USA.

Candidate gene approach revisited

Does this massive genome wide association approach mean that candidate gene studies are no longer needed? On the contrary. Finding an association of a tagging SNP with disease can be interpreted in different ways. The SNP itself can be associated with disease (direct association), but most likely the SNPs is a marker that is linked to the true disease allele that still needs to be determined (indirect association). Finding the association with the tagging SNP thus narrows the search region. It is still needed to identify the candidate gene and the disease or phenotype codetermining SNP(s) in the region covered by the SNP. A candidate gene approach based on the association with a marker SNP can then be conducted. This includes not only genotyping but preferably also functional studies to establish functional relevance of polymorphisms. These functional studies could be observational, but should ideally also include controlled (*in vitro*) studies, while at the same time recognizing the limitations of this approach in complex diseases where multiple genetic, both from the host as well as the microbe, and other environmental factors codetermine the phenotype. The logistic obstacles encountered in a disease like meningococcal infection, with its acute and rapidly evolving pathogenesis (a few hours to one day) for example, are well recognized. Another limitation of functional studies, specifically in paediatric populations is the relatively small number of patients available for inclusion and the limited volume of blood that can be drawn, allowing only few assays to be performed. Nonetheless, research in these populations is important to elucidate pathophysiological processes in a disease which still has a high mortality and morbidity. Furthermore, functional studies using RNA expression profiling provide additional information on the pathways involved in the pathogenesis of disease. This may direct the search for candidate genes. For example, *myosin* associated with celiac disease, rheumatoid arthritis, and systemic lupus erythematodes (SLE), also seems a good candidate for other inflammatory diseases.(34, 35) The MYO9B AAA haplotype was consistently associated with autoimmune disease. In celiac disease the aberrant myosin is thought to result in a compromised intestinal barrier enabling gluten peptides to enter the deeper mucosal layers where an inflammatory reaction can be elicited.(34)

Developments in genetic epidemiology

The requirement of large numbers of individuals has not always been met in past studies. Results of genetic association studies were contradicted, or at least the initially estimated odds ratio decreased in subsequent studies.(36, 37) This is not surprising given the large number of available SNPs in the human genome, and the extensive number of phenotypes that can be tested resulting in a low a-priori chance of a true association. Using meta-analysis a more realistic odds ratio can be estimated. (37) It must be taken into account, however, that special care has to be taken with regard to disease definition in pursuing this approach.(38) Outcome/phenotype

definition should be equal to or at least very similar to the original population, and at least the SNP that was observed to be associated with the phenotype studied should be included in the analysis to allow comparison. Additionally, for replicability, using the same genotyping platform in genome wide approaches facilitates comparison, in particular when raw data is made available. Furthermore, populations of different ethnic background should be tested to be able to generalize findings. Others, however, question whether replication makes sense at all. They argue that subdividing populations to meet the replication criteria results in a loss of power, and besides a reduced probability of finding false positives, also in a reduced opportunity to find true or universal associations.(39) Even despite the use of large populations and minimizing of between-study heterogeneity, a considerable portion of genuine associations may be found to be non-replicable.(13) Recently interim guidelines have been published to improve and assess quality of genetic association studies.(40) These provide a preliminary key for determining credibility of observed positive and negative observations, with regard to epidemiologic evidence. No consensus yet exists on guidance for rating clinical relevance and biological plausibility.(40)

Larger number of statistical tests performed, especially in the genome-wide microarray approach required the development of statistical methods capable of dealing with these massive amounts of data and to correct for multiple testing.(41) Bonferroni correction, the usually applied method is overly conservative and has already been rejected, since it assumes that all tests performed are independent, while in genetic association studies this is not the case, for example for tightly linked SNPs or for related phenotypes. Since it may be that not so much the number of analyses but the low a-priory probability of a true SNP-disease association is the problem, Bayesian methods may be more appropriate to determine the posterior probability of an association.(42)

Although different methods to correct for multiple testing have been compared by Tyrer et al. suggesting the use of a novel admixture maximum likelihood approach, no definite answer has been given so far.(43) The search continues for a test that allows correction for multiple testing, without substantial loss of statistical power. For now it is thought best to give p values as they are. This means that some findings that are now considered positive may in the future be regarded as false positives. A way to circumvent this is to use smaller p-values than 0.05 to be considered statistically significant. The size of p will then of course depend on the number of SNPs and the number of analyses performed. In addition, new insights, and the availability of increased computational capacity, resulted in progressively more stringent p values being accepted. For genome wide association studies (GWAS) a *P* value of $5*10^{-8}$ is currently considered genome wide statistically significant. Many different statistical packages have been developed and are currently being developed and used, some easier to use than others.(44). Usually, the application of one package is not sufficient requiring data transformation for use in other relevant packages. In this thesis, SPSS, Stata, Thesias, Genehunter, Haplostat, and R program were used. Construction of a package that would incorporate different statistical approaches is therefore expected to ease the work of scientists markedly.

As mentioned before, complex diseases are considered to have a multifactorial origin and two of the issues that arise are first, that it is statistically challenging even given the large number of samples in consortia, and, second, the expected interaction between individual SNPs and between SNPs and environmental factors. This is usually considered too difficult for current computer systems, and adds to the problems with multiple testing. Tackling of these problems will most likely improve our understanding of the pathogenesis of diseases and possibly even explain differences observed with regard to the effect of SNPs in different populations.

