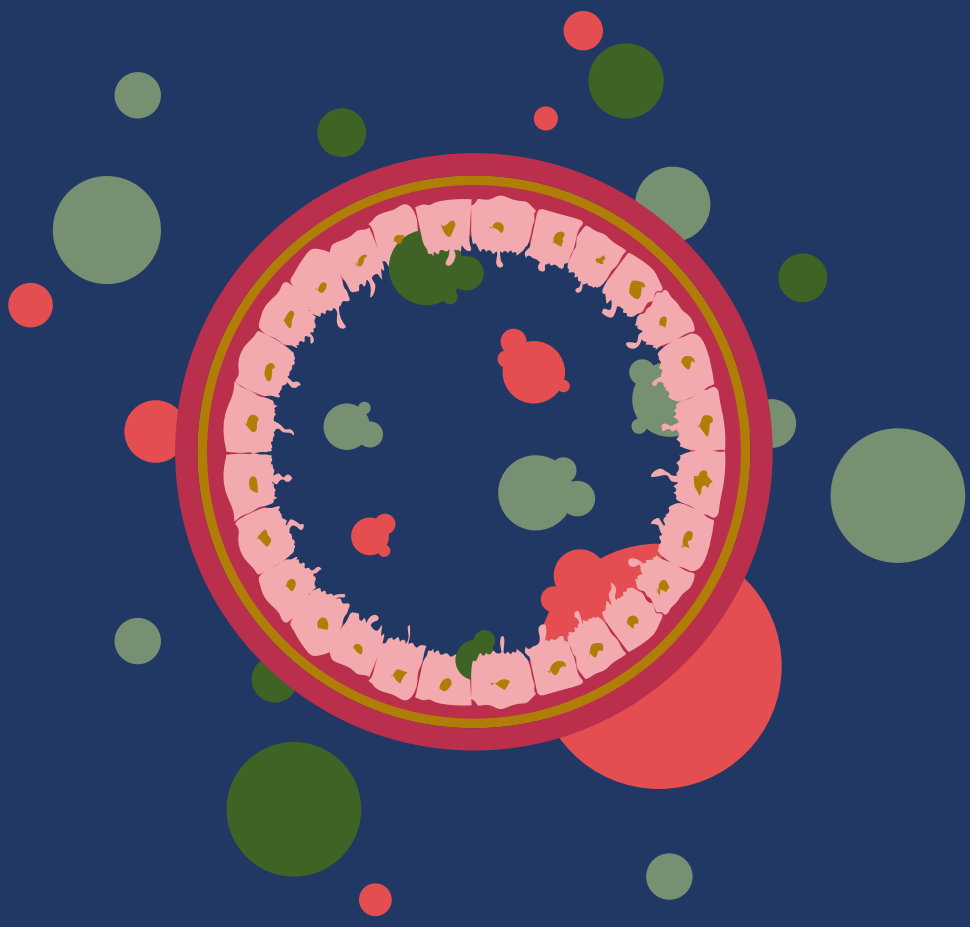
Pediatric Sepsis:

Determinants of outcome



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Voor mijn ouders;

Dankbaar voor de offers die
zij hebben gebracht

om hun kinderen een betere
toekomst te gunnen.

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Chapter 1

General introduction

In children younger than 5 years of age, infectious diseases kill almost three million children annually, accounting for almost half of all child deaths worldwide.[1] (Figure 1A) Sepsis is life-threatening due to organ dysfunction as a result of a dysregulated host response to infection.[2] The *World Health Organization* urges member states in a very recent resolution to take specific actions on prevention, diagnosis, and management to reduce the burden of sepsis, including *promoting research to develop innovative means to prevent, diagnose, and treat sepsis*.[3]

In developed regions, the total number of deaths and the proportion of deaths caused by infectious diseases is much lower than globally (10.0000 deaths caused by infectious diseases, which is approximately 10% of all causes of death).[1] (Figure 1B) Although the outcome of pediatric sepsis improved the past decades, this merely has been attributed to improved prevention and overall improvement of pediatric critical care instead of newly developed therapeutic strategies.[4, 5] To date, the cornerstone of sepsis management still is early recognition, aggressive fluid therapy/circulation support, early antibiotic treatment, and optimal supportive care.[6]

Determinants of pediatric sepsis

The incidence and outcome of sepsis is determined by multiple factors, such as host factors (eg genetic predisposition, immune response to bacteria), pathogen factors, and health-care system factors. (Figure 2) A yet under recognized determinant of outcome could be circadian variation, since clustering of fatal meningococcal cases in the morning hours has been reported.[7] Because of many contributing determinants, sepsis studies often involve heterogeneous study populations, making it difficult to apply findings to whole groups of sepsis patients.

Outcome has commonly been defined as *mortality* in the majority of pediatric sepsis studies. However, there is increasing evidence showing that acquired cognitive impairment, functional disability, and impaired quality of life are common amongst sepsis survivors.[8-10] Data on disability in pediatric sepsis survivors is lacking, but is essential to reflect the impact of sepsis from a societal point of view.[11, 12]

Genetic predisposition

The first study, from 1988, reporting a link between genetics and infectious diseases was based on 960 families that included children who were placed with adoptive parents unrelated to them early in life.[13] Adoptees and biological parents had more often an *infectious cause of death* in common, whereas the proportion of *infectious cause of death* in adoptees and adoptive parents did not differ from *all causes of death*.

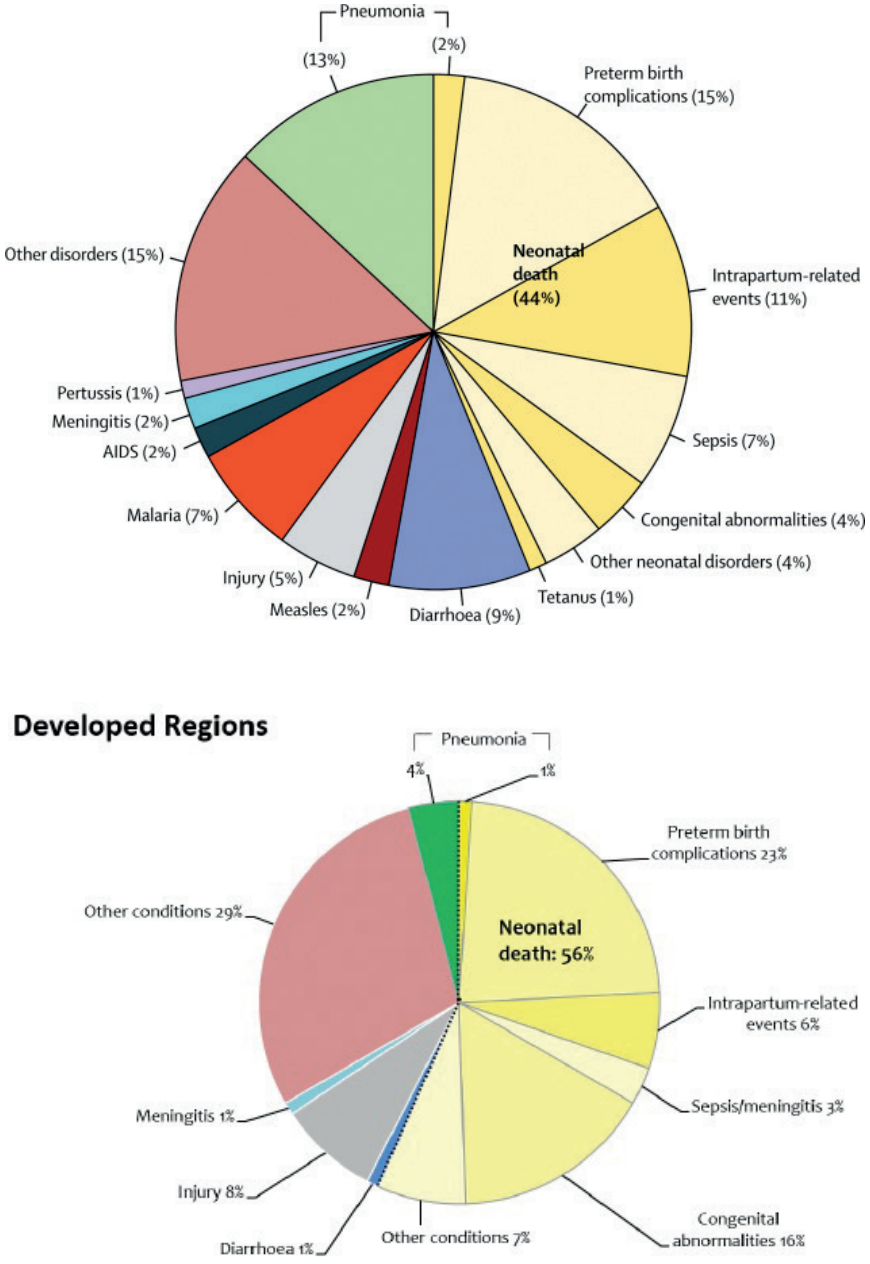

Figure 1: Causes of deaths in children younger than 5 years of age.

(Reprint with permission from [1])

A) Infectious diseases account for almost half of all child deaths worldwide.

B) In developed regions, approximately 10% of child deaths are attributable to infectious causes.

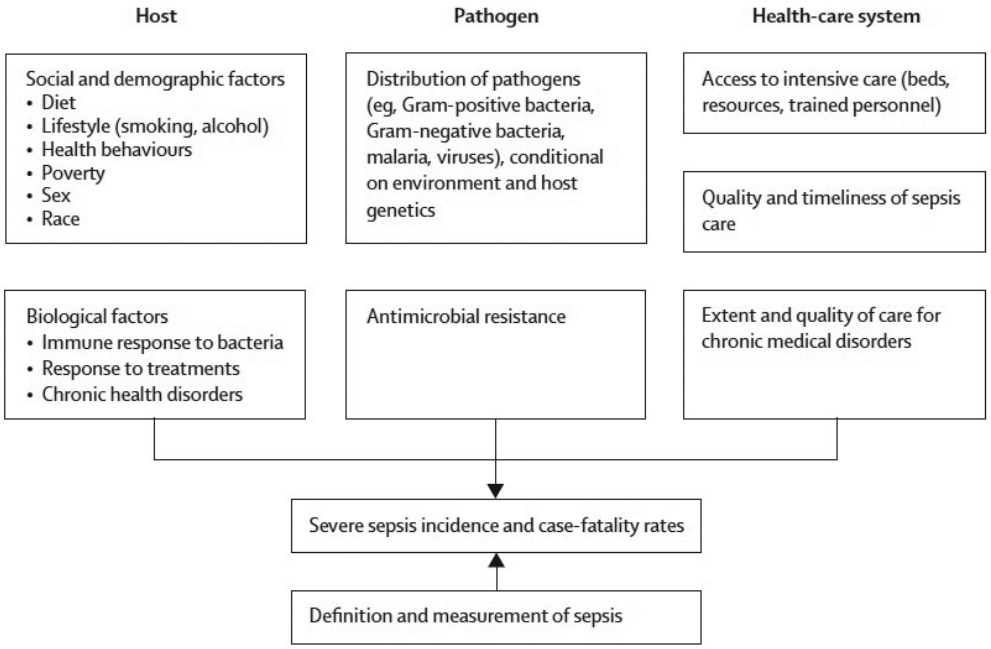

Figure 2: Multiple factors determining the incidence and outcome of sepsis.
(Reprint with permission from [11])

Previous studies have tried to identify which specific polymorphisms in genes are associated with susceptibility and severity of sepsis.[14-17] However, these studies were mainly based on a candidate-gene approach in relatively small patient cohorts. Due to the exponential increase of possibilities in the field of genetic research, genome-wide association studies (GWAS) are now able to overcome this biased approach. The first GWAS study in meningococcal disease patients has identified polymorphisms in the *CFH* region, which plays a role in complement activation, and therefore may be associated with meningococcal sepsis susceptibility.[18]

The host response to sepsis: inflammation and hemostasis

The host response to infection involves complex interplays between inflammation, coagulation, and fibrinolysis.

The inflammatory response[19-21] to infection is characterized by two stages and includes innate and adaptive immune responses. (Figure 3) A pro-inflammatory response is initiated by pattern-recognition receptors of the innate immune system (e.g. monocytes, macrophages, neutrophils, and dendritic cells) sensing pathogens (pathogen-associated molecular patterns) or stress signals (danger-associated

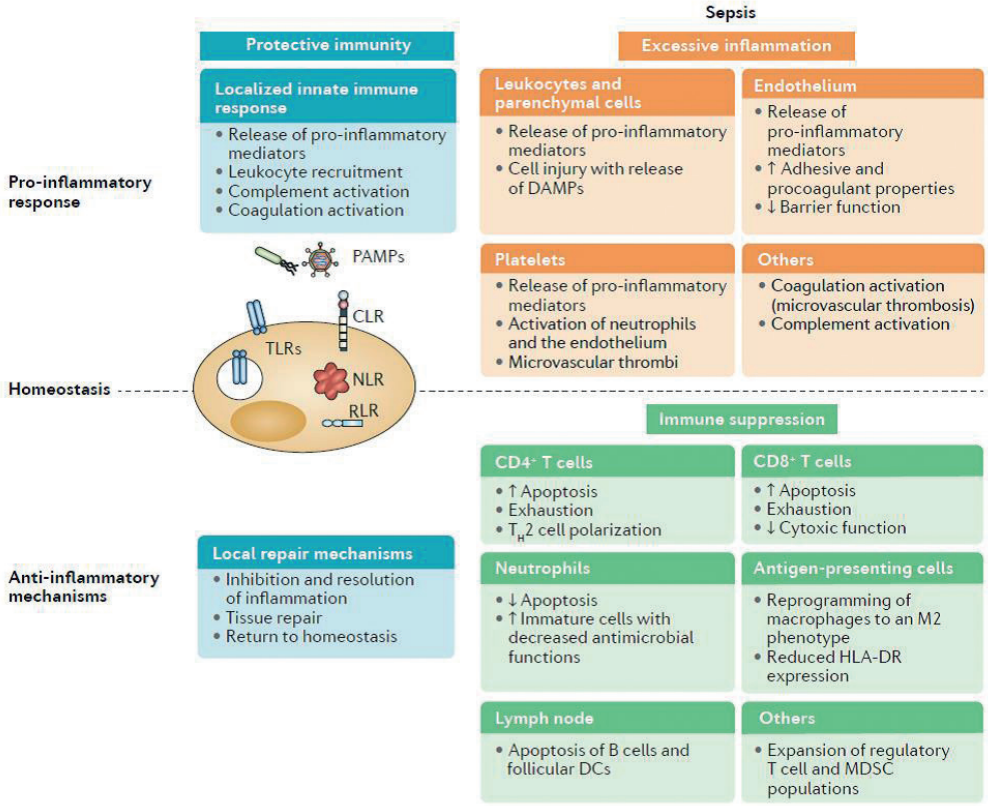

Figure 3: The inflammatory response to sepsis.

(Reprint with permission from [21])

The inflammatory response includes a pro-inflammatory and an anti-inflammatory response. An initial pro-inflammatory response is initiated by PAMPs sensed by immune cells (e.g. leukocytes and parenchymal cells, endothelial cells, and platelets) through an assortment of cell-surface and intracellular pattern recognition receptors (e.g. TLRs, NLRs, RLRs, and CLRs). Various pro-inflammatory cytokines and chemokines are released to neutralize the infection. Also, an anti-inflammatory compensatory mechanism restrains the initial inflammation, prevents collateral tissue damage, and restores homeostasis. An unbalanced and harmful response may result from prevailing and multiplying of the pathogen despite an activated immune response, leading to a concurrent excessive inflammation (top right). Extended release of anti-inflammatory mediators could result in immune suppression (bottom right).

Abbreviations: CLR = C-type lectin receptors, DCs = dendritic cells, PAMPs = pathogen-associated molecular patterns, DAMPs = danger-associated molecular patterns, MDSC = myeloid-derived suppressor cell, NLR = nucleotide-binding oligomerization domain-like receptors, RLR = retinoic acid-inducible gene-like receptors, TLRs = Toll-like receptors.

molecular patterns). This detection leads to an intracellular signal with activation of transcription factors, leading to the release of various pro-inflammatory cytokines (e.g., TNF-α, IL-1, IL-6) and chemokines that attract even more immune cells, enhancing phagocytosis. Additionally, proteins of the complement system (e.g. C1q and mannan-binding lectin) bind to the surface of pathogens and augment destruction. These pro-inflammatory factors also mount the more specific adaptive immune response, which depends on antigen presentation via major histocompatibility complex (MHC) molecules to lymphocytes. Two classes of lymphocytes, T cells and B cells, are responsible for cell-mediated immune responses and antibody responses, respectively. By cell-mediated immunity, T cells directly recognize and destroy infected cells, whereas the production of antibodies against one specific pathogen by B cells provides humoral immunity.

Simultaneous to the pro-inflammatory response, a systemic inhibition of the immune system occurs in order to restore homeostasis.[22] The result is that monocytes and macrophages have diminished capacity to release pro-inflammatory cytokines upon stimulation and blood monocytes are reprogrammed with reduced expression of HLA-DR. Additionally, there is an increase in T cell apoptosis and a release of anti-inflammatory mediators to counteract continual inflammation.

Usually, the combined pro- and anti-inflammatory response is able to combat the infection, without becoming unbalanced and harmful. However, an excessive pro-inflammatory response can result in early mortality in sepsis due to cardiovascular collapse and multiple organ dysfunction. Also, an extended release of anti-inflammatory mediators (termed immunoparalysis) can potentially result in failure to clear primary infections and increases susceptibility to new infections, resulting in late sepsis mortality.

The hemostatic response[23-25] initiates after the overwhelming pro-inflammatory response damages the microvascular endothelium. Subsequently, tissue factor is released and increasingly expressed by endothelial cells. (Figure 4) The tissue factor-factor VII pathway ultimately results in the generation of thrombin, and the conversion of fibrinogen to fibrin. In normal circumstances, activation of coagulation is controlled by three important physiological anticoagulant pathways: the antithrombin system, tissue factor pathway inhibitor (TFPI), and the activated protein C pathway. In sepsis, decreased activity of all three natural anticoagulant mechanisms results from a combination of impaired synthesis, ongoing consumption, leakage into the interstitial space, and proteolytic degradation. Lastly, fibrinolysis is impaired by sustained increase in plasminogen activator inhibitor, type 1 (PAI-1). Moreover, this increased production of PAI-1 leads to direct inhibition of activated protein C (APC). Altogether, these mechanisms result in coagulation abnormalities ranging from subtle derangements only detectable by highly sensitive assays to widespread deposition of fibrin throughout the microcirculation, manifesting as disseminated

intravascular coagulation (DIC), as typically seen in meningococcal sepsis under these particular circumstances. Ultimately, DIC contributes to the need to amputate extremities, to multiple organ dysfunction, and can eventually result in death.

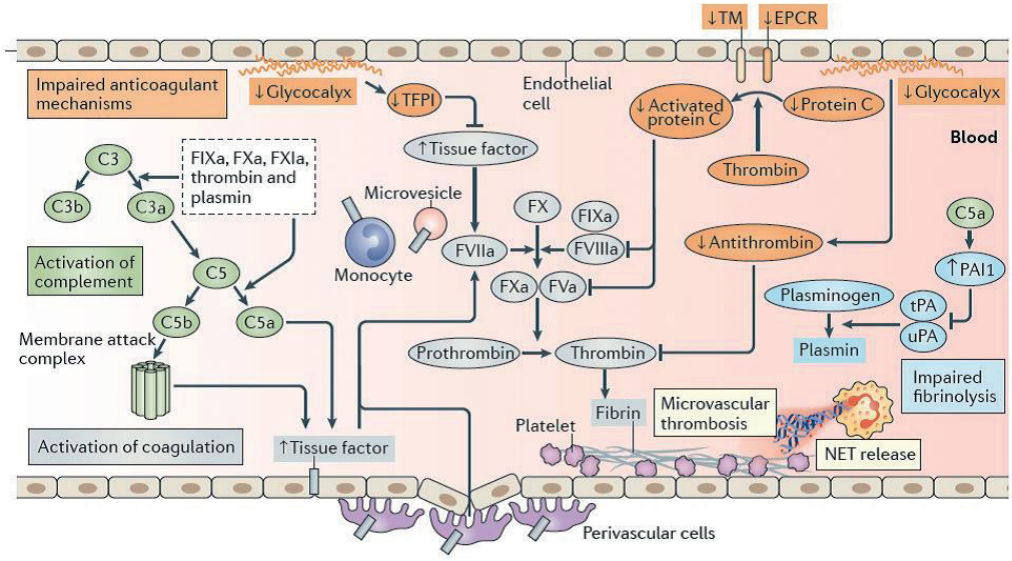

Figure 4: The hemostatic response to sepsis.

(Reprint with permission from [21])

Sepsis results in a net procoagulant state in the microvasculature by at least three mechanisms:

1. inflammatory cytokine-initiated activation of tissue factor generating thrombin (grey). Sepsis is accompanied by inflammation-induced vessel injury, which exposes tissue factor to blood coagulation factors, resulting in blood clotting. Tissue factor binds and activates FVII, after which a cascade of proteolytic reactions results in the formation of FXa, thrombin and fibrin.
2. insufficient control of anticoagulant pathways (orange). The tendency towards thrombosis during sepsis is augmented by the concurrently compromised activity of the three main anticoagulant pathways: antithrombin, TFPI and the protein C system. Antithrombin is the main inhibitor of thrombin and FXa, whereas TFPI is the main inhibitor of the tissue factor–FVIIa complex. Activated protein C is generated from protein C at the surface of resting endothelial cells, a process that is mediated by the binding of thrombin to TM and is amplified by the EPCR. Activated protein C proteolytically inactivates the coagulation cofactors FVa and FVIIIa, thereby inhibiting coagulation. During sepsis, the protein C system is impaired as a result of multiple factors, most notably the decreased synthesis of protein C by the liver, the increased consumption of protein C and the impaired activation of protein C as a result of diminished TM expression on endothelial cells.
3. PAI-1-mediated suppression of fibrinolysis (blue).

The interaction with the complement system (green) is outside the scope of this thesis.

Abbreviations: EPCR = endothelial protein C receptor , FVII = factor VII, PAI-1 = plasminogen activator inhibitor-1, TM = thrombomodulin, TFPI = tissue factor pathway inhibitor.

The problem in sepsis is to balance between a pro-inflammatory and pro-coagulant response to provide bacterial clearance, but to avoid an excessive response which leads to endothelial damage and extraordinary hemostatic response. However, the anti-inflammatory counter mechanism should not be too pronounced or persistent, as this could potentially result in ineffective bacterial clearance or vulnerability to secondary infections, respectively.

Thesis targets: place and rationale in the host response to sepsis

The aim of this thesis is to study determinants of pediatric sepsis outcome. We studied whether specific inflammatory, hemostatic, genetic, and environmental factors are associated with the severity of sepsis. We aimed to identify reliable prognostic markers in order to detect patients at risk for poor outcome at an early stage.

Neutrophils are an important part of the innate immune defense. They migrate to the site of infection to release regulatory cytokines, chemokines, and leukotrienes to contribute to microbial killing.[26] One of the tools actively contributing to microbial killing, is the release of neutrophil extracellular traps (NETs). NETs are extracellular DNA matrix, containing granule proteins and histones to degrade virulence factors and to kill bacteria.[27] Although NETs are primarily considered as a protective mechanism due to the toxicity of antimicrobial components of the NETs, NETs may contribute to disease severity by causing cell damage via cytotoxic effects of NET-bound histones and by promoting coagulation.[28-30] Our objective was to study the role of NETs in children with meningococcal sepsis. We measured levels of NETs, using myeloperoxidase (MPO)-DNA as a specific marker for NETs.[31] In addition, we investigated whether *N. meningitidis* isolates from patients are able to induce NETs. The inducing capacity of *N. meningitidis* is not known, in contrast to other bacterial species, such as *S. aureus* or *E. coli*.[32]

Monocyte recruitment from the bone marrow to peripheral blood and tissue is enhanced during infection to promote immune defense.[33] These monocytes express CD14 and CD16 on their surface, dividing monocytes into monocyte subsets, with each subset presumed to have specialized functions and phenotypes. The monocyte expression patterns are dynamic and monocytes differentiate from classical monocytes (CD14++CD16-) via intermediate monocytes (CD14++CD16+) to non-classical monocytes (CD14+CD16++).[34] Monocytes express HLA-DR (mHLA-DR), which is a MHC class II cell surface molecule needed to present antigens to T-cells. Numerous studies, mainly in adults, studied mHLA-DR in association with outcome.[35] However, longitudinal studies in critically ill children are limited so far and previous studies were restricted to one specific clinical diagnosis only. We longitudinally monitored monocyte subset distribution and mHLA-DR expression in children with sepsis, post-surgery, and trauma in relation to nosocomial infections and mortality.

Immunoglobulin G (IgG) is part of the adaptive immune response and plays a role in the humoral response against meningococcal infections.[36, 37] IgG is able to initiate complement-dependent lysis of the bacterium and leukocyte-mediated phagocytosis.[38, 39] It has been suggested that the severity of the disease is not determined by the abundance of (certain subclasses of) anti-meningococcal-IgG, but rather by either the specificity or affinity of the IgG molecule for the antigen or the IgG receptors.[36] Of great influence on the receptor affinity of IgG is the *N*-glycan on its fragment crystallizable (Fc) at Asn297.[40, 41] The Fc region is the tail region of an antibody that interacts with Fcγ receptors (FcγR) and proteins of the complement system. Functional studies have shown the effect of alterations in IgG Fc glycosylation on the binding affinity to both FcγR and complement factor C1q, mediating different immune effects of antibodies.[42] We studied differences in IgG Fc glycosylation between meningococcal sepsis patients and controls. In addition, we evaluated the potential of specific glycosylation features to serve as a predictive marker for disease outcome.

The main function of the PC pathway is to control coagulation by inactivation of activated (a) factor V (cofactor of factor Xa) and factor VIIIa (cofactor of factor IX), subsequently preventing thrombin generation.[43] PC also neutralizes PAI-1, concomitantly increasing fibrinolytic capacity. Thrombomodulin (TM), an endothelial cell surface glycoprotein, binds circulating thrombin and forms a TM-thrombin-complex. (Figure 5) This complex rapidly activates PC bound to the endothelial cell protein C receptor (EPCR). Activated PC (aPC) then dissociates from the EPCR, binds to protein S (PS), and forms a complex that inactivates factor Va and factor VIIIa.

The fibrinolytic pathway actively degrades existing fibrin clots. Plasmin is the major fibrinolytic protease and degrades fibrin into soluble fibrin degradation products (FDPs). (Figure 6) Plasminogen (Plg) is cleaved into plasmin by Plg activators. Inhibition of fibrinolysis occurs on several levels: by Plg activator inhibitors (PAI), by thrombin-activatable fibrinolysis inhibitor (TAFI), and by other plasmin inhibitors such as α2-antiplasmin and α2-macroglobulin. In addition, factor XIII stabilizes fibrin, thereby making the fibrin clot more resistant to fibrinolysis.[44, 45]

The PC and fibrinolytic pathways are activated in sepsis and have been associated with outcome.[25] More specifically, decreased levels of protein C[46] and increased levels of PAI-1[15, 47] are associated with a negative outcome in sepsis. Genetic polymorphisms are reported to affect both the amount and functional quality of these proteins. We reviewed genetic polymorphisms in PC and fibrinolytic pathway potentially affecting host susceptibility and severity of pediatric sepsis. In addition, we identified candidates for future molecular genetic research.

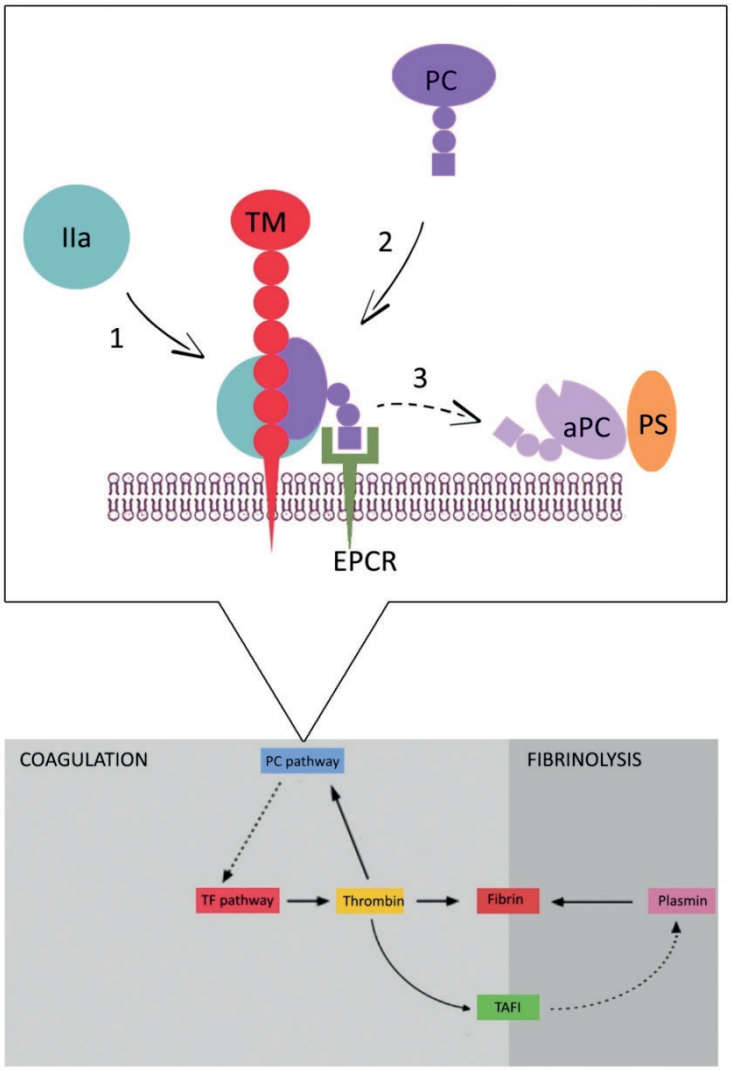

Figure 5: The protein C pathway

(Reprint with permission from [52])

TM binds circulating thrombin (1) and forms a TM-thrombin-complex, which activates PC bound to EPCR into aPC (2). APC then dissociates from the EPCR, binds to PS, and forms a complex that inactivates factor Va and factor VIIIa (3).

Abbreviations: IIa = thrombin, TM = thrombomodulin, EPCR = endothelial cell protein C receptor, (a)PC = (activated) protein C, PS = protein S.

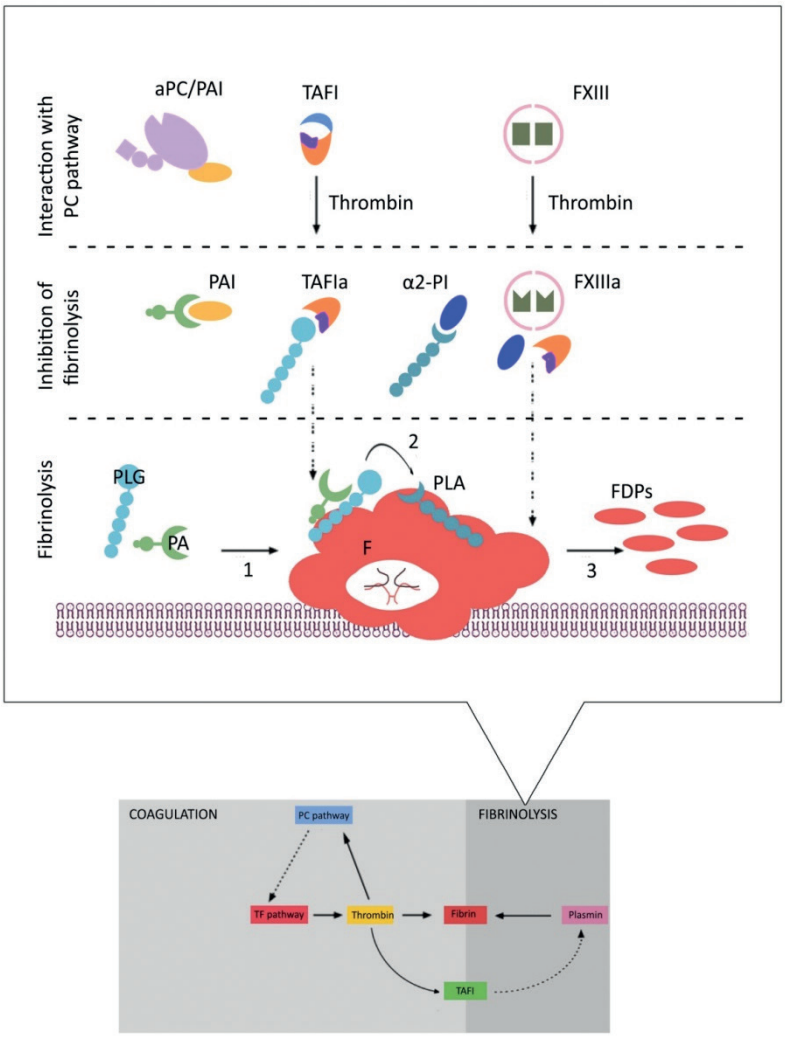

Figure 6: The fibrinolytic pathway

(Reprint with permission from [52])

Fibrinolysis occurs after both PLG and PA bind to F (1). The conversion of Plg to Pla by PA (2) results in degradation of F into FDPs (3). Fibrinolysis is inhibited (A) by PAI, which binds to PA; (B) by Plg-bound TAFIa, which attenuates the binding of Plg and PA to F; (C) by α2-PI, which binds to free circulating Pla; and (D) by FXIIIa which stabilizes fibrin by incorporation of α2-PI and TAFI, making the fibrin clot more resistant to fibrinolysis. PC pathway and fibrinolytic pathway interact via aPC-PAI complex, and via thrombin activating TAFI and FXIII.

Abbreviations: Plg = plasminogen, PA = plasminogen activators, Pla = plasmin, F = fibrin, FDPs = fibrin degradation products, PAI = plasminogen activator inhibitors, TAFI(a) = (activated) thrombin-activatable fibrinolysis inhibitor, α2-PI = α2-plasmin inhibitor, FXIII(a) = (activated) factor XIII, aPC = activated protein C.

The ADAMTS family includes 19 proteases, which play various roles in for example coagulation and inflammation.[48] ADAMTS-13, the von-Willebrand factor (vWF)-cleaving protease processing large multimeric vWF into an optimal size for normal coagulation, has been the most extensively studied ADAMTS-protein in sepsis. Previous studies demonstrated that decreased ADAMTS-13 levels, presumably leading to increased formation of thrombi, are associated with more severe disease and poor outcome.[49, 50] Unpublished data from a candidate gene study, performed by the EUCLIDS consortium[51], in 245 severe meningococcal sepsis patients identified a SNP in ADAMTS-1 (rs9975310) to be associated with skin graft or amputation, and a SNP in ADAMTS-18 (rs149698955) to be associated with death. Pooled analysis with GWAS data from approximately 1500 meningococcal sepsis patients confirmed the finding in ADAMTS-1. The SNP in ADAMTS-18 was not genotyped in the GWAS and could therefore not be verified. We studied ADAMTS-1 and ADAMTS-18 protein levels in pediatric meningococcal sepsis, and studied the association with outcome.

EUCLIDS consortium

The GWAS study on meningococcal disease has been undertaken by a consortium, which thereafter successfully applied to a European Union's Seventh Framework Programme (FP7) grant. This consortium designed the European Childhood Life-threatening Infectious Disease Study (EUCLIDS, FP7 grant number 279185), which is a large-scale prospective, multicenter (195 hospitals from 15 mainly European countries), cohort study aimed to identify genes, and biological pathways, which determine susceptibility and severity in life-threatening bacterial infections of childhood. Although the EUCLIDS consortium was specifically interested in patients with invasive meningococcal, pneumococcal, staphylococcal, salmonella and group A streptococcal infections, representing the most common causes of community-acquired sepsis in children, presentations due to other organisms were included as well. Patients were recruited as early as possible in the illness within a time window from presentation to the time when culture results became available. Eventually, the consortium managed to recruit approximately 3.000 patients and samples prospectively. This thesis has partially been funded by EUCLIDS and partly includes data from the EUCLIDS consortium.

Outline of this thesis

In **chapter 1**, we present the problems regarding pediatric sepsis and identify our research questions.

In **chapter 2**, we characterized clinical presentations, pathogens, mortality, and disability of hospitalized children with life-threatening bacterial infections, recruited through the multinational prospective EUCLIDS study **[chapter 2.1]**. Additionally, we focused on children admitted to European PICUs for community-acquired sepsis and we studied risk factors for mortality and disability. **[chapter 2.2]**

In **chapter 3**, we studied factors of the inflammatory response to sepsis; neutrophil extracellular traps (NETs) **[chapter 3.1]**, human leukocyte antigen-DR (HLA-DR) expression on monocyte subsets **[chapter 3.2]**, and IgG Fc glycosylation **[chapter 3.3]**, and studied associations with outcome.

In **chapter 4**, we focused on the hemostatic response to sepsis. We explored genetic polymorphisms in the protein C and fibrinolytic pathways in association with severity of sepsis. **[chapter 4.1]** In **chapter 4.2**, we translated preliminary genetic findings into a functional study. We studied A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS)-1 and ADAMTS-18, and studied the association with outcome. Thirdly, we assessed if circadian variation could be a relevant determinant of pediatric sepsis outcome by studying plasminogen activator inhibitor, type 1 (PAI-1) circadian variation. **[chapter 4.3]**

Lastly, in **chapter 5**, we discuss our main findings and give prospects for future studies.

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Chapter 2

Sepsis in children

Chapter 2.1

Life-threatening infections in children in Europe (the EUCLIDS Project): a prospective cohort study

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Abstract

Background: Sepsis and severe focal infections (SFI) represent a significant burden of disease in hospitalized children. To understand the burden of disease and outcome of childhood infection in Europe, children with life-threatening bacterial infections were studied in a multi-centre study in six countries in Europe.

Methods: Children aged 1 month-to-18 years old with sepsis or SFI, admitted to 98 European EUCLIDS network hospitals were prospectively recruited during July 2012-December 2016. Demographic, clinical, microbiological data and outcomes were collected.

Findings: A total of 2,844 patients were included (53.2% male; median age: 39.1 months). 43.2% of patients (*n*=1229) had sepsis and 56.8% (*n*=1615) SFI. Sepsis was diagnosed predominantly in younger children and SFI in older ones (*P*-value<0.0001). Main SFI were pneumonia (*n*=511, 18%), central nervous system infection (*n*=469, 16.5%) and skin and soft tissue infection (*n*=247, 8.7%). Causal microorganism was identified in 47.8% of children (*n*=1,359). Most prevalent causative agent was *Neisseria meningitidis* (9.1%, *n*=259) followed by *Staphylococcus aureus* (7.8%, *n*=222), *Streptococcus pneumoniae* (7.7%, *n*=219) and *Group A streptococcus* (5.7%, *n*=162). Mortality rate was 2.2% (*n*=57); and 37.6% of patients (*n*=1,070) required intensive care.

Interpretation: Mortality rate in European children hospitalised due to sepsis or SFI is low. Burden of disease lies predominantly in children under 5 years and is largely due to vaccine-preventable infections by meningococcus and pneumococcus. More than a third of children required intensive care. Despite availability and application of current clinical methods for microbiological diagnosis, the causative organism remained unidentified in approximately 50% of the patients.

Introduction

The Confidential Enquiry into Maternal and Child Health (CEMACH) report 'Why Children Die' demonstrated that infectious illness was 'the single largest cause of death in children dying of an acute physical illness', constituting '20% of the deaths overall' with the 1-4 year old group the most affected [1]. Amongst all the infectious agents, bacteria represent the principal cause of death in young children, accounting for over a third of all child deaths globally [2].

The World Health Organization (WHO) recently issued a resolution on sepsis in all age groups, recognizing deaths by severe infection as a main target for global and national prioritization in healthcare delivery [3]. This burden on childhood morbidity and mortality persists despite of the substantial reduction in vaccine-preventable invasive bacterial infections after the introduction of conjugate vaccines in childhood and the availability of antimicrobial agents [4-6], highlighting the need for a better understanding of the host response to infection, novel treatments of acute infection, new methods to identify those at risk, and better preventative strategies.

Currently, information regarding the global epidemiology of severe infections in the paediatric population is scarce. Most published studies on sepsis and severe focal infection (SFI) are biased towards a predominantly paediatric intensive care unit (PICU) population. Reported mortality and morbidity from recent large paediatric sepsis and septic shock studies ranged from 17% to 25% [7, 8].

In this paper, we present data from the European Union Childhood Life-threatening Infectious Disease Study (EUCLIDS), which aimed to describe the current burden of severe paediatric infectious diseases, with respect to demographic, clinical, microbiological data and outcomes, across Europe.

Materials and methods

Study design and recruitment criteria

This prospective, multicenter, observational study of children with life-threatening bacterial infection presenting to hospital was conducted between July 2012-December 2016 by the EUCLIDS Consortium (http://www.euclids-project.eu/). This network included 194 hospitals in Europe (in 9 countries) and one hospital in Africa (The Gambia). Data from Switzerland were not included in the analysis because they used different inclusion criteria. The African partner was also excluded because the present study focuses on the European burden of disease.

Eligible participants were children from 1 month to 18 years of age admitted to hospital with sepsis (or suspected sepsis) and/or severe focal infection including but not limited to pneumonia, soft tissue infection, meningitis, encephalitis, osteomyelitis, and septic arthritis (Appendix: Full definitions document, page 43). In order to enrol children as early as possible during the infection, potential recruits were identified from their clinical characteristics on presentation often before the results from confirmatory microbiology tests were available. Additionally, children admitted with proven infections due to *N. meningitidis, S. pneumoniae, S. aureus* and *Group A streptococcus (GAS)* who had not been included in the study on initial presentation to hospital were specifically targeted for recruitment. For this reason our findings cannot be used to accurately establish the relative prevalence of other potentially causative pathogens. although recruitment mostly took place before any causal pathogen was identified. Patients with hospital-acquired infections were not included.

The study used harmonised procedures for patient recruitment, sample processing and sample storage. A common clinical protocol agreed by EUCLIDS Clinical Network and approved by the Ethics Comitte was implemented at all hospitals. All clinical staff were trained in the projects proceedures, and specified criteria were used for clinical definitions and assignment of patients to diagnostic categories. Written informed consent was obtained from a parent or legal guardian for each subject before study inclusion.

Among 7,276 eligible patients included in the EUCLIDS database, we excluded 2,012 patients labelled as controls, 706 patients recruited retrospecively, 1,479 patients from the Swiss and Gambian Cohorts, and 235 that did not meet eligibility criteria or were incomplete (Figure 1). Analysis was limited to the remaining 2,844 subjects with a complete minimal dataset including patient age and discharge diagnosis.

Clinical data collection

The clinical information for each patient was collected using a secured web-based platform, including data on demographics, comorbidity, immunisation status, selected laboratory results, and past medical and family history of severe infectious diseases defined as: (a) any infection requiring hospitalization, if outpatient at onset; (b) any infection requiring oxygen, pressors or fluids to support blood pressure, or intubation; or (c) deep tissue (invasive) infection requiring intravenous or oral antibiotics to treat infection. Discharge diagnosis, clinical course, treatments and specific procedures during admission and outcomes (such as death or sequelae) were recorded.