Differences in populations may in their turn also result in a form of confounding, known as population stratification. This is defined by the fact that disease risk is more similar in related individuals, particularly when they are from the same ethnic group, when compared to less or even unrelated individuals. Ethnicity itself does not per se explain the risk but may be a marker for lifestyle or socioeconomic status determining access to healthcare.(45) This may result in both false positive but also false negative findings. The role of population stratification is debated and is thought to cause real problems not when present, but only when the magnitude of its effect is essential. (45, 46) The potential impact of population stratification was demonstrated by Kidd et al. who reported a deletion in the APOBEC3A and APOBEC3B genes that was differentially distributed amongst Europeans and Africans when compared to East Asians and Amerindians or Oceanic populations.(47) No single SNP in the HapMap project tagged the deletion, which may result in false negative results in association studies.

Ways to approach this problem take into account that allele frequencies can differ between populations.(32, 33) Ideally an outbred population is used as a representative for the general population; however, this requires very large numbers of individuals. Another strategy is to use ethnically matched controls. In addition genomic control can be used to control for ethnic differences in subgroups. This method uses null SNPs (> 100), expected not to be involved in disease, genotyped in both cases and controls, to confirm descent of individuals, and to determine if population stratification or admixture plays a role. This method also provides ways to correct for population stratification or admixture: If a population is composed of a recent admixture of different ethnic groups that differ in marker allele frequencies and disease frequencies (or the quantitative trait means), spurious associations may result between the marker genotypes (or alleles) and the complex traits. However, it does not control for cultural or socio-economic aspects of ethnicity, which may result in differential penetrance of the disease associated allele.(45)

Implementation of polymorphisms in treatment and diagnosis

Genetic association studies are currently directed towards understanding disease susceptibility and severity in populations. Since multiple factors, both genetic and environmental are involved in codetermining the phenotype, the contribution, if present, of each individual SNP will be small as outlined above. Presence of a certain

allele will increase or decrease risk or chance for a certain phenotype but does not automatically result in disease. Application of the results to individual patients is still rare and currently restricted to less common diseases such as adapted therapy in AML patients or dosage adaptations of drugs such as warfarin in pharmacogenomics.(48-50) If any association is observed, specific therapy is not yet possible and intervention is usually directed at prevention and behaviour modification, a measure that is likely to benefit all individuals and not just those at increased risk for diseases like diabetes or cardiovascular disease.

SNPs can identify groups of patients at increased risk for severe disease and may assist in selecting patients for experimental therapeutic trials. Especially for rapid onset diseases, such as meningococcal disease this, however, requires the development of bedside assays, for instant identification of patients that might potentially profit from additional, experimental therapies. Additionally, functional studies of allelic variants may provide better understanding of pathophysiologic processes. This currently appears to be the major contribution of genetic association studies. Speculations on individualized medicine, testing each individual, arise. But without better understanding of the magnitude of these genetic variations, which is likely small of individual variants but might become substantial upon combining them, and the current lack of therapeutic intervention, adequate screening programs are not to be expected in the near future, even though extensive progress is being made. Scientific evidence is insufficient for useful assessment of genetic risk for most common diseases or for providing lifestyle recommendations using the predictive genomic profiling tests commercially available at the moment.(51) Currently in complex diseases, only 2-3% and in some maybe 5% of the estimated genetic variance, which is the proportion of differences between individuals explained by genetic differences, is explained by genes actually found to be associated with this disease.

To complicate matters, infectious diseases are not only determined by host susceptibility, but also by exposure, and microbe diversity and microbe genetic heterogeneity. As an increasing number of bacterial genomes is becoming available, assessing the role of microbial genes in virulence and pathogenesis provides a great opportunity.(52, 53)

In summary one can conclude that despite the marked progress that has been made in revealing genotype-phenotype associations in complex diseases, there are still several barriers, such as incoherent disease definition, unrecognized clinical subpopulations, insufficient population size, multiple testing, and non replication. Recently, guidelines for genetic association studies for both candidate gene and genome-wide approaches have been published.(54, 55) In addition, a network of investigator networks is being formed between researchers and together with the Human Genome Epidemiology Network (HuGENet™) they serve to facilitate replication possibilities, exchange of information and standardization of analyses (http://www.cdc.gov/genomics/hugenet/). (56, 57) These initiatives stress the need for good quality data and sample collection, development of adequate statistical packages, public availability of both positive and negative data, and replicating findings in other ethnic populations, and thereby will contribute to the improvement of the quality of genetic association studies.