Patients were categorised into two main groups according to the clinical characteristics during the hospital admission: sepsis or SFI. Sepsis was defined as suspected or confirmed infection (infectious organisms or toxins) plus systemic inflammatory response syndrome

(SIRS) [9], and SFI included those illnesses with a suspected or confirmed infection but without SIRS. Patients were assigned one or more pre-defined clinical syndromes. (Appendix: Full definitions document, page 43).

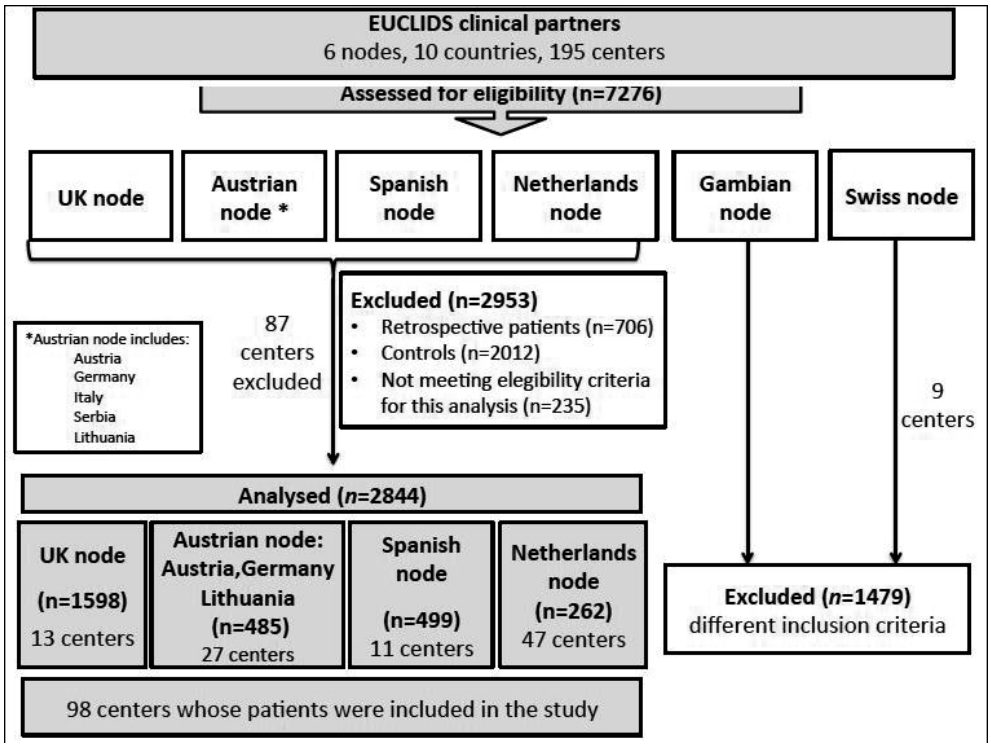


Figure 1: Consort Flow Diagram

Laboratory methods

Microbiological diagnosis was undertaken as part of clinical care using locally available clinical diagnostic procedures, including, as appropriate, bacterial culture from normally sterile sites (blood, cerebrospinal fluid, urine and invasive diagnostic samples), and from non-sterile sites (throat and wound swabs); bacterial and viral molecular diagnostics were applied to blood, cerebrospinal fluid and respiratory secretions, according to local availability.

In order to assign microbiological aetiology of infection in prospective patients recruited to the study, each patient was phenotyped according to their likelihood of bacterial infection, using an agreed algorithm, when all the results of investigations were available (Figure 2).

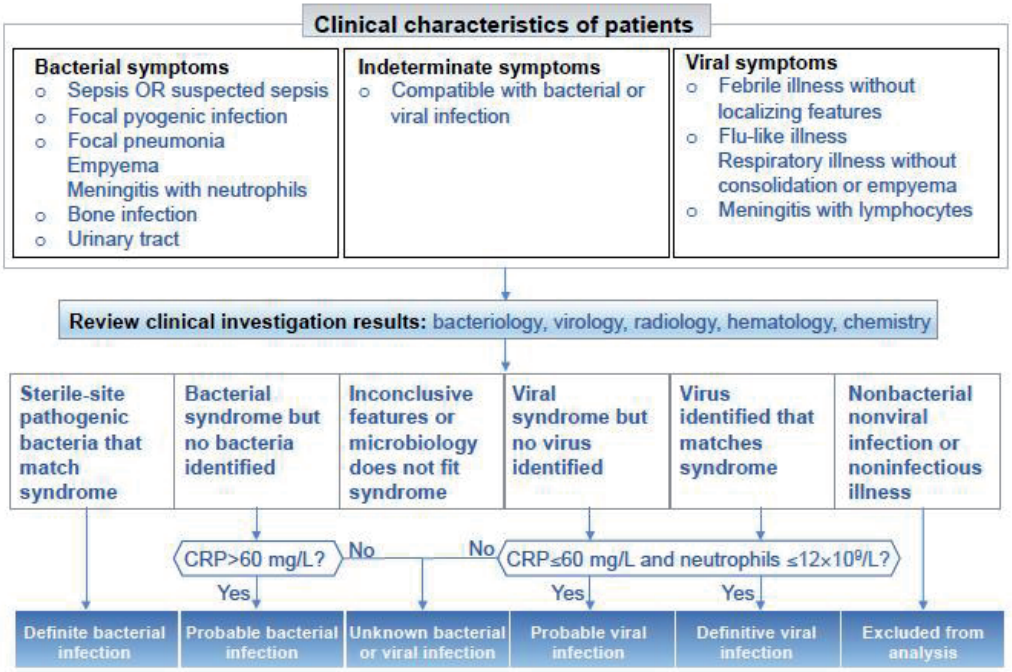


Figure 2: Phenotyping algorithm.
Figure adapted from Herberg et al. [10]

Specific inflammatory parameters: maximum levels of serum C-reactive protein (CRP) and neutrophil counts were compared to further assess their utility and sensitivity in discriminating focal *vs.* sepsis, PICU *vs.* non-PICU admission, and prognosis (survivors *vs.* death). For CRP values, all cohort values were used; while for neutrophil counts only UK values were available. Sensitivity and specificity was assessed using pre-agreed cut offs and numeric values were used to obtain receiver operating characteristic curves (ROC) Figure 2 [10].

Statistical analysis

General data are presented as percentages and odds ratios (OR) computed from contigency tables, and medians and interquartile ranges (IQR). Analysis was performed using R version 3.3.1 (www.r-project.org). The level of statistical significance was set at 0.05. Bonferroni correction was used in order to reduce the likelihood of false positive results caused by multiple testing. Associations were assessed using non-parametric tests: Fisher's exact test for discrete variables and Wilcoxon test for continuous variables (package *stats*). ROC curves and areas under curve (AUC) were calculated with *P*-values to test the null hypothesis that the AUC equals 0.50 (package *pROC*).

Role of the funding source

This project has received funding from the European Union's seventh Framework program under EC-GA no.279185 (EUCLIDS). The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Characteristics of the EUCLIDS cohort

A total of 2,844 subjects were analysed. 53.2% (1512/2841) were male and the median age was 39.1 months (IQR=12.4-93.9). Characteristics of the patients are summarised in Table 1.

A history of previous severe infection was found in 432 (16.9%) cases, whilst 240 cases had 1st or 2nd order family members with a history of serious infection (11.0%, 240/2174). Previous infections included meningitis (32.9%, 79/240), pneumonia (20.4%, 49/240), severe sepsis (11.3%, 27/240) and meningococcemia (7.5%, 18/240). 2.4% of cases (51/2127) had parental consanguinity and 2.1% (45/2150) had first- or second-degree relatives with an immunodeficiency. Prematurity was present in 9.8% (230/2343) of the cases. 30.1% (497/1652) of the patients lived with smokers at home (Table 1).

Immunisations were up-to-date according to the local schedules in 93.0% (2240/2409) of the patients. Nevertheless, we found that 89.5% (204/228) of the meningococcus isolated and serotyped could be eventually covered by vaccines that were not available or not included in the immunization calendars implemented in Europe at that time.

Sepsis was diagnosed predominantly in younger children and SFI in older ones (Figure 3A), with significant statistical differences in the age distribution between those in whom a causative organism was identified and those with no organism identified (Figure 3B, Table 1).

Most of patients (93.4%, 2282/2444) had a favourable clinical course (no death, skin grafts, amputations, hearing loss >40dB) with complete recovery from the illness. The mortality rate was 2.2% (57/2569) in the entire cohort, 0.5% (7/1549) in SFI *vs.* 4.9% (50/1020) for sepsis. The cause of death for patients included in the SFI sub-cohort is specified in Appendix: Cause of death for patients with SFI, page 63.

A total of 37.6% (1070/2844) patients were admitted to PICU of which 62.1% (763/1229) admissions presented with sepsis.

Table 1: Description of the main characteristics of the EUCLIDS study cohort.

Comparision between (A) no organism and organism identified, and (B) focal infection and sepsis. Data are expressed as % (*n*) or median (IQR). * *P*-values lower than Bonferroni correction threshold (0.05/37=0.0014).

A)

Variables	All patients	No organism identified	Organism identified	*P*-value
Total cohort	2844	52.2% (1485/2484)	47.8% (1359/2844)	
Demographic characteristics				
Sex (male)	53.2% (1512/2841)	53.9% (800/1484)	52.5% (712/1357)	0.4517
Age	39.1 (12.4-93.9)	42.8 (14.9-95.5)	33.2 (10.25-91.05)	0.0007*
0-12 months	24.3% (691/2844)	21.1% (313/1485)	27.8% (378/1359)	0.0005*
12-24 months	14.8% (421/2844)	14.5% (215/1485)	15.2% (206/1359)	–
24-48 months	17.1% (487/2844)	18.3% (272/1485)	15.8% (215/1359)	–
>48 months	43.8% (1245/2844)	46.1% (685/1485)	41.2% (560/1359)	–
Weight (kg)	14.8 (9.9-25.8)	15.4 (10.3-26.5)	14.0 (9.2-25.5)	0.0005*
Family history				
Severe infections	11.0% (240/2174)	10.1% (115/1143)	12.1% (125/1031)	0.1319
Immunodeficiency	2.1% (45/2150)	2.1% (24/1133)	2.1% (21/1017)	1.0000
Consanguinity	2.4% (51/2127)	2.6% (29/1122)	2.2% (22/1005)	0.5734
Smoker in the household	30.1% (497/1652)	28.3% (250/883)	32.1% (247/769)	0.0957
Patient medical history				
Premature birth	9.8% (230/2343)	9.9% (123/1244)	9.7% (107/1099)	0.9446
Past severe infections	16.9% (432/2563)	18.9% (252/1336)	14.7% (180/1227)	0.0051
Immunisations up-to-date	93.0% (2240/2409)	93.5% (1194/1277)	92.4% (1046/1132)	0.2998
Clinical data				
Antibiotics before culture	34.1% (714/2091)	34.4% (393/1142)	33.8% (321/949)	0.7813
PRISM Score	11 (5-20)	10.5 (4-16)	12.0 (5.0-21)	0.1097
Full recovery expected	93.4% (2282/2444)	95.7% (1219/1274)	90.9% (1063/1170)	<0.0001*
PICU admission	37.6% (1070/2844)	30.0% (445/1485)	46.0% (625/1359)	<0.0001*
Oxygen needed	36.3% (923/2546)	32.0% (426/1333)	41.0% (497/1213)	<0.0001*
Respiratory support	28.1% (720/2564)	23.3% (313/1345)	33.4% (407/1219)	<0.001*
Inotropes	11.8% (304/2578)	10.3% (138/1346)	13.5% (166/1232)	0.0122
Hospital LOS	7 (4-13)	6 (3-10)	10 (6-16)	<0.0001*
Death	2.2% (57/2569)	1.4% (19/1345)	3.1% (38/1224)	0.0045
Clinical syndrome				
CLABSI	0.5% (13/2844)	0.1% (2/1485)	0.8% (11/1359)	0.0099
CNS infection	16.5% (469/2844)	8.8% (130/1485)	24.9% (339/1359)	<0.0001*
Bronchiolitis	2.7% (78/2844)	2.1% (31/1485)	3.5% (47/1359)	0.0287
Pneumonia	18.0% (511/2844)	22.5% (334/1485)	13.0% (177/1359)	<0.0001*
LRTI	3.5% (100/2844)	4.7% (70/1485)	2.2% (30/1359)	0.0003*
Lung effusion or empyema	7.4% (210/2844)	6.3% (94/1485)	8.5% (116/1359)	0.0261
Soft tissue infection	8.7% (247/2844)	9.2% (136/1485)	8.2% (111/1359)	0.3518

Toxic shock syndrome	2.3% (64/2844)	1.1% (16/1485)	3.5% (48/1359)	<0.0001*
Endocarditis	0.7% (20/2844)	0.1% (2/1485)	1.3% (18/1359)	0.0001*
Osteomyelitis	6.7% (191/2844)	5.2% (77/1485)	8.4% (114/1359)	0.0010
Scarlet fever	0.3% (9/2844)	0.3% (5/1485)	0.3% (4/1359)	1.0000
Septic arthritis	5.2% (149/2844)	3.4% (50/1485)	7.3% (99/1359)	<0.0001*
Gastroenteritis	1.6% (45/2844)	1.3% (19/1485)	1.9% (26/1359)	0.1800
UTI–pyelonephritis	3.8% (109/2844)	2.6% (39/1485)	5.2% (70/1359)	0.0006*
ENT	6.3% (178/2844)	7.8% (116/1485)	4.6% (62/1359)	0.0003*
Abdominal condition	1.3% (38/2844)	1.5% (22/1485)	1.2% (16/1359)	0.5166
Severe sepsis	5.5% (157/2844)	3.6% (54/1485)	7.6% (103/1359)	<0.0001*
Septic shock	9.3% (264/2844)	6.2% (92/1485)	12.7% (172/1359)	<0.0001*

B)

Variables	**Focal infection**	**Sepsis**	***P*-value**
Total cohort	56.8% (1615/2844)	43.2% (1229/2844)	
Demographic characteristics			
Sex (male)	53.5% (863/1612)	52.8% (649/1229)	0.7045
Age	46.5 (15.8-100.4)	27.6 (9.0-80.2)	<0.0001*
0-12 months	19.8% (319/1615)	30.3% (372/1229)	0.0005*
12-24 months	13.6% (220/1615)	16.4% (201/1229)	–
24-48 months	18.1% (293/1615)	15.8% (194/1229)	–
>48 months	48.5% (783/1615)	37.6% (462/1229)	–
Weight (kg)	15.8 (10.7-28.0)	13.0 (8.7-23.1)	<0.0001*
Family history			
Severe infections	11.2% (137/1220)	10.8% (103/954)	0.7828
Immunodeficiency	2.1% (25/1211)	2.1% (20/939)	1.0000
Consanguinity	1.9% (22/1186)	3.1% (29/941)	0.0859
Smoker in the household	31.7% (301/951)	28% (196/701)	0.1155
Patient medical history			
Premature birth	8.5% (112/1316)	11.5% (118/1027)	0.0173
Past severe infections	18.7% (270/1441)	14.4% (162/1122)	0.0041
Immunisations up-to-date	93.2% (1282/1375)	92.6% (958/1034)	0.5740
Clinical data			
Antibiotics before culture	29.8% (359/1204)	40% (355/887)	<0.0001*
PRISM Score	5 (4-11.75)	14 (6-22)	<0.0001*
Full recovery expected	97.2% (1369/1409)	88.2% (913/1035)	<0.0001*
PICU admission	19.0% (307/1615)	62.1% (763/1229)	<0.0001*
Oxygen needed	22.1% (323/1463)	55.4% (600/1083)	<0.0001*
Respiratory support	11.8% (172/1454)	49.4% (548/1110)	<0.0001*
Inotropes	10.3% (151/1472)	44.4% (498/1121)	<0.0001*
Hospital LOS	6 (3-12)	9 (5-15)	<0.0001*
Death	0.5% (7/1549)	4.9% (50/1020)	<0.0001*

Clinical syndrome			
CLABSI	0.1% (1/1615)	1.0% (12/1229)	0.0001*
CNS infection	12.1% (196/1615)	22.2% (273/1229)	<0.0001*
Bronchiolitis	2.7% (44/1615)	2.8% (34/1229)	1.0000
Pneumonia	20.4% (329/1615)	14.8% (182/1229)	<0.0001*
LRTI	4.3% (69/1615)	2.5% (31/1229)	0.0134
Lung effusion or empyema	8.4% (136/1615)	6.0% (74/1229)	0.0168
Soft tissue infection	11.5% (185/1615)	5.0% (62/1229)	<0.0001*
Toxic shock syndrome	0.3% (5/1615)	4.8% (59/1229)	<0.0001*
Endocarditis	0.2% (4/1615)	1.3% (16/1229)	0.0011
Osteomyelitis	9.6% (155/1615)	2.9% (36/1229)	<0.0001*
Scarlet fever	0.4% (7/1615)	0.2% (2/1229)	0.3150
Septic arthritis	7.5% (121/1615)	2.3% (28/1229)	<0.0001*
Gastroenteritis	1.9% (31/1615)	1.1% (14/1229)	0.1285
UTI-pyelonephritis	4.0% (64/1615)	3.7% (45/1229)	0.6947
ENT	9.0% (145/1615)	2.7% (33/1229)	<0.0001*
Abdominal condition	1.4% (22/1615)	1.3% (16/1229)	1.0000
Severe sepsis	0% (0/1615)	12.8% (157/1229)	<0.0001*
Septic shock	0% (0/1615)	21.5% (264/1229)	<0.0001*

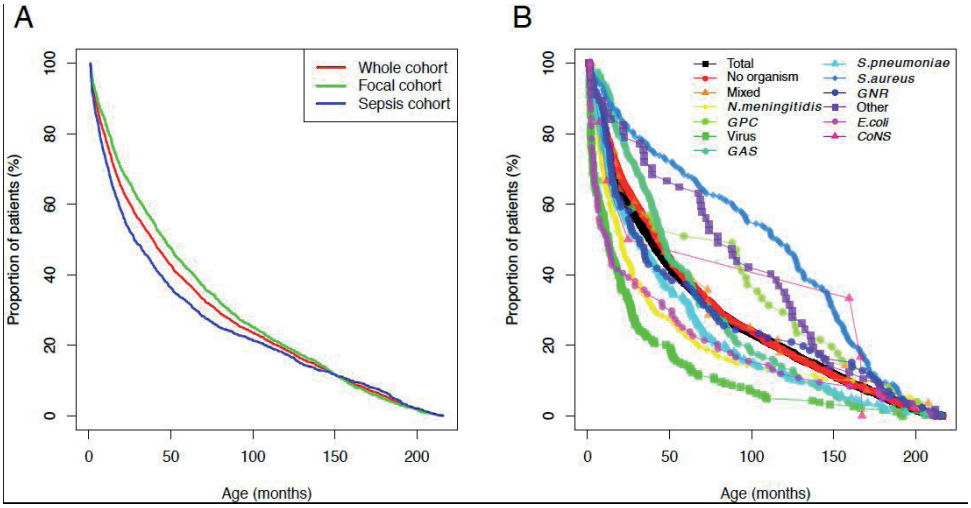


Figure 3: A) Age distribution in the EUCLIDS cohort and in those diagnosed with sepsis or a focal illness. B) Age distribution by causative organism.

GPC: gram positive cocci, GAS: *Group A Streptococcus*, GNR: gram negative rods, CoNS: *Coagulase Negative Staphylococci*.

Microbiological and clinical diagnosis

A total of 44.8% of children (1155/2581) had definite bacterial infection; 5.9% (152/2581) had definite viral; and 47.9% (1202/2509) suffered from uncertain type of infection (454 probable bacterial, 65 probable viral and 683 unknown) (Figure 2).

A causative microorganism was identified in 47.8% (1359/2844) of the cases. The most prevalent bacterial causative agent was *Neisseria meningitidis* in 9.1% (259/2844) followed by *Staphylococcus aureus* (7.8%, 222/2844), *Streptococcus pneumoniae* (7.7%, 219/2844) and *GAS* (5.7%, 162/2844) (Figure 4). Viruses were identified as causative agents in 6.5% (185/2844) of the patients with the most common ones being: enterovirus, rhinovirus and respiratory syncytial virus.

In patients admitted to PICU, the main identified bacteria were: *N. meningitidis* (16.5%, 162/981), *S. pneumoniae*, (9.9%, 97/981), *GAS* (8.1%, 79/981) and *S. aureus* (5.5%, 54/981). Viruses were the causative pathogen in the 8.1% (79/981) of the cases, and there was no organism identified in 41.6% (408/981) of the patients. Ward and PICU clinical syndromes, and causal agents are shown in Appendix Figure 1, page 64.

Significant differences were found in *N. meningitidis* rates in patients with a family history of severe bacterial infection [OR: 2.02 (95%CI: 1.31-3.04), *P*-value=0.0011], and in patients exposed to tobacco [OR: 3.21 (95%CI: 2.19-4.74), *P*-value<0.0001]. In premature patients there is a significant difference for viral infection rates [OR: 2.13 (95%CI: 1.38-3.22), *P*-value=0.0005].

Those patients in whom a causative organism was identified were more likely to have severe disease: a higher proportion was admitted to PICU (*P*-value<0.0001) and had a prolonged hospital length of stay (LOS) (*P*-value<0.0001), furthermore, they required more respiratory support (*P*-value<0.0001), and supplemental oxygen (*P*-value<0.0001). Additionally, inotropes (*P*-value=0.0122) and mortality were higher in patients with an identified causative organism (*P*-value=0.0045) although this was not statistically significant after Bonferroni adjustment (Table 1A).

Among patients with bacterial SFI, the most prevalent clinical syndromes were pneumonia (20.4%, 329/1615), central nervous system (CNS) infection (12.1%, 196/1615), skin and soft tissue infection (11.5%, 185/1615) and osteomyelitis (9.6%, 155/1615).

No correlation was found between administration of antimicrobial agents before cultures and organism identification (*P*-value=0.7813).

Children whose immunisations were not up to date (7.0%, 169/2409) were admitted mainly due to pneumonia (18.9%, 32/169), CNS infections (15.4%, 26/169) and urinary tract infections–pyelonephritis (11.8%, 20/169); with *S. pneumoniae* and *Escherichia coli* being the main causative microorganisms (6.5%, 11/169; and 5.9%, 10/169, respectively).

We further analysed the main presenting clinical syndromes according to the presence of a microorganism. For the main pathologies studied we found that CNS infections were caused mainly by *N. meningitidis* (29.9%, 140/469) and *S. pneumoniae* (19.0%, 89/469); soft tissue infection, osteomyelitis, toxic shock syndrome and septic arthritis by *S. aureus* and *GAS*, and abdominal conditions and urinary tract infections-pyelonephritis by *E. coli*. (Figure 4A)

Infection with *N. meningitidis* (22.8%, 13/57) was the most prevalent among the fatal cases, mainly associated with severe sepsis, followed by *S. pneumoniae* (19.3%, 11/57) and *S. aureus* (10.5%, 6/57). In 33.3% (19/57) of the non-survivors no causative pathogen was identified (Figure 4B).

Sepsis vs. SFI

The main differences observed between patients with sepsis or SFI were that septic patients had a more severe course, with significant differences for all parameters including full recovery at discharge (*P*-value<0.0001), need for supplemental oxygen (*P*-value<0.0001), respiratory support requirement (*P*-value<0.0001), inotropes (*P*-value<0.0001), PICU admission (*P*-value<0.0091) and death outcome (*P*-value<0.0001) (Table 1B).

Antibiotics had been administrated before blood cultures were taken in 40.0% (355/887) of septic patients and in 29.8% (359/1204) patients with SFI (*P*-value<0.0001).

Utility of inflammatory markers

We compared maximum CRP and neutrophil counts levels between different groups (Table 2). Patients with sepsis and those requiring intensive care, had an increased serum CRP (≥60 mg/L) compared to those with focal infection and non-PICU admission (*P*-value<0.0001). (Appendix Figure 2, page 65). No differences were found when comparing survivors *vs.* non-survivors.

ROC analysis for CRP to discriminate sepsis *vs.* SFI showed an AUC of 0.655 (95%CI 0.616-0.694, *P*-value<0.0001) and 0.661 (95%CI 0.621-0.701, *P*-value<0.0001) for distinguishing between PICU *vs.* non-PICU admission. The CRP AUC for discriminating between survivors and death was also significant (0.655, 95%CI 0.535-0.776, *P*-value=0.0153) (Appendix Figure 3, page 66).

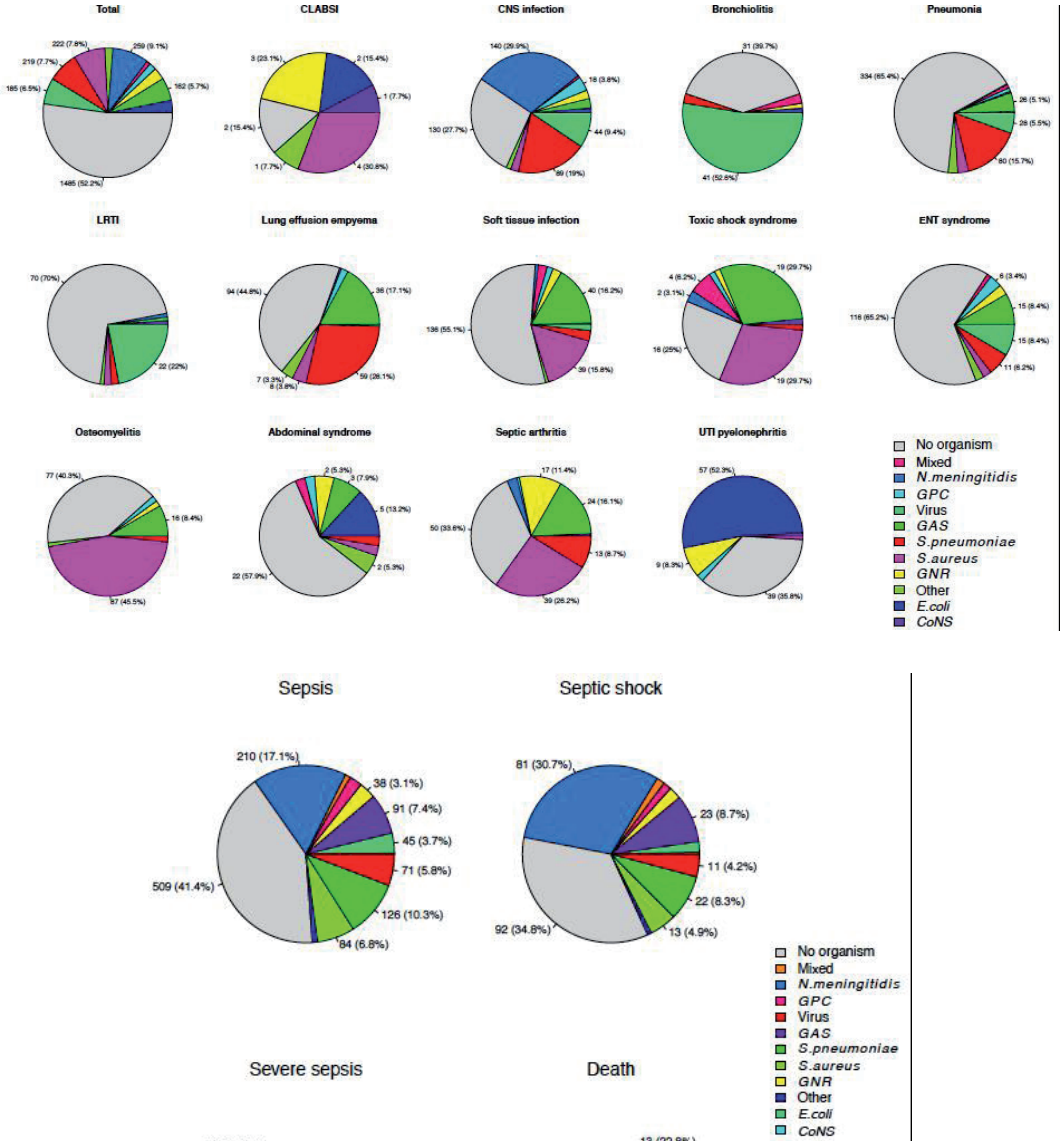


Figure 4. Causative microorganisms identified in EUCLIDS by syndrome.

(A) patients with severe focal infections and (B) sepsis. Data are expressed as (*n*) %. CNS infection: central nervous system infection, LRTI: lower respiratory tract infection, ENT syndrome: ear, nose, throat syndrome, UTI-pyelonephritis: urinary tract infection with pyelonephritis, GPC: gram positive cocci, GAS: *Group A Streptococcus*; GNR: gram negative rods, CoNS: *Coagulase Negative Staphylococci*.

Table 2: Description of serum levels of C-reactive protein (CRP) and neutrophil counts in different group of patients.

Data are expressed as % (*n*). * *P*-values lower than Bonferroni correction threshold (0.05/4=0.0125). SFI: Severe focal infection; PICU: paediatric intensive care unit.

	CRP≥60 mg/L	CRP<60 mg/L	*P*-value	Neutrophilis≥12´10⁹/L	Neutrophils<12´10⁹/L	*P*-value
Total	39.7 (966/2432)	60.3 (1466/2432)		68.2 (977/1432)	31.8 (455/1432)	
Sepsis *vs.* focal						
Sepsis	71.6 (755/1054)	28.4 (299/1054)	<0.0001*	35.8 (226/631)	64.2 (405/631)	0.0042*
SFI	51.6 (711/1378)	48.4 (667/1378)		28.6 (229/801)	71.4 (572/801)	
PICU *vs.* not PICU						
PICU	70.9 (654/922)	29.1 (268/922)	<0.0001*	36.3 (190/524)	63.7 (334/524)	0.0067*
Non-PICU	53.8 (812/1510)	46.2 (698/1510)		29.2 (265/908)	70.8 (643/908)	
Survivors *vs.* death						
Survivors	59.5 (1273/2139)	40.5 (866/2139)	0.0878	32.3 (397/1230)	67.7 (833/1230)	0.5039
Death	72.7 (32/44)	27.3 (12/44)		39.1 (9/23)	60.9 (14/23)	

ROC analysis for neutrophil count to discriminate sepsis *vs.* SFI showed an AUC of 0.553 (95%CI 0.523-0.583, *P*-value<0.0001) and 0.550 (95%CI 0.518-0.582, *P*-value=0.0015) for discriminating between PICU *vs.* non-PICU admission. The neutrophil AUC for discriminating between survivors and death was not significant (0.522, 95%CI 0.390-0.655, *P*-value=0.7158) (Appendix Figure 3, page 66).

Discussion

Our study highlights the burden of severe childhood infections, drawing on detailed clinical information from the largest prospective cohort of children with severe infection in Europe, recruited at 98 hospitals in 6 European countries. We demonstrate the continued importance of severe illness and mortality caused by vaccine-preventable infections (*N. meningitidis* and *S. pneumoniae*), and by pathogens for which vaccines are urgently required (*S. aureus* and *GAS*).

Laboratory tests failed to identify a causative pathogen in over half of children with severe illness, in line with data from the previous two decades [8, 11], despite the introduction of more sensitive and precise techniques in diagnostics in recent years. In over 50% of paediatric patients admitted with suspected life-threatening infections, decisions on need, type and duration of antimicrobial therapy thus have to be made with no clear guidance from the microbiological findings, indicating an urgent need for improved diagnostics. Patients with an identified microorganism suffered from more severe disease, which may suggest a higher pathogen load and more successful detection in these patients, but may be associated as well to increased diagnostic effort in the sickest patients.

Mortality

In our study, the case fatality ratio was 2.2%, significantly lower than that recently reported by two recent large studies [7, 8], although it should be noted that these studies were restricted to PICU patients with a more severe population (sepsis/septic shock). Mortality was highest in children with sepsis as defined by the International Paediatric Sepsis consensus conference [9]. The new sepsis definitions from 2016 [12] were not established for children, hence were not used in our study. Delay in timely treatment has been considered to increase the mortality risk in sepsis [6, 13]. Esteban et al. [14] reported a trend towards reduction in mortality after implementing an educational intervention for appropriate empiric antibiotic administration within the first hour of admission in children with sepsis. However, we were not able to assess this in our data. Our results are consistent with the reported mortality rates of patients with sepsis after the introduction of adequate treatment guidelines (hospital mortality 1%–3% in previously healthy, and 7%–10% in chronically ill children) [15], and with a recent population-based study on blood culture-proven bacterial sepsis [16]. As previously described [15], we found that mortality in community-acquired severe infections [6] was associated with the identification of the causative organism, the presence of sepsis, higher PICU admission rates, oxygen and/or respiratory support requirement, inotrope administration and prolonged LOS.

Severity and pathogen type

Though our study was not designed to reliably establish the relative prevalence of potentially causative pathogens; our results show the relative frequency of *N. meningitidis, S. pneumoniae*, *S. aureus* and GAS are roughly equal. Overall, the most frequent clinical syndromes were meningitis and pneumonia. Almost half of the patients admitted to hospital with a bacterial infection required intensive care admission. These findings are consistent with the reported leading causes of morbidity and mortality in children worldwide [1, 2]. The causative pathogens in our study differed from findings in Asia: were *Salmonella enterica* serotype Typhi was the most common bacterial pathogen, followed by *S. pneumoniae* and *Haemophilus influenzae* [17] and Africa: were *S. pneumoniae* is the most common isolate in children, followed by *S.aureus* and *E. coli* [18]. We also observed differences form studies in the United States were *S. aureus*, Pseudomonas species and Enterobacteriacae (mainly *E.coli)* were the main pathogens isolated. [19]

Vaccinations are an essential tool in our fight against infectious disease [4, 20, 21], and they have greatly reduced the global burden of infectious disease [21]. Although most patients were up-to-date according to their local immunisation schedule, we found that there was a considerable burden of mortality and morbidity caused by vaccine preventable infections, particularly meningococcal and pneumococcal disease. Vaccines for meningococcal serogroup B, Y, W and for a major proportion of pneumococcal

serotypes are not available or implemented in Europe. Thus, improved vaccines and implementation of current vaccines may yield further health gain. Wider implementation of existing vaccines and development of vaccines for *S. aureus* or *GAS* could contribute to a further decline in the burden of paediatric infectious diseases.

Tobacco smoke exposure

We found an increased risk of meningococcal infections in children exposed to tobacco (*P*-value<0.0001). In previous studies tobacco smoke exposure was associated with increased susceptibility to infections including tuberculosis, pneumonia, meningitis or otitis media. This could be explained by increased nasopharyngeal colonization with pathogens including *S. pneumoniae, H. influenzae* (non-type b), *M. catarrhalis*, *GAS and S. aureus* [22]. Furthermore, increased infection risk can also be explained by the reported interference of tobacco smoke with the antibacterial function of leukocytes (e.g. neutrophils, monocytes, T cells and B cells) [23].

Family history

The huge variation in clinical response to identical infecting pathogens is most likely the result of the combined effects of genetic variation in both the infecting pathogen and the infected host [24]. There is now strong evidence that host genetic factors influence occurrence of meningococcal disease, and a number of genes controlling susceptibility and severity of meningococcal disease have been identified in candidate gene association studies [25-28]. We found a significant association between family history of severe infectious diseases and meningococcal infection (*P*-value=0.0011),

Inflammatory biomarkers

In our study, higher CRP levels were associated with an increased risk of severe outcomes. Biomarkers may contribute to outcome prediction in life threatening infections [29]. However, there is still a need for improved host biomarker and pathogen diagnostics that can establish the clinical diagnosis, direct appropriate therapy and enable prediction of outcome [10]. Improved diagnostic discrimination in this group could have major implications for tackling rising antimicrobial resistance.

Sepsis outcomes for children in high-income countries have not changed dramatically over the past decade [6-8, 13]. Additional diagnostic approaches may help to establish the clinical diagnosis, direct early and adequate therapy and enable a more reliable outcome prognosis. It has been proposed that an approach combining sensitive pathogen diagnostics and novel host response biomarkers may improve treatment and clinical outcomes for children with serious infection [10]

PIRO concept

We identified specific variables associated with each of the components of PIRO concept [30] (predisposition, infection characteristics, host response and organ dysfunction): including age, gender, family history of severe infection, tabacco exposure, type of microorganism, infection focus, inflammatory biomarkers and a dynamic view of the patient's clinical course and outcomes. All of these variables described, contribute as a proof of concept of this novel approach and as a predictor of mortality for patients with community-acquired sepsis.

Limitations

Although children were recruited early in their clinical course, before a pathogen diagnosis was known, a limitation of our study is that children with known infection due to *N. meningitidis, S. pneumoniae, S. aureus,* and *GAS* were targeted for recruitment. The reason behind this targeted recruitment was to study the genetic basis of these pathogens as one of the main objectives of EUCLIDS Project (GA 279185). Overall the majority of patients were recruited unbiased, providing a cross section of different etiologies in children presenting with severe infection. But specific targeting of the four core pathogens will have caused bias towards these infections. The burden of disease from these selectively-target pathogens is within this study cohort but our study design limits the ability to generalize this to the broader population. Our findings thus cannot be used to establish population prevalence of each organism. Of note, one of EUCLIDS centres (Switzerland), where the recruitment strategy was to enroll children at a later time point, solely after confirmation of positive blood culture, were not included in this paper. In order to determine the disease burden and to elucidate the contributing factors to severe infection outcome, large epidemiological studies are needed. Recruitment was restricted to the hospital setting and did not capture out-of-hospital deaths, or severe focal infections managed as outpatients; our data therefore under-represent less severe infections. Eventhough the study used harmonised procedures for patient recruitment, sample processing, and sample storage, microbiological diagnosis was undertaken as part of clinical care using locally available clinical diagnostic procedures which could have limited in some way the assignment of patients as having viral infection, bacterial infection or co infection. We will report separately on additional viral studies undertaken as part of EUCLIDS using molecular diagnosis for a wide range of viruses.

Conclusions

This is the largest reported prospective study of severe childhood infections in Europe. Data collection was made possible by a diverse and widely representative European network. Recommendations or interventions based on our data are likely to reflect generalised patterns of illness and to be widely relevant across Europe. Although the mortality rate due to sepsis or SFI was low, we found considerable morbidity associated with severe childhood infection and more than a third of children required PICU admission. The burden of disease lies predominantly in children under 5 years and was predominantly caused by infections where vaccines are available: pneumococcus and meningococcus. We found that children had infections by common pathogens such as *S. aureus* (7.8%) and *GAS* (5.7%) for which there are no effective vaccines, and that 11.0% of the bacterial microorganisms were Gram-negative bacilli. Both of which, should have important implications for vaccine development and for empirical antimicrobial strategies implementation in Europe.

Appendix: Full definitions document

Focal infections

Lung infections

Pneumonia

It is an inflammation of one or both lungs: Lobar or segmental or multilobar collapse/ consolidation on CXR. Do not include perihilar consolidation or patchy consolidation.

Clinical symptoms compatible with acute respiratory infection and following radiological findings of consolidation/pleural effusion: alveolar consolidation (defined as a dense or fluffy opacity that occupies a portion or whole of a lobe or of the entire lung that may or may not contain air-bronchograms) or pleural effusion (defined as fluid in the lateral pleural space and not just in the minor or oblique fissure) that was spatially associated with a pulmonary parenchymal infiltrate (including other infiltrate) or obliterated enough of the hemithorax to obscure an opacity.

Pleural Effusion / Empyema

Simple parapneumonic effusion is defined as pleural effusion associated with lung infection (ie, pneumonia). These effusions result from the spread of inflammation and infection to the pleura. Much less commonly, infections in other adjacent areas (eg, retropharyngeal, vertebral, abdominal, and retroperitoneal spaces) may spread to the pleura resulting in the development of effusion.

Empyema is defined as the presence of grossly purulent fluid in the pleural cavity. In practice:

- Thoracentesis with microbial growth from pleural fluid or
- Thoracentesis with no growth on culture of pleural fluid but elevated protein, or cell count (normal and abnormal reference values as determined by clinical laboratory at each center)
- Ultrasound or other diagnostic imaging evidence of pleural fluid assessed by the radiologist as empyema or
- Diagnosis at time of thoracic surgery.

Whooping cough (*Bordetella pertussis*)

Clinical diagnosis — For endemic or sporadic cases, a clinical case of pertussis is defined as an acute cough illness lasting at least 14 days accompanied by one of the following:

- Paroxysms of coughing
- Inspiratory whoop
- Post-tussive vomiting

In an outbreak or following household contact to a known case, a clinical case is defined as a cough illness for at least 14 days; presence of the typical pertussis- associated features is not required.

Definite diagnosis — Clinical diagnosis confirmed by bacterial culture, polymerase chain reaction (PCR) or serology. Note that direct fluorescent antibody should not be considered due to variable specificity.

Bronchiolitis

Bronchiolitis is diagnosed clinically by the presence of viral upper respiratory prodromes followed by increased respiratory effort (eg, tachypnea, nasal flaring, chest retractions) and wheezing and/or rales in children younger than two years of age.

Neurological infections:

Meningitis

Meningitis is an infection of the membranes covering the brain and spinal cord (leptomeninges).

Compatible clinical syndrome: Any child (0-18 years) with clinical symptoms compatible with meningitis (a severe headache, fever, nausea, vomiting and feeling generally unwell). The symptoms in babies and young children are: becoming floppy and unresponsive, or stiff with jerky movements, becoming irritable and not wanting to be held, unusual crying, pale and blotchy skin, refusing feeds, loss of appetite, a staring expression, very sleepy and reluctant to wake up.

Definite bacterial meningitis

Compatible clinical syndrome, plus

- All ages: fever, 94%
- 1–5 mos: irritability, 85%
- 6–11 mos: impaired consciousness, 79%
- >12 mos: vomiting, 82%; neck rigidity, 78%
- (note: many other compatible signs and symptoms) plus

Positive culture of cerebrospinal fluid (CSF), or positive CSF Gram stain, PCR or bacterial antigen.

Probable bacterial meningitis

Compatible clinical syndrome, plus

Positive culture of blood, plus

One of the following CSF changes

- >5 leukocytes
- Protein of >100 mg/dL
- Glucose of <40mg/dl (<2.2mmol/l) or 0.5 CSF/serum ratio

Possible bacterial meningitis

Compatible clinical syndrome, plus

- One of the following CSF changes
  - >100 leukocytes
  - Glucose of < 40 mg/dl (<2.2mmol/l) or CSF/serum glucose ratio 0.5
  - Protein of > 100 mg/dL plus
- Negative cultures or antigen for bacteria, viral, fungal, or mycobacteria

Confirmed: A case that is laboratory-confirmed by growing (i.e. culturing) or identifying (i.e. by PCR or Gram stain or antigen detection methods) a bacterial pathogen (Hib, pneumococcus or meningococcus) in the CSF or from the blood in a child with a clinical syndrome consistent with bacterial meningitis

Note: Any persons with *H. influenzae*, meningococcus or pneumococcus isolated from CSF or blood may be considered as confirmed cases of meningitis if their clinical syndrome was meningitis (i.e. culture from normally sterile fluids is the gold standard). Culture of Hib, pneumococcus or meningococcus from a non-sterile site, such as the throat, does not confirm a case of disease, since the bacteria can grow in these other areas without causing disease.

Bacterial brain abscess

Brain abscess is a focal collection within the brain parenchyma caused by a bacterial infection, which can arise as a complication of a variety of infections, trauma, or surgery.

The diagnosis of focal collection is confirmed by CT scan with contrast or MRI.

The diagnosis of bacterial brain abscess is confirmed by a positive culture or positive Gram stain or positive 16s or bacterial antigen in specimen obtained from stereotactic CT-guided aspiration or surgery.

For EUCLIDS purposes, possible bacterial brain abscess can be included only on clinical and radiological findings.

Bone and Joint infection

Osteomyelitis:

It is defined as an inflammation or an infection in the bone marrow and surrounding bone.

In the EUCLIDS study both acute, subacute and chronic bacterial osteomyelitis are of interest and to be included:

- Acute osteomylitis is defined by a duration of symptoms < 14 days.
- Subacute osteomyelitis is definedy by a duration of symptoms between 14 days and 1 month.
- Chronic osteomyelits is defined by a duration of symptoms more than 1 month.

The diagnosis of acute/subacute/chronic osteomyelitis is based on the following criteria:

- Presence of localized pain/tenderness and other typical features of osteomyelitis (warmth and/or swelling of the affected region)

AND/OR

- Imaging findings conistent with osteomyelitis (typical MRI findings and/or a positive bone scan)

AND/OR

- Bacteriologic evidence of infection (positive blood and/or bone culture).

AND/OR

- Histopathological finding consistent with osteomyelitis (intraoperative specimen)

For diagnosis of osteomyelitis at least two criteria must be positive.

Septic arthritis:

It is diagnosed when a microorganism is isolated from blood with clinical arthritis, from the synovial fluid and/or purulent fluid is aspirated from the joint. Synovial fluid with white blood cell count (WBC) 50,000/mm3 is considered purulent.

MRI findings consistent with acute/subacute/chronic osteomyelitis:

- On unenhanced images, osteomyelitis is characterized by focally decreased marrow signal intensity on T1-weighted images AND focally increased marrow signal intensity on fluid-sensitive images (fat-suppressed T2-weighted and STIR sequences).

OR

- After contrast administration, osteomyelitis is described as focal abnormal bone marrow enhancement on fat-suppressed T1-weighted images.

AND/OR

- Complications of osteomyelitis can include abscesses: Intraosseous, subperiosteal, and soft-tissue abscesses are defined as well circumscribed areas of focally decreased signal intensity on T1-weighted images with increased signal intensity equal to that of fluid on fluid-sensitive sequences and/or rim enhancement on contrast-enhanced fat-suppressed T1-weighted images.
- Subacute osteomyelitis can manifest as Brodie abscess characterized by a central abscess cavity filled with fluid, an inner ring of enhancing high signal intensity granulation tissue on T1-weighted sequences, an outer ring of very low signal intensity sclerosis, and a peripheral halo of edema.
- In chronic osteomyelitis, imaging might reveal an involucrum (thick periosteal new bone), sequestrum (necrotic bone fragment), or cloaca (draining tract through a defect in the cortex and involucrum).

Bone scan consistent with osteomyelitis (Technetium-99m bone scan):

- The most definitive phase is the delayed phase: There is no osteomyelitis without abnormal radionuclide uptake on the images obtained during the delayed phase, even if there is increased activity on blood flow or blood pool images.

AND/OR

- The hallmark feature of osteomyelitis at 99mTc scintigraphy is increased activity in all three phases (1. angiographic or blood flow phase, 2. blood pool or tissue phase and 3. delayed phase).

Histopathological finding consistent with acute/subacute/chronic osteomyelitis:

- Inflammatory cells
  - Inacute osteomyelitis: predominately polymorphonuclear leucocytes
  - In chronic osteomyelitis: mononuclear cells including plasma cells and macrophage/monocyte cells
  -

AND/OR

- Destruction/necrosis of bone (necrotic marrow and bone, osteoclastic activity)

AND/OR

- Granulation tissue (hemorrhage, polymorphonuclear leucocytes, lymphocytes, and macrophages)

AND/OR

- In implant-associated infections, tissue specimens obtained for histopathology either by biopsy or during surgery as frozen section are important because the presence of neutrophils in significant amounts is indicative of infection. More than five neutrophils per high-power field indicates infection, with sensitivity of 43–84% and specificity of 93–97%. These infections will be considered as "community acquired" depending on the onset of symptoms after implantation: if >24months have elapsed it is considered a community acquired infection.

Diskitis/spondilodiskitis

Diskitis is an inflammatory process involving the intervertebral disks and the endplates of the vertebral bodies, and associated with characteristic clinical and radiologic findings.

Mastoiditis

Mastoiditis is a suppurative infection of the mastoid air cells, and the most common suppurative complication of acute otitis media. In acute mastoiditis, symptoms are of less than 1 month's duration.

There is a lack of consensus regarding the criteria and strategies for diagnosing acute mastoiditis in the paediatric population. The diagnosis is usually made clinically, without need for imaging studies.

- Clinical features:
  - Fever
  - Otalgia
  - Post-auricular erythema, tenderness, swelling, fluctuance or mass
  - Displacement of the auricle (down and out: children <2years); up and out in children ≥2years
- Imaging: CT, MRI: Haziness or destruction of the mastoid outline; and loss of or decrease in the sharpness of the bony septa that define the mastoid air cells.
- Microbiology: Positive culture or gram stain of a specimen obtained from the middle ear either by tympanocentesis through an intact eardrum or by aspiration through a tympanostomy tube or perforation.

Soft tissue infections:

Cellulitis

Acute, diffuse, spreading infection of the skin, involving the deeper layers of the skin and the subcutaneous tissue.

Ecthyma /erysipelas

Ecthyma is a bacterial infection of the dermis and epidermis characterized by a vesicle or vesico-pustule with an erythematous base that erodes through the epidermis into the dermis to form a crusted ulcer with elevated margins up to 4 cm in diameter.

Clinical diagnosis is confirmed by a positive culture or Gram stain of the lesion. Erysipelas is a superficial form of cellulitis with lymphatic involvement.

Necrotizing fasciitis

Necrotizing fasciitis is an infection of the deeper tissues that results in rapidly progressive destruction of the muscle fascia, overlying subcutaneous fat and epidermis. The definite diagnosis is surgical.

Myositis / pyomyositis

Myositis is an inflammation of the skeletal muscles, often caused by infection or autoimmune disease. Pyomyositis is a bacterial infection of the skeletal muscle that is usually caused by Staphylococcus aureus.

Pyomyositis is suspected by the clinical presentation (fever and pain with cramping usually localized to a single muscle group) and compatible findings in image techniques (Rx, CT, US, MRI). Definite diagnosis is made by culture and gram stain of drainage specimen.

Deep neck infections

Suppurative infection of the neck, including:

- Peritonsillar abscess: Collection of pus located between the capsule of the palatine tonsil and the pharyngeal muscles.
- Retropharyngeal abscess: Collection of pus located in the retropharyngeal space (extending from the base of the skull to the posterior mediastinum, between the middle layer and the deep layer of the deep cervical fascia).
- Lateral pharyngeal space infection: Collection of pus located in the lateral pharyngeal space (bounded laterally by the carotid sheath).
- Suppurative cervical lymphadenitis: Enlarged, inflamed and tender lymph node with or without fluctuance, usually unilateral. Clinical diagnosis is confirmed by positive culture or Gram stain of specimen obtained by needle aspiration or incision and drainage.

Intra-abdominal infections:

Acute appendicitis

Acute inflammation of the appendix, usually resulting from bacterial infection.

Clinical presentation is variable, often consisting of abdominal pain and tenderness in periumbilical region (early) migrating to the right lower quadrant of the abdomen, vomiting, fever and signs of localized or generalized peritoneal irritation.

Definite diagnosis is made by demonstration of an inflamed or perforated appendix on pathology after surgical removal.

Infectious peritonitis

Infection of the peritoneum, usually secondary to inoculation of the peritoneal cavity with bacteria and other inflammatory debris following intestinal perforation or postoperative anastomotic leak. Acute appendicitis is the most commonly associated condition leading to secondary peritonitis in older children.

Clinical diagnosis is confirmed by positive culture or Gram stain of peritoneal fluid.

Pyelonephritis

Urinary tract infection affecting the renal parenchyma and pelvis. In a patient with fever in absence of another source of infection:

- Possible pyelonephritis: presence of positive leukocyte esterase test results or nitrite test or microscopic analysis results positive for leukocytes or bacteria in a urine specimen collected by the most convenient means and compatible findings in renal ultrasonography, voiding cystourethrography or nuclear scanning with technetium- labeled dimercaptosuccinic acid.
- Definite pyelonephritis:
  - Presence of both pyuria and at least 50,000 colonies per mL of a single uropathogenic organism in an appropriately collected specimen of urine (by urethral catheterization or suprapubic aspiration)
  - and compatible findings in renal ultrasonography, voiding cystourethrography or nuclear scanning with technetium-labeled dimercaptosuccinic acid.

Toxic shock definition:

Staphylococcal toxic shock syndrome clinical case definition

1. Fever ≥38,9°C
2. Rash—diffuse macular erythroderma
3. Desquamation—1–2 weeks after onset of illness, especially of palms and soles
4. Hypotension—systolic blood pressure ≤90 mm Hg for adults
5. Multi-system involvement—3 or more of the following:
   a) Gastrointestinal—vomiting or diarrhoea at the onset of illness
   b) Muscular—severe myalgia or elevated creatine phosphokinase
   c) Mucous membranes—vaginal, oropharyngeal, conjunctival hyperaemia
   d) Renal—blood urea nitrogen or creatinine twice-upper limit of normal
   e) Hepatic—total bilirubin twice-upper limit of normal
   f) Haematological—platelets ≤100×109/L
   g) CNS—disorientation or alterations in consciousness without focal neurological signs
6. Negative results on the following tests:
   a) Blood, throat, or cerebrospinal fluid culture for another pathogen (blood culture may be positive for *Staphylococcus aureus*)
   b) Rise in titre to Rocky Mountain spotted fever, leptospirosis, or measles

Case classification:

- Probable: case with five of the six clinical findings described
- Confirmed: case with all six of the clinical findings described

Streptococcal toxic shock syndrome clinical case definition

1. Isolation of group A β-haemolytic streptococci:
   a) From a normally sterile site—blood, CSF, peritoneal fluid, tissue biopsy
   b) From a non-sterile site—throat, vagina, sputum 2. Clinical signs of severity:
   a) Hypotension—systolic blood pressure ≤90 mm Hg in adults or below normal age adjusted levels in children
   b) Two or more of the following signs:
      i) Renal impairment—creatinine >2 mg/dL (>177 μmol/L)
      ii) Coagulopathy—platelets ≤100×109/L or disseminated intravascular coagulation
      iii) Hepatic involvement—alanine aminotransferase, aspartate aminotransferase, or total bilirubin twice the upper limit of normal
      iv) Adult respiratory distress syndrome
      v) Generalised, erythematous, macular rash that may desquamate
      vi) Soft-tissue necrosis, including necrotising fasciitis, myositis, or gangrene

Case classification:

- Probable: case fulfils 1b and 2 (a and b) if no other cause for the illness is found –
- Confirmed: case fulfils 1a and 2 (a and b)

Clinical syndromes

Bacteraemia/septicaemia:

Systemic Inflammatory Response Syndrome (SIRS)

As per clinical criteria established by Goldstein et al, SIRS is defined by at least two of the following four criteria:

1.- Core (rectal, bladder, oral or central catether probe) temperature of > 38.5°C or < 36°C.
2.- Tachycardia, defined as a mean heart rate >2 SD above normal for age in the absence of external stimulus, chronic drugs, or painful stimuli; or otherwise unexplained persistent elevation over a 0.5- to 4-hr time period or OR for children <1 yr old: bradycardia, defined as a mean heart rate <10th percentile for age in the absence of

external vagal stimulus, B-blocker drugs, or congenital heart disease; or otherwise unexplained persistent depression over a 0.5-hr time period.

3.- Mean respiratory rate >2 SD above normal for age or mechanical ventilation for an acute process not related to underlying neuromuscular disease or the receipt of general anesthesia.

4.- Leukocyte count elevated or depressed for age (not secondary to chemotherapy-induced leukopenia) or > 10% immature neutrophils.

Sepsis

Defined as suspected infection plus SIRS, as per clinical criteria established by Goldstein et al, as long as temperature or leukocyte count is abnormal.

Severe sepsis

Sepsis plus one of the following:

- Acute respiratory distress syndrome OR
- Two or more other organ dysfunctions.
- NOTE: severe sepsis + cardiovascular organ dysfunction =septic shock (see below)

Respiratory dysfunction

- PaO2/FIO2 < 300 in absence of cyanotic heart disease or preexisting lung disease, OR
- $PaCO_2$ >65 torr or 20 mm Hg over baseline $PaCO_2$, OR
- Proven need or >50% FIO2 to maintain saturation >92%, OR
- Need for nonelective invasive or noninvasive mechanical ventilation Neurologic dysfunction
- Glasgow Coma Score <11, OR
- Acute change in mental status with a decrease in Glasgow Coma Score >3 points from abnormal baseline

Hematologic dysfunction

- Platelet count <80,000/mm3 or a decline of 50% in platelet count from highest value recorded over the past 3 days (for chronic hematology/oncology patients), OR
- International normalized ratio >2

Renal dysfunction

- Serum creatinine >2 times upper limit of normal for age or 2-fold increase in baseline creatinine

Hepatic dysfunction

- Total bilirubin >4 mg/dL (not applicable for newborn) OR
- ALT 2 times upper limit of normal for age

It is the presence of bacteria, other infectious organisms, or toxins created by infectious organisms in the bloodstream with spread throughout the body.

Septic shock

Sepsis and cardiovascular organ dysfunction.

Cardiovascular dysfunction

Despite administration of isotonic intravenous fluid bolus >40 mL/kg in 1 hr

- Decrease in BP (hypotension) <5th percentile for age or systolic BP <2 SD below normal for age, OR
- Need for vasoactive drug to maintain BP in normal range (dopamine >5 microg/kg/min or dobutamine, epinephrine, or norepinephrine at any dose), OR
- Two of the following
  - Unexplained metabolicacidosis:basedeficit >5.0mEq/L
  - Increasedarteriallactate >2timesupperlimitofnormal
  - Oliguria: urineoutput <0.5mL/kg/hr
  - Prolongedcapillaryrefill:>5secs
  - Coretoperipheraltemperaturegap>3°C

CLABSI (CDC definition)

Central line-associated BSI (CLABSI): A laboratory-confirmed bloodstream infection (LCBI) where central line (CL) or umbilical catheter (UC) was in place for >2 calendar days when all elements of the LCBI infection criterion were first present together, with day of device placement being Day 1,

AND

CL or UC was in place on the date of event or the day before. If the patient is admitted or transferred into a facility with a central line in place (e.g., tunnelled or implanted central line), day of first access is considered Day1.

Must meet one of the following criteria:

1. Patient has a recognized pathogen cultured from one or more blood cultures and organism cultured from blood is not related to an infection at another site.

2. Patient has at least one of the following signs or symptoms: fever (>38oC), chills, or hypotension

AND

positive laboratory results are not related to an infection at another site

AND

common commensal (i.e., diphtheroids [*Corynebacterium spp*. not *C. diphtheriae*], *Bacillus spp*. [not *B. anthracis*], Propionibacterium spp., coagulase-negative staphylococci [including *S. epidermidis*], viridans group streptococci, *Aerococcus spp., and Micrococcus spp.*) is cultured from two or more blood cultures drawn on separate occasions. Criterion elements must occur within a timeframe that does not exceed a gap of 1 calendar day.

(See complete list of common commensals at http://www.cdc.gov/nhsn/XLS/master-organism-Com-Commensals-Lists.xls)

3. Patient ≤1 year of age has at least one of the following signs or symptoms: fever (>38°C core) hypothermia (<36°C core), apnea, or bradycardia

AND

positive laboratory results are not related to an infection at another site

AND

common skin commensal (i.e., diphtheroids [*Corynebacterium spp. not C. diphtheriae*], *Bacillus spp*. [not *B. anthracis*], *Propionibacterium spp.*, coagulase- negative staphylococci [including *S. epidermidis], viridans group streptococci, Aerococcus spp., Micrococcus spp.)* is cultured from two or more blood cultures drawn on separate occasions. Criterion elements must occur within a timeframe that does not exceed a gap of 1 calendar day.

(See complete list of common commensals at http://www.cdc.gov/nhsn/XLS/master-organism-Com-Commensals-Lists.xlsx)

Scarlet Fever (positive throat swab, admitted)

Scarlet fever is an infection that is caused by *Group A streptococcal* bacteria (*S. pyogenes*). The disease is characterized by a sore throat, fever, and a sandpaper-like rash on reddened skin.

In the EUCLIDS study, scarlet fever (positive throat swab) is of interest and to be included.

Gastroenteritis by salmonella (salmonellosis)

Salmonellosis is a disease caused by the bacteria salmonella. It is usually characterized by acute onset of fever, abdominal pain, diarrhoea, nausea and sometimes vomiting.

In the EUCLIDS study, gastroenteritis for salmonella (positive culture stool or blood?) is of interest and to be included.

Endocarditis:

It´s an inflammation of one or more of the heart valves and lining tissues of the heart. Symptoms are nonspecific and include fever, chills, and weakness

In the EUCLIDS study, bacterial endocarditis (positive culture) is of interest and to be included.

Dukes Clinical Criteria for Diagnosis of Infective Endocarditis

DEFINITE INFECTIVE ENDOCARDITIS

Pathologic Criteria

- Microorganisms: demonstrated by culture or histology in a vegetation, in a vegetation that has embolized or in an intracardiac abscess
- Pathologic lesions: vegetation or intracardiac abscess present, confirmed by histology showing active endocarditis

Clinical Criteria (see below)

- Two major criteria, OR
- One major and three minor criteria, OR
- Five minor criteria

POSSIBLE INFECTIVE ENDOCARDITIS

- One major criterion and one minor criterion OR
- Three minor criteria

REJECTED

- Firm alternative diagnosis for manifestations of endocarditis, OR
- Resolution of manifestations of endocarditis with antibiotic therapy for ≤ 4 days, OR
- No pathologic evidence of infective endocarditis at surgery or autopsy, after antibiotic therapy for < 4 days, OR
- Does not fulfill criteria above

Definitions of Major and Minor Criteria Used in the Duke Schema for the Diagnosis of Infective Endocarditis (IE)

MAJOR CRITERIA

1. Positive blood culture for IE

a. Typical microorganism consistent with IE from two separate blood cultures:
- *Viridans streptococci*
- *Streptococcus bovis*
- HACEK group [a]
- *Staphylococcus aureus*
- Community-acquired enterococci (without a primary focus)
- Single positive blood culture for *Coxiella burnetii* or IgG antibody titer > 1:800

2. Evidence of endocardial involvement.

a. Positive echocardiogram for IE, defined as:
- Oscillating intracardiac mass on valve or supporting structures, in the path of regurgitant jets, or on implanted material in the absence of an alternative anatomic explanation
- Abscess
- New partial dehiscence of prosthetic valve

b. New valvular regurgitation (worsening or changing of pre-existing murmur not sufficient)

MINOR CRITERIA

1. Predisposition: predisposing heart condition or intravenous drug use
2. Fever: temperature ≥38.0°C
3. Vascular phenomena: major arterial emboli, septic pulmonary infarcts, mycotic aneurysm, intracranial hemorrhage, conjunctival hemorrhages, and Janeway lesions
4. Immunologic phenomena: glomerulonephritis, Osler nodes, Roth spots, and rheumatoid factor
5. Microbiologic evidence: positive blood culture but does not meet a major criterion as noted above [b] or serologic evidence of active infection with organism consistent with IE

[a] HACEK: *Haemophilus aphrophilus, Actinobacillus actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens, Kingella kingae.*

[b] Excludes single positive cultures for coagulase-negative staphylococci and organisms that do not cause endocarditis.

Influenza-like illness

Sudden-onset fever (>38°C) with headache, myalgia, malaise and manifestation of URTI, such as cough, sore throat or rhinitis, in the absence of other diagnoses.

Fever without source (FWS)

Children with fever lasting for one week or less without adequate explanation after a careful history and thorough physical examination.

It is also known as fever without localizing signs or fever without focus.

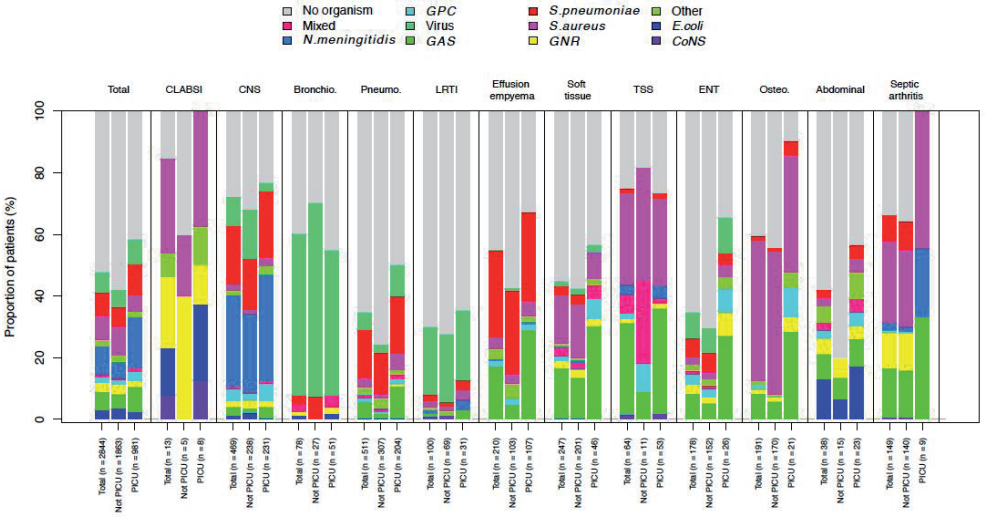
Fever of unknown origin (FUO)

Children with fever >38.3°C of at least 8 days' duration, in whom no diagnosis is apparent after initial outpatient or hospital evaluation that includes a careful history and physical examination and initial laboratory assessment.

Appendix: Causes of death for patients with SFI.

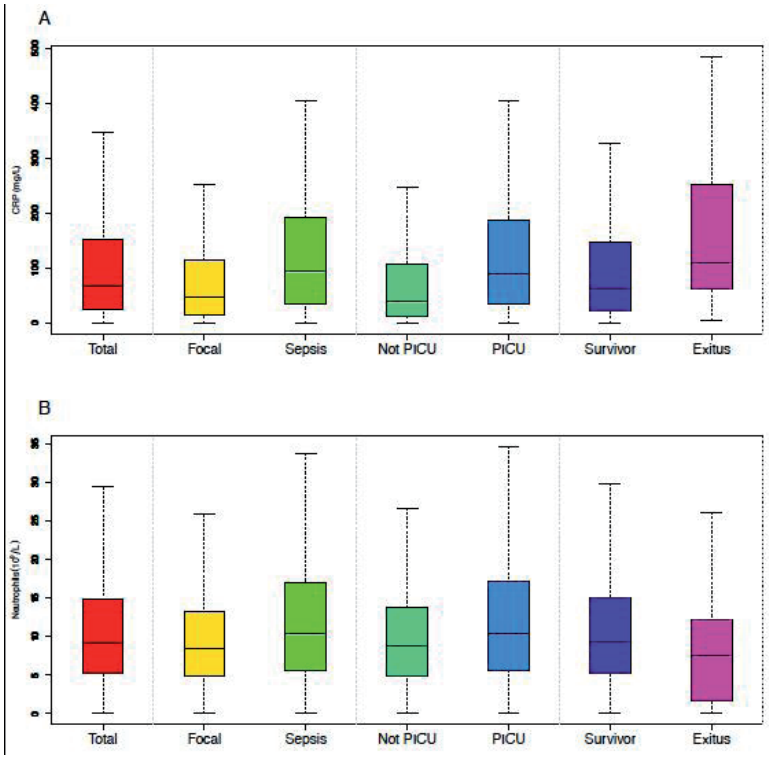
The 7 patients with SFI who died were due to a decompensation of a chronic disease or a complication from the initial infection as follows:

- Patient 1: acute necrotizing encephalopathy
- Patient 2: pneumonia in the context of a complex congenital heart disease
- Patient 3: lower respiratory tract infection on background of chronic lung disease
- Patient 4: acute respiratory distress syndrome and pulmonary haemorrhage in the context of an RSV infection
- Patient 5: cerebral infarction and cardiac failure in the context of a complex congenital disease operated
- Patients 6: respiratory failure in a patient with Leigh's disease
- Patient 7: pulmonary haemorrhage in a patient with epilepsy, scoliosis, deformity of spine and lissencephaly



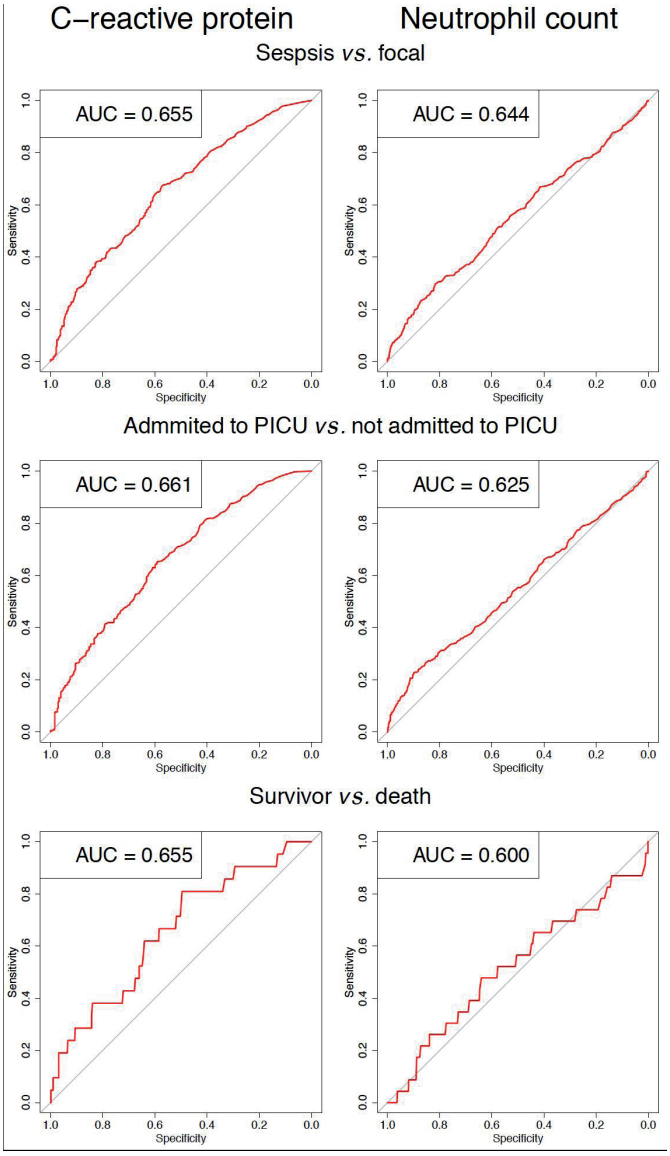
Appendix Figure 1: Differences between the identified organisms in whole cohort, those admitted to PICU and those admitted to wards by syndrome.

GPC: gram positive cocci, GAS: *Group A Streptococcus*, GNR: gram negative rods, CoNS: *Coagulase Negative Staphylococci*.



Appendix Figure 2: Serum levels of (A) C-Reactive protein (CRP) and (B) neutrophils counts in different group of patients on admission.

Data are expressed as mg/L for CRP and x10^9/L for neutrophils count.



Appendix Figure 3: Receiver operating characteristic curve of CRP, and neutrophil count in different settings.

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Chapter 2.2

Mortality and morbidity in community-acquired sepsis in European pediatric intensive care units: a prospective cohort study from the European Childhood Life-threatening Infectious Disease Study (EUCLIDS)

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Abstract

Background: Sepsis is one of the main reasons for non-elective admission to pediatric intensive care units (PICUs), but little is known about determinants influencing outcome. We characterized children admitted with community-acquired sepsis to European PICUs and studied risk factors for mortality and disability.

Methods: Data were collected within the collaborative Seventh Framework Programme (FP7)-funded EUCLIDS study, which is a prospective multicenter cohort study aiming to evaluate genetic determinants of susceptibility and/or severity in sepsis. This report includes 795 children admitted with community-acquired sepsis to 52 PICUs from seven European countries between July 2012 and January 2016. The primary outcome measure was in-hospital death. Secondary outcome measures were PICU-free days censured at day 28, hospital length of stay, and disability. Independent predictors were identified by multivariate regression analysis.

Results: Patients most commonly presented clinically with sepsis without a source (n = 278, 35%), meningitis/encephalitis (n = 182, 23%) and pneumonia (n = 149, 19%). Of 428 (54%) patients with confirmed bacterial infection, *Neisseria meningitidis* (n=131, 31%) and *Streptococcus pneumoniae* (n=78, 18%) were the main pathogens. Mortality was 6% (51/795), increasing to 10% in the presence of septic shock (45/466). Of the survivors, 31% were discharged with disability, including 24% of previously healthy children who survived with disability. Mortality and disability were independently associated with *S. pneumoniae* infections (mortality OR 4.1, 95% CI 1.1-16.0, $P = 0.04$; disability OR 5.4, 95% CI 1.8-15.8, $P < 0.01$) and illness severity as measured by Pediatric Index of Mortality (PIM2) score (mortality OR 2.8, 95% CI 1.3-6.1, $P < 0.01$; disability OR 3.4, 95% CI 1.8-6.4, $P < 0.001$).

Conclusions: Despite widespread immunization campaigns, invasive bacterial disease remains responsible for substantial morbidity and mortality in critically ill children in high-income countries. Almost one third of sepsis survivors admitted to the PICU were discharged with some disability. More research is required to delineate the long-term outcome of pediatric sepsis and to identify interventional targets. Our findings emphasize the importance of improved early sepsis recognition programs to address the high burden of disease.

Background

Pediatric sepsis represents one of the most common reasons for Pediatric Intensive Care Unit (PICU) admission, and the prevalence and mortality in high-income countries has become comparable to that in adults.[1-5] In 2013, 10% of childhood deaths under the age of 5 years in high-income countries were attributable to infections, with the majority of acute infection-related deaths occurring in PICU.[6] Recent reports have demonstrated the major impact of comorbidities with increasing rates of healthcare-associated infections.[1, 2, 4, 5, 7-9]

In contrast, recent data on community-acquired sepsis are limited. Community-acquired sepsis represents specific patterns, affecting different hosts, and involving different pathogens, which may translate into different outcomes compared to healthcare-associated infections.[10, 11] In view of the need to develop improved strategies for early recognition and treatment of sepsis, as demanded by the recent resolution of the World Health Organization[12], it is imperative to assess contemporary characteristics of epidemiology and severity predictors for community-acquired sepsis.[13] Previous larger epidemiological sepsis studies have been predominantly based on hospital coding or PICU databases with mortality as the main outcome.[1, 5] A recent roadmap for future sepsis research highlighted the inherent limitations of such approaches, identifying the need to define the longer-term impact on survivors.[14] While increasing evidence in neonatal and adult patients demonstrate that new cognitive impairment, functional disability, and impaired quality of life are common amongst sepsis survivors[15-18], little is known about disability in pediatric sepsis survivors.[19, 20]

The aim of this study was to characterize clinical presentations, pathogens, mortality, and disability in children admitted to European PICUs with community-acquired sepsis, based on patients recruited through the multinational prospective European Childhood Life-threatening Infectious Disease Study (EUCLIDS).

Methods

Consortium and study sites

The EUCLIDS is a Seventh Framework Programme (FP7) project in the context of the European Union's Research and Innovation funding programme for 2007-2013. This large-scale prospective, multicenter, cohort study aimed to identify genes, and biological pathways that determine susceptibility and severity in life-threatening bacterial infections of childhood. The EUCLIDS clinical network includes predominantly academic pediatric hospitals that host a total of 52 PICUs from 7 European countries; Austria (9), Germany (7), Lithuania (1), The Netherlands (5), Spain (9), Switzerland (8) and the UK (13).

Study patients

From July 2012 to January 2016, patients aged 29 days to 18 years admitted with community-acquired sepsis to PICUs in participating centers were prospectively enrolled in the study. The 2005 pediatric consensus criteria for sepsis were used, dividing patients into those with sepsis, severe sepsis, or septic shock.[21] Healthcare-associated infections[22], patients undergoing bone marrow transplant, and patients already recruited who were readmitted within the same illness episode were excluded. Children with a central venous catheter at admission were not excluded. Although the consortium was specifically interested in patients with invasive meningococcal, pneumococcal, staphylococcal, salmonella, and group A streptococcal infections, representing the most common causes of community-acquired sepsis in children, presentations due to other organisms were included as well. Patients were recruited as early as possible in the illness within a time window from presentation to the time when culture results became available.

Ethical aspects

This study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. The study protocol was approved by at least one ethical review board in every country (Coordinating Center Research Ethics Committee reference: 11/LO/1982).[23] Written informed consent was obtained from parents or legal guardians. In the Swiss study[24, 25], consent was obtained for collection of blood for research, but waiver of consent for collection of anonymized epidemiological data was approved.

Clinical data collection

Data on clinical presentation, underlying disease, illness severity, management, microbiological results, and outcome were collected prospectively. Children were split into four age categories; infants (29 days to <1 year), toddlers (≥1 year to <5 years), school-aged children (≥5 years to <12 years), and adolescents (≥12 year to <18 years). Underlying conditions at admission to the PICU were classified following the pediatric complex chronic conditions classification system.[26] Illness severity was measured by the Pediatric risk of mortality score (PRISM)[27] and Pediatric Index of Mortality (PIM2)[28]. We studied lactate values obtained on admission, concomitant with PIM2 data collection. Invasive bacterial infections were defined as isolation by culture or PCR of a bacterial organism from a normally sterile site. We considered blood, cerebrospinal fluid, urine, bronchoalveolar lavage, joint aspirate, abscess aspirate, intraoperative swabs, and pleural aspirate as sterile sites. Urine positive for pneumococcal antigen was also considered as an invasive bacterial infection if patients met sepsis criteria. Positive cultures from sites such as endotracheal tube aspirate, nasopharyngeal aspirate, throat/nasal swabs, and wounds were not considered as sterile sites. We defined potentially vaccine-preventable infections as infections caused by pathogens that are included in currently

available national immunization programs, with a focus on *Haemophilus influenzae* type B (HiB), meningococcus serogroups ACWY (MenACWY), meningococcus serogroup B (MenB), meningococcus serogroup C (MenC), pneumococcal conjugate vaccine 7 (PCV7, Prevnar, serotypes 4, 6B, 9V, 14, 18C, 19F and 23F), pneumococcal conjugate vaccine 10 (PCV10, Synflorix, additional serotypes 1, 5, 7F), pneumococcal conjugate vaccine 13 (PCV13, Prevnar 13, additional serotypes 3, 6A, 19A) and pneumococcal polysaccharide vaccine 23 (PPSV23, additional serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, 33F). Data on routine immunization schedules and uptake in the countries involved are presented in Additional file 1: Table S1. We classified patients as primary bloodstream infection and sepsis without a known source (grouped as *no focus*) versus patients with a clinical focus of infection. Patients admitted with systemic inflammatory response syndrome (SIRS) in the presence of suspected infection (i.e. sepsis), in whom a bacterial, viral or fungal infection eventually could not be confirmed, were categorized as clinical presentation *other*.

Outcomes

The primary outcome measure was death in hospital, recorded as alive or death status at the time of hospital discharge. Secondary outcomes were assessed at time of hospital discharge and included disability, PICU-free days censored at day 28 (days alive and free from the need for intensive care), and hospital length of stay. Disability was defined as a Pediatric Overall Performance Category (POPC) scale > 1 [29], need for skin graft, amputation, or hearing loss. The POPC scale was determined either by direct observation or by chart review and ranges from 1 to 6: (1) good overall performance, (2) mild overall disability, (3) moderate overall disability, (4) severe overall disability, (5) coma or vegetative state, and (6) brain death. A description of these categories is presented in Additional file 1: Table S2.[29] PICU-free days in patients who died were considered zero. All data were collected in web-based case report forms. Monthly telephone conferences, biannual meetings, clinical protocols including case definitions, data audits, and monitoring ensured uniform procedures among study sites.

Statistical analysis

Categorical variables are presented as counts (percentages). We used the chi-Square test or Fisher's exact test to compare frequency distributions between two categorical variables. Post-hoc Bonferroni correction for multiple testing was applied when we compared age groups with features on clinical presentation or pathogens. Continuous variables are presented either as mean (± standard deviation (SD)) for data with a parametric distribution or as median (interquartile range (IQR)) for non-parametric data. We tested differences between groups with analysis of variance (ANOVA) or Kruskal-Wallis and Student's *t* test or Mann-Whitney U test, as appropriate. Logistic regression (of binary outcome measures) and linear regression (of continuous outcome measures) were

used to identify independent predictors. Variables with a *P*-value <0.20 in the univariable analysis were included in the multivariable analysis. In the multivariable analysis, we only included one parameter of illness severity (PIM2), because of multicollinearity of the illness severity parameters. Area under the receiver operating characteristic (AUROC) curve analysis was applied to determine the Youden index. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), and negative likelihood ratio (NLR) were calculated for the optimal cut-off value of lactate. Statistical analyses were performed with SPSS version 21 (Armonk, USA). Graphs were created with GraphPad Prism 5.00. A *P* value <0.05 was considered statistically significant.

Results

From July 2012 to January 2016, 795 children (54% male, median age 2.2 years (IQR 8 months to 6years)) admitted with community-acquired sepsis to 52 PICUs in 7 European countries were enrolled (Figure 1). Baseline characteristics by age category are presented in Table 1. An underlying condition was present in 288 (36%) patients, of which prematurity and neonatal conditions (n = 87, 11%) and neurologic and neuromuscular conditions were most common (n = 70, 9%). A total of 466 (59%) patients presented with septic shock.

Clinical presentations and pathogens

Primary bloodstream infection and sepsis without a known source among patients with community-acquired sepsis accounted for 278 (35%) admissions to the PICU. The other most common clinical illnesses were meningitis/encephalitis (n = 182, 23%) and pneumonia (n = 149, 19%) (Additional file 1: Figure S1). Clinical presentations were similar across age groups, apart from Osteomyelitis/septic arthritis, which was diagnosed more frequently in school-aged children than in infants (7.3% versus 0.4%, *P* value = 0.002).

Bacterial etiology was confirmed in 428 (54%) patients, including 334 (42%) patients with a positive blood culture, and pathogen distribution was associated with age (Fig. 2). *Neisseria meningitidis* was the most commonly identified pathogen (n = 131, 31%), of which serogroup B was most prevalent (n = 89, 68%), followed by *Streptococcus pneumoniae* (n = 78, 18%, of which serotypes 3 (n = 7, 14%) and 10A (n = 6, 12%) were most commonly identified in those with serotyping information available (n=51)). (Additional file 1: Table S3)

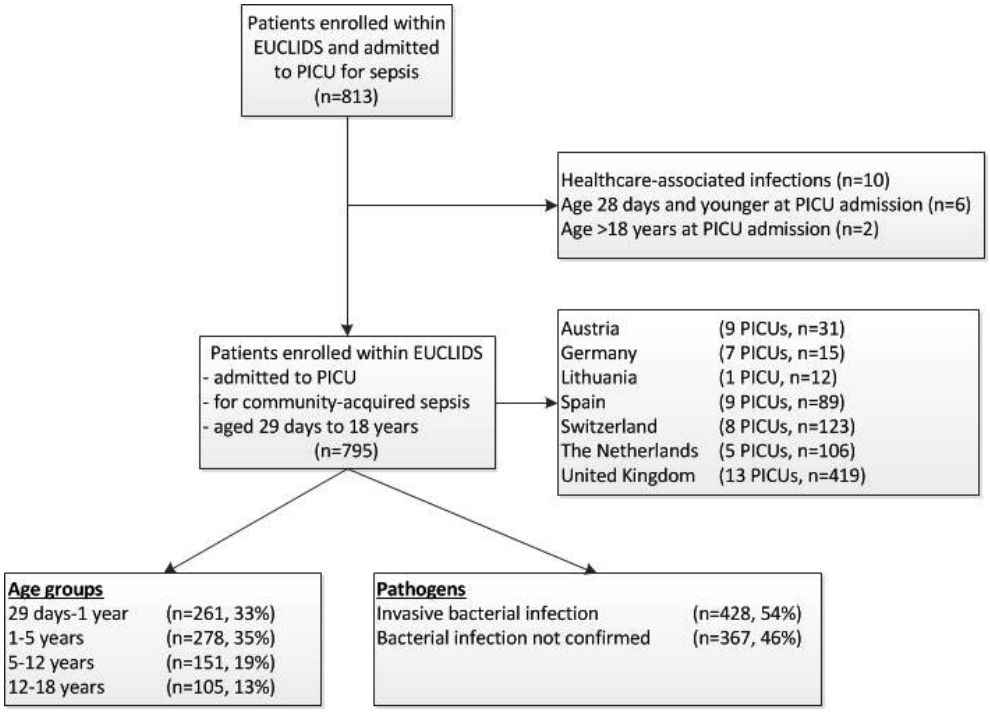


Figure 1: patients admitted with community-acquired sepsis to European PICUs

Of the 466 patients with septic shock, an invasive bacterial infection was confirmed in 255 (55%) patients. *N. meningitidis* (n = 91, 36%) and group A streptococcus (n = 49, 19%) were the most commonly identified pathogens, followed by *Streptococcus pneumoniae* (n=33, 13%).

Therapy

Invasive ventilation was used in 519 (69%) patients (median length of invasive respiratory support 5 days, IQR 3-8, n = 43 with missing data) and vasoactive agents for 418 (57%) patients (median 3 days, IQR 2-5 days, n = 56 with missing data). Infants needed invasive ventilation more frequently than adolescents (73% versus 58%, *P* value = 0.03).

Mortality and PICU-free survival

Of the 795 children admitted to PICU with community-acquired sepsis, 51 patients (6%) died. Mortality increased to 10% (n = 45) in patients with septic shock. Univariable analysis showed that the presence of bacteremia (odds ratio (OR) 4.4, 95% confidence interval (CI) 2.3-8.4, p < 0.001) and infections caused by *S. pneumoniae* (OR 2.5, 95% CI 1.2-5.1, p = 0.01) were associated with mortality in patients with sepsis (Table 2). In addition, illness severity, as measured by PRISM score, PIM2 score, invasive ventilation,

the need for inotropes, and higher lactate at PICU admission, were also associated with sepsis mortality. The AUROC for lactate as a predictor for mortality was 0.723 (95% CI 0.624-0.822), with an optimal cut-off value of 2.2 mmol/L (sensitivity 0.78, specificity 0.60).(Additional file 1: Figure S2).