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Chapter 6.3

Inflammatoire ziekten: Parallelen in de pathogenese

Nederlandse samenvatting

Inleiding

Dit proefschrift is het resultaat van een samenwerking tussen diverse klinische en preklinische afdelingen van het Erasmus MC, en enkele Nederlandse en buitenlandse centra. Het project werd geïnitieerd vanwege een gemeenschappelijke interesse in de rol van genetische variaties bij de gevoeligheid voor en de pathogenese van inflammatoire ziekten. Van de gekozen ziektebeelden werd verwacht dat er tenminste een gedeeltelijke overlap was in de pathogenese. Alle geïncludeerde ziektebeelden hebben inflammatoire componenten, die veroorzaakt worden door een gemeenschappelijke innate immuunrespons. Deze immuunrespons kan geïnitieerd worden door infectie of kolonisatie met bacteriën of virussen, of kan een gevolg zijn van een auto-immuun reactie. De beschikbaarheid van goed gedefinieerde, homogene patiëntencohorten was een voorwaarde voor inclusie in de studie, om de introductie van bias door slecht gedefinieerde heterogene cohorten te voorkomen.
In dit hoofdstuk zijn de resultaten uit dit proefschrift samengevat. Genetische polymorfismen werden geanalyseerd in patiënt cohorten met inflammatoire ziekten: meningokokken infecties, chronische hepatitis C virus (HCV) infectie en recidiverende acute middenoor ontsteking (AOM) als infectie ziekten; reumatoïde artritis (RA) uit de groep van auto-immuun ziekten; en Guillain Barré syndroom (GBS) en Barrett oesophagus als intermediair tussen infectie ziekten en auto-immuun ziekten. Daarnaast werden nasale dragerschap van *S. aureus* als risicofactor voor infectie, en sepsis na ernstig multitrauma als een inflammatoire aandoening bestudeerd.
In Tabel 1 worden de resultaten van de studies samengevat. Polymorfismen in *IL4*, *PAI1*, *TNFA*, *IL6*, *IL10* and *C1INH* waren geassocieerd met meer dan een van de bestudeerde ziektebeelden. Dit betekent dat de strategie, waarbij op basis van de literatuur bij aanvang van de studies werd gehypothetiseerd welke factoren gemeenschappelijk zijn in inflammatoire ziekten, correct is gebleken. Aangetekend moet worden dat sommige polymorfismen alleen werden bestudeerd in een of twee, maar niet alle patiënt cohorten (*CRP*, *CFH*, *MBL*, *CD14*, *IL1RN*, *TAFI*, and *MMP9*). Dit werd veroorzaakt door de specifieke interesse in bepaalde genen bij de betrokken afdelingen en centra.

IL4 -542 C/T polymorfisme in meningokokken infectie en nasale kolonisatie door *S. aureus*

Het *IL4* -524 C allel werd vaker door ouders aan patiënten met meningokokken infectie doorgegeven dan verwacht onder Mendeliaanse overerving (Hoofdstuk 2.4). In oudere individuen met het *IL4* -524 C/C genotype werd, ook na correctie voor confounders, vaker persisterend *S. aureus* dragerschap gevonden dan in individuen met het *IL4* -524 T allel (Hoofdstuk 5.3). De *IL4* hoge productie variant is geassocieerd met atopie, astma en ernstige respiratoire infecties.(1-3) Het *IL4* -524 C/C genotype is geassocieerd met lage *IL4* expressie, en lage IL-4 serum concentraties zijn

Tabel 1. Associaties tussen SNP en ziekten beschreven in dit proefschrift

Ziektebeeld	Gevoeligheid	Ernst	Effect van behandeling
Meningokokken infectie	*DEFB1*[1] G/G > UK *IL4* -542 C >, tdt *C1INH* 480 Val >, tdt	*DEFB1* G/G > DIC, UK[1] *DEFB1* C/C > mortaliteit, UK[1] *IL4* -524 T/T > mortaliteit, NL *TAFI* 325Ile/Ile > DIC, NL[2]	NB
Sepsis in trauma patiënten	*PAI1* 4G/4G >	-	NB
Guillain Barré syndroom	-	*MMP9* -1562 T > ernstig[3] *TNFA* -863 A/A > ernstig	NB
Reumatoïde artritis	*IL6* -174 G/G > *IL8* 781C/C > *PAI1* 5G/5G >	*TNFA* -308 G/G > ernstig *TNFA* -238 A/A > ernstig	NB
Barrett oesophagus	*IL1RN* 2018C >[4] *CD14* -260 T/T <[4] *CD14* -260 T/T en *TLR4* +896 A/A gecombineerd < *IL1B* -511 C/C + *IL1RN* 2081 T/T + CD14 -260T/C + TLR4 896 A/G gecombineerd <	NB	NB
Chronische HCV infectie	*IL6* -174 G/G > *TNFA* -857 T >	-	*IL10* -819 T *IL10* -1082A;-819T > virale respons *PAI1* 5G/5G SVR *PARP* 762 A < ETR[5]
Recidiverende acute otitis media	*IL6* -174 G/G >	*PAI1* 4G/4G > *TNFA* -238 G/G > *TNFA* -376 G/G > *TLR4* 299 A/A >	*TNFA* -238 G/G *TNFA* -863 A lagere specifieke antistof respons na vaccinatie *IL10* -1082 A/A: beschermt tegen toename AOM na complete vaccinatie
Staphylococcus aureus nasale kolonisatie	*C1INH* 480 Val > MBL haplotype A >[6] *IL4* -524 C/C > *CRP* haplotype 1184C; 2042C; 2911C <[6]	-	NB
Staphylococcus aureus infectie: steenpuisten	*CFH* Tyr402 > *CRP* 2911 C/C >		

NB, niet beschikbaar.
[1]Het *DEFB1* polymorfisme werd alleen gegenotypeerd voor meningokokken infectie en voor *S. aureus* dragerschap.
[2]*TAFI* polymorfismen werden alleen bepaald in patiënten met meningokokken sepsis DIC, gedissemineerde intravasculaire coagulatie; tdt, transmission disequilibrium test.
[3]*MMP9* werd alleen gegenotypeerd in patiënten met Guillain Barré syndroom en controles.
[4]*IL1RN* and *CD14* polymorfismen werden alleen gegenotypeerd in Barrett oesophagus patiënten en controles.
[5]ETR, 'end of treatment' respons.
[6]*MBL, CFH* and *CRP* polymorfismen werden alleen bepaald voor *S. aureus* dragerschap analyses.

geassocieerd met lage mucine productie.(4-6) Dit zou kunnen betekenen dat zowel een verhoogde gevoeligheid voor infectie met meningokokken als kolonisatie met *S. aureus* mede veroorzaakt worden door een verminderde mucociliaire klaring.
Niet zozeer lang bestaande, maar vooral recente kolonisatie met bacteriën geeft een verhoogd risico op infectie. Kolonisatie met micro-organismen genereert een

specifieke antistof productie; dit neemt echter enige tijd in beslag. Wanneer de kolonisatie pas recent heeft plaatsgevonden is er onvoldoende tijd geweest voor de gastheer om specifieke antistoffen te produceren, en gebrek aan deze specifieke antistoffen resulteert in een verhoogd risico op infectie.(7) De rol van IL-4 in systemische infecties is cruciaal, hetgeen gedemonstreerd wordt door de verhoogde gevoeligheid van IL-4 deficiënte muizen voor shock geïnduceerd door een combinatie van stafylokokken enterotoxine B en D-galactosamine.(8) IL-4, in combinatie met IL-10, beschermt muizen tegen een overactieve pro-inflammatoire immuunrespons tijdens infectie met *S. aureus*.(9) In volwassen sepsis patiënten zijn de IL-4 mRNA concentraties verlaagd in vergelijking met patiënten met bacteriemie of controles.(10)

Gezien deze resultaten zou men kunnen verwachten dat co-kolonisatie met *N. meningitidis* en *S. aureus* voorkomt in daarvoor gevoelige individuen. Alhoewel voor kinderen een positieve correlatie werd vastgesteld tussen kolonisatie door *N. meningitidis* en *S. pneumoniae* kolonisatie, bestaat er geen correlatie tussen *S. aureus* en meningokokken dragerschap.(11) Mogelijk is er slechts een beperkte interactie tussen *S. aureus* en meningokokken die co-kolonisatie van deze twee microben mogelijk maakt, zonder dat kolonisatie van de ander positief of negatief beïnvloed wordt door de aanwezigheid van de eerste. De interactie van de microben en de gastheer in een bepaalde niche, bijvoorbeeld de nasopharynx, zal de uitkomst bepalen na blootstelling van de gastheer aan het micro-organisme: asymptomatische kolonisatie, infectie, ernst van infectie, en uitkomst van infectie.(12)

IL-4 is een typisch Th2 cytokine.(6) Individuen met lage IL-4 productie vertonen een verhoogde gevoeligheid voor microbiële kolonisatie en infectie, terwijl hoge IL-4 productie genotypen geassocieerd zijn met astma en atopie. Dit onderstreept het belang van een adequate regulatie van zowel Th1 als Th2 respons, om beide ziekte-fenotypen hierboven te voorkomen.

PAI1 4G/5G polymorfisme is belangrijk in weefsel integriteit

PAI-1 inhibeert migratie en invasie van cellen, processen die essentieel zijn voor weefsel herstel bij inflammatoire ziekten.(13, 14) Hogere PAI-1 concentraties zouden kunnen resulteren in persisterende weefsel inflammatie, of abnormaal gerepareerd weefsel, dat gevoeliger is voor bacteriële kolonisatie en nieuwe infecties. Het -675 4G/4G promoter genotype is geassocieerd met hoge PAI-1 expressie.(15, 16) Dit hoge productie genotype is geassocieerd met een slechte uitkomst van meningokokken infectie.(17)

Kinderen met het *PAI1* 4G/4G genotype hadden, ook na correctie voor co-factoren, een verhoogd risico op meer frequente AOM episodes vergeleken met kinderen die homozygoot zijn voor de 5G variant. Dit geldt voornamelijk voor kinderen onder de leeftijd van 4 jaar (Hoofdstuk 4.1). Daarnaast was het *PAI1* 4G/4G genotype in patiënten met HCV infectie geassocieerd met een niet-permanente respons na therapie (Hoofdstuk 3.4) Na een aanvankelijk goede response was later toch weer

virus detecteerbaar. In multitrauma patiënten is dit genotype geassocieerd met gevoeligheid voor steriele sepsis (Hoofdstuk 2.9). De *PAI1* 5G homozygote variant was bovendien geassocieerd met verhoogde gevoeligheid voor RA (Hoofdstuk 3.2).
Er zijn minstens twee mogelijke mechanismen voor PAI-1 interactie en inhibitie van weefselherstel. Ten eerste zou PAI-1 de proteolytische cascade aan het celoppervlak kunnen inhiberen, die normaal resulteert in matrixdestructie, waardoor migratie en invasie van cellen, nodig voor weefsel herstel, mogelijk is. Tijdens deze proteolytische cascade wordt oppervlakte-gebonden plasminogeen geactiveerd door urokinase plasminogen activator (uPA). PAI-1 kan binden aan uPA op het celoppervlak en inhibeert zo de proteolytische activiteit en dus weefselherstel.(18-20) Het tweede mechanisme betreft een competitieve binding van PAI-1 en integrine aan vitronectine. Beide maken gebruik van dezelfde bindingsplaats op vitronectine. Vitronectine/integrine interactie is noodzakelijk voor cel migratie en adhesie. Het inhiberende effect van PAI-1 wordt ongedaan gemaakt wanneer een overmaat aan niet oppervlakte gebonden uPA een complex vormt met PAI-1 wat resulteert in dissociatie van PAI-1 van vitronectine, dat dan beschikbaar komt voor interactie met integrine, waardoor celadhesie weer mogelijk is.(21)
Een mogelijke rol voor hoge PAI-1 concentraties is de versterkte inhibitie van herstel van ontstoken otolaryngeaal weefsel, wat kan resulteren in een verhoogde gevoeligheid voor otitis. Vijftien dagen na myringectomie is er een vertraagd herstel van het geperforeerde trommelvlies in plasminogeen-deficiënte muizen, vergeleken met het trommelvlies van wild type muizen.(22) Intraveneuze toediening van plasminogeen aan plasminogeen deficiënte muizen resulteerde zelfs tot 30 dagen na de perforatie in herstel. De verdeling van inflammatoire cellen in het geperforeerde trommelvlies verschilde significant tussen plasminogeen-deficiënte en wild type muizen. Plasminogeen-deficiënte muizen met aanvankelijk normale trommelvliezen ontwikkelden spontaan middenoor effusie. In een otitis media model in de rat is de expressie van uPA verhoogd tijdens infectie met *Streptococcus pneumoniae*.(23-26) Interactie van PAI-1 met uPA voorkomt de katalytische activatie van plasminogeen naar plasmine. Hoge PAI-1 concentraties kunnen daarom resulteren in dezelfde klinische fenotypen als bij plasminogeen deficiëntie. Bovendien wordt bij weefsel herstel van de huid expressie van PAI-1, uPA en uPA-receptor gevonden ter plaatse van de regeneratieve epitheel uitgroei aan de rand van de wond.(27, 28) Plasminogeen deficiënte muizen hebben een verstoorde wondgenezing.(29)
Het pleiotrope PAI-1 molecuul is ook betrokken bij fibrose.(30) Bovendien beschermt PAI-1 deficiëntie tegen fibrosering in diverse diermodellen.(31) Plasminogeen deficiëntie interfereert met de klaring van debris na acuut letsel, en resulteert in de activatie van hepatische stellaire cellen die betrokken zijn bij progressie van fibrose. (32, 33) Omdat verhoogde PAI-1 concentraties wondgenezing inhiberen en fibrose induceren, is het redelijk aan te nemen dat dit mechanisme bijdraagt aan de niet-permanente respons op de behandeling met peg-interferon alfa-2a plus ribavirine in *PAI1* 4G homozygote HCV geïnfecteerde patiënten (Hoofdstuk 3.4).
Of de rol van PAI-1 in pathofysiologische processen in otitis media en HCV infectie gelijk is aan die voor gevoeligheid voor sepsis in multitrauma patiënten is nog niet