Table 1: Baseline characteristics of children admitted with community-acquired sepsis to PICU

	All patients (n=795)	**29dy - 12m (n=261)**	**1-5y (n=278)**	**5-12y (n=151)**	**12-18y (n=105)**	***P***
Sex (male n, %)	428 (54%)	151 (58%)	148 (53%)	76 (50%)	53 (51%)	ns
Age	2y [8m-6y]	5m [2m-8m]	2y [18m-3y]	8y [6y-10y]	15y [14y-16y]	-
Ethnicity[*]						ns
African/North African	46 (6%)	15 (6%)	12 (4%)	14 (10%)	5 (5%)	
Asian	57 (7%)	22 (9%)	18 (7%)	10 (7%)	7 (7%)	
European	607 (79%)	195 (76%)	222 (82%)	106 (75%)	84 (82%)	
Meso/South American	7 (1%)	1 (0%)	3 (1%)	1 (1%)	2 (2%)	
Middle Eastern	10 (1%)	5 (2%)	1 (0%)	2 (1%)	2 (2%)	
Other/mixed	45 (6%)	18 (7%)	15 (6%)	9 (6%)	3 (3%)	
Time interval onset symptoms to hospital admission[†] (days)	1 [1-3]	1 [0-3]	2 [1-4]	2 [0-4]	1 [1-3]	ns
Immunizations up to date[‡]	585 (89%)	177 (82%)	219 (89%)	114 (95%)	75 (97%)	<0.001
Number of underlying conditions						<0.01
None	507 (64%)	176 (67%)	192 (69%)	85 (56%)	54 (51%)	
1	175 (22%)	55 (21%)	53 (19%)	36 (24%)	31 (30%)	
≥2	113 (14%)	30 (12%)	33 (12%)	30 (20%)	20 (19%)	
Underlying conditions						<0.01
Neurologic and neuromuscular	70 (9%)	6 (2%)	17 (6%)	27 (18%)	20 (19%)	
Cardiovascular	52 (7%)	14 (5%)	27 (10%)	8 (5%)	3 (3%)	
Respiratory	40 (5%)	14 (5%)	12 (4%)	9 (6%)	5 (5%)	
Renal and urologic	18 (2%)	5 (2%)	8 (3%)	2 (1%)	3 (3%)	
Gastrointestinal	35 (4%)	9 (3%)	11 (4%)	8 (5%)	7 (7%)	
Hematologic or immunologic	11 (1%)	2 (0%)	4 (1%)	4 (3%)	1 (1%)	
Metabolic	20 (3%)	5 (2%)	6 (2%)	5 (3%)	4 (4%)	
Other congenital or genetic defect	56 (7%)	9 (3%)	18 (6%)	17 (11%)	12 (11%)	
Malignancy	9 (1%)	1 (0%)	1 (0%)	2 (1%)	5 (5%)	
Premature and neonatal	87 (11%)	56 (21%)	19 (7%)	9 (6%)	3 (3%)	
Other	52 (7%)	10 (4%)	16 (6%)	14 (9%)	12 (11%)	

Illness severity						
PRISM score[§]	14 [7-21]	14 [7-22]	15 [8-22]	14 [8-21]	11 [4-16]	<0.01
PIM2 score[ll] (predicted death, %)	4.0 [1.1-9.5]	4.0 [1.0-8.2]	4.8 [1.1-10.3]	4.2 [1.1-17.1]	3.3 [0.9-9.0]	ns
Lactate at PICU admission[**](mmol/L)	1.8 [1.1-3.4]	1.6 [1.0-3.2]	1.6 [1.0-3.2]	2.2 [1.1-3.7]	2.3 [1.2-4.7]	ns
Septic shock (n, %)	466 (59%)	133 (51%)	168 (60%)	94 (62%)	71 (68%)	<0.05

Definition of abbreviations: PRISM=Pediatric Risk of Mortality[27]; PIM2=Pediatric Index of Mortality 2[28].
Values are reported as counts (percentages) or medians [interquartile ranges], unless stated otherwise.

[*] Ethnicity data were available for 772/795 patients; 256/261 infants, 271/278 toddlers, 142/151 school-aged children, and 103/105 adolescents.

[†] Time interval from onset of symptoms to hospital admission was available for 642/795 patients; 212/261 infants, 229/278 toddlers, 111/151 school-aged children, and 90/105 adolescents.

[‡] Immunization data were available for 657/795 patients; 215/261 infants, 245/278 toddlers, 120/151 school-aged children, and 77/105 adolescents.

[§] PRISM score was available for 672/795 patients; 223/261 infants, 240/278 toddlers, 118/151 school-aged children, and 91/105 adolescents.

[ll] PIM2 score was available for 681/795 patients; 224/261 infants, 243/278 toddlers, 123/151 school-aged children, and 91/105 adolescents.

[**] Lactate at PICU admission was available for 444/795 patients; 146/261 infants, 167/278 toddlers, 76/151 school-aged children, and 55/105 adolescents.

Infections caused by *S. pneumoniae* (OR 4.1, 95% CI 1.1-16.0, p = 0.04) and illness severity (PIM2 score OR 2.8, 95% CI 1.3-6.1, p < 0.01) remained independently significantly associated with mortality in multivariable analysis. A trend towards higher mortality was observed for bacteremia (OR 7.4, 95% CI 1.0-56.6, p = 0.06). PICU mortality did not differ significantly across age categories or countries. Also, the presence of an underlying condition at admission to the PICU was not associated with mortality.

The median PICU-free days to day 28 were 23 days (IQR 18-25) and the median hospital length of stay was 12 days (IQR 8-21). PIM2 score (B = -0.202, p < 0.001), invasive *S. pneumoniae* infections (B = -0.161, p = 0.02), and invasive *Staphylococcus aureus* infections (B = -0.163, p = 0.01) were independent predictors of PICU-free days. PIM2 score (B = 0.270, p < 0.001), pneumonia (B = 0.145, p = 0.04), and invasive *Staphylococcus aureus* infections (B = 0.234, p = 0.001) were independent predictors of hospital length of stay (Additional file 1: Table S4).

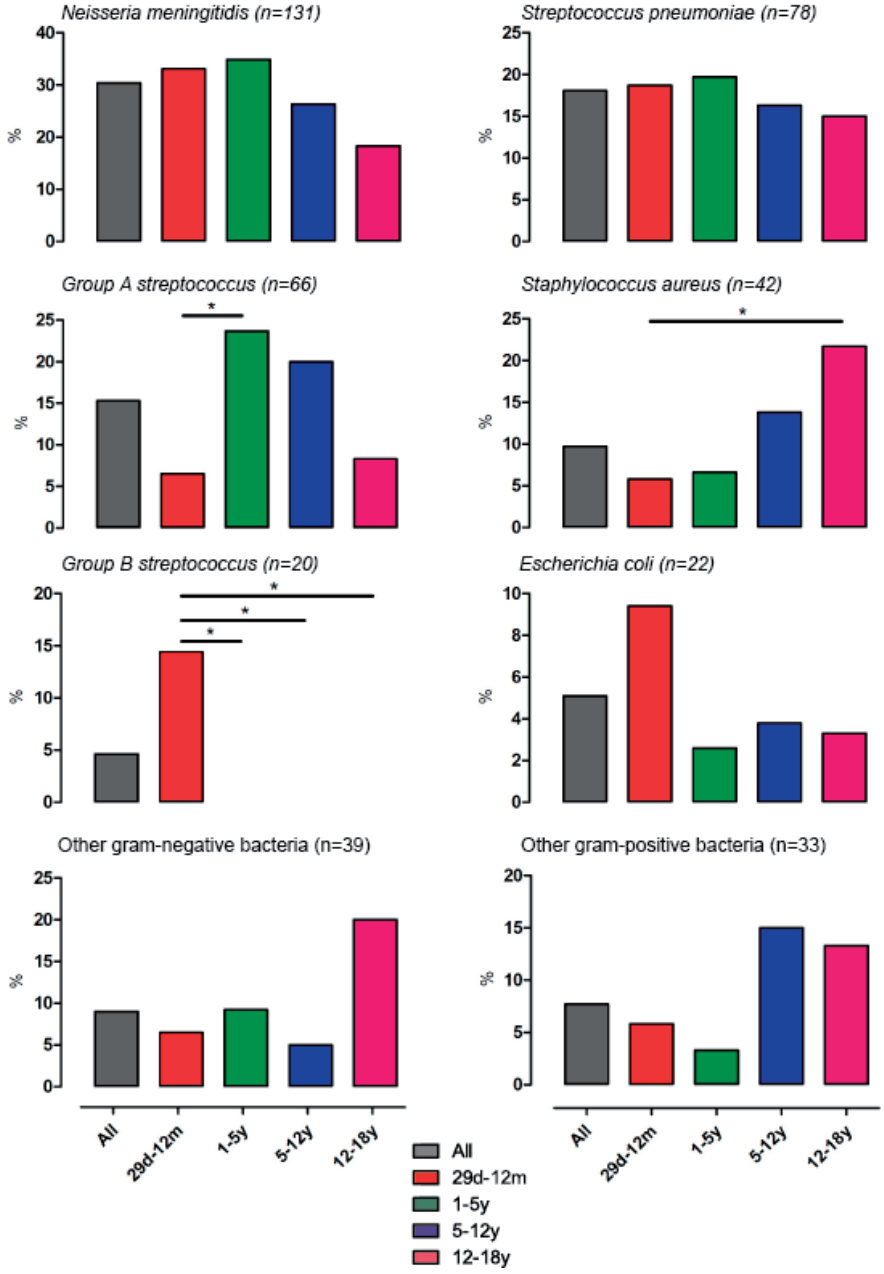


Figure 2: invasive pathogens in patients with community-acquired sepsis admitted to PICU.

Invasive pathogens (n = 428) by age category. Numbers are higher as three patients had mixed bacterial infections (one patient with *Staphylococcus aureus*/gram-negative bacteria, two patients with gram-negative/gram-positive bacteria). The y-axis represents the percentage of respective pathogen within the age category, i.e. percentages of all pathogens within an age category add up to 100%.

Disability

Data on disability at discharge were available on 558/744 (75%) survivors,. Of these patients, 173/558 (31%) were discharged with disability including 71 (13%) patients with mild overall disability, 39 (7%) with moderate overall disability, 50 (9%) with severe overall disability, 4 (0.7%) who had undergone amputation, 2 (0.4%) with hearing loss, and 7 (1.3%) who had undergone skin graft. Toddlers (34%) and school-aged children (42%) were more often discharged with disability than infants (21%, *P*-value <0.05).

Among survivors who did not have an underlying condition at admission to PICU, i.e. previously healthy children, 24% (83/349 patients whose data were available) were discharged with some disability. Disability data were available on 339/421 (81%) survivors of septic shock. In these, 120/339 (35%) patients had disability at PICU discharge, including 45 (13%) with mild overall disability, 29 (9%) moderate overall disability, 35 (10%) with severe overall disability, 4 (1.2%) who had undergone amputation, and 7 (2.0%) who had undergone skin graft. Outcome as measured by mortality and POPC score was worst in patients admitted with pneumonia (Additional file 1: Figure S3) and in patients with invasive bacterial infections caused by *Streptococcus pneumoniae* (Additional file 1: Figure S4). When comparing patients discharged with and without disability, by univariable and multivariable analysis, the PIM2 score (OR 3.4, 95% CI 1.8-6.4, p <0.001) and infections caused by *Streptococcus pneumoniae* (OR 5.4, 95% CI 1.8-15.8, p <0.01) were independent predictors of disability.(Table 3)

Economic impact of vaccine-preventable infections

Assuming an average cost per PICU day of €4000 and €1000 per day on a general ward, we estimate an average cost of €42000 per vaccine-preventable episode of severe community-acquired infection requiring admission to a PICU. This calculation was based on the mean hospital length of stay (18 days), including mean PICU length of stay (8 days), of the subgroup of patients with vaccine-preventable infections (n = 149). Within our consortium, a total of 149 vaccine-preventable cases reflect 43 cases per year. The impact on cost and resource utilization for the hospitals included was estimated at almost €2 million for potentially vaccine-preventable infections annually.

Table 2: Predictors of death in children with community-acquired sepsis

	Sepsis survivors (n=744)	Deaths (n=51)	Univariable odds ratio for death (95% CI)	*P*	Multivariable odds ratio for death (95% CI)	*P*
Sex						
Male	399/744 (54%)	29/51 (57%)	Reference			
Female	345/744 (46%)	22/51 (43%)	0.9 (0.5-1.6)	0.65	..	
Age						
29d-12m (infants)	248/744 (33%)	13/51 (26%)	Reference			
1-5y (toddlers)	259/744 (35%)	19/51 (37%)	1.4 (0.7-2.9)	0.37	..	
5-12y (school aged children)	140/744 (19%)	11/51 (22%)	1.5 (0.7-3.4)	0.34	..	
12-18y (adolescents)	97/744 (13%)	8/51 (16%)	1.6 (0.6-3.9)	0.33	..	
Time interval onset symptoms to hospital admission[*] (days)	1 [1-3]	2 [1-4]	1.0 (1.0-1.1)	0.47	..	
Immunizations up-to-date						
No	70/621 (11%)	2/36 (6%)	Reference			
Yes	551/621 (89%)	34/36 (94%)	2.2 (0.5-9.2)	0.30	..	
Underlying condition						
No	479/744 (64%)	28/51 (55%)	Reference			
Yes	265/744 (36%)	23/51 (45%)	1.5 (0.8-2.6)	0.18	0.7 (0.2-2.0)	0.46
Illness severity						
PRISM score[†]	14 [7-20]	22 [15-30]	1.1 (1.0-1.1)	<0.001	..	
PIM2 score[‡] (predicted death, %)	3.9 [1.0-9.1]	14.7 [3.8-48.0]	3.9 (2.1-7.2)	<0.001	2.8 (1.3-6.1)	<0.01
Lactate at PICU admission[§] (mmol/L)	1.7 [1.0-3.3]	3.3 [2.3-5.4]	8.9 (2.7-29.1)	<0.001	..	
Invasive ventilation	474/705 (67%)	45/47 (96%)	11.0 (2.6-45.6)	0.001	..	
Inotropes	377/693 (54%)	41/46 (89%)	6.9 (2.7-17.6)	<0.001	..	
Bacteremia						
No	445/741 (60%)	13/51 (26%)	Reference			
Yes	296/741 (40%)	38/51 (75%)	4.4 (2.3-8.4)	<0.001	7.4 (1.0-56.6)	0.06
Clinical syndromes						
No focus	254/744 (34%)	24/51 (47%)	1.7 (1.0-3.0)	0.06	3.0 (0.8-10.9)	0.09
Meningitis/encephalitis	172/744 (23%)	10/51 (20%)	0.8 (0.4-1.6)	0.56	..	
Pneumonia	138/744 (19%)	11/51 (22%)	1.2 (0.6-2.4)	0.59	..	
Other focus	180/744 (24%)	6/51 (12%)	0.4 (0.2-1.0)	0.05	1.8 (0.3-11.8)	0.52
Invasive pathogens[‖]						
N. meningitidis	120/386 (31%)	11/39 (28%)	0.8 (0.4-1.8)	0.88	..	
S. pneumoniae	65/386 (17%)	13/39 (33%)	2.5 (1.2-5.1)	0.01	4.1 (1.1-16.0)	0.04
Group A streptococcus	60/386 (16%)	6/39 (15%)	1.0 (0.4-2.5)	1.0	..	
S. aureus	37/386 (10%)	4/39 (10%)	1.1 (0.4-3.2)	0.88	..	
Other invasive pathogen	104/386 (27%)	5/39 (13%)	0.4 (0.2-1.0)	0.06	0.3 (0.0-2.2)	0.21

This study included 795 children admitted with community-acquired sepsis, of whom 51 patients died. Multivariable analysis included variables with a *P* value <0.20 in univariable analysis. Because parameters of illness severity are strongly correlated, only the PIM2 score has been included in multivariable analysis. Values are reported as counts (percentages) or medians [interquartile ranges], unless stated otherwise.

Definition of abbreviations: PRISM=Pediatric Risk of Mortality[27]; PIM2=Pediatric Index of Mortality 2[28].

* Time interval from onset of symptoms to hospital admission was available for 609/744 sepsis survivors and 33/51 non-survivors.

† PRISM score was available for 636/744 sepsis survivors and 36/51 non-survivors.

‡ PIM2 score was available for 645/744 sepsis survivors and 36/51 non-survivors. Data were log transformed for univariable and multivariable analysis.

§ Data on lactate at PICU admission were available for 421/744 sepsis survivors and 23/51 non-survivors. Data were log transformed for univariable analysis.

‖ Bacterial etiology was confirmed in 428 patients, including 3 patients with mixed invasive pathogens in culture results: these 3 patients have been excluded, leaving 425 patients for analysis.

Table 3: Predictors of disability in survivors of community-acquired sepsis

	No disability at discharge (n=385)	Disability at discharge (n=173)	Univariable odds ratio for disability (95% CI)	*P*	Multivariable odds ratio for disability (95% CI)	*P*
Sex						
Male	196/385 (51%)	90/173 (52%)	Reference			
Female	189/385 (49%)	83/173 (48%)	1.0 (0.7-1.4)	0.81	..	
Age						
29d-12m (infants)	145/385 (38%)	38/173 (22%)	Reference			
1-5y (toddlers)	135/385 (35%)	70/173 (41%)	2.0 (1.3-3.1)	<0.01	1.8 (0.8-4.1)	0.14
5-12y (school aged children)	55/385 (14%)	39/173 (23%)	2.7 (1.6-4.7)	<0.001	2.6 (1.0-7.0)	0.05
12-18y (adolescents)	50/385 (13%)	26/173 (15%)	2.0 (1.1-3.6)	0.02	2.0 (0.7-6.0)	0.20
Time interval onset symptoms to hospital admission* (days)	1 [1-3]	1 [1-3]	1.0 (0.9-1.0)	0.16	0.9 (0.9-1.0)	0.07
Immunizations up-to-date						
No	29/334 (9%)	17/157 (11%)	Reference			
Yes	305/334 (91%)	140/157 (89%)	0.8 (0.4-1.5)	0.45	..	
Underlying condition						
No	266/385 (69%)	83/173 (48%)	Reference			
Yes	119/385 (31%)	90/173 (52%)	2.4 (1.7-3.5)	<0.001	1.9 (0.9-3.6)	0.08
Illness severity						
PRISM score	12 [6-19]	16 [11-23]	1.1 (1.0-1.1)	<0.001	..	
PIM2 score† (predicted death, %)	3.1 [0.9-7.1]	6.8 [2.3-17.2]	2.7 (1.9-3.9)	<0.001	3.4 (1.8-6.4)	<0.001
Lactate at PICU admission‡ (mmol/L)	1.6 [1.0-3.2]	2.3 [1.2-4.5]	2.0 (1.0-3.8)	0.04	..	
Invasive ventilation	242/370 (65%)	139/167 (83%)	2.6 (1.7-4.2)	<0.001	..	
Inotropes	196/365 (54%)	112/166 (68%)	1.8 (1.2-2.6)	<0.01	..	
Bacteremia						
No	262/385 (68%)	112/173 (65%)	Reference			
Yes	123/385 (32%)	61/173 (35%)	1.2 (0.8-1.7)	0.44	..	
Clinical syndromes						
No focus	146/385 (38%)	48/173 (28%)	0.6 (0.4-0.9)	0.02	0.9 (0.3-2.4)	0.83
Meningitis/encephalitis	74/385 (19%)	49/173 (28%)	1.7 (1.1-2.5)	0.02	1.0 (0.3-3.2)	0.95
Pneumonia	57/385 (15%)	49/173 (28%)	2.3 (1.5-3.5)	<0.001	1.2 (0.4-3.8)	0.73
Other focus	108/385 (28%)	27/173 (16%)	0.5 (0.3-0.8)	<0.01	1.0 (0.3-3.0)	0.96
Invasive pathogens						
N. meningitidis	81/177 (45%)	18/87 (21%)	0.3 (0.2-0.6)	<0.001	0.5 (0.2-1.3)	0.16
S. pneumoniae	12/177 (7%)	27/87 (31%)	6.3 (3.0-13.2)	<0.001	5.4 (1.8-15.8)	<0.01
Group A streptococcus	31/177 (18%)	15/87 (17%)	1.0 (0.5-2.0)	1.0	..	
S. aureus	16/177 (9%)	11/87 (13%)	1.5 (0.7-3.4)	0.34	..	
Other invasive pathogen	37/177 (21%)	16/87 (18%)	0.9 (0.4-1.6)	0.63	..	

This study included 795 children with community-acquired sepsis, of whom 173 patients were discharged with disability, i.e. POPC score 2 to 5[29], need of skin graft, hearing loss or need of amputation (51 deaths and 186 patients with missing data are not included in this analysis). Multivariable analysis included variables with a *P* value <0.20 in univariable analysis. Because parameters of illness severity are strongly correlated, only the PIM2 score has been included in multivariable analysis. Values are reported as counts (percentages) or medians [interquartile ranges], unless stated otherwise.

Definition of abbreviations: PRISM=Pediatric Risk of Mortality[27]; PIM2=Pediatric Index of Mortality 2[28].

* Time interval from onset of symptoms to hospital admission was available for 372/385 patient without disability and for 154/173 patients with disability at discharge.

† Data were log transformed for univariable and multivariable analysis.

‡ Data on lactate at PICU admission were available for 247/385 patient without disability and for 109/173 patients with disability at discharge. Data were log transformed for univariable analysis.

Discussion

This prospective multicenter study of 795 children admitted with community-acquired sepsis to European PICUs demonstrates the substantial burden of severe invasive bacterial disease, despite widespread immunization programs, predominantly affecting previously healthy children.[30] Almost one third of survivors (31%) were discharged with disability ranging from mild to severe.

We observed a crude mortality of 6% in children admitted with sepsis. Other studies have reported higher mortality of up to 29% in high-income countries, which may relate to the high number of hospital-acquired infections with a disproportionate impact of high-risk patients such as those with oncologic conditions or those undergoing transplant in other cohorts.[1, 2, 4, 5, 8, 31-33] The enrolment criteria in our study were based on the 2005 consensus pediatric sepsis definition, and we included patients with sepsis in addition to patients with severe sepsis and septic shock, which may account for the lower mortality observed. However, most study patients were admitted to the PICU because of single or multiple organ dysfunction, and hence would be expected to meet the Sepsis-3-based sepsis definitions too.[21, 34] The limitations of current pediatric sepsis definitions including the low predictive accuracy of SIRS[35], and the need to adapt Sepsis-3 for pediatric age groups, have been highlighted recently.[36]

We observed that 1 out of 3 sepsis survivors were discharged with a disability, including 1 in 10 with severe disability and/or amputation. Notably, 24% of previously healthy children left the hospital with some form of disability. While there is a lack of large studies on pediatric sepsis long-term outcomes, similar incidence of disability has been reported in two other studies, with a decline in functional status observed in 28% to 34% of pediatric sepsis survivors.[4, 20] Others have observed impaired neuropsychological performance and impaired educational functioning.[19] Our findings highlight the need to include disability as an outcome measure in pediatric sepsis trials in the future. More research is required to delineate the nature of the disabilities and to study the add-on effect of sepsis when underlying conditions are already present. Disability in children with underlying conditions could be evaluated more accurately in the future by reporting changes in performance scales between admission and discharge.

Independent risk factors for death, disability, and PICU-free days were illness severity - reflected by severity scores - and invasive pneumococcal infections. Our findings indicate that while current PICU severity scores were calibrated against mortality, PIM performs very well to predict disability as well, which indicates that some patients predicted to die survive, yet with a major impact on functional status. Larger studies are urgently needed to assess long-term impact, as this patient group is at high risk of prolonged dependency on health

support, reduced school and work life performance, and reduced quality of life, resulting in an under recognized disproportionate impact of sepsis on our society.[14] Lactate was associated with mortality and the optimal cutoff value of 2.2 mmol/L supports using lactate as a trigger threshold in NICE guidelines.[37] Previous studies have demonstrated the strong association between lactate and mortality, and indicated that both arterial and venous serum lactate level can be used for risk stratification.[38-41] Importantly, our study demonstrated that increased lactate levels at PICU admission were associated with disability too.

An invasive bacterial infection was confirmed in half of the children, which is comparable to pathogen detection rates from 30% to 65% in other studies.[1, 4, 5, 33] The most common community-acquired invasive pathogens in our study were meningococci - especially serogroup B (menB) - and pneumococci - especially serotypes 3 and 10A. Recently, a menB vaccine (Bexsero®) has been licensed for active immunization against menB and this vaccine has been implemented in the Czech Republic and UK routine immunization schedules.[42, 43] It had been anticipated that this vaccine would cover approximately 70% to 80% of MenB strains, depending on geographical region and age.[30, 42] Preliminary data report 93% vaccine uptake of two doses by 12 months of age.[44] Future studies should determine the impact on disease and herd protection. Meningococcal serogroup C immunization resulted in a significant drop in incidence of over 80% in the UK and over 90% in The Netherlands.[45-47] In contrary, invasive MenW disease is increasing and careful monitoring in the coming years is necessary.[48] Immunization against pneumococcal disease is recommended in almost all European countries, and has been proven effective in the decline of invasive pneumococcal infections.[49] In the post-immunization era, the incidence of non-vaccine serotypes has however increased, suggesting serotype replacement.[49] Additionally, vaccine failure does occur. Primary immunodeficiency is present in up to 26% of children >2 years of age with invasive pneumococcal infections after introduction of vaccination, indicating that infected patients should undergo immunological investigations.[50] Pneumococcal serotype 3 is included in Prevnar 13 (PCV13), but not in Synflorix (PCV10). Limited vaccine effectiveness for serotype 3 has been reported previously.[51, 52] Pathogen detection is complicated by early administration of antibiotics and by low circulating microbial loads.[53] Therefore, new diagnostics to improve pathogen detection and optimal antimicrobial therapy are urgently needed.[54]

Despite widespread vaccination campaigns in Europe effectively targeting invasive pneumococcal disease[43, 55-57], the burden due to these potentially vaccine preventable infections has remained considerable: 17% of patients with pneumococcal infections have died, while 35% of the patients have been discharged with some form of disability. The pneumococcal mortality rate in our study is slightly higher than mortality rates from other PICU studies.[2, 8, 58] However, those studies relied on ICD-9 codes for organisms and did not evaluate specific culture results. Therefore, a number of infections

might have been classified based on non-sterile site cultures (e.g. nasopharyngeal aspirate), and thus could have identified a colonized location, whereas we confirmed each invasive pneumococcal infection by sterile site positive detection. On the other hand, our definition of invasive pneumococcal infection might have skewed the results away from respiratory infections, towards central nervous infections, as we considered blood, pleural aspirate and bronchoalveolar lavage as invasive detection sites for pneumonia. These sites are not routinely screened in patients with pneumonia. Nevertheless, our findings emphasizes the importance and the need to continuously improve current immunization programs. Additionally, reducing potentially vaccine-preventable infections will have a beneficial effect on economic resources. We estimated an average cost of €42000 per vaccine preventable episode of severe community-acquired infection requiring PICU. In comparison, a recent Australian and New Zealand study[5] estimated the mean cost per ICU and ward admission for sepsis and septic shock of AUS$62062 (equal to €39000). Beside direct costs in severe cases requiring PICU admission, the total economic impact of vaccine-preventable diseases encompasses direct costs in cases not requiring PICU admission, and indirect costs related to loss of revenue of caregivers and long-term costs related to permanent disability.

This study has several limitations, most of them relating to the design of this large, international consortium. First, the primary aim of our consortium was to identify genes associated with susceptibility and severity of invasive meningococcal, pneumococcal, staphylococcal, and group A streptococcal infections, which might have caused an enrollment bias in favor of infections caused by these organisms. Therefore, data on the prevalence and distribution of pathogens need to be interpreted with caution. Second, due to the genetic basis of this study, a bias towards enrollment of previously healthy children might have occurred. In our cohort, only 36% of patients had an underlying condition at admission, whereas in other studies percentages varying from 49 to 77% are reported, yet these studies included hospital-acquired infections too.[1, 2, 4, 5, 7, 8]. Third, we assessed disability by the easily applicable and well-validated pediatric overall performance scale at discharge.[29, 59] However, there is a fair amount of disability data missing, possibly because of loss to follow up after patients had been transferred back to the local hospital prior to discharge. For patients with disability data available, we did not take further recovery after discharge into account. Fourth, the EUCLIDS consortium represents a network of institutions active in infectious diseases research and was not designed to provide population-based coverage. Hence we are unable to compare findings between countries and we are unable to estimate the impact of vaccine-preventable disease on mortality, morbidity, and costs at population level. Finally, our consortium includes multiple centers from multiple countries, representing a different epidemiological context, healthcare structures, and case-mix. Nevertheless, this study includes the largest prospectively enrolled contemporary cohort of children with community-acquired sepsis

in high-income countries. Because previous pediatric sepsis studies included healthcare-associated infections, results from this study especially have implications for policy makers in public health, e.g. to develop immunization strategies. Last, this report differs from previous reports because we included disability as an outcome measure, thereby meeting the need to improve our understanding of the short-term physical effects of sepsis and understanding the implications for sepsis survivors.[14]

Conclusions

This report from high-income countries describes a large cohort of children admitted with community-acquired sepsis to European PICUs, providing contemporary assessment of the epidemiology and characteristics of one of the most common reasons for PICU admission. Our study demonstrates the substantial burden caused by community-acquired sepsis, predominantly affecting previously healthy children. One out of three survivors was discharged with disability, indicating an urgent need for improved recognition, treatment, and follow up of children with sepsis.

Additional files

Figure E1: clinical presentations (n=795) within age categories.

Figure E2: AUROC curve analysis for lactate as predictor for mortality.
Sensitivity, specificity, PPV, NPV, PLR, and NLR for 2.2 mmol/L as optimal cut-off value of lactate.

Figure E3: unadjusted mortality and POPC score of patients with meningitis/encephalitis, pneumonia, and no focus.

Figure E4: unadjusted mortality and POPC score of patients with invasive meningococcal, pneumococcal, Group A streptococcal, and S. aureus infections.

Table E1: routine immunization schedules and uptake.

Table E2: the Pediatric Overall Performance Category (POPC) scale.

Table E3:characteristics of *Haemophilus influenza*, meningococcal, and pneumococcal sepsis, including serotypes/serogroups and clinical presentation of deaths and patients with disability.

Table E4: predictors of PICU-free days and hospital length of stay in children with community-acquired sepsis.

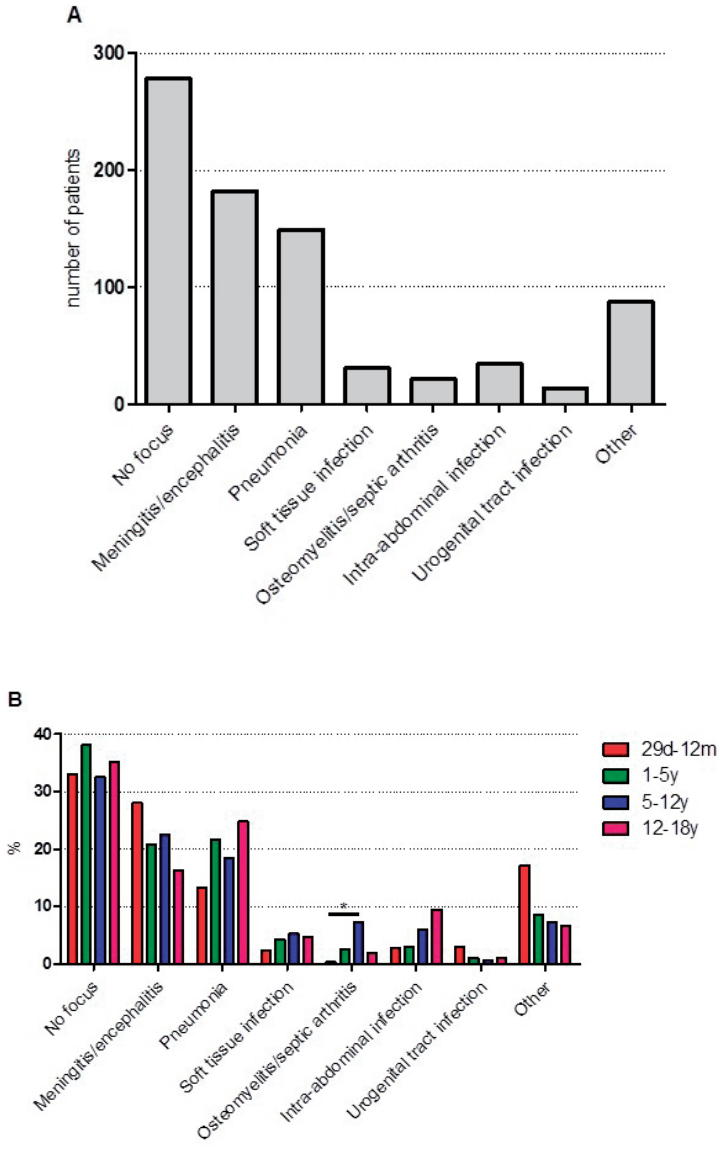
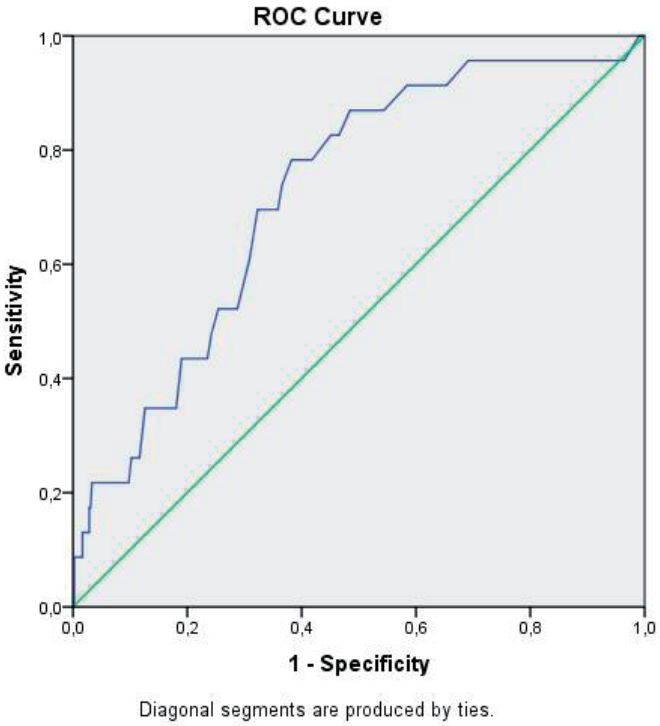


Figure E1: clinical presentations (n=795) within age categories.

A) Clinical presentations of patients admitted to PICU for community-acquired sepsis. Most common were patients in whom a focus of infection could not be identified (n=278, 35%), meningitis/encephalitis (n=182, 23%) and pneumonia (n=149, 19%).

B) The percentages of clinical presentations within four age categories were similar, apart from Osteomyelitis/septic arthritis. **P*-value<0.05.



	Death	No death	Total
Lactate ≥ 2.2 mmol/L	18	169	187
Lactate < 2.2 mmol/L	5	252	257
Total	23	421	444

Sensitivity: 18/23 = 78%
Specificity: 252/421 = 60%
PPV: 18/187 = 10%
NPV: 252/257 = 98%
PLR: (18/23)/(169/421) = 1.9
NLR: (5/23)/(252/421) = 0.4

Figure E2: AUROC curve analysis for lactate as predictor for mortality. Sensitivity, specificity, PPV, NPV, PLR, and NLR for 2.2 mmol/L as optimal cut-off value of lactate.

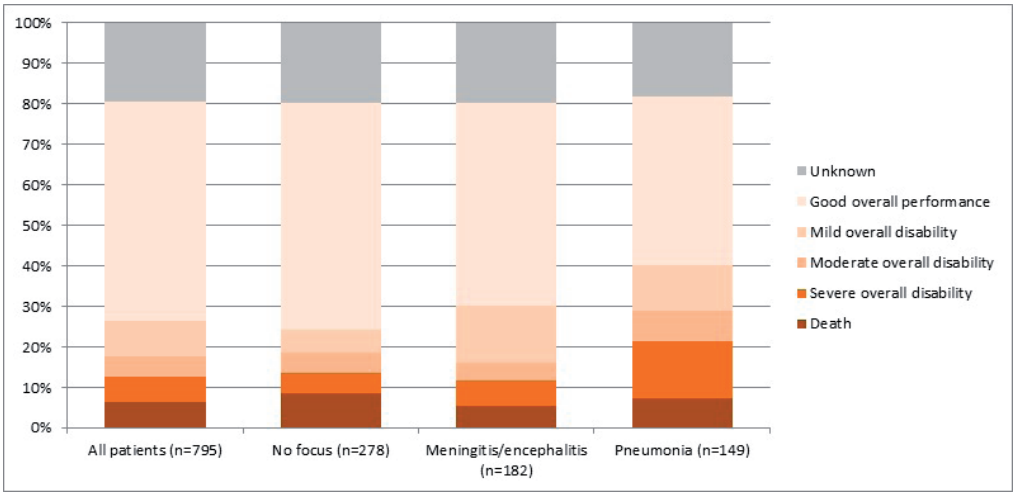


Figure E3: unadjusted mortality and POPC score of patients with meningitis/encephalitis, pneumonia, and no focus.
Patients admitted with pneumonia (n=149) had a crude mortality of 7% (n=11), while an additional 33% (n=49) were discharged with mild, moderate or severe disability.

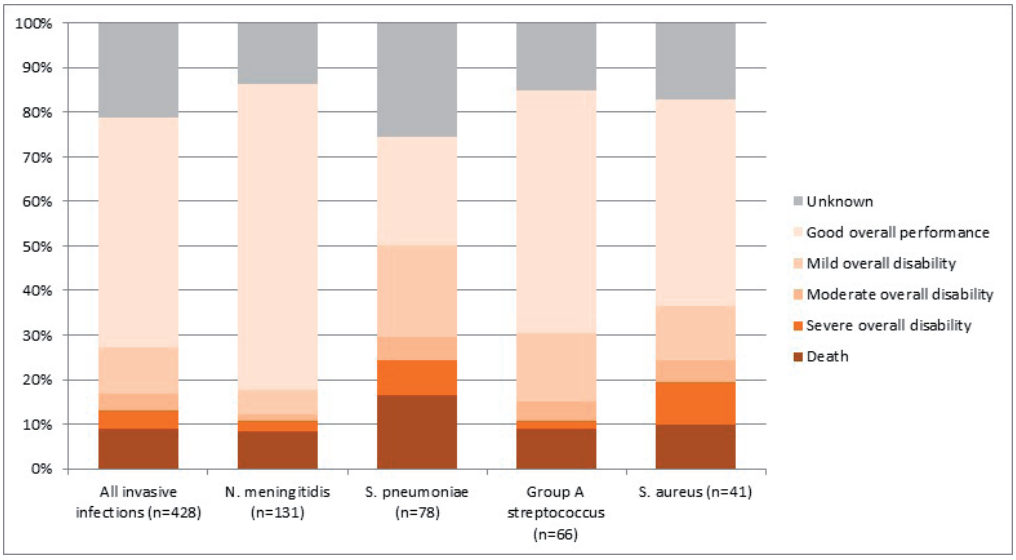


Figure E4: unadjusted mortality and POPC score of patients with invasive meningococcal, pneumococcal, Group A streptococcal, and S. aureus infections. Invasive bacterial infections caused by Streptococcus pneumoniae (n=78) resulted in the worst crude outcome; 17% mortality (n=13), while an additional 33% (n=26) were discharged with POPC scores mild, moderate or severe disability.

Table E1: Immunization schedules and uptake

	Austria[1]	Germany[1]	Lithuania[1]	Spain[2]	Switzerland[3]	The Netherlands[4]	United Kingdom[5,6]
HiB							
Introduced	1992	1990	2004	2000	1990	1993	1992
Uptake				96%	89%	95%	94%
MenACWY							
Introduced	2013	-	-	-	-	-	2015
Uptake							84%
MenB							
Introduced	2015	-	2018	-	-	-	2015
Uptake							93%
MenC							
Introduced	2009	2006	-	2002	2006	2002	1999
Uptake				98%	72%	95%	92%
PCV7							
Introduced	2004	2006	-	2006	2006	2006	2006
Uptake							
PCV10							
Introduced	2011	2009	2014	-	-	2011	2009
Uptake						94%	
PCV13							
Introduced	-	2009	-	2012	2011	-	2010
Uptake					80%		94%
PPSV23							
Introduced	-	-	-	-		-	
Uptake							

For immunizations where uptake is not reported, data are unavailable or unknown.

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5. Public Health England, Health Protection Report, 27 January 2018. Vaccine coverage estimates for the school based meningococcal ACWY (MenACWY) adolescent vaccination programme in England, to 31 August 2017 and Preliminary vaccine coverage estimates for the meningococcal B (MenB) immunisation programme for England, update from August to December 2017.
6. NHS digital, Childhood Vaccination Coverage Statistics: http://bit.ly/child_vaccstats_annual

Table E2: The Pediatric Overall Performance Category (POPC) scale.

Score	Category	Description
1	Good overall performance	Normal; at age-appropriate level; school-age child attending regular school classroom. Healthy, alert, and capable of normal activities of daily life.
2	Mild overall disability	Conscious; alert, and able to interact at age-appropriate level; school-age child attending regular school classroom but grade perhaps not appropriate for age; possibility of mild neurologic deficit. Possibility of minor physical problem that is still compatible with normal life; conscious and able to function independently.
3	Moderate overall disability	Conscious; sufficient cerebral function for age-appropriate independent activities of daily life; school-age child attending special education classroom and/or learning deficit present Possibility of moderate disability from noncerebral systems dysfunction alone or with cerebral system dysfunction; conscious and performs independent activities of daily life but is disabled for competitive performance in school.
4	Severe overall disability	Conscious; dependent on others for daily support because of impaired brain function. Possibility of severe disability from noncerebral systems dysfunction alone or with cerebral system dysfunction; conscious but dependent on others for activities of daily living support.
5	Coma or vegetative state	Any degree of coma without the presence of all brain death criteria; unawareness, even if awake in appearance, without interaction with environment; cerebral unresponsiveness and no evidence of cortex function (not aroused by verbal stimuli); possibility of some reflexive response, spontaneous eye-opening, and sleep-wake cycles.
6	Brain death	Apnea, areflexia, and/or electroencephalographic silence.

Table E3: characteristics of Haemophilus influenza, meningococcal, and pneumococcal sepsis, including serotypes/serogroups and clinical presentation of deaths and patients with disability.