duidelijk, maar wel aannemelijk. PAI-1 concentraties bij opname zijn verhoogd in multitrauma patiënten.(34) Dit resulteert in een verlaagde fibrinolyse, en de vorming van microthrombi in gedissemineerde coagulatie zoals in meningokokken sepsis. Het hoge productie *PAI1* 4G/4G promoter genotype is geassocieerd met verhoogde mortaliteit.(34) Dit laatste kon in de huidige studie niet bevestigd worden, mogelijk door een minder ernstig ziektebeeld en verbeterde behandelprotocollen in de huidige studiepopulatie.
De rol voor PAI-1 in reumatoïde artritis lijkt te verschillen van de mechanismen zoals hierboven beschreven voor otitis media, HCV infectie and sepsis in trauma patiënten. De *PAI1* 5G homozygote variant, gerelateerd aan lage PAI-1 concentraties en dus verminderde inhibitie van plasminogeen activator was geassocieerd met RA in vergelijking met controles. Er was geen associatie met de ernst van RA. Plasminogeen deficiëntie is geassocieerd met verlaagde gevoeligheid voor en verminderde ernst van artritis in muismodellen.(35) PAI-1 deficiëntie is echter ook geassocieerd met verminderde ernst in antigeen-geïnduceerde artritis in muizen.(36) Over het algemeen is de rol van factoren betrokken bij weefselherstel en integriteit, zoals filaggrine en myosine, de laatste jaren duidelijker geworden, zoals geïllustreerd wordt in respectievelijk atopische dermatitis en coeliakie.(37-39) Twee genetische varianten resulterend in functie verlies van filaggrine, zijn sterk geassocieerd met atopische dermatitis.(38) Dit onderstreept het belang van weefselherstel en integriteit in de pathogenese van inflammatoire ziekten.

IL6 en verhoogde gevoeligheid voor inflammatoire ziekten

Het *IL6* -174 G/G genotype was oververtegenwoordigd in reumatoïde artritis patiënten (Hoofstuk 3.2), en individuen met hepatitis C infectie (Hoofdstuk 3.4) vergeleken met gezonde controles. Bovendien werd het *IL6* -174 G/G promoter genotype frequenter gevonden in AOM patiënten (Hoofdstuk 4.2). Dit ondersteunt de vondst van verhoogde expressie van IL-6 tijdens experimentele otitis media in dieren.(40, 41) Het *IL6* G/G genotype is geassocieerd met verhoogde IL-6 concentraties vergeleken met het C/C genotype. Deze SNP draagt dus bij aan de complexe regulering van de IL-6 productie, waarbij meerdere polymorfismen betrokken zijn.(42-45) Bovendien wordt de IL-6 expressie beïnvloed door TNF-α, en interactie van polymorfismen in deze en andere genen zal het fenotype mede bepalen.
De hoge productie variant komt meer voor in persisterende HCV infectie vergeleken met spontane virale klaring.(46) Patiënten met chronische hepatitis C hebben een verhoogde IL-6 expressie vergeleken met gezonde controles. Of dit secundair is aan de infectie, of een verhoogde gevoeligheid of verminderde klaring van infectie met HCV weerspiegelt, is nog niet duidelijk.(47) De substantiële verbetering van RA door behandeling met anti-IL-6 receptor antistof onderstreept het belang van dit pleiotrope cytokine in de pathogenese. (48) De exacte functionele rol van IL-6 in de toename van de gevoeligheid voor deze ziekten is nog niet bekend. Experimentele modellen die de vroege fase van ziekte analyseren in normale en IL-6 deficiënte dieren kan een beter inzicht opleveren over de rol van IL-6 in de gevoeligheid voor RA.

IL10 lage productie varianten vergemakkelijken pro-inflammatoire responsen

IL-10 is een bekend anti-inflammatoir cytokine, dat samen met andere cytokinen zoals IL-4 zorgt voor een tegenbalans bij de pro-inflammatoire respons die veroorzaakt wordt door infectie. Het promoter -1082A;-819T haplotype is eerder geassocieerd met een verminderde expressie van IL-10.(49-51)
In de huidige studie was dragerschap van het *IL10* -819T allel geassocieerd met een snelle virale respons (een vermindering of ondetecteerbaar worden van virale titers) in HCV patiënten na 6 weken behandeling met peg-interferon alfa-2a plus ribavirine (Hoofdstuk 3.4). Het lage productie haplotype van de *IL10* promoter was geassocieerd met virale respons aan het eind van de behandeling en persisterende virale respons 24 weken na het beëindigen van de behandeling.
Bovendien was het *IL10* -1082 A/A genotype geassocieerd met bescherming tegen AOM na vaccinatie (Hoofdstuk 4.2). Deze observaties wijzen op een verbeterde pro-inflammatoire response in lage IL-10 producerende patiënten met recidiverende AOM gevaccineerd tegen pneumokokken en patiënten behandeld in verband met HCV infectie. Het eerste concept wordt ondersteund door observaties in proefdier studies. In IL-10 deficiënte muizen, geïmmuniseerd met een niet-virulente ongekapselde *S. pneumoniae* (stam R36A), werd een verhoogde inductie van pro-inflammatoire cytokinen geobserveerd, wat de hypothese ondersteunt dat individuen met lage IL-10 productie een betere respons hebben op vaccinatie. Antistof titers tegen pneumokokken eiwitten waren verhoogd in IL-10 deficiënte muizen vergeleken met wild type muizen.(52) De huidige resultaten impliceren dat het mogelijk is individuen te identificeren die meer waarschijnlijk dan anderen een matige respons hebben op vaccinatie. Deze individuen kunnen in de toekomst mogelijk geselecteerd worden voor aangepaste vaccinatie regimes, waardoor optimale vaccinatie voor meerdere individuen mogelijk is.

C1INH polymorfisme in meningokokken infectie en nasale kolonisatie met *S. aureus*

De *C1INH* 480 Val variant was geassocieerd verhoogde gevoeligheid voor meningokokken infectie en ook met nasale dragerschap van *S. aureus* (Hoofdstukken 2.4 en 5.1).
Het complementsysteem is essentieel bij de afweer tegen bacteriële infecties. Dit wordt geïllustreerd door de verhoogde gevoeligheid voor meningokokken infectie van mensen met een complement deficiëntie, en door de mechanismen die *S. aureus* heeft ontwikkeld om het complement systeem te kunnen ontwijken.(53, 54) Overactivatie van de complement cascade wordt voorkomen door de serine protease C1 inhibitor (C1-Inh), dat een belangrijke regulator is. Dit multifunctionele eiwit inhibeert ook de activiteit van contactsysteem proteases, zoals kallikrein en stollingsfactor XII, en is betrokken bij het binden van leukocyten aan het endotheel bij de extravasatie.(55-57)

Omdat de functionele relevantie van de V480M substitutie nog bepaald moet worden, is de rol van dit polymorfisme in stafylokokken kolonisatie en de pathogenese van meningokokken infectie onbekend.(58) Eerder werd geen verschil gevonden tussen de V480 C1-Inh en de M480 C1-Inh met betrekking tot structuur, eiwit stabiliteit, serum concentratie of inhibitie van C1.(59) Het is daarom onwaarschijnlijk dat er een verschil is in de directe interactie tussen microben en C1-Inh.(60) Het is mogelijk dat het polymorfisme een effect heeft op een van de andere functies van C1-Inh.(56, 59) Toekomstige opsonofagocytose experimenten of karakterisering van complement depositie op bacteriële cellen met behulp van serum monsters van donoren met verschillende *C1INH* genotypen zijn noodzakelijk om de moleculaire basis van (indirecte) microbe- C1-Inh interacties te bepalen.

TNFA expressie: te veel, of te weinig?

De *TNFA* -863 A variant was geassocieerd met ernstiger beloop van GBS en een verminderde specifieke antistof respons na anti-pneumokokken vaccinatie (Hoofdstukken 3.1 en 4.2). Een eerdere studie toonde dat het A-allel op positie -863 van *TNFA* geassocieerd was met hogere TNF-α concentraties door een inadequate inhibitie van de productie.(61) Eerdere publicaties toonden ook een associatie aan tussen hoge serum concentraties van TNF-α en de ernst van GBS.(62-66) Dit wordt mogelijk gemedieerd door de mate van macrofaag activatie, die onvoldoende gecontroleerd kan zijn, en dus sterk pro-inflammatoir.
Daarnaast was het *TNFA* -238 G/G genotype geassocieerd met frequenter recidiverende AOM, terwijl het -238 A/A genotype geassocieerd was met ernstiger RA (Hoofdstukken 3.2 en 4.2).
De interpretatie van deze bevindingen is complex. Zowel een te hoge als een insufficiënte *TNFA* expressie kan ernstiger ziekte bij AOM verklaren. Een overschot aan TNF-α kan resulteren in verhoogde weefselschade en gevoeligheid voor recidiverende infecties, terwijl een verlaagde TNF-α respons kan verhinderen dat een adequate klaring of inactivatie van microben optreedt, resulterend in persisterende infectie. Het effect van het TNFA -238 polymorfisme op TNF-α expressie is onduidelijk. In het recente verleden zijn diverse studies uitgevoerd naar de associatie tussen *TNFA* promoter polymorfismen en TNF-α levels in verschillende inflammatoire- en infectie ziekten, die tegenstrijdige resultaten rapporteerden.(67) TNF-α expressie wordt door een combinatie van meerdere polymorfismen in *TNFA* en -geassocieerde genen bepaald. Dit wordt nog eens gecompliceerd door de lage mutant allelfrequentie. Kleine veranderingen in de aantallen kunnen de resultaten substantieel wijzigen, wat het risico van vals-positieve en vals-negatieve resultaten vergroot. Bovendien kunnen verschillende pathogenen een variabele cytokine signatuur induceren. Omdat diverse pathogenen, zowel bacterieel als viraal, otitis kunnen veroorzaken, is het bepalen van de rol van de polymorfismen in TNF-α productie in de humane situatie erg moeilijk. Bovendien is timing van monstername een erg belangrijke determinant van de resultaten, omdat TNF-α concentraties zullen veranderen gedurende de infectie.