	H. influenza (n=12)	**Meningococcal (n=131)**	**Pneumococcal sepsis (n=78)**
Sex (male n, %)	7 (58%)	73 (56%)	43 (55%)
Age	12m [10m-3y]	20m [8m-5y]	21m [8m-6y]
Country			
Austria	-	15 (12%)	1 (1%)
Germany	-	6 (5%)	1 (1%)
Lithuania	-	10 (8%)	-
Spain	1 (8%)	32 (24%)	6 (8%)
Switzerland	8 (67%)	16 (12%)	31 (40%)
The Netherlands	1 (8%)	9 (7%)	21 (27%)
United Kingdom	2 (17%)	43 (33%)	18 (23%)
Serotype/-group (n, %)	B (10, 83%)	B (89, 68%)	***7-valent:***
	Non-B (1, 8%)	C (9, 7%)	6B (1, 1%)
	Unspecified (1, 8%)	W135 (8, 6%)	***10-valent:***
		Y (1, 1%)	1 (2, 3%)
		Unspecified (24, 18%)	7F (2, 3%)
			13-valent:
			3 (7, 9%)
			19A (4, 5%)
			23-valent:
			8 (2, 3%) 15B (1, 1%),
			10A (6, 8%) 17F (1, 1%)
			11A (2, 3%) 22F (2, 3%)
			12F (1, 1%) 33F (1, 1%)
			Other:
			12 (1, 1%) 23 (1, 1%) 24F (1, 1%)
			15C (2, 3%) 23A (1, 1%) 27 (1, 1%)
			17 (1, 1%) 23B (1, 1%) 33 (1, 1%)
			21 (1, 1%) 24 (4, 5%) 38 (1, 1%)
			15B or 15C unspecified (3, 4%)
			Unknown (27, 35%)

Immunization status of potentially VPI* (n, %)	10 (83%)	107 (82%)	32 (41%)		
Complete immunization	2 (20%)	1 (1%)	0 (0%)		
Incomplete immunization - age	5 (50%)	5 (5%)	1 (3%)		
Incomplete immunization - avail.	0 (0%)	82 (77%)	25 (78%)		
No immunization	1 (10%)	2 (2%)	0 (0%)		
Unknown	2 (20%)	17 (16%)	6 (19%)		
Clinical presentation					
No focus	1 (8%)	68 (52%)	5 (6%)		
Meningitis/encephalitis	4 (33%)	61 (47%)	51 (66%)		
Pneumonia	4 (33%)	1 (1%)	21 (27%)		
Other	3 (25%)	1 (1%)	1 (1%)		
Deaths sub-analysis	**0 (0%)**	**11 (8%)**	**13 (17%)**		
Serotype/-group (n, %)	-	B (7, 64%)	3 (1, 8%)	15C (2, 15%)	23A (1, 8%)
		C (2, 18%),	8 (1, 8%)	21 (1, 8%)	24 (2, 15%)
		Unspecified (2, 18%)	12F (1, 8%)	22F (1, 8%)	unkn. (3, 23%)
Clinical presentation (n, %)	-	No focus (10, 91%)	No focus (2, 15%)		
		Meningitis/enceph (1, 9%)	Meningitis/encephalitis (7, 54%)		
			Pneumonia (3, 23%)		
			Other (1, 8%)		
Disability sub-analysis	**0 (0%)**	**18 (14%)**	**27 (35%)**		
Serotype/-group (n, %)	-	B (11, 61%)			
		W135 (3, 17%),	6B (1, 4%)	11A (1, 4%)	17F (1, 4%)
		Unspecified (4, 22%)	7F (1, 4%)	12 (1, 4%)	19A (1, 4%)
			8 (1, 4%)	15B (1, 4%)	27 (1, 4%)
			10A (3, 11%)	17 (1, 4%)	33 (1, 4%)
			15B or 15C unspecified (1, 4%)		
			Unknown (12, 44%)		
Clinical presentation (n, %)	-	No focus (12, 67%)			
		Meningitis/enceph (6, 33%)	Meningitis/encephalitis (25, 93%)		
			Pneumonia (2, 7%)		

Values are reported as counts (percentages) or medians [interquartile ranges], unless stated otherwise. VPI = vaccine preventable infections.

*Immunization: complete = immunizations up-to-date according to local schedule and sufficient doses for protection received; incomplete age = immunizations up-to-date according to local schedule, but sufficient doses for protection not received because of young age; incomplete - availability = immunizations up-to-date according to local schedule, but immunization not received because of unavailability in local schedule; no = immunizations not up-to-date according to local schedule, and therefore doses not received.

Table E4: predictors of PICU-free days and hospital length of stay in children with community-acquired sepsis.

	PICU-FREE DAYS				HOSPITAL LENGTH OF STAY			
	Univariable analysis Standardized B	***P***	**Multivariable analysis Standardized B**	***P***	**Univariable analysis Standardized B**	***P***	**Multivariable analysis Standardized B**	***P***
Sex (Female)	-0.015	0.67	..		-0.035	0.50	..	
Age								
29d-12m (infants)	0.030	0.41	..		-0.034	0.51	..	
1-5y (toddlers)	0.020	0.58	..		-0.004	0.93	..	
5-12y (school aged children)	-0.030	0.40	..		0.054	0.30	..	
12-18y (adolescents)	-0.035	0.33	..		-0.006	0.91	..	
Time onset symptoms to hospital admission[*] (days)	-0.071	0.08	-0.060	0.30	0.061	0.25	..	
Immunizations up-to-date	-0.004	0.92	..		0.012	0.83	..	
Underlying condition	-0.183	<0.001	-0.067	0.25	0.085	0.10	0.062	0.36
Illness severity								
PRISM score	-0.326	<0.001	..		0.303	<0.001	..	
PIM2 score[-] (predicted death, %)	-0.244	<0.001	-0.202	0.001	0.226	<0.001	0.268	<0.001
Lactate PICU admission[@] (mmol/L)	-0.265	<0.001	..		0.179	<0.01	..	
Invasive ventilation	-0.338	<0.001	..		0.245	<0.001	..	
Inotropes	-0.331	<0.001	..		0.147	<0.01	..	
Bacteremia	-0.066	0.07	-0.061	0.32	0.094	0.07	0.107	0.13
Clinical syndromes								
No focus	0.019	0.60	..		-0.083	0.11	-0.060	0.43
Meningitis/ encephalitis	0.104	<0.01	0.109	0.12	-0.052	0.31	..	
Pneumonia	-0.197	<0.001	-0.129	0.06	0.138	<0.01	0.148	0.04
Other focus	0.058	0.11	0.030	0.67	0.027	0.60	..	

Invasive pathogens	0.163	0.001	0.025	0.79	-0.195	<0.01	-0.012	0.89
N. meningitidis	-0.144	<0.01	-0.161	0.02	-0.015	0.84	..	
S. pneumoniae	-0.078	0.11	-0.020	0.80	0.103	0.15	0.072	0.37
Group A streptococcus	-0.096	0.05	-0.163	0.01	0.239	0.001	0.249	0.001
S. aureus	0.080	0.11	-0.018	0.81	-0.024	0.74	..	
Other pathogen								
***R*²**			0.17				0.20	
ANOVA: *df, F*			11, 4.920	<0.001			8, 5.664	<0.001

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Chapter 3

Inflammatory response to sepsis

Chapter 3.1

Neutrophil extracellular traps in children with meningococcal sepsis

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Abstract

Background: Children with meningococcal sepsis are highly at risk for fulminant disease, multi-organ failure and death. Recently, neutrophil extracellular traps (NETs) levels have been indicated as a marker for severity in different kinds of sepsis. Our aim was to study the role of NETosis in meninogococcal sepsis in children.

Methods: We measured MPO-DNA, a marker for NETs, in serum of meningococcal sepsis patients upon admission to PICU, at 24 hours, and at 1 month and studied the association with clinical outcome. Subsequently, we tested whether *N. meningitidis*, isolated from children with meningococcal sepsis, were able to induce NETosis, using confocal microscopy live imaging.

Results: MPO-DNA levels at admission (n=35, median 0.21 AU/mL, IQR 0.12-0.27) and at 24 hours (n=39, median 0.14 U/mL, IQR 0.09-0.25) were significantly higher than the MPO-DNA levels after 1 month (controls, n=36, median 0.07 AU/mL, IQR 0.05-0.09, $p<0.001$). We did not observe a correlation between MPO-DNA levels and mortality, cell-free DNA or other inflammatory markers. In addition, *N. meningitidis* are fast and strong inducers of NETosis.

Conclusion: Children admitted to PICU for meningococcal sepsis have higher NETs levels at admission and after 24 hours than controls. NETs levels were not associated with outcome, cell-free DNA or other inflammatory markers. These NETs may be induced by *N. meningitidis*, since these are strong NETosis inducers.

Introduction

Meningococcal sepsis is notorious for its rapid progression to fulminant disease, multi-organ failure and death [1,2]. Complex interplays between host, pathogen, and environmental factors, including immune evasion mechanisms, determine the severity of *Neisseria meningitidis* infections, ranging from harmless colonization to lethal disease [3-5].

A recently identified mechanism that may play a role in the pathology of meningococcal sepsis is NETosis. The release of neutrophil extracellular traps (NETs) is an important part of innate immune defense [6,7]. NETs are an extracellular DNA matrix, containing also granule proteins and histones, released by neutrophils to degrade virulence factors and to kill bacteria [8]. NETs are primarily considered as a protective mechanism against a broad range of microorganisms, including gram-negative and gram-positive bacteria, because they prevent bacteria from spreading and contain toxic histones [8,9]. Several inducers of NETosis are known, including bacterial species such as *Staphylococcus aureus* [10]. Multiple studies describe a negative influence on outcome in mice and humans [11,12], since the tissue damage caused by NETs may contribute to disease severity [13,14]. Also cell free DNA (cfDNA), the backbone of a NET, has been described to contribute to disease via the formation of microthrombi and to inhibit fibrinolysis [15].

Studies on the relationship between NETs and *Neisseria meningitidis* in sepsis are lacking. Our objective was to study the role of NETs in children with meningococcal sepsis. We measured levels of NETs, cell-free DNA and other inflammatory markers in serum from children with meningococcal sepsis and studied the association with severity of disease. We also investigated whether *N. meningitidis* isolates from patients are able to induce NETosis.

Material and methods

Patients and samples

From 1988 to 2005, children with meningococcal sepsis presenting to the pediatric intensive care unit (PICU) of Erasmus MC-Sophia Children's Hospital (Rotterdam, The Netherlands) were prospectively enrolled in meningococcal studies [16-19]. These studies were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All individual meningococcal studies as well as the current laboratory study (MEC-2015-497) were approved by the ethical committee of Erasmus MC, and written informed consent was obtained from parents or legal guardians. All patients fulfilled internationally agreed criteria for sepsis [20]. Blood samples were taken on admission to PICU, at 24 hours after PICU admission, and at 1 month after PICU admission. Since all children were healthy at 1 month, the 1 month convalescence samples were used as

controls. Samples were processed on ice and serum was stored at -80°C until analysis. This is a retrospective laboratory study in remaining samples of prospectively collected samples.

Clinical data collection

Clinical data were collected prospectively. Disease severity was indicated by Pediatric Risk of Mortality (PRISM) [21], predicted death based on the Rotterdam score [17], disseminated intravascular coagulation (DIC) score [22], and the base excess and platelet count at presentation (BEP) score [23]. Patients were classified as *death* if death occurred during PICU-stay. PICU-free days in patients who died were considered zero.

NETosis measurements

Our MPO-DNA ELISA assay was performed as reported earlier [24]. For detection of NETs in serum, we adjusted the commercial human ELISA kit that measures cell death (Cell death detection ELISA^{PLUS}, Cat. No 11920685001, Roche Diagnostics Nederland B.V., Almere, the Netherlands) [25]. Briefly, ELISA plates were coated with a mouse-anti-human myeloperoxidase (MPO) monoclonal antibody (AbD Serotec, Oxford, UK), a NETs marker, overnight at 4 °C. The plates were then washed with Phosphate Buffered Saline (PBS) containing 0,05 % v/v Tween®-20 and incubated with blocking solution (1% BSA/PBS) overnight at 4 °C. Next, samples were added to the plate and incubated with MPO-DNA immunoreagens for 2 hours at 300 rpm at RT, washed and incubated with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) reagens for 30 min at 250 rpm at RT. The reaction was stopped with stop solution from the kit and plates were measured using a Biotek reader (FLX 800, Austria) at 405 nm with a 490 nm reference filter. The NETs reference curve was created from neutrophils that were transferred to FBS serum and stimulated with phorbol-myristate-acetate (PMA) for 4 hours. Samples were serially diluted and stored at -80°C. Values are expressed as arbitrary units (AU/mL).

Inflammatory markers CRP, fibrinogen, soluble TNFr, IL-1B, IL-6, and IL-8 and cell-free DNA (nucleosomes) were measured in previous studies from our research group [16-19,26].

Neutrophil isolation

Neutrophils were isolated as previously described [10]. Briefly, red blood cells and granulocytes were isolated from blood from adult healthy donors, as approved by the Medical Ethics Committee of the Erasmus MC, using Lymphoprep™ (Stem cell Technologies, Grenoble, France). Granulocytes were further purified by lysing the erythrocytes with erythrolysis buffer (3.1M NH_4Cl, 0.2M $KHCO_3$, 0.02M EDTA, pH 7.4) and washing the neutrophil rich pellet twice with HEPES (0.115M NaCl, 0.012mM $CaCl_2$, 1.51mM $MgCl_2$, 4mM KCl, 0.01M HEPES, pH 7.4) buffer.

In vitro NETosis

For experiments with meningococcal bacteria, neutrophils were transferred to Dulbecco's Modified Eagle Medium (DMEM) culture medium without any additions (Biowhittaker, Lonza, Walkersville, USA) in a confocal ring. Propidium Iodide (PI, 1:400, Sigma Aldrich, Zwijndrecht, The Netherlands) was added to visualize extracellular DNA during live imaging.

To visualize extracellular DNA (NETs), neutrophils were stained for DNA with Hoechst 34580 (1: 10000, Life Technologies, Landsmeer, The Netherlands) and PI (1:400, Sigma Aldrich, Zwijndrecht, The Netherlands) prior to contact with *N. meningitidis* as described previously by our group [27]. Clinical isolates of *N. meningitidis* bacteria were cultured as described previously [28]. After 4 hours of culturing, 100 µl $1x10^8$/ml bacteria were added to 500 µl $2x10^6$/ml neutrophils. NETs were visualized using confocal microscopy (Leica SP5 AOBS). Excitation of Hoechst with a 405 laser and a BP 420-500 emission filer, and excitation of PI with a 561 141 and BP 580-620 emission filter. Elongated PI positive structures larger than 10 µm (the average diameter of a neutrophil) were defined as NETs. Round PI positive structures equal or smaller than 10 µm were defined as necrotic neutrophils.

Statistical analysis

Patient characteristics and MPO levels are presented as numbers and proportions (means and percentages) or medians and interquartile ranges. To compare MPO-DNA levels between the three time points, we used the Kruskal-Wallis test, including the post-hoc Mann-Whitney rank-sum test. To compare MPO-DNA level between survivors and non-survivors, we used the Mann-Whitney rank-sum test. Correlations were assessed using Spearman's rank correlation. Linear regression analyses were performed on logarithmically transformed data. Graphs were created with GraphPad Prism 7.00. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS, IBM, version 21). Two-sided p-values <0.05 were considered to indicate statistical significance.

Results

NETs levels in meningococcal sepsis

In this study we included 60 children with meningococcal sepsis (58% male, median age 2 years and 10 months [IQR 21 months-9 years]), of whom 35, 39, and 36 serum samples were available originating from admission to PICU, after 24 hours, and at 1 month, respectively. Patient characteristics are presented in Table 1.

Table 1: Patient characteristics at admission to PICU (n=60). Characteristics for 2 patients are unknown.

	Total group (n=60)	Non-survivors (n=11)	Survivors (n=49)	P-value
Male gender (%)	36 (58%)	7 (64%)	29 (59%)	ns
Age (years)	2.9 (1.8-9.6)	2.2 (0.8-2.8)	3.5 (1.9-10)	0.08
PRISM score	17 (10-25)	25 (21-34)	17 (12-24)	0.003
P (death Rotterdam)*	12 (2- 72)	98 (83-99)	5 (1-25)	<0.001
Presence of DIC$ (%)	26 (52%)	10 (100%)	16 (40%)	<0.001
DIC score#	6 (4-7)	7 (6-7)	5 (4-6)	ns
P (death BEP)~	6 (3-19)	30 (20-59)	5 (3-12)	<0.001

* Data are available for 49 patients; 11-non-survivors and 38 survivors. $ Data are available for 50 patients; 10 non-survivors and 40 survivors. These data represent the values collected at t=0. # Data are available for 36 patients; 6 non-survivors and 30 survivors. ~ Data are available for 53 patients; 11 non-survivors and 42 survivors . Categorical variables are presented as percentages, continuous variables are presented as median (IQR). Abbreviations: PRISM = pediatric risk of mortality[21], P (death Rotterdam) = predicted death rate based on the Rotterdam score[17], DIC=Disseminated intravascular coagulation[22], P (death BEP) = predicted death rate based on the BEP score [23].

MPO-DNA levels at admission (n=35, median 0.21 AU/mL, IQR 0.12-0.27) and at 24 hours (n=39, median 0.14 AU/mL, IQR 0.09-0.25) were significantly higher than the convalescent MPO-DNA levels in the control group of survivors after 1 month (n=36, median 0.07 AU/mL, IQR 0.05-0.09, p<0.001) (Figure 1A). The MPO-DNA level in children who eventually died did not differ significantly from survivors at admission to PICU (non-survivors: n=11, median 0.23 AU/mL, IQR 0.12-0.47; survivors: n=23, median 0.16 AU/mL, IQR 0.12-0.25, p=0.14) and after 24 hours (non-survivors: n=3, median 0.14 AU/mL; survivors: n=34, median 0.15 AU/mL, IQR 0.09-0.26, p=0.48) (Figure 1B).

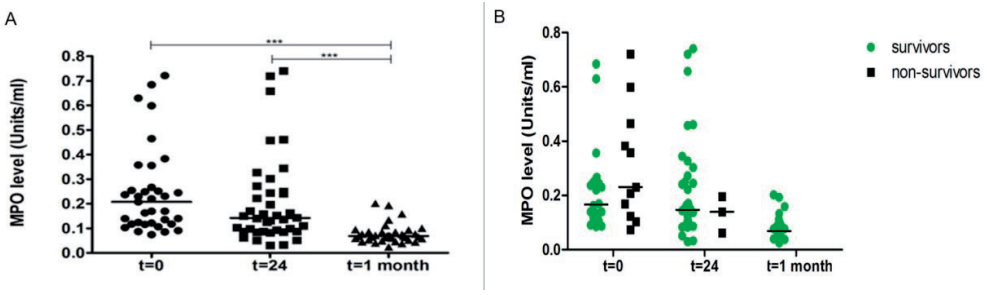

Figure 1: MPO-DNA levels measured at different time points (A) and in relation to survival (B).

NETs and inflammatory markers in sepsis

Since NETs can be induced by inflammatory markers, and NETs in turn can lead to an increased inflammatory response, we explored the correlations between inflammatory markers (neutrophil count, and serum C-reactive protein (CRP), fibrinogen, soluble Tumor Necrosis Factor (TNFr), Interleukin (IL)-1B, IL-6 and IL-8 levels) and MPO-DNA levels in our samples. We did not observe any significant correlation of NETs levels with any of these markers (Table 2).

Table 2: Correlation of MPO-DNA levels with pro-inflammatory factors.

Laboratory parameter	R-Spearman T=0	P-value	R-Spearman T=24	P-value
Neutrophils	0.043 (23)	0.85	0.211 (27)	0.29
CRP	0.105 (28)	0.60	-0.114 (29)	0.86
Fibrinogen	0.035 (29)	0.86	0.037 (36)	0.82
Soluble TNFr	0.238 (17)	0.36	-	-
IL-1B	0.391 (17)	0.12	-	-
IL-6	0.326 (17)	0.20	-0.068 (20)	0.78
IL-8	0.35 (17)	0.17	-0.291 (20)	0.21

Number of patients are indicated between brackets.

NETs and cell-free DNA

Because nucleosome levels in serum reflect cell-free DNA, we tested how serum levels of nucleosomes were correlated to serum levels of NETs. We did not find a significant correlation between nucleosomes and MPO-DNA at any of the time points (t=0: r=0.162, p= 0.54; t=24: r=0.044, p=0.86; t=1m: r=0.025, p=0.93).

In vitro formation of NETs in the presence of *Neisseria meningitidis*

In our pilot experiment, we saw that clinical isolates from *N. meningitidis* induce NETs *in vitro* in adult neutrophils. NETs were visible after 15 minutes of incubation of neutrophils with the bacteria (Fig. 2).

In the control experiment with unstimulated neutrophils without bacteria, no NETs were seen.

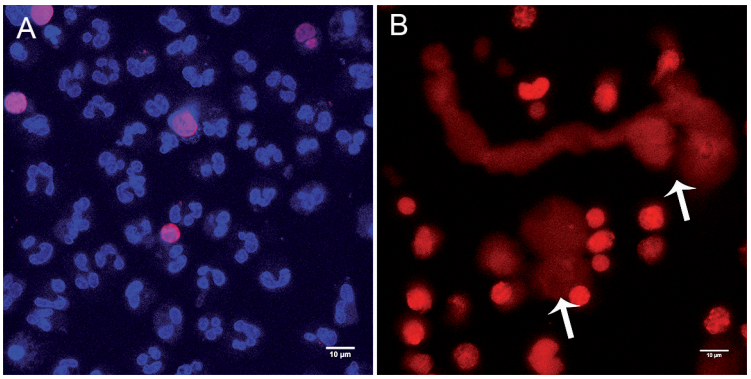

Figure 2: Unstimulated neutrophils (A) vs NETs (see arrows) formed by *N. meningitidis* (B). Blue: Hoechst staining for DNA. Red: PI staining for extracellular DNA (NETs) and dead cells.

Since neutrophils started dying approximately 30-40 minutes after contact with *N. meningitidis,* we only observed NETs within this time frame.

Discussion

This is the first study to describe NETs in children with meningococcal sepsis, describing both *in vitro* and *in vivo* data. The main findings of our study are that NETs levels in these children are higher at admission to PICU and after 24 hours compared to levels after one month. We also found that NETs levels were not associated with clinical outcome in our cohort. Lastly, in a pilot experiment we found that *N. meningitidis,* isolated from children with meningococcal sepsis, are strong NETs inducers in adult neutrophils.

In our study the NETs levels, measured by MPO-DNA levels, in children with meningococcal sepsis are higher in the acute phase of disease, i.e. at admission to PICU and at 24 hours after admission, compared to 1 month. Similar results were previously observed in adult studies, reporting increased NETs levels during the acute phase of sepsis [11,13,29].

Multiple animal and patient studies associated NETs levels with sepsis severity and outcome [13,14]. This effect of NETs in sepsis likely results from the interplay between NETs, platelets and thrombin, which activates coagulation and inhibits fibrinolysis, leading to more severe DIC [13,14,30]. In our study, we did not find an association between NETs, severity parameters, and outcome. Our study group was relatively small, and may have been underpowered to detect these effect. Alternatively, the positive and negative effects of NETosis, respectively containing meningococcal infection and inducing tissue damage and deregulate coagulation might be balanced in meningococcal sepsis in children.

Previously, Zeerleder et al (2013) [31] measured nucleosomes in this cohort and reported that nucleosomes are correlated with several factors, such as organ dysfunction, several cytokines and patient outcome. In some studies, nucleosomes are used as marker for NETosis [32]. We did not observe a correlation between MPO-DNA and nucleosome levels in this study, indicating that the nucleosomes in these patients are probably cell-free DNA originating of other cell death mechanisms than neutrophils in NETosis. In sepsis patients, severe tissue and organ damage are the main cause of death. Cell-free DNA is therefore very likely originating from these damaged cells.

We have shown in a pilot experiment that *N. meningitidis* isolated from sepsis patients, are able to induce NETosis in neutrophils *in vitro*. This is in line with a previous study on NETs formation by *N. meningitidis* [28]. This article describes that *N. meningitidis* is also able to evade NETs different from *S. aureus*, which excretes nucleases to break down the NETs [33]. *N. meningitidis* secretes small outer membrane vesicles (SOMVs) that bind to the NETs, blocking the binding of the bacteria themselves. In our experiments, we observed that after 30-40 minutes, neutrophils underwent necrosis next to NETosis, as expected since the bacteria are known to be toxic. Altogether, our results indicate that *N. meningitidis* has developed multiple mechanisms to avoid and destroy neutrophils, which might contribute to the severe pathogenesis of these bacteria in sepsis.

This is the largest prospective cohort of children with severe meningococcal disease of which detailed clinical and extensive laboratory data are available. Although a total number of 60 patients might be relatively small compared to adult sepsis cohorts, the advantage from our study is that these children are all affected by the same bacterium, *N. meningitidis*. The detailed assays performed and measurements of several factors over time allowed us to study correlations with markers assessed in both the current and previous studies [26,31]. Also, we were able to follow these patients over time and collect data on many variables, including NETs and inflammatory markers.

We have shown that *N. meningitidis* is capable of inducing NETosis in neutrophils isolated from healthy adult donors in vitro. Unfortunately age matched donors were not available, as volumes obtained for diagnostic tests are limited in children. Therefore we cannot exclude that neutrophils of children might react differently [34]. However, as NETs were present in our patient serum samples, such a difference *in vitro* is not expected.

Conclusions

N. meningitidis are strong NETs inducers. Children admitted to PICU for meningococcal sepsis have higher NETs levels at admission and after 24 hours than controls. NETs levels

were not associated with positive or negative outcome or other inflammatory markers, indicating that the beneficial and detrimental effects of NETosis in meningococcal sepsis might be balanced. We also did not find a correlation between MPO-DNA and nucleosomes, indicating that nucleosomes are not only NETs but all cell free DNA.

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Chapter 3.2

HLA-DR expression on monocyte subsets in critically ill children

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Abstract

Background: To longitudinally study blood monocyte subset distribution and human leukocyte antigen-DR (HLA-DR) expression on monocyte subsets in children with sepsis, post-surgery, and trauma in relation to nosocomial infections and mortality.

Methods: In 37 healthy children and 37 critically ill children (12 sepsis, 11 post-surgery, 10 trauma, and 4 admitted for other reasons) - participating in a randomized controlled trial on early versus late initiation of parenteral nutrition - monocyte subset distribution and HLA-DR expression on monocyte subsets were measured by flow cytometry upon admission and on day 2, 3, and 4 of PICU stay.

Results: Upon PICU admission, critically ill children had a higher proportion of classical monocytes (CD14++CD16-) than healthy children [PICU 95% (IQR 88-98%); controls 87% (IQR 85-90%), p<0.001]. HLA-DR expression was significantly decreased within all monocyte subsets and at all time points, being most manifest on classical monocytes and in patients with sepsis. Percentage of HLA-DR expressing classical monocytes [upon PICU admission 67% (IQR 44-88%); controls 95% (IQR 92-98%), p<0.001] as well as the HLA-DR mean fluorescence intensity [upon PICU admission 3219 (IQR 2650-4211); controls 6545 (IQR 5558-7647), p<0.001] decreased during PICU stay. Patients who developed nosocomial infections (n=13) or died (n=6) had lower HLA-DR expression on classical monocytes at day 2 (p=0.002) and day 3 (p=0.04), respectively.

Conclusions: Monocytic HLA-DR expression decreased during PICU stay and was lower compared to controls on all examined time points, especially on classical monocytes and in children admitted for sepsis. Low HLA-DR expression on classical monocytes was associated with nosocomial infections and death.

Introduction

Infection, major surgery, and severe injury are frequently associated with a period of immunosuppression.(1-3) Immunosuppression in its most severe form is often referred to as immunoparalysis and is associated with increased risk of nosocomial infections and death.(1-4) Therefore, there is a growing interest in immunostimulatory therapy, such as interferon-gamma and granulocyte-macrophage colony-stimulating factor, as potential treatment options to improve outcome in severely immunosuppressed patients.(4-8) Laboratory markers that guide the need for immunostimulatory therapy are needed.

Human blood monocytes are a heterogeneous cell population and are subdivided into three main subsets based on expression of cell surface markers: 1) classical monocytes (CD14++CD16- monocytes), which represent the major monocyte subset, 2) intermediate monocytes (CD14++CD16+ monocytes), and 3) non-classical monocytes (CD14+/-CD16++ monocytes).(9, 10) Classical monocytes migrate from bone marrow to peripheral blood circulation and differentiate into intermediate monocytes and sequentially into non-classical monocytes.(11) In different types of diseases, mostly in infection or inflammatory conditions, expansion of intermediate and/or non-classical monocytes have been described.(12)

Human leukocyte antigen-DR (HLA-DR) is a MHC class II cell surface molecule that is expressed, amongst others, by monocytes in order to present antigens to T-cells. Numerous studies, mainly in adults, have identified decreased monocytic HLA-DR (mHLA-DR) expression as marker for immunosuppression and showed that low mHLA-DR expression is associated with nosocomial infections and death.(8, 13-16) In addition, measurement of mHLA-DR expression has been proven effective to identify septic adults eligible for immunostimulatory therapy.(17-19)

Studies on blood monocyte subset distribution and mHLA-DR expression in critically ill children are limited so far, especially studies focusing on longitudinal monitoring of mHLA-DR expression during the course of disease.(13-15, 20-22) In addition, previous studies were restricted to one specific patient category, thus studies comparing mHLA-DR expression between children admitted for different clinical reasons are lacking. The primary objective of this study was to longitudinally monitor monocyte subset distribution and mHLA-DR expression in children with sepsis, post-surgery, and trauma in relation to nosocomial infections and mortality.

Materials and Methods

Patients and controls

Critically ill children, from term newborns to 17 years of age, who had been enrolled to a randomized controlled trial on macronutrient management in the PICU of Erasmus MC-Sophia Children's Hospital, were eligible for this study.(23, 24) Patients had been randomized to early (within 24 hours) supplementation of parenteral nutrition (PN) or late (not before day 8) supplementation of PN when enteral nutrition was insufficient to reach caloric targets. 0.5 mL whole blood was obtained in EDTA tubes on admission to the PICU and on the mornings of day 2, day 3, and day 4. Subsequently, samples were stored at 4 ^{0}C until analysis, which was performed within 4 hours after blood sampling. The number of patients that could be included was limited due to blood sample volume restrictions and logistic reasons at our hospital.

For comparison, blood from 37 healthy controls was sampled immediately after placement of an intravenous catheter prior to minor elective surgery.

Laboratory analysis

Monocyte subsets (classical monocytes: CD14++CD16-, intermediate monocytes: CD14++CD16+, non-classical monocytes: CD14+/-CD16++) and mHLA-DR expression were determined by flow cytometry. The following antibodies were used: CD45 PO (Invitrogen, clone HI30), CD14 APC-H7 (Becton Dickinson, clone MO-P9), CD16 PeC7 (Becton Dickinson, clone 3G8) and HLA-DR PB (Biolegend, clone L243). Antibodies were added to 50 ml of whole blood and incubated for 15 minutes at room temperature in the dark. Subsequently, 500 ml NH4Cl was added for 10 minutes at room temperature in the dark to lyse erythrocytes. Eventually, the sample was measured on a flow cytometer (FACSCanto II machine; Becton Dickinson) that was calibrated according to a standardized instrument setting described in detail in the EuroFlow protocols.(25) Analysis was performed with Infinicyt 1.7 flow cytometry software. Monocytes, lymphocytes and granulocytes were identified on the basis of CD45 expression along with side-scatter (SSC) characteristics. Monocytes were analyzed for CD14 and CD16 expression and the three monocyte subsets were identified on the basis of CD14 and CD16 expression levels.

HLA-DR expression was determined as percentage of HLA-DR positive cells within the monocyte subsets, using HLA-DR negative lymphocytes (T-cells) and HLA-DR positive lymphocytes (B-cells) as an internal control(26), as well as the mean fluorescence intensity (MFI) within each monocyte subset (see Figure, Supplemental Digital Content 1). Absolute counts of monocytes and monocyte subsets were calculated based on the total white blood cell count. Total white blood cell count in the peripheral blood was determined by a standard hematology analyzer (Sysmex XP-300).

Clinical data collection

The presence of infection on admission to the PICU and the identification of nosocomial infections was determined by consensus opinion of two infectious disease specialists, who made their decision on the basis of study protocol guidelines.(23, 24) Infectious disease specialists selected all patients receiving antimicrobial agents for more than 48 hours, after excluding all patients who received prophylaxis. Each patient who fulfilled the criteria for infection, as well as the type of infection, was identified as such based on thorough review of the medical record.(27) Patients for whom antimicrobial agents were initiated prior to PICU admission or within the first 48 hours of admission while the criteria for infection were fulfilled, were classified in the "sepsis" subgroup. When antimicrobial agents were initiated after randomization and beyond the first 48 hours in the PICU, and were given for more than 48 hours while the criteria for infection were fulfilled, the patient was labeled as having acquired a "nosocomial infection".(23, 27) The PELOD score indicated illness severity.(28) We defined death as death within 90 days after admission to PICU.

Statistical analysis

Variables are presented as numbers and proportions, means and standard deviations, or medians and interquartile ranges. Characteristics of patients from different diagnostic categories were compared by chi squared tests or by Kruskal-Wallis tests, including post-hoc Mann-Whitney U tests. In controls, the distribution of cells between five age categories was tested by the Kruskal-Wallis test. Spearman's correlation coefficient was used to analyze the correlation between monocyte subsets and age. Monocyte subsets and HLA-DR expression in patients were compared to controls using the Mann-Whitney U test. The relation between mHLA-DR expression and nosocomial infections, death, and the effect of early versus late PN was analyzed by Mann-Whitney U tests. Statistical analyses were performed with SPSS version 21. Graphs were created with GraphPad Prism 7.00. Two-sided p-values <0.05 were considered to indicate statistical significance.

Ethical aspects

This study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Protocols were approved by the Central Committee on Research Involving Human Subjects and our institutional ethical review board.

Results

Subjects

We enrolled 37 patients of which 12 patients were admitted for sepsis, 11 post-surgery, 10 after trauma, 3 for severe asthma, and 1 patient for renal failure. Baseline characteristics are presented in Table 1; patients with sepsis included a lower percentage of males (33%) compared to patients post-surgery (82%, p=0.019). Also, patients with sepsis had higher illness severity compared to patients post-surgery, as reflected by higher PELOD score at day 1 (p=0.011) and more days on ventilator (p=0.008). Patients admitted after trauma were older than patients from the two other categories (p=0.025 compared to sepsis, p=0.024 compared to post-surgery). None of the enrolled patients had a primary immunodeficiency.

Table 1: patient characteristics

Patient characteristics	All (n=37)	Sepsis (n=12)	Post-surgery (n=11)	Trauma (n=10)	Significance
Male gender (n [%])	22 (60%)	4 (33%)	9 (82%)	7 (70%)	#[1]
Age in years (median [IQR])	9 (5-13)	8 (3-11)	8 (3-11)	14 (10-14)	&[1], ¶[1]
Illness severity					
PELOD score at day 1 (median [IQR])	11 (11-22)	12 (11-33)	11 (2-11)	16 (2-22)	#[1]
Ventilatory support at day 1 (n [%])	33 (89%)	11 (92%)	11 (100%)	7 (70%)	ns
Days on ventilator (median [IQR])	4 (2-9)	6 (3-12)	2 (2-5)	14 (2-21)	#[2]
Hemodynamic support at day 1 (n [%])	17 (46%)	5 (42%)	5 (46%)	5 (50%)	ns
Days on hemodynamic support (median [IQR])	6 (2-13)	7 (5-14)	4 (2-16)	8 (3-13)	ns
Outcome					
Mortality (n [%])	6 (16%)	2 (17%)	1 (9%)	1 (10%)	ns
PICU days (median [IQR])	5 (3-17)	5 (3-19)	5 (2-9)	11 (4-28)	ns

#: Significant difference (#[1]: p<0.05, #[2]: p<0.01) between patients with sepsis and post-surgery; &[1]: Significant difference (p<0.05) between patients with sepsis and trauma; ¶[1]: Significant difference (p<0.05) between patients post-surgery and trauma.

Furthermore, we enrolled 37 healthy controls who underwent a minor elective surgical procedure (21 males, median age 3y (IQR 11m-8y)).

Effect of age in controls

In Figure 1, panel A, we show that the absolute number of white blood cells decreased significantly (p=0.001) with age, from 7580 cells/uL in infants to 3099 cells/uL in children aged 12 to 18 years. No differences were observed between males and females (data not shown). The absolute number of total monocytes and the percentage total monocytes did not differ between age groups.(Figure 1, panel B) The relative distribution of the

three monocyte subsets showed no correlation with age (classical monocytes, r=0.11, p=0.51; intermediate monocytes, r=-0.09, p=0.60; non-classical monocytes, r=-0.14, p=0.42).(Figure 1, panel C) However, the absolute numbers of classical monocytes and non-classical monocytes were significantly and negatively correlated to age (classical monocytes, r=-0.44, p=0.007; non-classical monocytes, r=-0.41, p=0.01).(Figure 1, panel D) Therefore, for further analyses, the relative proportion of monocyte subsets of the control group could be compared to that of patients.

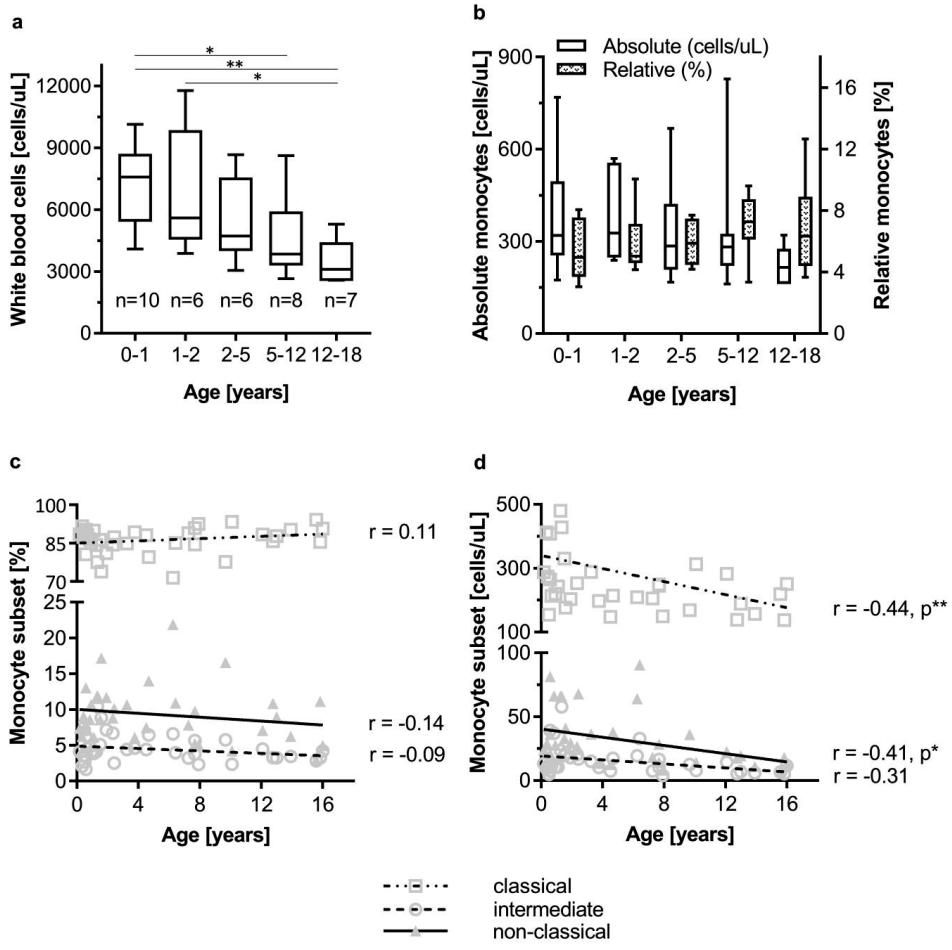


Fig. 1 White blood cell count, monocytes and monocyte subsets in controls by age. The relative distribution of the three monocyte subsets showed no correlation with age.
a) White blood cell count in relation to age; b) Total monocyte numbers and relative proportion of total white blood cell population in relation to age; c) Relative monocyte subset distribution in relation to age; d) Absolute monocyte numbers per subset in relation to age. a-d: Horizontal line indicates the median value, box represents IQR, and the whiskers represent 95% CI. *p-value<0.05, **p-value≤0.01.

Reference values for absolute numbers as well as relative distribution of blood monocyte subsets and HLA-DR expression are derived from our control cohort (see Table, Supplemental Digital Content 2). The percentage of HLA-DR expressing monocytes within the three monocyte subsets as well as the HLA-DR MFI within the monocyte subsets did not differ between age groups, thus, for further analysis, we did not have to adjust for age.

Monocyte subset distribution in critically ill children

Figures, Supplemental Digital Content 3, 4, and 5 depict monocyte subset distributions according to the main diagnostic categories (sepsis, post-surgery, and trauma) over a period of four days following admission to the PICU. On admission, for the whole group of patients, the percentage of classical monocytes was significantly ($p<0.001$) higher (95%, IQR 88-98%) compared to controls (87%, IQR 85-90%), while the percentage of non-classical monocytes was significantly lower in patients (patients 2%, IQR 1-5%; controls 9%, IQR 7-11%; $p<0.001$).

The most prominent differences in monocyte subset distribution were found in patients admitted for sepsis. In the sepsis group, the percentage of classical monocytes tend ($p=0.09$) to be lower (82%, IQR 73-88%) compared to controls (87%, IQR 85-90%). The percentage of intermediate monocytes at admission in sepsis patients (8%, IQR 5-14%) was significantly ($p=0.003$) higher compared to controls (4%, IQR 3-5%) and increased even further to 14% (IQR 5-28%) at day 2 ($p=0.001$ compared to controls).

HLA-DR expression on monocyte subsets

For the total group of patients, HLA-DR expression was significantly decreased within all monocyte subsets and at all time points, being most manifest on classical monocytes where 67% (IQR 44-88%) expressed HLA-DR on admission compared to 95% (IQR 92-98%) of this population in controls ($p<0.001$).(Figure 2, panel A) Also the MFI of HLA-DR expression on classical monocytes, presented in panel B, was significantly ($p<0.001$) lower in patients at PICU admission (MFI: 3219, IQR 2650-4211) compared to controls (MFI: 6545, IQR 5558-7647). A further drop in both the percentage of HLA-DR expressing classical monocytes and HLA-DR MFI on this population was evident during the following three days after PICU admission.

The HLA-DR decrease on classical monocytes was most prominent in the patients with sepsis.(Figure 2, panel C-H) Therefore we focused our analysis on this monocyte subset. Intermediate and non-classical monocytes have a largely comparable decrease of HLA-DR expression, as shown in Figures, Supplemental Digital Content 6 and 7.

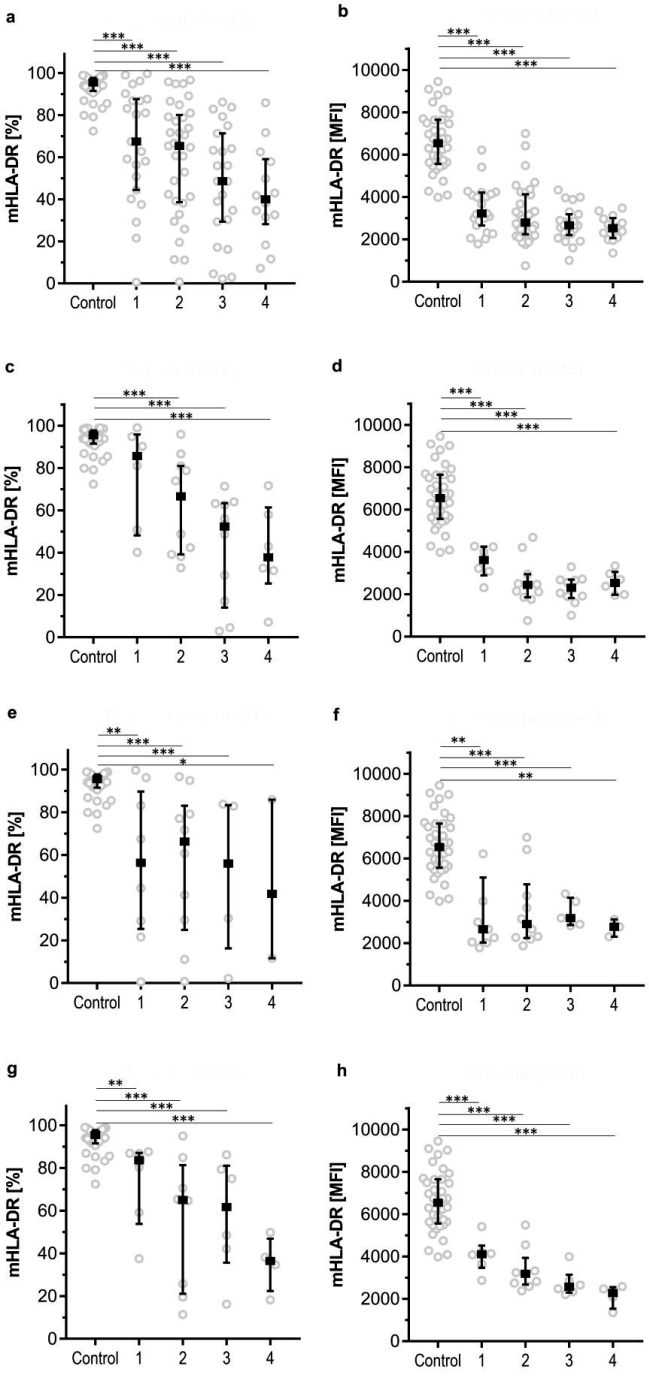


Fig. 2 HLA-DR expression on classical monocytes during PICU stay was significantly decreased at all time points.

a) Percentage of classical monocytes expressing HLA-DR (mHLA-DR %), all patients (n=37); b) HLA-DR MFI of classical monocytes (mHLA-DR MFI), all patients (n=37); c-d) mHLA-DR (%, c; MFI, d) in patients admitted for sepsis (n=12); e-f) mHLA-DR (%, e; MFI, f) in post-surgery patients (n=11); g-h) mHLA-DR (%, g; MFI, h) in trauma patients (n=10). a-h: Square indicates median value and whiskers represent IQR. *p-value<0.05, **p-value≤0.01, ***p-value≤0.001. X-axis 1, 2, 3, 4 represent admission, day 2, day 3, day 4, respectively.

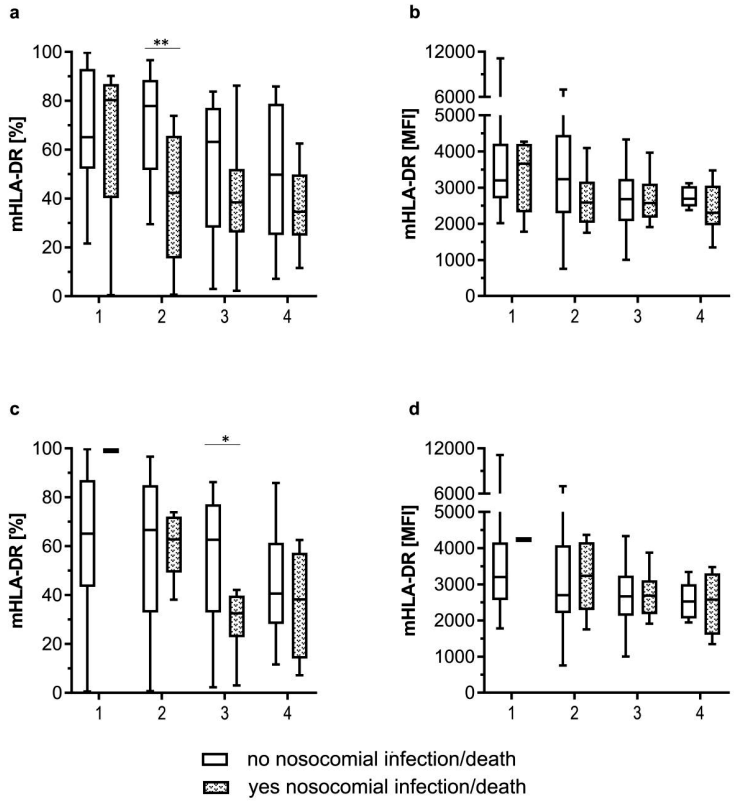


Fig. 3 HLA-DR expression (all patients, n=37) on classical monocytes during PICU stay in association with the occurrence of nosocomial infections and mortality.

a-b) Percentage of classical monocytes expressing HLA-DR (mHLA-DR %) (a) and HLA-DR MFI (mHLA-DR MFI) of classical monocytes (b) in relation to nosocomial infection (n=13); c-d) mHLA-DR (%, c; MFI, d) in relation to death (n=6). a-d: Horizontal line indicates the median value, box represents IQR, and the whiskers represent 95% CI. *p-value<0.05, **p-value≤0.01. X-axis 1, 2, 3, 4 represent admission, day 2, day 3, day 4, respectively.

HLA-DR expression in relation to nosocomial infections and death

Thirteen patients (35%) acquired at least one nosocomial infection during PICU stay. At day 2 after PICU admission, the percentage of HLA-DR expressing classical monocytes was significantly (p=0.002) lower in patients who acquired nosocomial infections (42%, IQR 16-66%) than in patients who did not acquire infections (78%, IQR 52-89%, Figure 3, panel A). Patients who died (n=6, 16%) had a significantly (p=0.04) lower percentage of HLA-DR expressing classical monocytes at day 3 after PICU admission (33%, IQR 23-40%) compared to survivors (63%, IQR 33-77%, Figure 3, panel C). Clinical

characteristics of patients with complications are summarized in Table, Supplemental Digital Content 8. One patient admitted with sepsis acquired an infection caused by an opportunistic pathogen (Candida albicans). In 54% of the infections, no pathogen could be identified.

HLA-DR expression in relation to parenteral nutrition

Patients participated in a randomized controlled trial on early versus late supplementation of PN. Patients still in the PICU on day 4 and exposed to early PN had a lower proportion of classical monocytes on day 4 compared to children who did not receive PN (Early PN: n=5, median 81%, IQR 44-88; Late PN: n=9, median 93%, IQR 90-96; p=0.007) (Figure, Supplemental Digital Content 9). HLA-DR expression within all monocyte subsets and at all time points did not differ between the two randomization groups (Figure, Supplemental Digital Content 10)

Discussion

The percentage of HLA-DR expressing cells within all monocyte subsets as well as the HLA-DR MFI within the monocyte subsets in critically ill children was lower compared to controls, and decreased further during PICU stay. These findings were most pronounced for classical monocytes and in patients admitted for sepsis. In addition, low HLA-DR on classical monocytes on day 2 and day 3 after PICU admission was significantly associated with the occurrence of nosocomial infections and with mortality, respectively.

Our study confirms previous findings, mainly in adults, of decreased HLA-DR on monocytes after admission for sepsis, trauma, burns or surgery, and that such a decreased mHLA-DR expression is associated with nosocomial infections and/or death.(13-16, 20, 21, 29, 30) However, our study clearly demonstrates for the first time that the decrease in HLA-DR expression is most prominent on classical monocytes. It remains unclear whether this decrease results from down-regulation of HLA-DR expression on the monocyte or from emergency myelopoiesis; i.e. increased recruitment of immature, low HLA-DR expressing cells from bone marrow in a pro-inflammatory state, including myeloid-derived suppressor cells.(31-34)

The decreased mHLA-DR was most prominent in children admitted for sepsis. Although clinical outcome did not differ significantly between diagnostic groups, patients with sepsis had higher illness severity at admission to PICU (compared to post-surgery patients), which could partly explain decreased mHLA-DR.(15, 16, 30) Prolonged sepsis-induced immunosuppression is considered to contribute to long-term morbidity and mortality in sepsis survivors.(2, 35, 36) An explorative study in 8 adult long-term sepsis

survivors hinted towards recurrent infections for months to years after surviving sepsis and also infections caused by opportunistic infections.(37) However, laboratory analysis in that study showed no substantial differences in mHLA-DR expression between sepsis survivors and controls. Further studies in larger cohorts are therefore needed to draw firm conclusions on the relation between mHLA-DR expression and long-term complications in sepsis survivors. Furthermore, future studies have to determine whether associations are present between mHLA-DR expression and specific clinical infectious syndromes and/or specific pathogens.

In addition to decreased mHLA-DR expression, our study revealed shifts in monocyte subset distribution in critically ill children. For the total group of 37 patients, on admission to PICU, a shift towards increased classical monocytes and decreased non-classical monocytes was observed. However, we observed a different pattern in sepsis patients compared to post-surgery patients and trauma patients as a considerable increase in the percentage of intermediate monocytes occurred in the sepsis patients. However, since sepsis patients usually develop symptoms days before admission to PICU, these patients may reflect a progressed course of disease compared to patients post-surgery or trauma, who usually are admitted to PICU at short notice. In adults, an increase in intermediate monocytes has been observed in a variety of infections, including bacterial and viral infections.(12, 38) Also in children with sepsis, a higher proportion of intermediate monocytes has been reported compared to healthy controls.(22) However, in 30 children and young adults after hematopoietic stem cell transplantation, 11 of these patients developed sepsis, but no shift in monocyte subset distribution was observed.(20) Thus, although we do not understand the clinical significance of monocyte subsets, these subsets might have specific functions and alterations in monocyte subset distribution could be disease-specific.(12)

Parenteral nutrition, in particular fatty acids and lipids, may adversely affect immune function, resulting in lower mHLA-DR expression.(39-41) Patients included in this study participated in a randomized controlled trial on early versus late initiation of PN.(23) At day 4 of PICU stay, early supplementation of PN was associated with a lower proportion of classical monocytes compared to withholding PN. We did not find significant differences in mHLA-DR expression between the randomization groups. However, this sub-study was not aimed and thus not sufficiently powered to detect such differences, thus the lower incidence of nosocomial infections reported in patients with late initiation of parenteral nutrition(23) could potentially be associated with mHLA-DR expression.

We generated a small reference cohort for blood monocyte subset distribution and mHLA-DR expression. Previous studies in healthy individuals suggested dynamic changes of monocyte subsets and mHLA-DR expression in neonates(42), children(21)

and adults(43). In our cohort, the relative proportion of monocyte subsets and the percentage as well as MFI of HLA-DR expressing monocytes did not vary with age. Also, no impact of gender on monocyte subset or mHLA-DR expression was observed. Despite the small number of controls, our data could be used for future studies because reference values of monocyte subsets and their HLA-DR expression in children are scarce.

This is the first study that examined HLA-DR expression on all three major blood monocyte subsets in a population of critically ill children, allowing us to compare children admitted for sepsis, post-surgery, and after trauma. So far, a few other studies in children examined these major blood monocyte subsets in a longitudinal way, but were all limited to one specific patient group.(14, 15, 20, 22) Our study is limited by the small number of patients included (resulting in high standard deviations) and missing values in a not-normally distributed dataset of repeated measures. Therefore we unfortunately could not study alterations in mHLA-DR expression between different time points during PICU stay. However, we observed a clear trend towards decreasing mHLA-DR expression during PICU stay. For this same limitation, our findings on shifts in monocyte subset distribution need to be interpreted witch caution. We found relatively small changes in monocyte subset distribution, which need to be validated in larger cohorts. A second important limitation is that we only examined four time points. For the total group of patients, nadir mHLA-DR expression was detected on day 4, but the declining trend was still ongoing. Therefore, the validity of our findings is limited by our inability to show a time to recovery of mHLA-DR expression. It is essential that future studies monitor mHLA-DR for a longer period of time. Prolonged decrease in mHLA-DR expression might be relevant for outcome and could be useful in predicting long-term complications. Also, we determined the proportion of HLA-DR expressing monocytes - which has a potential of inter-observer variability - as well as the HLA-DR MFI on monocytes - that may depend on the type of flow cytometer and instrument settings used - making comparison between different laboratories troublesome. Therefore a more standardized flow cytometry protocol, based on the generation of a calibration curve and an anti-HLA-DR antibody, conjugated 1:1 with a fluorochrome, that allows measurement of bound HLA-DR molecules per cell independently of the type of flow cytometer and instrument settings used, would allow better comparison of data between centers and studies.(44, 45) Lastly, our data needs to be interpreted with caution, because we did not specifically exclude patients on immunosuppressive therapy which might have influenced HLA-DR expression. Also, we found a significant association between low HLA-DR expression on classical monocytes and clinical complications only at day 2 and 3, and only in the percentage HLA-DR positive cells.

Conclusions

In critically ill children, HLA-DR expression on all monocyte subsets decreased the first four days of PICU stay and was lower compared to controls on all examined time points, especially on classical monocytes and in children admitted for sepsis. Low HLA-DR expression on classical monocytes was associated with nosocomial infections and death. Future studies should include a larger cohort of children, including subgroups of different clinical infectious syndromes and different pathogens, and more time points to study the utility of mHLA-DR expression as a prognostic marker and a marker to guide future immunomodulatory therapy trials.

Supplemental Digital Content

Supplemental Digital Content 1. Figure
Flow cytometric identification of monocyte subsets and HLA-DR expression.

Supplemental Digital Content 2. Table
Reference values of white blood cells, monocytes, monocyte subsets and HLA-DR expression on monocyte subsets by age group.

Supplemental Digital Content 3. Figure
Distribution of classical monocytes during PICU stay by diagnosis.

Supplemental Digital Content 4. Figure
Distribution of intermediate monocytes during PICU stay by diagnosis.

Supplemental Digital Content 5. Figure
Distribution of non-classical monocytes during PICU stay by diagnosis.

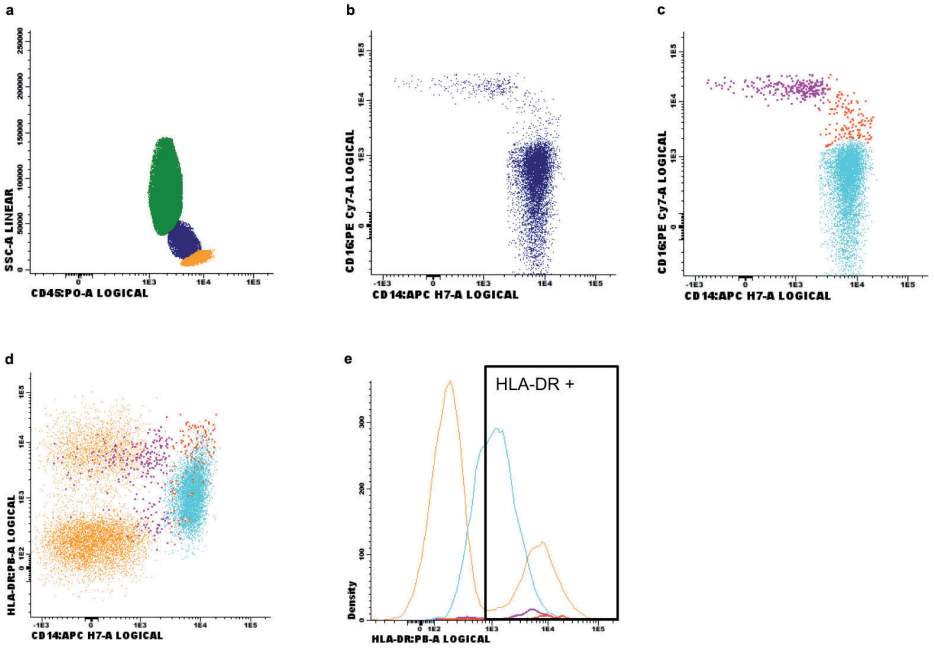
Supplemental Digital Content 6. Figure
HLA-DR expression on intermediate monocytes during PICU stay.

Supplemental Digital Content 7. Figure
HLA-DR expression on non-classical monocytes during PICU stay.

Supplemental Digital Content 8. Table
Characteristics of patients with clinical complications.

Supplemental Digital Content 9. Figure
Monocyte subset distribution in relation to parenteral nutrition.

Supplemental Digital Content 10. Figure
HLA-DR expression on monocyte subsets in relation to parenteral nutrition.



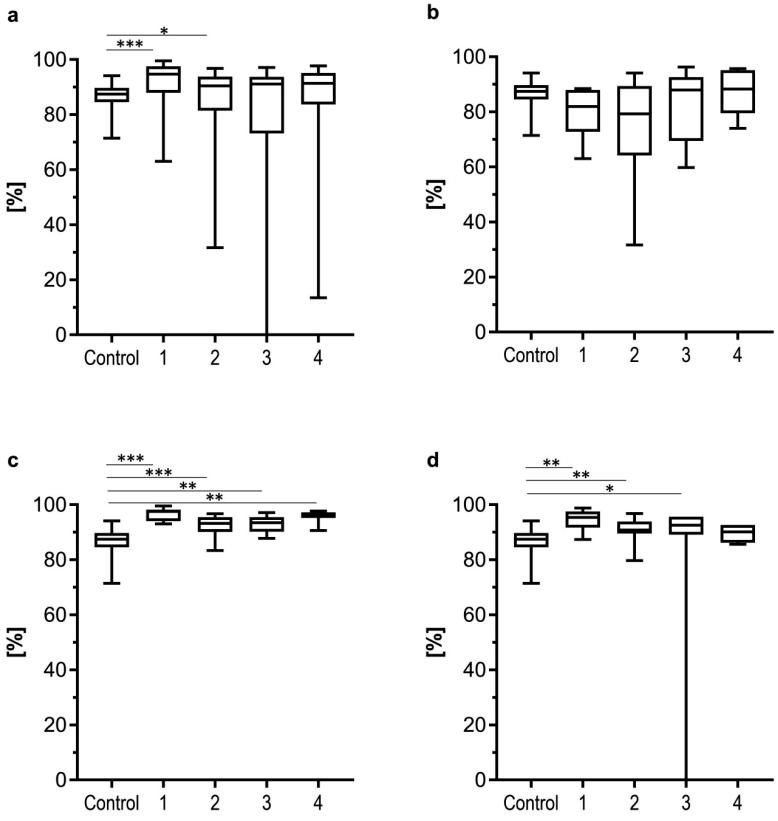
Figure, Supplemental Digital Content 1. Flow cytometric identification of monocyte subsets and HLA-DR expression.

A) Monocytes (blue), lymphocytes (orange), and granulocytes (green) were identified on the basis of CD45 expression along with side-scatter (SSC) characteristics; B) Monocytes were analyzed for CD14 and CD16 expression; C) Three monocyte subsets were identified on the basis of CD14 and CD16 expression levels; classical monocytes: CD14++CD16- (light blue), intermediate monocytes: CD14++CD16+ (red), and non-classical monocytes: CD14+/-CD16++ (purple); D and E) HLA-DR positivity within the monocyte subsets (classical monocytes (light blue), intermediate monocytes (red), non-classical monocytes (purple)) was determined by using HLA-DR negative lymphocytes (T-cells; lower orange population in figure D and left orange peak in figure E) and HLA-DR positive lymphocytes (B-cells; upper orange population in figure D and right orange peak in figure E) as reference. In Figure E, HLA-DR positivity is indicated by the square.

Table, Supplemental Digital Content 2.

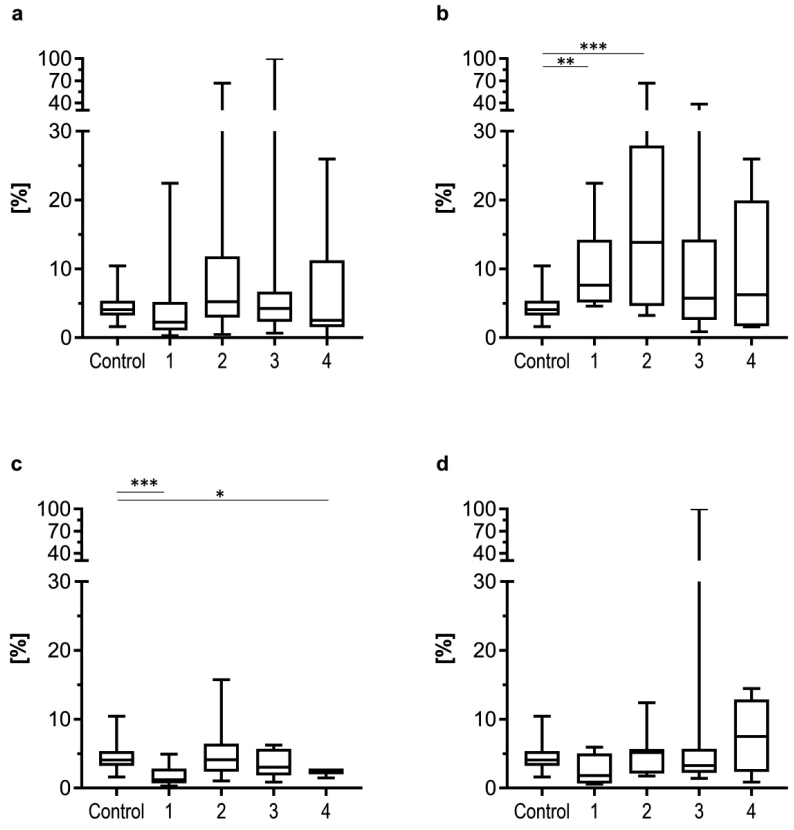
Reference values of white blood cells, monocytes, monocyte subsets and HLA-DR expression on monocyte subsets by age group. HLA-DR expressing monocytes within the three monocyte subsets do not differ between age groups. *p-value<0.05, **p-value≤0.01.

	All (n = 37)	0-1y (n = 10)	1-2y (n = 6)	2-5y (n = 6)	5-12y (n = 8)	12-18y (n = 7)	P
White blood cells (cells/uL)	**4770**	**7580**	**5600**	**4717**	**3837**	**3099**	**
Median (95% CI)	(4222-6189)	(4433-9522)	(3872-11770)	(3049-8660)	(2646-8622)	(2526-5290)	
Monocytes (cells/uL)	**276**	**319**	**327**	**285**	**282**	**215**	**ns**
Median (95% CI)	(242-320)	(242-625)	(239-570)	(168-668)	(162-829)	(161-320)	
Monocytes (%)	**6.2**	**5.0**	**5.0**	**5.9**	**7.3**	**6.3**	**ns**
Median (95% CI)	(4.8-7.3)	(3.5-8.0)	(4.2-10.1)	(4.2-7.7)	(3.4-9.6)	(3.7-12.7)	
Classical monocytes (cells/uL)	**245**	**281**	**287**	**234**	**227**	**189**	**ns**
Median (95% CI)	(209-283)	(214-504)	(176-480)	(148-583)	(150-705)	(138-283)	
Classical monocytes (%)	**87.5**	**88.4**	**82.7**	**86.1**	**87.0**	**88.4**	**ns**
Median (95% CI)	(85.2-88.6)	(85.2-90.3)	(74.0-90.0)	(79.6-89.3)	(71.5-93.4)	(85.6-94.2)	
HLA-DR expression (%)	**95.5**	**93.5**	**96.0**	**97.1**	**92.0**	**97.4**	**ns**
Median (95% CI)	(92.8-96.6)	(83.3-98.3)	(90.6-98.6)	(91.4-98.8)	(72.4-98.3)	(79.2-98.8)	
Intermediate monocytes (cells/uL)	**11**	**11**	**20**	**16**	**11**	**7**	*
Median (95% CI)	(10-16)	(8-37)	(11-58)	(7-20)	(4-33)	(5-14)	
Intermediate monocytes (%)	**4.1**	**3.8**	**6.1**	**4.5**	**3.9**	**3.3**	**ns**
Median (95% CI)	(3.5-4.6)	(2.1-4.8)	(3.9-10.5)	(2.5-6.7)	(2.3-6.6)	(2.8-4.5)	
HLA-DR expression (%)	**98.8**	**98.2**	**98.8**	**99.6**	**99.4**	**98.8**	**ns**
Median (95% CI)	(98.4-99.5)	(97.0-99.1)	(95.4-100)	(98.8-99.9)	(93.9-99.7)	(97.9-100)	
Non-classical monocytes (cells/uL)	**27**	**28**	**37**	**31**	**23**	**18**	**ns**
Median (95% CI)	(18-33)	(18-66)	(17-66)	(13-68)	(8-91)	(7-23)	
Non-classical monocytes (%)	**8.7**	**8.3**	**11.6**	**9.5**	**8.8**	**7.1**	**ns**
Median (95% CI)	(7.4-10.7)	(7.1-10.7)	(6.1-17.2)	(6.1-14.0)	(4.2-21.9)	(3.0-11.2)	
HLA-DR expression (%)	**99.5**	**99.3**	**99.7**	**99.7**	**98.9**	**99.3**	**ns**
Median (95% CI)	(99.1-99.6)	(98.7-99.6)	(98.0-100)	(99.1-100)	(97.1-100)	(95.6-100)	



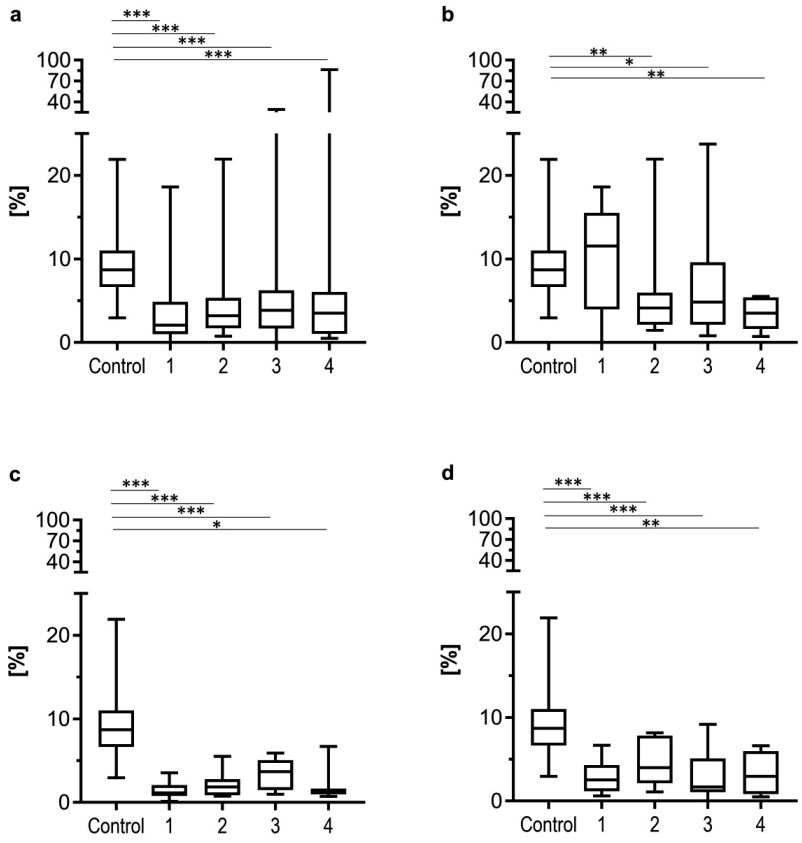
Figure, Supplemental Digital Content 3.

Distribution of classical monocytes during PICU stay a) in all patients (n=37); b) in patients admitted for sepsis (n=12); c) in post-surgery patients (n=11); and d) in trauma patients (n=10). On admission, the percentage of classical monocytes in all patients was higher (95%, IQR 88-98%) compared to controls (87%, IQR 85-90%). a-d: Horizontal line indicates the median value, box represents IQR, and the whiskers represent 95% CI. *p-value<0.05, **p-value≤0.01, ***p-value≤0.001. X-axis 1, 2, 3, 4 represent admission, day 2, day 3, day 4, respectively.



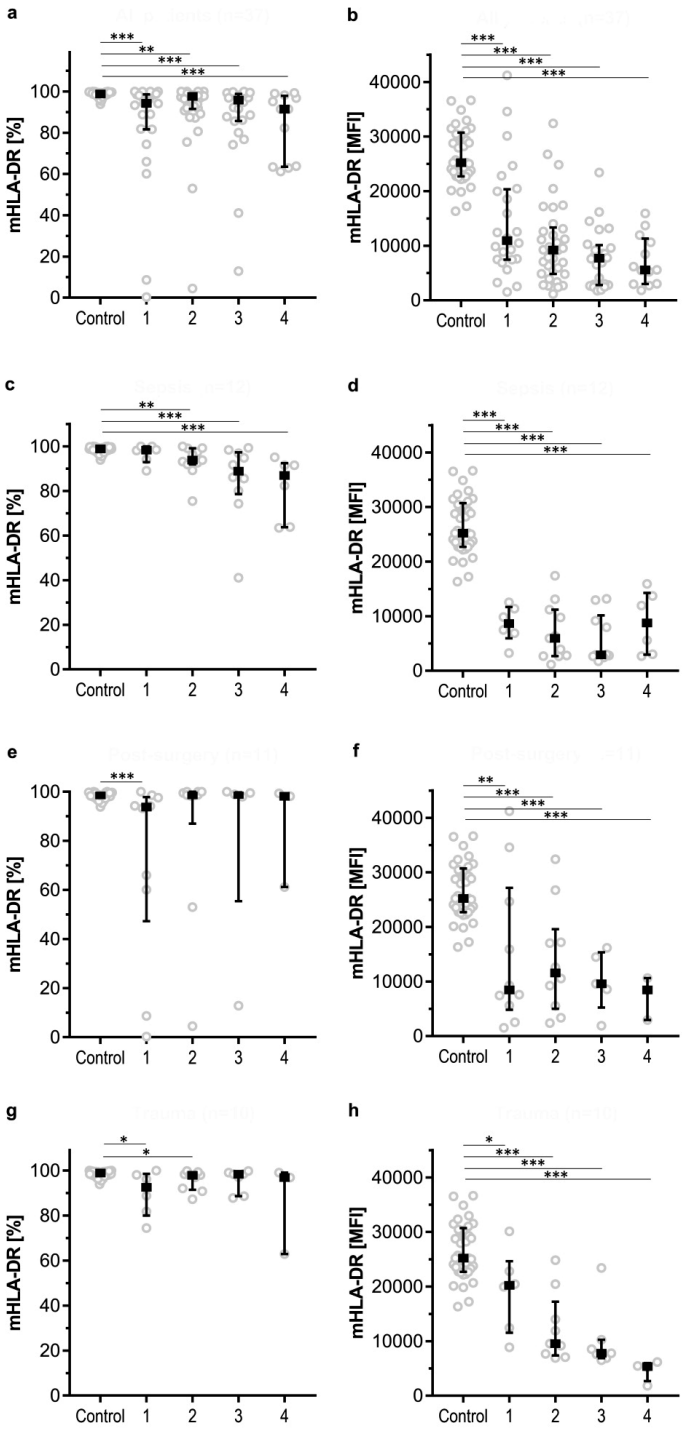
Figure, Supplemental Digital Content 4.

Distribution of intermediate monocytes during PICU stay in all patients (n=37); b) in patients admitted for sepsis (n=12); c) in post-surgery patients (n=11); and d) in trauma patients (n=10). The percentage of intermediate monocytes at admission in sepsis patients (8%, IQR 5-14%) was higher compared to controls (4%, IQR 3-5%) and increased even further to 14% (IQR 5-28%) at day 2. a-d: Horizontal line indicates the median value, box represents IQR, and the whiskers represent 95% CI. *p-value<0.05, **p-value≤0.01, ***p-value≤0.001. X-axis 1, 2, 3, 4 represent admission, day 2, day 3, day 4, respectively.



Figure, Supplemental Digital Content 5.

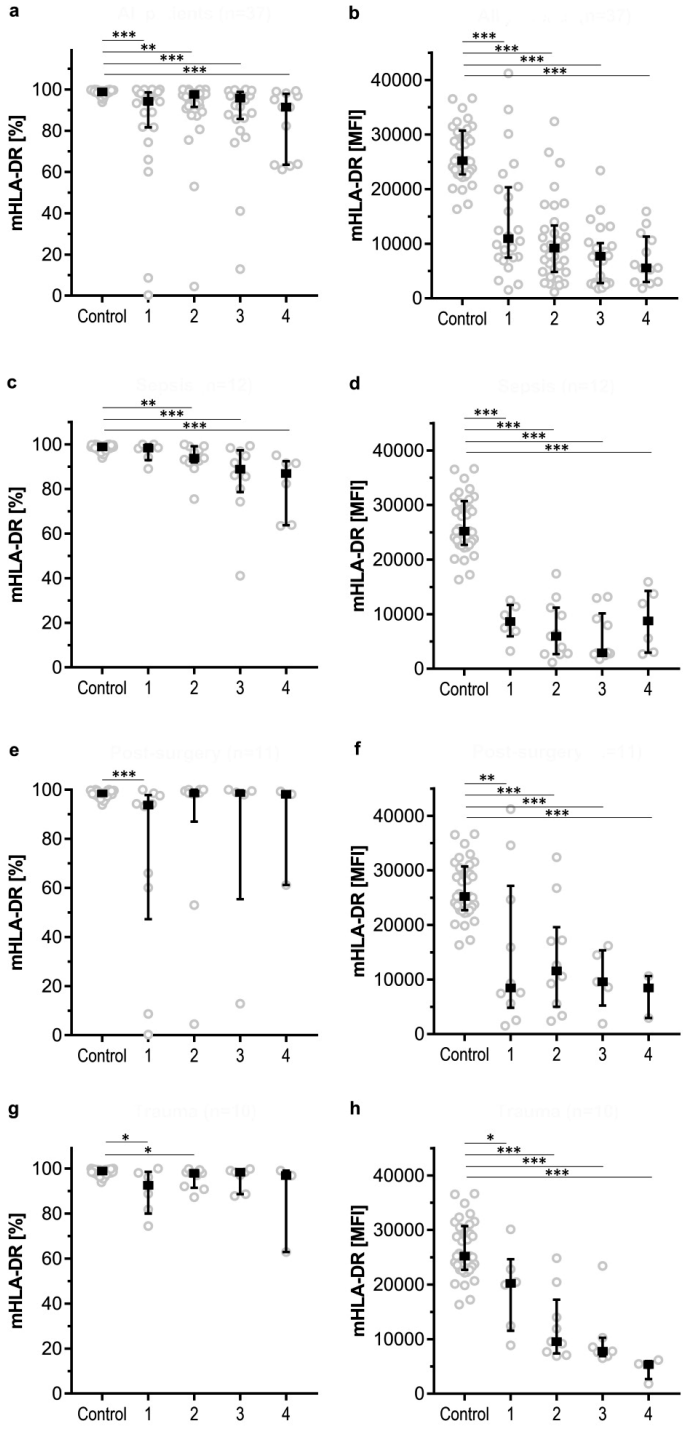
Distribution of non-classical monocytes during PICU stay in all patients (n=37); b) in patients admitted for sepsis (n=12); c) in post-surgery patients (n=11); and d) in trauma patients (n=10). The percentage of non-classical monocytes was significantly lower in patients compared to controls. a-d: Horizontal line indicates the median value, box represents IQR, and the whiskers represent 95% CI. *p-value<0.05, **p-value≤0.01, ***p-value≤0.001. X-axis 1, 2, 3, 4 represent admission, day 2, day 3, day 4, respectively.



Figure, Supplemental Digital Content 6.

HLA-DR expression on intermediate monocytes during PICU stay.

a) Percentage of intermediate monocytes expressing HLA-DR (mHLA-DR %), all patients (n=37); b) HLA-DR MFI of intermediate monocytes (mHLA-DR MFRI), all patients (n=37); c-d) mHLA-DR (%, c; MFI, d) in patients admitted for sepsis (n=12); e-f) mHLA-DR (%, e; MFI, f) in post-surgery patients (n=11); g-h) mHLA-DR (%, g; MFI, h) in trauma patients (n=10). a-h: Square indicates median value and whiskers represent IQR. *p-value<0.05, **p-value≤0.01, ***p-value≤0.001. X-axis 1, 2, 3, 4 represent admission, day 2, day 3, day 4, respectively.



Figure, Supplemental Digital Content 7.

HLA-DR expression on non-classical monocytes during PICU stay.

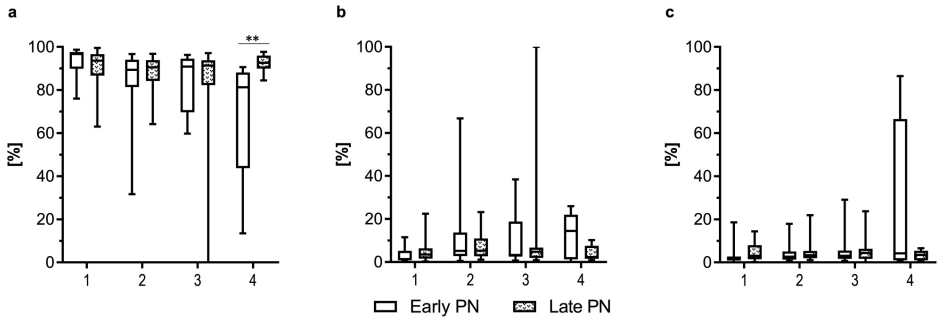
a) Percentage of non-classical monocytes expressing HLA-DR (mHLA-DR %), all patients (n=37); b) HLA-DR MFI of non-classical monocytes (mHLA-DR MFRI), all patients (n=37); c-d) mHLA-DR (%, c; MFI, d) in patients admitted for sepsis (n=12); e-f) mHLA-DR (%, e; MFI, f) in post-surgery patients (n=11); g-h) mHLA-DR (%, g; MFI, h) in trauma patients (n=10). a-h: Square indicates median value and whiskers represent IQR. *p-value<0.05, **p-value≤0.01, ***p-value≤0.001. X-axis 1, 2, 3, 4 represent admission, day 2, day 3, day 4, respectively.

Table, Supplemental Digital Content 8.
Characteristics of patients with clinical complications.

	Sex	Age	Diagnosis	Complication	Day at compli-cation	If infection, clinical syndrome	If infection, pathogen	Comments
1	F	20m	Sepsis	Death	5			Multi-organ failure due to MSS
2	M	6y	Sepsis	Infection	5	No focus identified	No pathogen identified	
				Death	27			End of life decision after severe morbidity due to MSS
3	M	9y	Sepsis	Infection	6	Pneumonia	No pathogen identified	
4	F	12y	Sepsis	Infection	8	Post-operative wound infection	Candida albicans (wound)	
5	F	16y	Sepsis	Infection	7	Bloodstream infection	S. aureus (blood)	
6	F	5y	Post-surgery	Infection	7	Urinary tract infection	E. coli (urine)	Immunosuppressive therapy after heart transplant
7	M	8y	Post-surgery	Infection	4	Pneumonia	S. aureus (ETT)	
				Death	65			Palliative therapy post-PICU
8	M	9y	Post-surgery	Infection	4	Pneumonia	No pathogen identified	
9	F	5y	Trauma	Infection	4	Pneumonia	Haemophilus influenzae (blood and ETT); Moraxella catarrhalis, Streptococcus pneumoniae, and S. aureus (ETT)	
10	F	11y	Trauma	Infection	4	Pneumonia	S. aureus and S. pneumoniae (ETT)	
11	M	13y	Trauma	Infection	4	Probable CVC infection	No pathogen identified	
12	M	14y	Trauma	Infection	8	No focus identified	No pathogen identified	
13	F	16y	Trauma	Infection	4	No focus identified	No pathogen identified	
				Death	9			Brain herniation after head injury

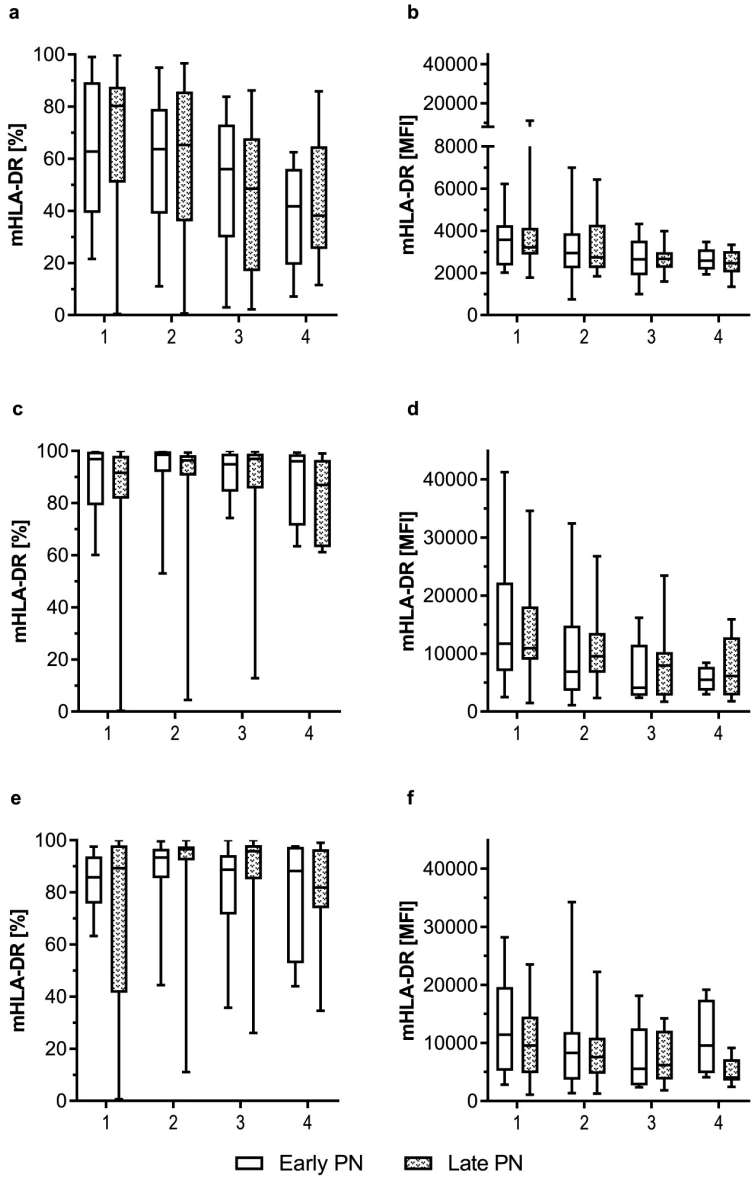
14	M	13y	Severe asthma	Death	1			Hypoxemia following acute, severe asthma
				Infection	3	No focus identified	No pathogen identified	
15	F	15y	Renal failure	Death	77			Palliative therapy after relapsed acute myeloid leukemia

Abbreviations: F=Female, M=Male, m=month(s), y=year(s), MSS = meningococcal septic shock, CVC=central venous catheter, ETT=endotracheal tube.



Figure, Supplemental Digital Content 9.

Monocyte subset distribution in patients with early PN (n=18) and patients with late PN (n=19), a) classical monocytes; b) intermediate monocytes; c) non-classical monocytes. Patients on day 4 and exposed to early PN had a lower proportion of classical monocytes compared to children who did not receive PN. a-c: Horizontal line indicates the median value, box represents IQR, and the whiskers represent 95% CI. **p-value≤0.01. X-axis 1, 2, 3, 4 represent admission, day 2, day 3, day 4, respectively.



Figure, Supplemental Digital Content 10.