Verschil in expressie van TNF-α beïnvloedt waarschijnlijk de gevoeligheid en ernst van ziekte. TNF-α stimuleert bijvoorbeeld immunoglobuline en mucine productie. Lage TNF-α concentraties compromitteren deze afweermechanismen.(68) TNF-α concentraties in nasofaryngeaal secreet zijn verlaagd in kinderen met recidiverende otitis media vergeleken met gezonde kinderen.(69)
De associaties tussen diverse *TNFA* polymorfismen en AOM, RA, en GBS beschreven in dit proefschrift tonen dat er waarschijnlijk een rol is voor deze polymorfismen in TNF-α productie *in vivo*. Analyse van TNF-α expressie in onze patiënt cohorten in relatie tot de *TNFA* haplotypen, zou het effect van de polymorfismen getypeerd in deze patiënten kunnen verhelderen.

Conclusie en perspectieven

Bovenstaande toont aan dat diverse polymorfismen beschreven in dit proefschrift inderdaad geassocieerd zijn met twee of meer van de bestudeerde fenotypen. Dit ondersteunt de hypothese dat soortgelijke pathogenetische processen betrokken zijn in verschillende ziekten. Terwijl de observaties consistent waren voor enkele polymorfismen, lijken er ook voor bijvoorbeeld SNPs in TNFA verschillen te bestaan tussen de diverse ziektebeelden. Een lage allelfrequentie, gevonden in relatief kleine patiënt populaties, met daardoor een verhoogd risico op zowel vals-positieve als vals-negatieve resultaten kan hieraan bijgedragen hebben. Dit wordt verder geïllustreerd door het verlies van statistische significantie na correctie voor multiple testen. Men moet zich echter bedenken dat het effect van genetische polymorfismen beperkt is, en dat er meerdere SNPs en ook omgevingsfactoren betrokken zijn bij complexe ziekten.
Observationele studies in samenhang met gecontroleerde (*in vitro*/proefdiermodellen) experimenten, naar eiwit concentraties te, zoals beschreven voor TAFI, ADAMTS13, VWF, and MIF in meningokokken infectie (Hoofdstukken 2.5 tot en met 2.7) zijn noodzakelijk om de relevantie van SNPs die geassocieerd zijn met ziekte te bepalen. Daarnaast kunnen RNA expressie profielen zoals beschreven in hoofdstuk 2.8 een genetische associatie ondersteunen, en nieuwe gezichtspunten genereren en kandidaat genen alsmede biologische processen identificeren die betrokken zijn bij de gen regulatie in de pathogenese van inflammatoire ziekten.
De toenemende interesse en het belang van weefselintegriteit worden ondersteund door de observaties voor het *PAI1* polymorfisme in AOM, HCV infectie, en mogelijk sepsis in multitrauma patiënten. Daarnaast verdient de rol van *IL4* and *C1INH* in gevoeligheid voor nasale *S. aureus* dragerschap en gevoeligheid voor infectie met meningokokken verdere aandacht. Functionele studies die de bijdrage van deze moleculen aan de pathogenese bestuderen zijn derhalve noodzakelijk.

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Curriculum vitae

Name: Marieke Emonts
Date of birth: January 20, 1975
Place of birth: Venray, the Netherlands

Education

1987 – 1993	Secondary education, Jerusalem College, Venray, the Netherlands (Dutch, English, French, Latin, Mathematics, Chemistry, Physics, Biology).
1993 – 2001	Biomedical Science and Medicine, Leiden University Medical Centre, Leiden, the Netherlands
2001	MSc degree cum laude.
1995 – 1998	Masters degree in Medicine, University Leiden.
2000	State qualification Medical School, University of Leiden, the Netherlands, MD degree cum laude.
2003-2007	Training for Biomedical Scientist Immunology, followed by registration as immunologist; Foundation for Scientific Training in Medical Biology (SMBWO) Immunology, finished with thesis.

Courses

1994	Radiation safety course, level 4B, University of Leiden, the Netherlands.
1994	Laboratory Animal Science, article 9, University of Leiden, the Netherlands.
2001	Boerhaave course on pneumococcal infections, University of Leiden, the Netherlands.
2002	PhD course Molecular Immunology, department of Immunology, Erasmus MC, Rotterdam, the Netherlands
2003	Erasmus MC Summer Program: data-analysis, regression-analysis and statistical models in epidemiology.
2004	Course 'Infection and Immunity in Children 2004', St Catherine's College, Oxford, United Kingdom.
2005	Erasmus MC Summer Program, Bioinformatics in medicine, Genetic epidemiology of complex diseases.
2006 & 2008	Royal Dutch Society of Science (KNAW) colloquium and master classes: "The role of DNA polymorphisms in complex traits and diseases".
2007	Advanced Paediatric Life Support (APLS).