HLA-DR expression on monocyte subsets in patients with early PN (n=18) and patients with late PN (n=19). a) Percentage of classical monocytes expressing HLA-DR (mHLA-DR %) in relation to parenteral nutrition; b) HLA-DR MFI of classical monocytes (mHLA-DR MFRI) in relation to parenteral nutrition; c-d) mHLA-DR (%, c; MFI, d) of intermediate monocytes in relation to parenteral nutrition; e-f) mHLA-DR (%, e; MFI, f) of non-classical monocytes in relation to parenteral nutrition. HLA-DR expression within all monocyte subsets and at all time points did not differ between the two randomization groups. a-f: Horizontal line indicates the median value, box represents IQR, and the whiskers represent 95% CI. X-axis 1, 2, 3, 4 represent admission, day 2, day 3, day 4, respectively.

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Chapter 3.3

Differences in IgG Fc Glycosylation are Associated with Outcome of Pediatric Meningococcal Sepsis

de Haan N, Boeddha NP, Ekinci E, Reiding KR, Emonts M, Hazelzet JA, Wuhrer M, Driessen GJ.

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Abstract

Pediatric meningococcal sepsis often results in morbidity and/or death, especially in young children. Our understanding of the reasons why young children are more susceptible to both the meningococcal infection itself and a more fulminant course of the disease is limited. Immunoglobulin G (IgG) is involved in the adaptive immune response against meningococcal infections and its effector functions are highly influenced by the glycan structure attached to the fragment crystallizable (Fc) region.

It was hypothesized that IgG Fc glycosylation might be related to the susceptibility and severity of meningococcal sepsis. Because of this, the differences in IgG Fc glycosylation between 60 pediatric meningococcal sepsis patients admitted to the pediatric intensive care unit and 46 age-matched healthy controls were investigated, employing liquid chromatography with mass spectrometric detection of tryptic IgG glycopeptides. In addition, Fc glycosylation profiles were compared between patients with a severe outcome (death or the need for amputation) and a non-severe outcome.

Meningococcal sepsis patients under the age of 4 years showed lower IgG1 fucosylation and higher IgG1 bisection as compared to age-matched healthy controls. This might be a direct effect of the disease, however, it can also be a reflection of previous immunological challenges and/or a higher susceptibility of these children to develop meningococcal sepsis. Within the young patient group, levels of IgG1 hybrid-type glycans and IgG2/3 sialylation per galactose were associated with illness severity and severe outcome. Future studies in larger groups should explore whether IgG Fc glycosylation could be a reliable predictor for meningococcal sepsis outcome.

Importance

Meningococcal sepsis causes significant mortality and morbidity worldwide, especially in young children. Identifying risk factors for a more fulminant infection would help to decide on appropriate treatment strategies for the individual patients. Immunoglobulin G (IgG) plays an essential role in humoral immune responses and is involved in the adaptive immune response against meningococcal infections. Of great influence on the receptor affinity of IgG is the N-glycan on its fragment crystallizable (Fc) portion. In the current study we analyze IgG glycosylation during the fast development of meningococcal sepsis in children, and we were able to identify glycosylation features that are different between meningococcal sepsis patients and healthy controls. These features might be indicative for a higher susceptibility to meningococcal sepsis. In addition, we found glycosylation features in the patients that were associated with illness severity and severe disease outcome, having the potential to serve as a disease outcome predictor.

Introduction

Meningococcal infections continue to cause significant mortality and morbidity, despite important reductions in the number of cases as a result of vaccination programs (1, 2). Several factors associated with susceptibility and/or severity have been identified, e.g. living in crowded conditions, passive smoking and antecedent viral infections (3, 4). In addition, younger age (<4 years) is an important risk factor for both disease susceptibility and severity, presumably due to an immature immune system (1, 5, 6). However, the exact mechanism causing young children to be more vulnerable and the factors determining the course of disease are largely unknown (5). Besides identifying risk factors for the susceptibility of children to be admitted to the pediatric intensive care unit (PICU) with meningococcal sepsis, there is a great interest in identifying prognostic markers to predict the course of the disease and severe outcomes (e.g. death or the need for amputation). These markers would help to decide on appropriate treatment strategies for the individual patients (7). Several laboratory markers have proven to be of predictive value for the course of meningococcal sepsis, including levels of procalcitonin, C-reactive protein (CRP), leukocytes, thrombocytes, plasminogen activator inhibitor 1 (PAI-1), fibrinogen and various cytokines (8-10). In addition to the individual markers, predictive scores were developed and validated for the course of meningococcal sepsis, among which the pediatric risk of mortality (PRISM) score (11), the Rotterdam score (9) and the base rate and platelet count (BEP) score (12), all reported to have a good predictive performance for meningococcal sepsis mortality with an AUC between 0.80 and 0.96 (12).

Immunoglobulin G (IgG) plays an essential role in humoral immune responses and is highly involved in the adaptive immune response against meningococcal infections (13, 14). IgG specific for meningococcal serogroup B is able to initiate complement-dependent lysis of the bacterium and leukocyte-mediated phagocytosis (15, 16). Both the complement- and the leukocyte-mediated effector functions are mainly induced by IgG1 and IgG3 while the other two IgG subclasses show less activity (IgG2) or no activity at all (IgG4) (14). It was suggested that the severity of the disease in young children in particular is not determined by the abundance of (certain subclasses of) antimeningococcal-IgG, but rather by either the specificity or affinity of the IgG molecule for the antigen or the IgG receptors (13).

Of great influence on the receptor affinity of IgG is the N-glycan on its fragment crystallizable (Fc) at Asn297 (17, 18). The Fc portion of the IgG molecule is N-glycosylated in the endoplasmic reticulum (ER) and Golgi apparatus of B lymphocytes, a process which is under the regulation of both genetic factors and environmental B cell stimuli (19-21). Functional studies have shown the effect of alterations in IgG Fc glycosylation on the binding affinity to both Fcγ receptors (FcγR) and complement factor

C1q (22). For example, increased IgG1 Fc galactosylation showed increased C1q binding and complement-dependent cytotoxicity (CDC) (22, 23). Of note, afucosylation of IgG1 Fc glycans resulted in substantially increased binding of the antibody to FcγRIIIa and FcγRIIIb, which resulted in increased antibody-dependent cellular cytotoxicity (ADCC) (18, 22).

In addition to the influence of IgG Fc glycosylation on receptor interaction, changes in the glycosylation are also associated with various inflammatory diseases, like active tuberculosis infections (24), HIV (25), alloimmune cytopenias (26, 27), and autoimmune diseases like rheumatoid arthritis (28) and inflammatory bowel disease (29, 30). Because of the large influence of IgG glycosylation on the antibody function and its association with inflammatory processes, we hypothesize that the fast development of meningococcal sepsis could be associated with Fc glycosylation profiles in the total plasma IgG pool. The aim of this study was to identify differences in IgG Fc glycosylation between pediatric meningococcal sepsis patients and age-matched healthy controls. In addition, we evaluated the potential of specific glycosylation features to serve as a predictive marker for disease outcome.

Materials and Methods

Patients and controls

In the current retrospective study, plasma or serum samples of prospective cohorts of children with meningococcal sepsis were used. Samples were collected from patients recruited for pediatric meningococcal sepsis studies (1988 to 2005) at the PICU of Erasmus MC-Sophia Children's Hospital (Rotterdam, The Netherlands) (9, 46-48). These studies were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All individual meningococcal studies as well as the current study were approved by the ethical committee of Erasmus MC (MEC-2015-497), and written informed consent was obtained from parents or legal guardians.

Blood samples from 60 children with meningococcal sepsis were taken within 6 hours after admission to the PICU. All patients fulfilled internationally agreed criteria for sepsis (49). Most patients already received antibiotics treatment at the moment of sampling and had a central venous catheter in situ. In addition, treatment and medication assisting in resuscitation were generally given (such as fluids and inotropes). Samples were processed on ice and were stored at -80 °C until analysis. The samples types comprised a variety of serum, citrate plasma, heparin plasma and EDTA plasma. For several patients, multiple materials taken at the same time point were available.

Plasma samples of 46 healthy controls, selected to be in the same age range as the patients, were collected in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines (31, 50). The collection of the samples was approved by the ethical committee of Erasmus MC (MEC-2005-137), and written informed consent was obtained from parents or legal guardians.

Clinical data collection

Various clinical data were collected, illness severity was indicated by Pediatric Risk of Mortality (PRISM) (11), predicted death based on the Rotterdam score (9), and the disseminated intravascular coagulation (DIC) score (51). Coagulation (thrombocytes, fibrinogen and PAI-1) and inflammation (leukocytes, procalcitonin, CRP, tumor necrosis factor (TNFα), interleukins (IL)-6 and -8) markers were measured for clinical reasons or were obtained in previous meningococcal sepsis studies (9, 46, 48). Patients were classified to have died if death occurred during PICU-stay. The need for amputation and/or the occurrence of death during the PICU-stay were together classified as a severe disease outcome.

Chemicals

Disodium hydrogen phosphate dihydrate (Na2HPO4·2H2O), potassium dihydrogen phosphate (KH2PO4), NaCl, and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Formic acid, ammonium bicarbonate, and TPCK-treated trypsin from bovine pancreas were obtained from Sigma-Aldrich (Steinheim, Germany). Furthermore, HPLC SupraGradient acetonitrile (ACN) was obtained from Biosolve (Valkenswaard, The Netherlands) and ultra-pure deionized water (MQ) was generated by the Purelab Ultra, maintained at 18.2 MΩ (Veolia Water Technologies Netherlands B.V., Ede, The Netherlands). Phosphate-buffered saline (PBS; pH 7.3) was made in-house, containing 5.7 g/L Na2HPO4·2H2O, 0.5 g/L KH2PO4 and 8.5 g/L NaCl.

IgG isolation and glycopeptide preparation

The 98 clinical samples and 46 healthy control samples were randomized in a 96-well plate format, together with 32 VisuCon pooled plasma standards (Affinity Biologicals Inc., Ancaster, ON, Canada; eight per plate) and 16 PBS blanks (minimally two per plate). Randomization was performed in a supervised way, selecting an optimal distribution of age, sex and case-control-ratio per plate. IgG was isolated using protein G affinity beads (GE Healthcare, Uppsala, Sweden) as described previously (52). Briefly, 2 µL of plasma was incubated with 15 µL of beads in 100 µL of PBS for 1 h with agitation. Beads were then washed three times with 200 µL of PBS and three times with 200 µL of MQ, after which the antibodies were eluted with 100 µL 100 mM formic acid. Eluates were dried for 2 h at 60 ˚C in a vacuum concentrator and dissolved in 40 µL 25 mM ammonium bicarbonate containing 25 ng/µL trypsin. Samples were shaken for 10 min and incubated at 37 ˚C for 17 h.

LC-MS analysis of glycopeptides

The IgG digest was separated and analyzed by an Ultimate 3000 high-performance liquid chromatography (HPLC) system (Dionex Corporation, Sunnyvale, CA), coupled to a Maxis Impact HD quadrupole time-of-flight mass spectrometer (q-TOF-MS; Bruker Daltonics) as described before (52) and explained in detail in the Supplementary Materials and Methods (Text S1).

Data processing

The raw LC-MS data was extracted and curated using LacyTools v0.0.7.2 as described previously (52, 53), cohort specific parameters are provided in the Supplementary Materials and Methods (Text S1). Using the described separation methods, glycopeptides with the same peptide portion co-eluted. This resulted in three glycopeptide clusters: one for IgG1, one for IgG4 and one for the combination of IgG2 and 3. As the study population was mainly of the Caucasian ancestry, the tryptic Fc glycopeptides of IgG2 and 3 were assumed to have identical masses, and could therefore not be distinguished by our profiling method. However, it is possible that for part of the samples, the IgG3 glycopeptides are co-analyzed with the ones of IgG4, due to the presence of different IgG3 allotypes (54). All chromatograms were aligned based on the exact mass and the average retention time of the three most abundant glycoforms of each IgG subclass. After alignment, sum spectra were created per glycopeptide cluster, and then calibrated based on at least five glycopeptides per cluster with a signal-to-noise ratio (S/N) higher than nine. For the targeted extraction, analyte lists were created by manual annotation of summed spectra per biological class (healthy or meningococcal sepsis), covering both doubly charged and triply charged species. Compositional assignments were made on the basis of accurate mass and literature (20, 55). Glycopeptide signals were integrated by including enough isotopomers to cover at least 95% of the area of the isotopic envelope. Spectra were excluded from further analysis when the total spectrum intensity was below ten times the average spectrum intensity of the blanks. In this way, no spectra were excluded for IgG1, 15 spectra were excluded for IgG2/3 and 29 spectra were excluded for IgG4. Analytes were included in the final data analysis when their average S/N (calculated per biological class) was above nine, their isotopic pattern did not, on average, deviate more than 20% from the theoretical pattern and their average mass error was within ± 10 parts per million (ppm). This resulted in the extraction of 22 IgG1, 15 IgG2/3 and 10 IgG4 glycoforms (Table S2 in Supplementary Material).

Data analysis

The absolute intensities of the extracted glycoforms were total area normalized per IgG subclass and derived glycan traits were calculated based on specific glycosylation features (Table 2 and Table S2 in Supplementary Material). Data quality was evaluated based on the 32 pooled plasma standards that were randomly included in the cohort.

These resulted in highly repeatable profiles showing a median relative standard deviation of 3.6% for the IgG1 glycopeptides, 2.9% for the IgG2/3 glycopeptides and 2.4% for the IgG4 glycopeptides (Figure S4 in Supplementary Material). For ten patients, serum, citrate plasma and EDTA plasma of the same time point were available. For these samples, the relative intensities of the individual glycoforms were averaged over the different materials and relative standard deviations were calculated and compared to the standard deviations obtained from the technical replicates. This revealed in general no higher relative standard deviation over the different materials than over the technical replicates (Figure S4 in Supplementary Material). For patients that had different materials available at the same time point, the data of the samples were averaged. Statistical analysis was performed using R 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria) and RStudio 0.98.1091 (RStudio, Inc.). First, outliers were removed, which were defined as values outside the 99% confidence interval per biological group (healthy or meningococcal sepsis). Samples were excluded from further statistical analysis when two or more of the derived traits per IgG subclass were marked as outlier. This resulted in the exclusion of one IgG1 and IgG2/3 sample and two IgG4 samples. The derived glycosylation traits per subclass were tested to correlate with age and the continuous clinical variables using Spearman's correlation test. Mann-Whitney U tests were performed to assess glycosylation differences between cases and controls and between patients with a severe and a non-severe disease outcome. All statistical tests were performed both on the whole dataset, and on the subsets of children below the age of 4 and above the age of 4. The tests for disease outcome were exclusively performed on the younger age category (below 4). The significance threshold (α) was adjusted for multiple testing by the Benjamini Hochberg false discovery rate (FDR) method, with an FDR of 5%. This resulted in $\alpha = 0.0027$ throughout the study.

Results

IgG Fc glycosylation was analyzed in a subclass-specific way for 60 pediatric patients with a meningococcal infection admitted to the PICU, as well as for 46 age and sex matched healthy controls (Table 1 and Table S1 in Supplementary Material). For all samples 22 IgG1 Fc glycoforms were quantified, for 57 cases and 34 controls 15 IgG2/3 glycoforms were quantified and for 48 cases and 29 controls 10 IgG4 glycoforms were quantified (Figure 1 and Table S1 and S2 in Supplementary Material). From these directly measured glycan traits, derived traits were calculated per IgG subclass. This was done based on glycan type (complex or hybrid-type), bisection, fucosylation, galactosylation and sialylation (Table 2 and Table S2 in Supplementary Material). The samples of the healthy controls, remeasured in the current study, were a subset of a larger cohort previously analyzed using a different technique, based on the matrix assisted laser/desorption ionization time-of-

flight mass spectrometric (MALDI-TOF-MS) detection of derivatized glycopeptides (31). The healthy controls featured a lower IgG1 fucosylation and IgG1 and IgG2/3 sialylation with higher age as already previously reported for this control cohort (Figure S1 and Table S3 in Supplementary Material). In addition, we detected a lower relative abundance of IgG1 and IgG2/3 hybrid-type glycans with higher age in the healthy controls, as well as a higher abundance of bisected glycans on IgG2/3 with higher age, both not previously described for healthy children in this age category (Figure S1 in Supplementary Material)(31, 32).

Table 1. Baseline characteristics of children admitted to PICU with meningococcal sepsis and of their age-matched healthy controls.

Median and interquartile ranges are presented unless indicated differently. PRISM: Pediatric risk of mortality (11); P (death Rotterdam): Predicted death rate (%) based on the Rotterdam score (9); DIC: Disseminated intravascular coagulation (51), PAI-1: Plasminogen activator inhibitor-1; TNFα: Tumor necrosis factor. The number of samples for which specific clinical data was available can be found in Table S1 in Supplementary Material.

	Patients (n=60)	**Patients <4 years (n=37)**	**Patients ≥4 years (n=22)**
Age (years)	2.5 [1.5-8.8]	1.8 [1.2-2.4]	10.1 [6.7-12.3]
Sex (male n, %)	35 (59%)	23 (62%)	12 (55%)
Illness severity			
PRISM score	20 [12-25]	21 [14-25]	19 [9-27]
P (death Rotterdam)	11 [1-82]	32 [2-89]	5 [1-14]
DIC score	5 [4-6]	5 [4-7]	5 [4-7]
Coagulation markers			
Thrombocytes ($x10^6$/L)	97 [54-150]	92 [49-166]	109 [82-138]
Fibrinogen (g/L)	2.3 [0.9-3.2]	2.3 [0.9-4.0]	2.2 [1.1-2.9]
PAI-1 (µg/mL)	4.8 [2.7-6.9]	5.4 [3.6-10.7]	4.3 [1.5-6.0]
Inflammatory markers			
Leukocytes ($x10^9$/L)	7.8 [4.0-15.3]	7.1 [3.4-14.3]	11.0 [5.5-17.2]
C-reactive protein (mg/L)	74 [44-119]	60 [39-115]	91 [69-128]
Procalcitonin (ng/mL)	281 [83-482]	361 [145-498]	243 [20-468]
TNF (pg/mL)	8.4 [5.0-19.8]	12.0 [5.3-23.0]	5.0 [5.0-17.5]
Interleukin-6 (ng/mL)	72 [18-383]	176 [42-723]	38 [1-258]
Interleukin-8 (ng/mL)	20 [4-119]	33 [5-219]	9 [1-58]
Outcome			
Mortality (n, %)	12 (20%)	10 (27%)	2 (9%)
Amputation (n, %)	7 (12%)	2 (5%)	5 (23%)
Severe outcome (n, %)	19 (32%)	12 (32%)	7 (32%)
	Controls (n=46)	Controls <4 years (n=24)	Controls ≥4 years (n=22)
Age (years)	3.9 [1.4-10.0]	1.5 [0.8-2.8]	10.0 [6.7-11.6]
Sex (male n, %)	28 (61%)	15 (63%)	13 (59%)

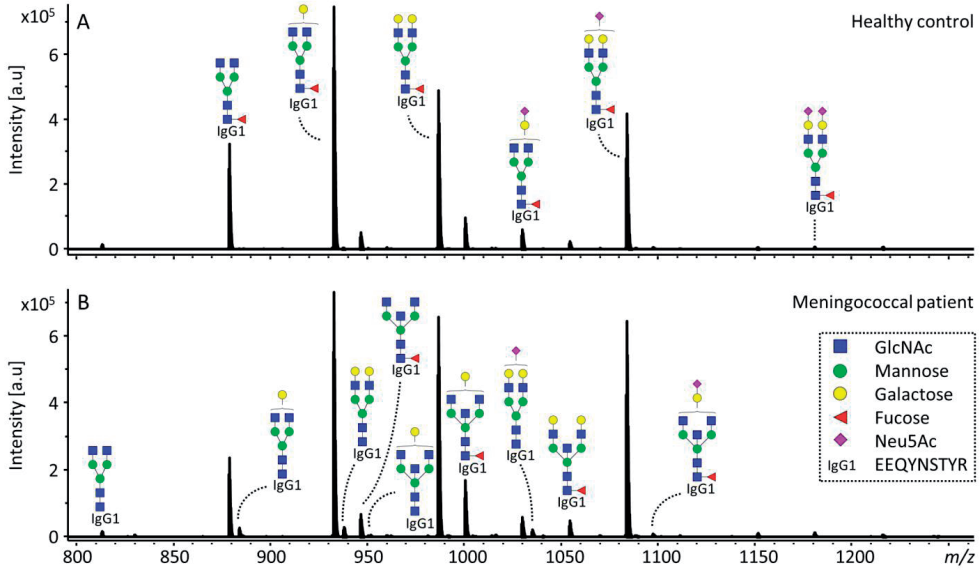


Figure 1. IgG1 glycoforms detected in healthy controls and meningococcal patients.
Representative mass spectra of a (A) healthy 2.8 year old boy and a (B) 2.8 year old male meningococcal patient. Annotated are the 15 overall most abundant IgG1 glycoforms, the glycoforms that were found to be higher in the meningococcal patients as compared to healthy controls (diantennary glycans without fucose or with a bisecting GlcNAc) are indicated in the spectrum of the patient (B). The proposed glycan structures are based on fragmentation and literature (20, 31). Green circle: mannose, yellow circle: galactose, blue square: N-acetylglucosamine (GlcNAc), red triangle: fucose, pink diamond: N-acetylneuraminic acid (Neu5Ac).

IgG Fc glycosylation differences between patients with meningococcal sepsis and healthy controls

Comparing the derived glycosylation traits for all IgG subclasses between the meningococcal patients and the healthy controls (Table S4 in Supplementary Material) revealed a lower IgG1 fucosylation in the children with a meningococcal infection (median cases: 96.1%, controls: 97.8%; Figure 2A). When comparing children in the younger age category (< 4 years old) separately form the older children (≥ 4 years old) this effect appeared to be strongly present in the younger children (cases: 96.1%, controls: 98.1%; Figure 2B, Table 3), while for the older children the difference in IgG1 fucosylation was only detected as trend (Figure 2C). Also IgG1 bisection showed to associate with disease in children below 4, being higher in meningococcal patients (11.0%) compared to healthy controls (8.4%; Figure 2E, Table 3). In the older age group, a corresponding trend was observed (Figure 2D and F).

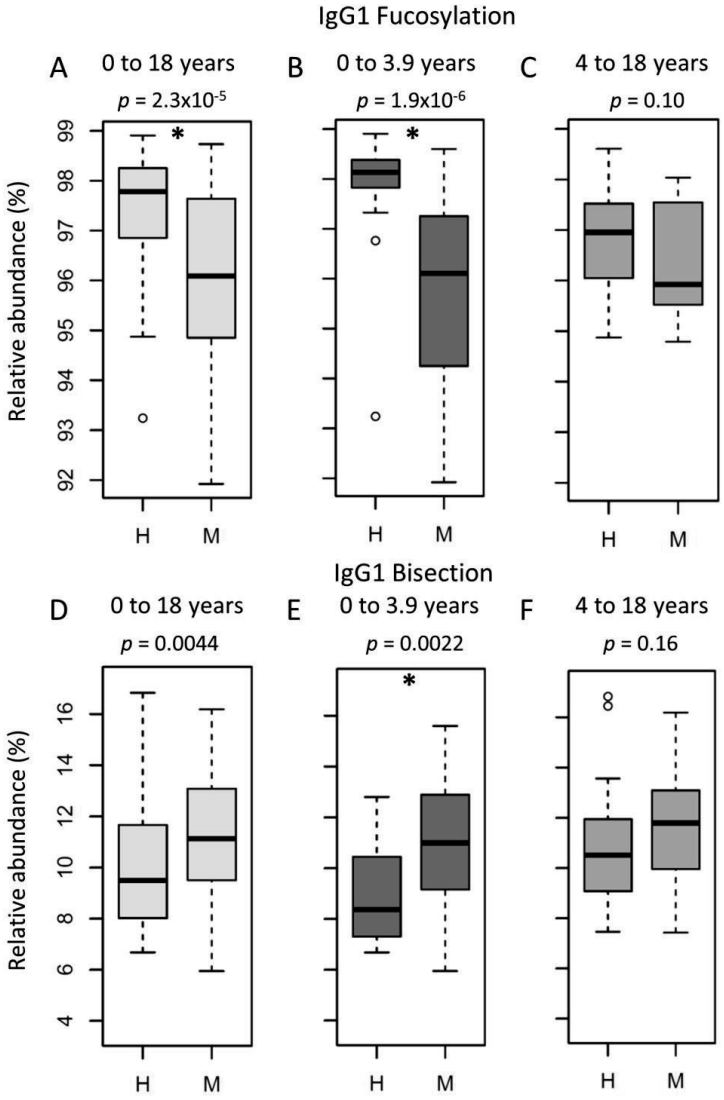
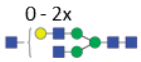
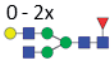
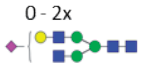
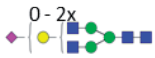


Figure 2. IgG1 Fc fucosylation and bisection are different between meningococcal patients (M) and healthy controls (H) below the age of 4.

IgG1 Fc fucosylation in meningococcal patients between 0 and 18 (A), 0 and 3.9 (B) and 4 and 18 (C) years old compared to age-matched healthy controls and IgG1 Fc bisection in meningococcal patients between 0 and 18 (D), 0 and 3.9 (E) and 4 and 18 (F) years old compared to age-matched healthy controls. Shown are box and whisker plots, where the boxes represent the inter quartile range (IQR) and the whiskers 1.5xIQR. After multiple testing correction, p-values below 2.7x10-3 were considered statistical significant (indicated by an asterisk). The number of samples for which subclass-specific glycosylation data was available can be found in Table S1 in Supplementary Material.

Table 2. Derived glycosylation traits.

The individual glycoforms were grouped based on their glycosylation features as described before for IgG glycopeptides (31). Green circle: mannose, yellow circle: galactose, blue square: N-acetylglucosamine, red triangle: fucose, pink diamond: N-acetylneuraminic acid. The depictions of the derived traits show the minimally required composition to contribute to the given trait. For detailed calculations of the traits, see Table S2 in the Supplementary Material.

Derived trait	Depiction	Description
Hybrid-type		Fraction of hybrid-type glycans
Bisection		Fraction of glycans with a bisecting *N*-acetylglucosamine
Fucosylation		Fraction of glycans with a core fucose
Galactosylation		Galactosylation per antenna of diantennary glycans
Sialylation		Sialylation per antenna of diantennary glycans
Sialylation per galactose		Sialylation per galactose of diantennary glycans

IgG Fc glycosylation associates with patient outcome in children below 4 years old

IgG Fc glycosylation differences between cases and controls were most pronounced in the young children (below the age of 4 years), a group known to behave clinically different from older meningococcal infected patients (5, 33). The glycosylation differences between patients with severe disease outcome (death or amputation) and non-severe disease outcome (full recovery) were only compared within this group (Table 3), as our cohort contained high data density in this age category (Figure S2 in Supplementary Material). The abundance of hybrid-type structures on IgG1 was found to be overall low (below 1%), however it appeared to be even lower in patients with a severe disease outcome (0.37%) as compared to the patients that fully recovered (0.50%; Figure 3A). A similar observation was done for the hybrid-type structures on IgG2/3 (Figure 3B), of which the levels correlated significantly with the levels of IgG1 hybrid-type glycans (Figure S3 in Supplementary Material). In addition, IgG2/3 sialylation per galactose was lower in patients with a severe disease outcome (19.3%) as compared to the patients with non-severe outcome (21.9%; Figure 3D). A similar trend was also observed for sialylation

per galactose on the other IgG subclasses (Figure 3C and E), which correlated positively with the levels on IgG2/3 (Figure S3 in Supplementary Material). The IgG1 hybrid-type glycans, as well as the sialylation per galactose on IgG2/3 associated negatively with the Rotterdam score (IgG1 hybrid-type correlation coefficient (r) = -0.6, IgG2/3 sialylation per galactose r = -0.7; Figure 4 and Table S5 in Supplementary Material), known to be predictive for patient outcome (9). Also IgG1 and IgG2/3 sialylation associated negatively with the Rotterdam score (IgG1 r = -0.5, IgG2/3 r = -0.6). In addition, IgG2/3 sialylation and IgG4 sialylation per galactose associated negatively with the other clinically used predictive score, PRISM (r = -0.6 for both; Figure 4) (11) which correlated positively with the Rotterdam score (Figure S3 in Supplementary Material). The described associations were less pronounced in the total dataset, and were not present for the older pediatric patients (Figure S3 in Supplementary Material).

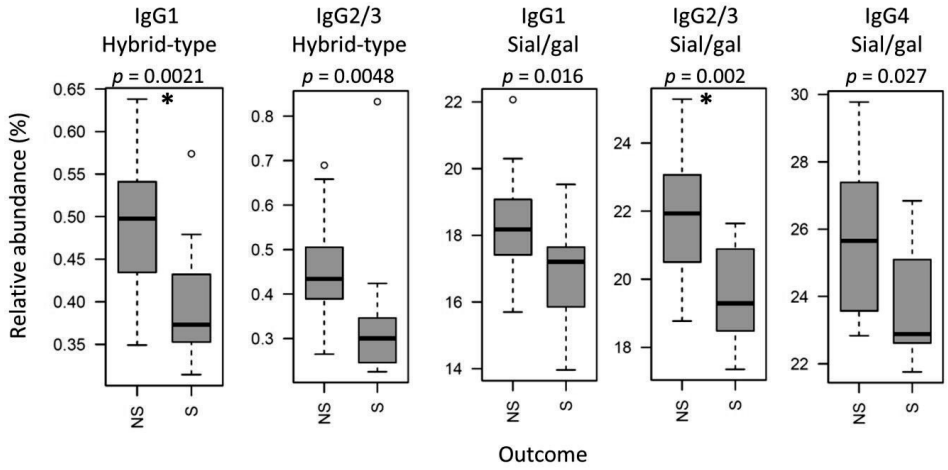


Figure 3. Levels of IgG Fc glycosylation features between meningococcal patients below the age of 4 with severe (S) and non-severe (NS) disease outcome.

Shown are box and whisker plots of the levels of (A) IgG1 hybrid-type glycans, (B) IgG2/3 hybrid-type glycans, (C) IgG1 sialylation per galactose, (D) IgG2/3 sialylation per galactose, (E) IgG4 sialylation per galactose, where the boxes represent the inter quartile range (IQR) and the whiskers 1.5xIQR. After multiple testing correction, p-values below 2.7x10-3 were considered statistically significant (indicated by an asterisk). The number of samples for which subclass-specific glycosylation data was available can be found in Table S1 in Supplementary Material.

Associations between IgG Fc glycosylation and inflammatory markers

Various inflammatory markers were measured in the patient samples, including levels of thrombocytes, fibrinogen, PAI-1, CRP, leukocytes, procalcitonin, TNFα and IL-6 and -8. Thrombocyte levels associated positively with IgG1 and IgG2/3 hybrid-type glycans in the young children (r = 0.6 for the hybrid-type glycans on both subclasses;

Figure 4 and Table S5 in Supplementary Material). The same effect was seen for IgG1 hybrid-type glycans and fibrinogen levels (r = 0.6). In addition, IgG2/3 sialylation per galactose correlated positively with fibrinogen and thrombocyte levels (r = 0.6 and 0.7, respectively). Furthermore, IgG2/3 sialylation associated negatively with PAI-1 levels (r = -0.7) and IgG1 fucosylation associated negatively with IL-6 (r = -0.7). In the older pediatric meningococcal patients, leukocyte levels were positively associated with IgG1 and IgG2/3 hybrid-type structures (r = 0.6 for both subclasses) and patients with lower CRP levels had lower levels of IgG2/3 hybrid-type structures (r: 0.7; Figure S3 and Table S5 in Supplementary Material).

Table 3. Glycosylation differences between pediatric meningococcal patients (0 to 4 years of age) and age and sex matched healthy controls and between meningococcal patients with a severe and a non-severe disease outcome. Mann Whitney U tests were performed to compare the groups. After multiple testing correction, p-values below 2.7x10-3 were considered statistical significant (indicated in bold). For detailed calculations of the traits, see Table S2 in Supplementary Material. The number of samples for which subclass-specific glycosylation data was available can be found in Table S1 in Supplementary Material.

	Cases and controls <4 years			Meningococcal patients <4 years		
	Healthy	Patients		Non severe	Severe	
Derived trait	Median % (IQR)	Median % (IQR)	*p*-value	Median % (IQR)	Median % (IQR)	*p*-value
IgG1 Hybrid-type	0.45 (0.43-0.52)	0.45 (0.41-0.53)	8.0E-01	0.50 (0.44-0.54)	0.37 (0.35-0.43)	2.1E-03
IgG1 Bisection	8.4 (7.4-10.3)	11.0 (9.2-12.9)	2.0E-03	10.0 (9.1-11.8)	13.1 (11.6-14.3)	3.0E-02
IgG1 Fucosylation	98.1 (97.8-98.4)	96.1 (94.2-97.3)	1.9E-06	96.4 (94.6-97.8)	94.5 (93.9-95.8)	2.7E-02
IgG1 Galactosylation	61.9 (56.7-63.6)	61.6 (58.4-63.3)	8.2E-01	62.2 (59.1-63.4)	59.7 (56.9-62.1)	2.1E-01
IgG1 Sialylation	11.3 (10.1-12.8)	10.6 (9.7-11.9)	1.5E-01	11.2 (10.3-11.9)	9.6 (9.2-10.4)	1.8E-02
IgG1 Sialylation per galactose	18.4 (17.7-19.7)	17.8 (16.9-18.7)	3.7E-02	18.2 (17.5-19)	17.2 (15.9-17.6)	1.6E-02
IgG2/3 Hybrid-type	0.43 (0.40-0.54)	0.39 (0.30-0.49)	9.5E-02	0.43 (0.39-0.51)	0.30 (0.25-0.35)	4.8E-03
IgG2/3 Bisection	7.4 (6.3-8.1)	9.7 (8.8-11.2)	4.0E-03	9.2 (8.5-10)	11.1 (9.2-11.6)	1.2E-01
IgG2/3 Fucosylation	98.4 (98.3-98.7)	97.7 (97.5-98.1)	5.7E-03	97.8 (97.5-98.4)	97.5 (97.5-98)	4.6E-01
IgG2/3 Galactosylation	54.5 (46.7-58.1)	51.4 (49.2-53.6)	5.1E-01	51.8 (49.3-54.4)	51.3 (49.8-52.6)	6.1E-01
IgG2/3 Sialylation	13.1 (9.3-14.3)	10.6 (9.7-11.7)	2.1E-01	11.0 (10.5-11.9)	9.6 (9.4-10.3)	8.3E-03
IgG2/3 Sialylation per galactose	23.7 (21.4-24.7)	21.1 (19.5-22.6)	1.4E-02	21.9 (20.5-23.1)	19.3 (18.5-20.9)	2.0E-03
IgG4 Bisection	13.2 (10.6-14.5)	15.5 (13.5-16)	1.7E-01	14.2 (12.4-15.8)	15.9 (15.7-16.1)	3.8E-02
IgG4 Galactosylation	51.6 (44.5-54.9)	54.5 (52.5-58.2)	7.0E-02	55.8 (52.4-59.1)	53.7 (52.7-55.4)	4.5E-01
IgG4 Sialylation	14.1 (11.3-14.9)	13.6 (12.4-14.7)	8.5E-01	14.4 (13.4-15.4)	12.5 (12.3-13)	2.3E-02
IgG4 Sialylation per galactose	26.5 (25.2-28.6)	24.9 (23-26.7)	1.5E-01	25.7 (23.6-27.3)	22.9 (22.6-25.1)	2.7E-02

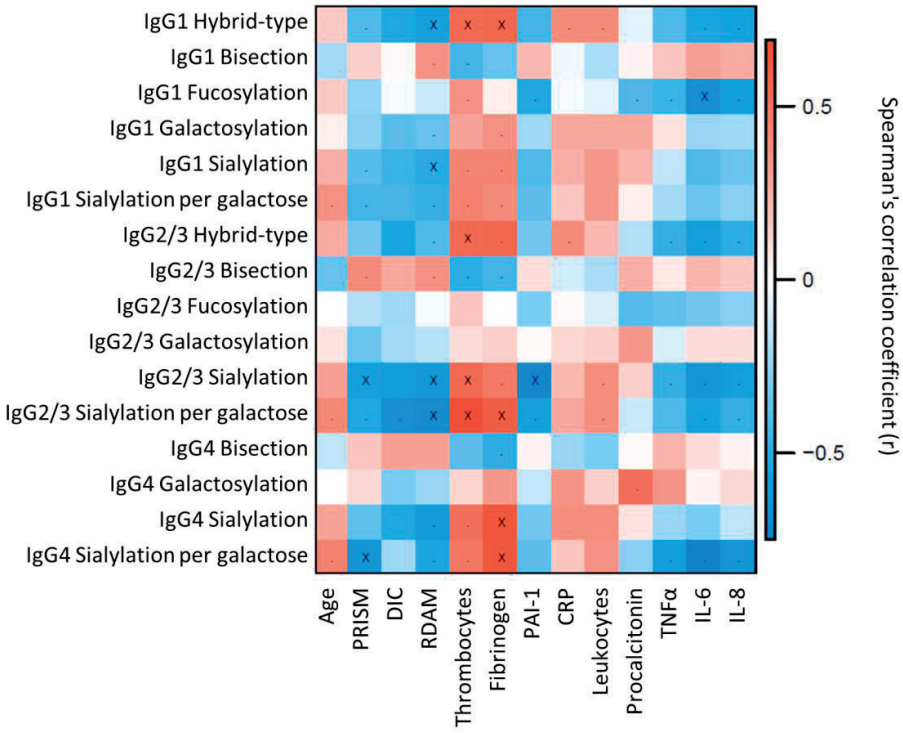


Figure 4. Correlation between IgG Fc glycosylation and clinical variables for meningococcal patients between 0 and 3.9 years old.

The Spearman correlation coefficient is represented in red for a positive correlation and in blue for a negative correlation between derived glycan trait and the outcome scores and inflammatory markers. Periods (.): $p < 0.05$, crosses (x): $p < 2.7x10\text{-}3$ ($\alpha = 2.7x10\text{-}3$ adjusted to allow an FDR of 5%).

Discussion

IgG Fc glycosylation in patients with meningococcal sepsis

Pediatric meningococcal infections resulting in septic shock often occur in young and previously healthy children (1). The reason why young children are more susceptible to this severe infection and the factors that determine the outcome of disease are largely unknown (5). In this study, we analyzed the IgG Fc glycosylation of children admitted to the PICU with meningococcal sepsis. Changes in IgG Fc glycosylation are known to have a large influence on the effector function of the antibody and are associated with various inflammatory conditions (18, 22-24, 28).

We found IgG1 Fc fucosylation to be lower in patients as compared to age-matched healthy controls. As previous research showed no sex-related IgG glycosylation effects in

healthy children between 3 months and 17 years (31), the boys and girls in the current study were not assessed separately. The observed difference between cases and controls was more pronounced in children below the age of 4 years. In addition, in children of this age category, IgG1 Fc bisection was higher in patients as compared to controls. Previous studies reported a different disease course and mortality rate in very young children, showing the relevance of studying this patient group separately from older pediatric meningococcal sepsis patients (1, 5). Additionally, we found that the cytokine levels in our cohort tended to be higher in younger patients as compared to the older ones. This might be explained by a trend of higher illness severity in young patients and consequently higher cytokine levels . During the maturation of the immune system, when children start to produce their own IgG molecules, the Fc fucosylation is relatively high compared to older children (above the age of 4 years), while the Fc bisection is relatively low (31). In young children with meningococcal sepsis, a change in the glycosylation of a disease-specific subset of their IgGs might be induced by the meningococcal infection itself in an early stage of the disease. IgG glycosylation is able to change fast as shown in patients experiencing acute systemic inflammation after cardiac surgery, where part of the patients showed an increased antibody galactosylation the first day after surgery (35). In addition, studies in mice showed that Fc sialylation could be regulated dynamically, by the interplay of soluble sialyltransferases and the accumulation of platelets providing CMP-sialic acid (36, 37). The fact that sialyl- and galactosyltransferases are also circulating in the human plasma suggests that glycosylation might be dynamically regulated in humans too (38).

Alternatively, low Fc fucosylation and high Fc bisection in young patients rather reflect the extent of exposure to previous infections and subsequent adaptive immune responses. Antigen-specific IgG Fc glycosylation was previously shown to differ substantially from the glycosylation of the total pool of IgG. For example gp120-specific IgG in HIV infected patients displays significantly lower Fc fucosylation compared to the total pool of IgG in the infected patients (25). Furthermore, in alloimmune cytopenias also low levels of Fc fucosylation were observed in anti-HPA-1a and anti-RhD IgGs, as compared to the total IgG pool (26, 27). Low IgG1 Fc fucosylation (i.e., high afucosylation) enhances binding of IgG to FcγRIIIa and FcγRIIIb 20- to 100-fold (22, 39), thereby increasing antibody-dependent cellular cytotoxicity (ADCC) (18, 22). The two-fold higher abundance of afucosylated IgGs that we observed for meningococcal sepsis patients might reflect the upregulation of antigen-specific groups of IgG. For IgG specific for meningococcal outer membrane vesicle (OMV) antigens obtained after OMV vaccination, no change in fucosylation was seen over time (40). However, no comparison was made between total IgG before and after vaccination and glycosylation changes on antigen-specific IgG might be substantially different between vaccinated and naturally infected individuals. We speculate that the low IgG fucosylation observed in young meningococcal patients

may reflect a history of exposure to (viral) infections, or in a broader sense antigenic stimuli, resulting in the build-up of low-fucosylation IgG against the respective antigens which manifests itself in a shift of the total IgG pool towards lower fucosylation. Hence, the low fucosylation may be a marker of a history of infections which may e.g. reflect a certain proneness to viral or other infectious diseases in these children.

Future studies should elucidate whether skewed IgG Fc glycosylation featuring low fucosylation and high bisection can be identified on meningococcal-specific IgG and whether the deviations from normal are induced by the meningococcal infection or were already present in the children before infection.

IgG Fc glycosylation associates with illness severity

Young patients with severe disease, defined by death or need for amputation, have a lower level of IgG1 hybrid-type glycans and IgG2/3 sialylation per galactose when admitted to the PICU. In addition, these glycosylation features correlate negatively with illness severity, as measured by the Rotterdam score, as well as positively with previously determined predictive factors in meningococcal sepsis, namely levels of thrombocytes and fibrinogen (9), which correlate negatively with severity. Thus, our data suggest that IgG1 hybrid-type glycans and IgG2/3 sialylation per galactose could be a predictor for meningococcal sepsis severity.

Hybrid-type glycans are known to be present on human IgG-Fc in minor amounts, and not much is known about their function (41, 42). We show here, for the first time, that the level of hybrid-type glycans on both IgG1 and IgG2/3 correlates negatively with age in healthy children. This is likely connected to maturation of the immune system as hybrid-type glycans are precursors of the usually found complex-type glycans on IgG and might originate from immature B-cells.

In the young patients, IgG2/3 Fc sialylation seems to change independently from the level of Fc galactosylation (serving as a substrate for sialylation), which is likely an effect of the higher availability of sialyltransferase ST6Gal1 or the increased presence of the substrate CMP-sialic acid, either inside the Golgi apparatus or outside the cell (36, 37, 43). IgG1 Fc sialylation is known to modulate antibody binding to C1q, and subsequent CDC, either positively (22) or negatively (23). This discrepancy is suggested to be caused by the spatial distribution of the monoclonal antibodies on the cell surface, which depends on the monoclonal antibody studied.