2007 Course 'Infection and Immunity in Children 2007', St Catherine's College, Oxford, United Kingdom.

2007 Diabetes course organised by Novo Nordisk, Hoevelaken, the Netherlands.

2007 Course on pharmacotherapy in children, Utrecht, the Netherlands.

Research projects

1998 Heatstroke, Internal Medicine LUMC, Leiden, the Netherlands. Supervisor: Prof.dr. A.E. Meinders, MD PhD.

1998 Characterisation of a case of atypical beta-thalassaemia, Department of Anthropogenetics, LUMC, Leiden, the Netherlands. Supervisor: Dr. C.L. Harteveld, PhD.

1998 Alternative translation of LAGE-1, Department of Clinical Oncology, LUMC, Leiden, the Netherlands. Supervisor: Dr. P.I. Schrier, PhD, and Dr. C.A. Aarnoudse, PhD.

1999 Adnexal torsion in young girls, Gynaecology, Groene Hart Hospital, Gouda. Supervisor: J.F. Admiraal, MD.

2000- 2001 Characterisation of PpmA, a surface protein of *Streptococcus pneumoniae*, Laboratory of Paediatrics, Division of Paediatric Infectious Diseases and Immunology, Department of Paediatrics, Erasmus MC-Sophia Children's Hospital, University Medical Centre Rotterdam, the Netherlands. Supervisors: Prof.dr. R. de Groot MD, PhD, Prof.dr. P.W.M. Hermans, PhD, and Dr. P.V. Adrian, PhD.

2002-present Polymorphisms in immune response genes in infectious diseases and autoimmune diseases. Laboratory of Paediatrics, Division of Paediatric Infectious Diseases and Immunology, Department of Paediatrics, Erasmus MC-Sophia Children's Hospital, University Medical Centre Rotterdam, the Netherlands. Supervisors: Prof.dr. R. de Groot MD PhD, Prof.dr. J.D. Laman PhD, and Prof.dr. P.W.M. Hermans PhD

Presently Genome wide association study in meningococcal infection, in collaboration with UK and Austrian research groups.

Employment

1996-1997 Ward attendant short stay and daycare, Hospitals Noord-Limburg

05-12/2001 Junior Registrar (AGNIO) at the Erasmus MC Sophia Children's Hospital, University Medical Centre Rotterdam, the Netherlands

2002-present Combined residency in Paediatrics and research training program (AGIKO) on polymorphisms in immune response genes in infectious diseases and autoimmune diseases.

AGIKO supervisor: Prof.dr. R. de Groot, MD PhD
Residency supervisors: Prof.dr. A.J. van der Heijden, MD PhD and Dr. M. de Hoog, MD PhD for the Erasmus MC-Sophia Children's Hospital, University Medical Centre Rotterdam, the Netherlands; and Dr. A.A.P.H. Vaessen-Verberne, MD PhD for the Amphia Hospital, Breda, the Netherlands. The Paediatric residency will be finished in December 2009.

2001-present Training of three medical students and a technician during their research projects.

Grants and awards

2004 Hoffmann La-Roche grant for a technician for project: Genetic polymorphisms in host and pathogen associated with infection: chronic hepatitis C.
2006 NVVI travel grant for ISPPD conference Australia.
2007 ESPID sponsored grant to attend Course on "Infection and Immunity in Children 2007," St Catherine's College, Oxford, United Kingdom.
2008 ESPID Young Investigator Award 2008.

List of publications

1. Giordano, P. C., C. L. Harteveld, H. L. Haak, D. Batelaan, P. van Delft, R. J. Plug, M. Emonts, R. Zanardini, and L. F. Bernini. 1998. A case of non-beta-globin gene linked beta thalassaemia in a Dutch family with two additional alpha-gene defects: the common -alpha3.7 deletion and the rare IVS1-116 (A-->G) acceptor splice site mutation. *Br J Haematol 103:370*.
2. Emonts, M., and A. E. Meinders. 2000. Hitteberoerte: pathofysiologie en pathogenese [Heat stroke: pathophysiology and pathogenesis]. *Ned Tijdschr Geneeskd 144:509*.
3. Emonts, M., J. A. Hazelzet, R. de Groot, and P. W. M. Hermans. 2003. Host genetic determinants of Neisseria meningitidis infections. *Lancet Infect Dis 3:565*.
4. Emonts, M., H. Doornewaard, and J. C. Admiraal. 2004. Adnexal torsion in very young girls: diagnostic pitfalls. *Eur J Obstet Gynecol Reprod Biol 116:207*.
5. Emonts, M., and R. de Groot. 2004. Sepsis syndrome in children: can we do better? *Adv Exp Med Biol 549:63*.
6. Emonts, M., R. De Groot, and P. W. M. Hermans. Genetic susceptibility to *Neisseria meningitidis* infections. The Netherlands Journal of Medicine, Supplement 62(3):28-35, 2004.
7. Emonts M., P. W. M. Hermans, R. De Groot, and J. A. Hazelzet. Genetische factoren van de gastheer in infecties met *Neisseria meningitidis*. Tijdschr Kindergkd.5:163-171, 2004
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The persons who are a master in the art of living make little distinction

between their work and their play, their labor and their leisure,
their mind and their body,

their education and their recreation, their love and their religion.
They hardly know which is which.

They simply pursue their vision of excellence and grace in whatever they do,

leaving others to decide whether they are working or playing.

To them, they are always doing both.

(Anonymous, Zen text)