Similar to the glycosylation differences observed between cases and controls, the question is whether the low levels of hybrid-type glycans and sialylation per galactose are emerging during the course of the disease (and are thus meningococcal sepsis specific)

or were already present on (a fraction of) the IgG-Fc of patients appearing to have a severe disease outcome. In both situations, the alterations can either be the cause of the severe outcome, or a bystander effect. In either situation IgG Fc glycosylation features have the potential to be used to predict meningococcal sepsis outcome in very young patients, which should be validated in larger study populations.

Interestingly, IgG1 fucosylation associated negatively with IL-6 levels in patients below the age of 4 years, while previous studies in healthy adults showed a positive correlation between IL-6 levels and fucosylation (44). IL-6 levels have been shown before to be elevated with meningococcal sepsis and to have a potential role in outcome prediction (45). Furthermore, none of the glycosylation features are associated with levels of CRP in the young patients, while CRP in the healthy adult population does associate with IgG Fc galactosylation (negatively) and Fc fucosylation (positively) (44). Low CRP levels at the time of admission at the PICU are a known predictor of mortality rate in meningococcal sepsis (9), indicating that the prediction based on CRP is grounded on different mechanisms than the prediction based on glycosylation features. This opens possibilities to combine these factors for an improved prediction tool.

Conclusion

We found IgG1 fucosylation and bisection to be associated with meningococcal sepsis in children below the age of 4 years. Within these young patients we found IgG1 hybrid-type glycans and IgG2/3 sialylation per galactose correlated to the severity of the disease. Further research is needed to determine whether the observed glycosylation differences between patients and controls are a result of the meningococcal infection itself or rather associated with increased susceptibility to meningococcal septic shock. Furthermore, glycosylation changes associated with illness severity have the potential to be used as outcome predictors, which should be validated in larger study populations.

Text S1. Supplementary Materials and Methods.

NanoLC-MS(/MS) analysis of glycopeptides

The IgG digest was separated and analyzed by an Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA), coupled to a Maxis Impact HD quadrupole time-of-flight mass spectrometer (q-TOF-MS; Bruker Daltonics) as described before (1). The HPLC system consisted of a gradient pump, an isocratic loading pump, an autosampler maintained at 4 °C and a column oven maintained at 30 °C. Of each sample 250 nL was injected into a flow of 25 µL/min of solvent A (aqueous 0.1 % TFA (*v/v*)) and trapped on the trap column (Dionex Acclaim PepMap100 C18, 5 mm × 300 µm; Thermo Fisher Scientific, Breda, The Netherlands). With a flowrate of 900 nL/min the analytes were eluted on a C18 nano-column (Ascentis Express C18 nanoLC column, 50 mm × 75 µm, 2.7 µm fused core particles; Supelco, Bellefonte, PA) and separated in a linear gradient from 3% to 30% solvent B (95% ACN (*v/v*)) in 5 min. The interface between the HPLC system and the q-TOF-MS was equipped with CaptiveSpray and nanoBooster technologies (Bruker Daltonics), using ACN-doped nebulizing gas (pressure: 0.2 bar; dry gas flow: 3.0 L/min; dry temperature: 180 °C). Profile spectra were recorded in *m/z* range 550 to 1800 with a frequency of 1 Hz. The collision energy was 7.0 eV, the transfer time 110 µs, and the pre-pulse storage 21 µs. The total analysis time per sample was 12 min. The HPLC system and the q-TOF-MS were operated under Chromeleon Client v6.80 build 3161 and otofControl v3.4 build 14, respectively.

Data processing

The raw LC-MS data were extracted and curated using LacyTools v0.0.7.2 as described previously (1, 2). Briefly, the chromatograms of each run were aligned based on the exact mass and the average retention time over all runs of the three most abundant glycoforms of each IgG subclass; H3N4F1, H4N4F1 and H5N4F1 (H: Hexose, N: *N*-acetylhexosamine, F: Fucose). An alignment time window of ± 10 s and a mass window of ± 0.1 Th were used. Glycopeptide peaks were used for alignment only when their signal-to-noise ratio (S/N) was above nine, with a minimum of seven glycopeptides per sample. Using the described separation methods, glycopeptides with the same peptide portion co-eluted. This resulted in three glycopeptide clusters: one for IgG1, one for IgG4 and one for the combination of IgG2 and 3. The tryptic Fc glycopeptides for IgG2 and 3 have identical peptide moieties in the Caucasian population and are therefore not distinguishable by this profiling method. After alignment, sum spectra were created per glycopeptide cluster, with a time window of ± 12 s per cluster. Sum spectra were calibrated based on at least five glycopeptides per cluster with a S/N higher than nine; for all IgG subclasses H3N4F1, H4N4F1, H5N4F1, H5N5F1 and H5N4F1S1 were used for calibration. The mass window used for calibration was 0.3 Da. For the targeted extraction, the areas of at least 95% of the isotopic envelope were integrated for the initial analytes, specified based on

manual annotation of summed spectra per biological class (healthy or meningococcal sepsis), both as doubly charged and as triply charged species. Background subtraction was performed based on local background calculations. Spectra were excluded from further analysis when the total spectrum intensity was below ten times the average spectrum intensity of the blanks. In this way, no spectra were excluded for IgG1, 15 spectra were excluded for IgG2/3 and 29 spectra were excluded for IgG4. Analytes were included in the final data analysis when their average S/N (calculated per biological class) was above nine, their isotopic pattern did not, on average, deviate more than 20% from the theoretical pattern and their average mass error was within ± 10 ppm. This resulted in the extraction of 22 IgG1, 15 IgG2/3 and 10 IgG4 glycoforms (**Table S2** in Supporting information).

1. Falck D, Jansen BC, de Haan N, Wuhrer M. 2017. High-Throughput Analysis of IgG Fc Glycopeptides by LC-MS. Methods Mol Biol 1503:31-47.
2. Jansen BC, Falck D, de Haan N, Hipgrave Ederveen AL, Razdorov G, Lauc G, Wuhrer M. 2016. LaCyTools: A Targeted Liquid Chromatography-Mass Spectrometry Data Processing Package for Relative Quantitation of Glycopeptides. J Proteome Res 15:2198-210.

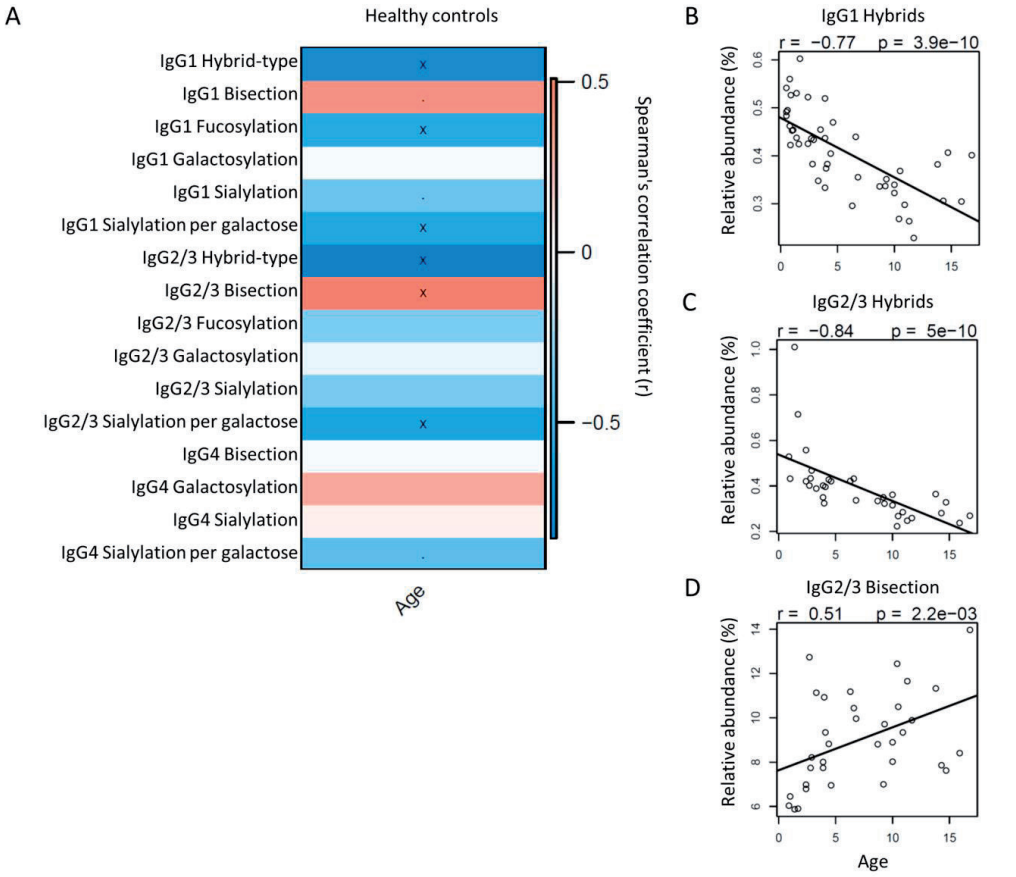
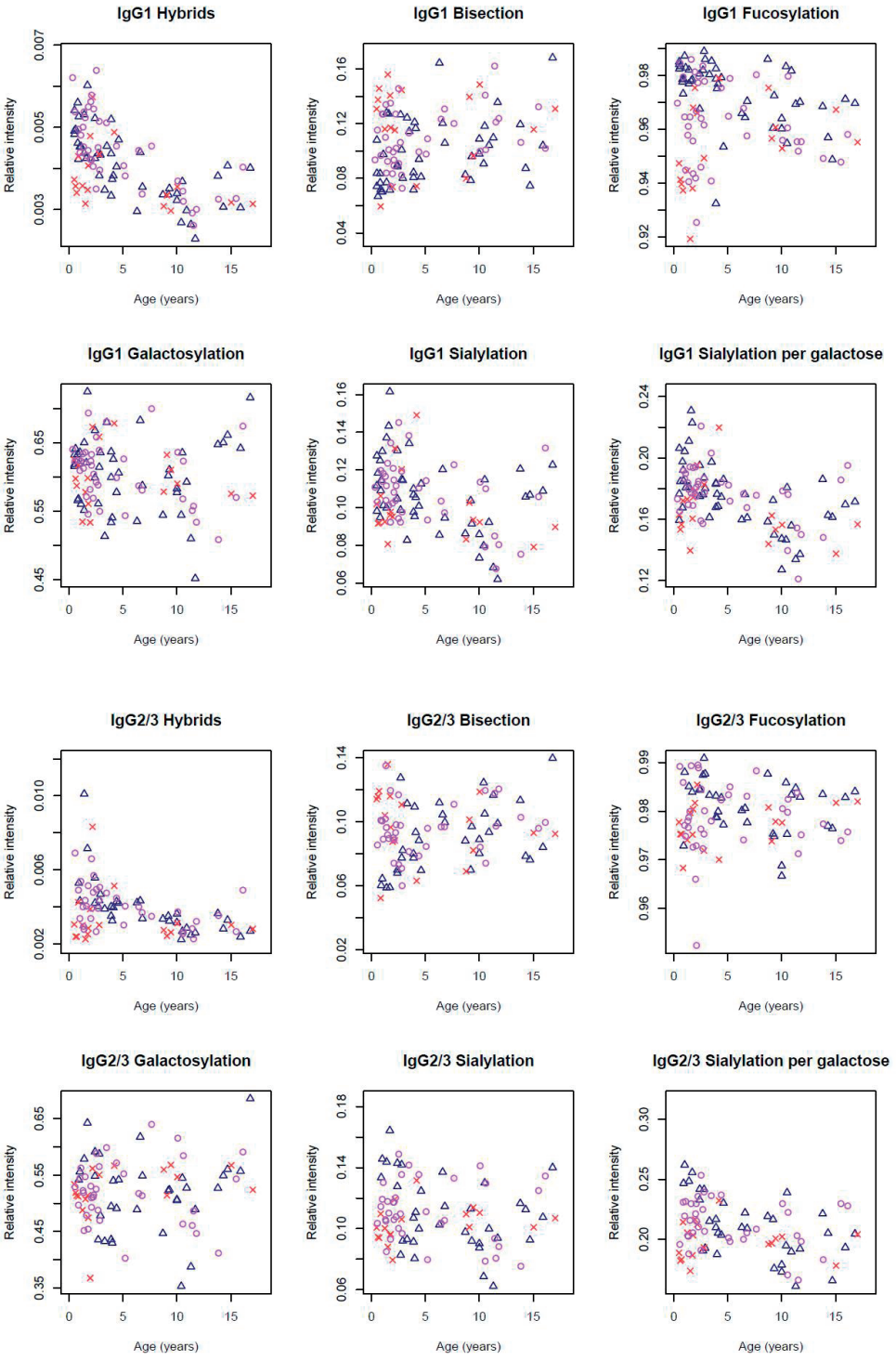


Figure S1. Correlation between IgG Fc glycosylation and age in the healthy controls.

The Spearman's correlation coefficient is represented in red for a positive correlation and in blue for a negative correlation between glycan trait and age (A). Periods (.): $p < 0.05$, crosses (x): $p < 2.7\text{x}10\text{-}3$ ($\alpha = 2.7\text{x}10\text{-}3$ after 5% FDR correction). Newly reported glycan correlations with age in healthy children were the negative association of levels of IgG1 and 2/3 hybrid-type glycans with age (B and C) and the positive association of IgG2/3 bisection with age (D). The number of samples for which subclass-specific glycosylation data was available can be found in Table S1 in Supporting information.


IgG1 Hybrids
IgG1 Bisection
IgG1 Fucosylation
IgG1 Galactosylation
IgG1 Sialylation
IgG1 Sialylation per galactose
IgG2/3 Hybrids
IgG2/3 Bisection
IgG2/3 Fucosylation
IgG2/3 Galactosylation
IgG2/3 Sialylation
IgG2/3 Sialylation per galactose
Relative intensity
Age (years)

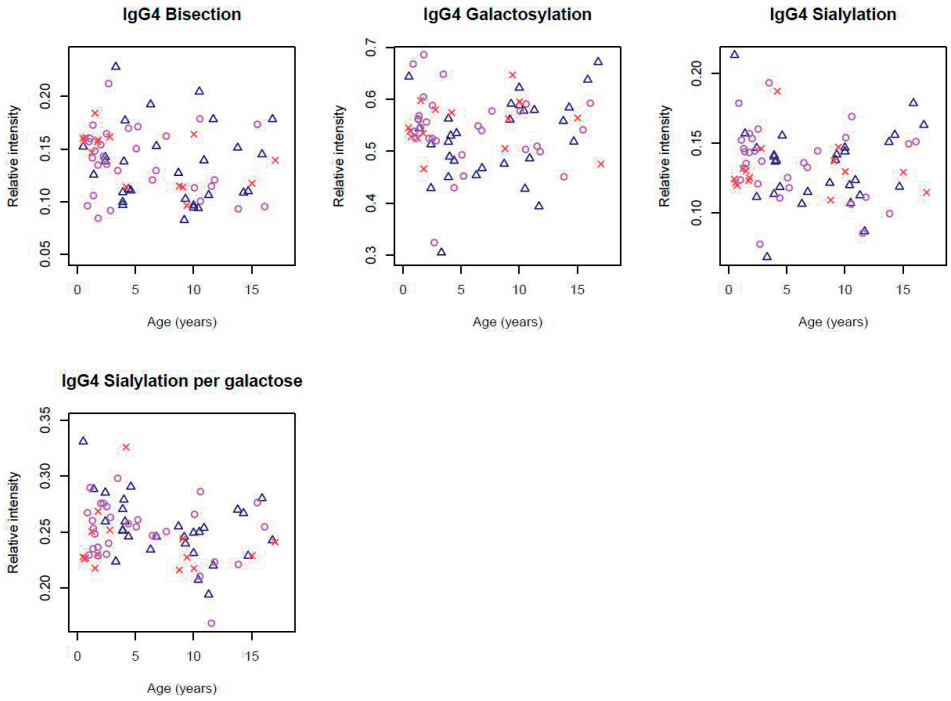


Figure S2. Glycosylation traits plotted versus age.

Data points were plotted against age per derived trait. Blue triangles: healthy controls, purple circles: meningococcal patients with non-severe (NS) disease outcome, red crosses: meningococcal patients with severe (S) disease outcome.

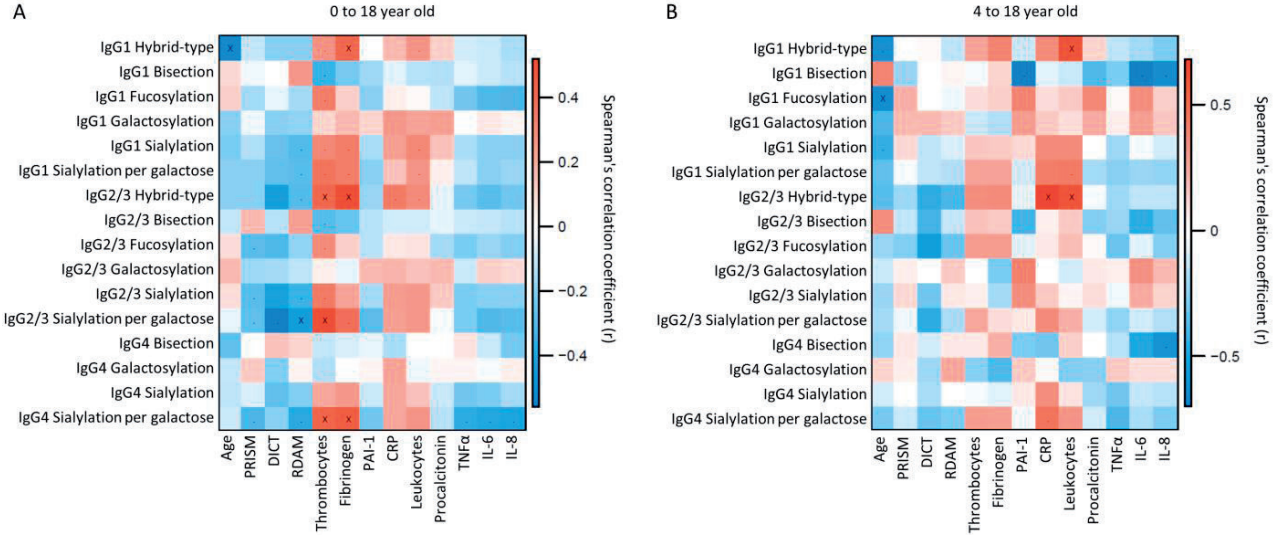


Figure S3. Correlation heat maps.

Correlation is shown between (A) IgG Fc glycosylation traits vs. IgG Fc glycosylation traits for meningococcal sepsis patients between 0 and 4 years old, (B) clinical variables vs. clinical variables for meningococcal sepsis patients between 0 and 4 years old, (C) IgG Fc glycosylation traits vs. clinical variables for meningococcal patients between 0 and 18 years old and (D) IgG Fc glycosylation traits vs. clinical variables for meningococcal patients between 4 and 18 years old. The Spearman's correlation coefficient is represented in red for a positive correlation and in blue for a negative correlation. Periods (.): $p < 0.05$, crosses (x): $p < 2.7x10\text{-}3$ ($\alpha = 2.7x10\text{-}3$ after 5% FDR correction).

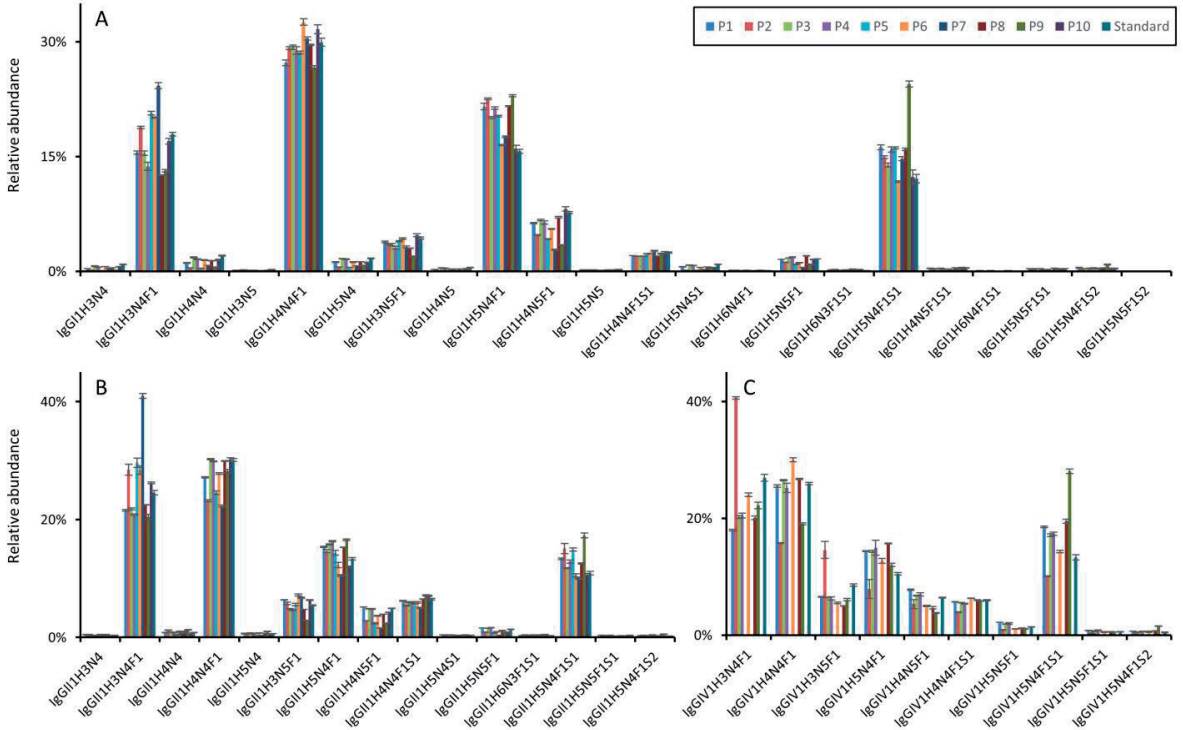


Figure S4. Method robustness.

Relative abundances of the extracted IgG1 (A), IgG2/3 (B) and IgG4 (C) glycoforms in the 32 pooled plasma standards included in the cohort (final bar, labeled "Standard") and for ten patients for which three materials (serum, citrate plasma and EDTA plasma) collected at the same time point were available (first ten bars, labeled "P1" to "P10"). Shown are the average relative abundances and standard deviations over all technical replicates (Standard) or over the measurements from the three materials (P1 to P10). The standards reveal highly repeatable profiles throughout the cohort measurements. The profiling in different materials shows relative standard deviations over the three materials in general not to be larger than the relative standard deviations over the technical replicates. H: Hexose, N: N-acetylhexosamine, F: Fucose, S: N-acetylneuraminic acid.

Table S1. Sample numbers.

The number of patients for whom specific clinical data was present is shown in the upper part of the table. Numbers are presented for the whole dataset as well as for the different age subgroups (below and above 4 years). The number of samples for which subclass-specific glycosylation data is present is shown in the bottom part of the table. Data was excluded based on spectra quality or after outlier removal, as described in the Materials and Methods section.

	Number of patients for which specific clinical data is present		
Clinical data	**All patients**	**Patients <4 years**	**Patients ≥4 years**
Total number of samples	60	37	22
Age	59	37	22
Sex	59	37	22
Illness severity			
PRISM score	58	36	22
P (death Rotterdam)	54	34	20
DIC score	22	14	8
Coagulation markers			
Thrombocytes	58	36	22
Fibrinogen	52	31	21
PAI-1	35	20	15
Inflammatory markers			
Leukocytes	58	36	22
C-reactive protein	56	36	22
Procalcitonin	38	24	14
TNFα	40	23	17
Interleukin-6	35	20	15
Interleukin-8	35	20	15
Outcome			
Mortality	59	37	22
Amputation	59	37	22
Severe outcome	59	37	22
	Number of samples for which subclass-specific glycosylation data is present		
Sample group	IgG1	IgG2/3	IgG4
Healthy	46	34	29
Healthy <4 years	24	12	8
Healthy ≥4 years	22	22	21
Patients	60	57	48
Patients <4 years	37	34	26
Patients ≥4 years	22	22	21
Patients severe outcome	19	19	16
Patients severe outcome <4 years	12	12	9
Patients severe outcome ≥4 years	7	7	7
Patients non-severe outcome	40	37	31
Patients non-severe outcome <4 years	25	22	17
Patients non-severe outcome ≥4 years	15	15	14

Table S2. Direct and derived glycosylation traits.

In the upper part of the table the theoretical mass of the triply charged glycopeptide is shown when the corresponding glycoform was detected for the specific IgG subclass. The individual glycoforms were grouped based on their glycosylation features as described before for IgG glycopeptides in human (De Haan et al., 2016) and shown in the lower part of the table. The depictions of the derived traits show the minimally required composition to contribute to a trait. H: Hexose, N: N-acetylhexosamine, F: Fucose, S: N-acetylneuraminic acid. H: Hexose, green circle: mannose, yellow circle: galactose, blue square: N-acetylglucosamine, red triangle: fucose, pink diamond: N-acetylneuraminic acid.

		[M+3H]$^{3+}$ when extracted		
Composition	**Depiction**	**IgG1**	**IgG2/3**	**IgG4**
H3N4		829,997	819,334	
H3N4F1		878,683	868,02	873,351
H4N4		884,015	873,351	
H3N5		897,694		
H4N4F1		932,7	922,037	927,369
H5N4		938,032	927,369	
H3N5F1		946,376	935,713	941,044
H4N5		951,708		
H5N4F1		986,718	976,055	981,386
H4N5F1		1000,394	989,73	995,062
H5N5		1005,729		
H4N4F1S1		1029,732	1019,069	1024,401
H5N4S1		1035,064	1024,401	
H6N4F1		1040,74		
H5N5F1		1054,411	1043,748	1049,08
H6N3F1S1		1070,074	1059,411	
H5N4F1S1		1083,75	1073,087	1078,418
H4N5F1S1		1097,425		
H6N4F1S1		1137,771		
H5N5F1S1		1151,443	1140,78	1146,111
H5N4F1S2		1180,782	1170,118	1175,45
H5N5F1S2		1248,479		

Derived trait	Depiction	Description	Calculation
IgG1 Hybrid-type		Fraction of hybrid glycans on IgG1	(IgG1 H6N4F1 + IgG1 H6N3F1S1 + IgG1 H6N4F1S1) / (IgG1 H3N4 + IgG1 H3N4F1 + IgG1 H4N4 + IgG1 H3N5 + IgG1 H4N4F1 + IgG1 H5N4 + IgG1 H3N5F1 + IgG1 H4N5 + IgG1 H5N4F1 + IgG1 H4N5F1 + IgG1 H5N5 + IgG1 H4N4F1S1 + IgG1 H5N4S1 + IgG1 H6N4F1 + IgG1 H5N5F1 + IgG1 H6N3F1S1 + IgG1 H5N4F1S1 + IgG1 H4N5F1S1 + IgG1 H6N4F1S1 + IgG1 H5N5F1S1 + IgG1 H5N4F1S2 + IgG1 H5N5F1S2)
IgG1 Bisection		Bisection on IgG1	(IgG1 H3N5 + IgG1 H3N5F1 + IgG1 H4N5 + IgG1 H4N5F1 + IgG1 H5N5 + IgG1 H5N5F1 + IgG1 H4N5F1S1 + IgG1 H5N5F1S1 + IgG1 H5N5F1S2 + IgG1 H6N4F1 + IgG1 H6N4F1S1) / (IgG1 H3N4 + IgG1 H3N4F1 + IgG1 H4N4 + IgG1 H3N5 + IgG1 H4N4F1 + IgG1 H5N4 + IgG1 H3N5F1 + IgG1 H4N5 + IgG1 H5N4F1 + IgG1 H4N5F1 + IgG1 H5N5 + IgG1 H4N4F1S1 + IgG1 H5N4S1 + IgG1 H6N4F1 + IgG1 H5N5F1 + IgG1 H6N3F1S1 + IgG1 H5N4F1S1 + IgG1 H4N5F1S1 + IgG1 H6N4F1S1 + IgG1 H5N5F1S1 + IgG1 H5N4F1S2 + IgG1 H5N5F1S2)
IgG1 Fucosylation		Fucosylation on IgG1	(IgG1 H3N4F1 + IgG1 H4N4F1 + IgG1 H3N5F1 + IgG1 H5N4F1 + IgG1 H4N5F1 + IgG1 H4N4F1S1 + IgG1 H6N4F1 + IgG1 H5N5F1 + IgG1 H6N3F1S1 + IgG1 H5N4F1S1 + IgG1 H4N5F1S1 + IgG1 H6N4F1S1 + IgG1 H5N5F1S1 + IgG1 H5N4F1S2 + IgG1 H5N5F1S2) / (IgG1 H3N4 + IgG1 H3N4F1 + IgG1 H4N4 + IgG1 H3N5 + IgG1 H4N4F1 + IgG1 H5N4 + IgG1 H3N5F1 + IgG1 H4N5 + IgG1 H5N4F1 + IgG1 H4N5F1 + IgG1 H5N5 + IgG1 H4N4F1S1 + IgG1 H5N4S1 + IgG1 H6N4F1 + IgG1 H5N5F1 + IgG1 H6N3F1S1 + IgG1 H5N4F1S1 + IgG1 H4N5F1S1 + IgG1 H6N4F1S1 + IgG1 H5N5F1S1 + IgG1 H5N4F1S2 + IgG1 H5N5F1S2)
IgG1 Galactosylation		Galactosylation per antenna of diantennary glycans on IgG1	(1/2 * (IgG1 H4N4 + IgG1 H4N4F1 + IgG1 H4N5 + IgG1 H4N5F1 + IgG1 H4N4F1S1 + IgG1 H4N5F1S1) + (IgG1 H5N4 + IgG1 H5N4F1 + IgG1 H5N5 + IgG1 H5N4S1 + IgG1 H5N5F1 + IgG1 H5N4F1S1 + IgG1 H5N5F1S1 + IgG1 H5N4F1S2 + IgG1 H5N5F1S2)) / (IgG1 H3N4 + IgG1 H3N4F1 + IgG1 H4N4 + IgG1 H3N5 + IgG1 H4N4F1 + IgG1 H5N4 + IgG1 H3N5F1 + IgG1 H4N5 + IgG1 H5N4F1 + IgG1 H4N5F1 + IgG1 H5N5 + IgG1 H4N4F1S1 + IgG1 H5N4S1 + IgG1 H5N5F1 + IgG1 H5N4F1S1 + IgG1 H4N5F1S1 + IgG1 H5N5F1S1 + IgG1 H5N4F1S2 + IgG1 H5N5F1S2)

IgG1 Sialylation	Sialylation per antenna of diantennary glycans on IgG1	(1/2 * (IgG1 H4N4F1S1 + IgG1 H5N4S1 + IgG1 H5N4F1S1 + IgG1 H4N5F1S1 + IgG1 H5N5F1S1) + (IgG1 H5N4F1S2 + IgG1 H5N5F1S2)) / (IgG1 H3N4 + IgG1 H3N4F1 + IgG1 H4N4 + IgG1 H3N5 + IgG1 H4N4F1 + IgG1 H5N4 + IgG1 H3N5F1 + IgG1 H4N5 + IgG1 H5N4F1 + IgG1 H4N5F1 + IgG1 H5N5 + IgG1 H4N4F1S1 + IgG1 H5N4S1 + IgG1 H5N5F1 + IgG1 H5N4F1S1 + IgG1 H4N5F1S1 + IgG1 H5N5F1S1 + IgG1 H5N4F1S2 + IgG1 H5N5F1S2)
IgG1 Sialylation per galactose	Sialylation per galactose of diantennary glycans on IgG1	IgG1 Sialylation / IgG1 Galactosylation
IgG2/3 Hybrid-type	Fraction of hybrid glycans on IgG2/3	(IgG2/3 H6N3F1S1) / (IgG2/3 H3N4 + IgG2/3 H3N4F1 + IgG2/3 H4N4 + IgG2/3 H4N4F1 + IgG2/3 H5N4 + IgG2/3 H3N5F1 + IgG2/3 H5N4F1 + IgG2/3 H4N5F1 + IgG2/3 H4N4F1S1 + IgG2/3 H5N4S1 + IgG2/3 H5N5F1 + IgG2/3 H6N3F1S1 + IgG2/3 H5N4F1S1 + IgG2/3 H5N5F1S1 + IgG2/3 H5N4F1S2)
IgG2/3 Bisection	Bisection on IgG2/3	(IgG2/3 H3N5F1 + IgG2/3 H4N5F1 + IgG2/3 H5N5F1 + IgG2/3 H5N5F1S1) / (IgG2/3 H3N4 + IgG2/3 H3N4F1 + IgG2/3 H4N4 + IgG2/3 H4N4F1 + IgG2/3 H5N4 + IgG2/3 H3N5F1 + IgG2/3 H5N4F1 + IgG2/3 H4N5F1 + IgG2/3 H4N4F1S1 + IgG2/3 H5N4S1 + IgG2/3 H5N5F1 + IgG2/3 H6N3F1S1 + IgG2/3 H5N4F1S1 + IgG2/3 H5N5F1S1 + IgG2/3 H5N4F1S2)
IgG2/3 Fucosylation	Fucosylation on IgG2/3	(IgG2/3 H3N4F1 + IgG2/3 H4N4F1 + IgG2/3 H3N5F1 + IgG2/3 H5N4F1 + IgG2/3 H4N5F1 + IgG2/3 H4N4F1S1+ IgG2/3 H5N5F1 + IgG2/3 H6N3F1S1 + IgG2/3 H5N4F1S1 + IgG2/3 H5N5F1S1 + IgG2/3 H5N4F1S2) / (IgG2/3 H3N4 + IgG2/3 H3N4F1 + IgG2/3 H4N4 + IgG2/3 H4N4F1 + IgG2/3 H5N4 + IgG2/3 H3N5F1 + IgG2/3 H5N4F1 + IgG2/3 H4N5F1 + IgG2/3 H4N4F1S1 + IgG2/3 H5N4S1 + IgG2/3 H5N5F1 + IgG2/3 H6N3F1S1 + IgG2/3 H5N4F1S1 + IgG2/3 H5N5F1S1 + IgG2/3 H5N4F1S2)
IgG2/3 Galactosylation	Galactosylation per antenna of diantennary glycans on IgG2/3	(1/2 * (IgG2/3 H4N4 + IgG2/3 H4N4F1 + IgG2/3 H4N5F1 + IgG2/3 H4N4F1S1) + (IgG2/3 H5N4 + IgG2/3 H5N4F1 + IgG2/3 H5N4S1 + IgG2/3 H5N5F1 + IgG2/3 H5N4F1S1 + IgG2/3 H5N5F1S1 + IgG2/3 H5N4F1S2)) / (IgG2/3 H3N4 + IgG2/3 H3N4F1 + IgG2/3 H4N4 + IgG2/3 H4N4F1 + IgG2/3 H5N4 + IgG2/3 H3N5F1 + IgG2/3 H5N4F1 + IgG2/3 H4N5F1 + IgG2/3 H4N4F1S1 + IgG2/3 H5N4S1 + IgG2/3 H5N5F1 + IgG2/3 H5N4F1S1 + IgG2/3 H5N5F1S1 + IgG2/3 H5N4F1S2)
IgG2/3 Sialylation	Sialylation per antenna of diantennary glycans on IgG2/3	(1/2 * (IgG2/3 H4N4F1S1 + IgG2/3 H5N4S1 + IgG2/3 H5N4F1S1 + IgG2/3 H5N5F1S1) + (IgG2/3 H5N4F1S2)) / (IgG2/3 H3N4 + IgG2/3 H3N4F1 + IgG2/3 H4N4 + IgG2/3 H4N4F1 + IgG2/3 H5N4 + IgG2/3 H3N5F1 + IgG2/3 H5N4F1 + IgG2/3 H4N5F1 + IgG2/3 H4N4F1S1 + IgG2/3 H5N4S1 + IgG2/3 H5N5F1 + IgG2/3 H5N4F1S1 + IgG2/3 H5N5F1S1 + IgG2/3 H5N4F1S2)

IgG2/3 Sialylation per galactose	Sialylation per galactose of diantennary glycans on IgG2/3	IgG2/3 Sialylation / IgG2/3 Galactosylation
IgG4 Bisection	Bisection on IgG4	(IgG4 H3N5F1 + IgG4 H4N5F1 + IgG4 H5N5F1 + IgG4 H5N5F1S1) / (IgG4 H3N4F1 + IgG4 H4N4F1 + IgG4 H3N5F1 + IgG4 H5N4F1 + IgG4 H4N5F1 + IgG4 H4N4F1S1 + IgG4 H5N5F1 + IgG4 H5N4F1S1 + IgG4 H5N5F1S1 + IgG4 H5N4F1S2)
IgG4 Galactosylation	Galactosylation per antenna of diantennary glycans on IgG4	(1/2 * (IgG4 H4N4F1 + IgG4 H4N5F1 + IgG4 H4N4F1S1) + (IgG4 H5N4F1 + IgG4 H5N5F1 + IgG4 H5N4F1S1 + IgG4 H5N5F1S1 + IgG4 H5N4F1S2)) / (IgG4 H3N4F1 + IgG4 H4N4F1 + IgG4 H3N5F1 + IgG4 H5N4F1 + IgG4 H4N5F1 + IgG4 H4N4F1S1 + IgG4 H5N5F1 + IgG4 H5N4F1S1 + IgG4 H5N5F1S1 + IgG4 H5N4F1S2)
IgG4 Sialylation	Sialylation per antenna of diantennary glycans on IgG4	(1/2 * (IgG4 H4N4F1S1 + IgG4 H5N4F1S1 + IgG4 H5N5F1S1) + (IgG4 H5N4F1S2)) / (IgG4 H3N4F1 + IgG4 H4N4F1 + IgG4 H3N5F1 + IgG4 H5N4F1 + IgG4 H4N5F1 + IgG4 H4N4F1S1 + IgG4 H5N5F1 + IgG4 H5N4F1S1 + IgG4 H5N5F1S1 + IgG4 H5N4F1S2)
IgG4 Sialylation per galactose	Sialylation per galactose of diantennary glycans on IgG4	IgG4 Sialylation / IgG4 Galactosylation

Table S3. Correlation between IgG Fc glycosylation and age in healthy children.

Spearman's correlation tests were performed, after multiple testing correction, p-values below 2.7x10-3 were considered statistical significant (indicated in bold).

	Age in healthy controls	
	Spearman's correlation coefficient (r)	***p*-value**
IgG1 Hybrid-type	-0,77	3,90E-10
IgG1 Bisection	0,41	0,0048
IgG1 Fucosylation	-0,57	**3,70E-05**
IgG1 Galactosylation	-0,025	0,87
IgG1 Sialylation	-0,38	0,0096
IgG1 Sialylation per galactose	-0,58	**2,10E-05**
IgG2/3 Hybrid-type	-0,84	**5,00E-10**
IgG2/3 Bisection	0,51	**0,0022**
IgG2/3 Fucosylation	-0,32	0,067
IgG2/3 Galactosylation	-0,047	0,79
IgG2/3 Sialylation	-0,34	0,052
IgG2/3 Sialylation per galactose	-0,6	**0,00019**
IgG4 Bisection	-0,022	0,91
IgG4 Galactosylation	0,31	0,099
IgG4 Sialylation	0,066	0,73
IgG4 Sialylation per galactose	-0,42	0,023
α	0,0027	

Table S4. Glycosylation differences between pediatric meningococcal patients and age- and sex- matched healthy controls.

Mann-Whitney U tests were performed to compare the groups, after multiple testing correction, p-values below 2.7x10-3 were considered statistical significant (indicated in bold).

	0 to 18 years old		
	Healthy	**Patients**	
	Median % (IQR)	**Median % (IQR)**	***p*-value**
IgG1 Hybrid-type	0.4 (0.3-0.5)	0.4 (0.3-0.5)	0,54
IgG1 Bisection	9.5 (8-11.6)	11.1 (9.6-13.1)	0,0044
IgG1 Fucosylation	97.8 (96.9-98.2)	96.1 (94.9-97.6)	2,30E-05
IgG1 Galactosylation	61.2 (56.6-63.7)	60.5 (57.2-62.7)	0,68
IgG1 Sialylation	10.6 (9.5-11.8)	10.4 (9.3-11.7)	0,67
IgG1 Sialylation per galactose	17.6 (16.1-18.6)	17.6 (16-18.6)	0,87
IgG2/3 Hybrid-type	0.4 (0.3-0.4)	0.4 (0.3-0.4)	0,72
IgG2/3 Bisection	8.8 (7.7-10.5)	9.6 (8.5-10.4)	0,14
IgG2/3 Fucosylation	98.3 (97.7-98.4)	97.8 (97.5-98.2)	0,043
IgG2/3 Galactosylation	52.7 (48.9-55.4)	51.8 (48.8-55.3)	0,92
IgG2/3 Sialylation	10.7 (9.2-12.9)	10.8 (9.7-11.9)	0,91
IgG2/3 Sialylation per galactose	20.9 (19.2-22.8)	20.5 (19.7-22.8)	0,87
IgG4 Bisection	12.7 (10.6-15.2)	14.5 (11.4-16.1)	0,27
IgG4 Galactosylation	53 (47.6-57.9)	54.3 (50.8-57.8)	0,34
IgG4 Sialylation	13.7 (11.5-14.7)	13.4 (12.1-15)	0,78
IgG4 Sialylation per galactose	25.1 (24-27)	24.7 (22.9-26.6)	0,47
	0 to 3.9 years old		
	Healthy	**Patients**	
	Median % (IQR)	**Median % (IQR)**	***p*-value**
IgG1 Hybrid-type	0.5 (0.4-0.5)	0.5 (0.4-0.5)	0,8
IgG1 Bisection	8.4 (7.4-10.3)	11 (9.2-12.9)	0,002
IgG1 Fucosylation	98.1 (97.8-98.4)	96.1 (94.2-97.3)	1,90E-06
IgG1 Galactosylation	61.9 (56.7-63.6)	61.6 (58.4-63.3)	0,82
IgG1 Sialylation	11.3 (10.1-12.8)	10.6 (9.7-11.9)	0,15
IgG1 Sialylation per galactose	18.4 (17.7-19.7)	17.8 (16.9-18.7)	0,037
IgG2/3 Hybrid-type	0.4 (0.4-0.5)	0.4 (0.3-0.5)	0,095
IgG2/3 Bisection	7.4 (6.3-8.1)	9.7 (8.8-11.2)	0,004
IgG2/3 Fucosylation	98.4 (98.3-98.7)	97.7 (97.5-98.1)	0,0057
IgG2/3 Galactosylation	54.5 (46.7-58.1)	51.4 (49.2-53.6)	0,51
IgG2/3 Sialylation	13.1 (9.3-14.3)	10.6 (9.7-11.7)	0,21
IgG2/3 Sialylation per galactose	23.7 (21.4-24.7)	21.1 (19.5-22.6)	0,014
IgG4 Bisection	13.2 (10.6-14.5)	15.5 (13.5-16)	0,17
IgG4 Galactosylation	51.6 (44.5-54.9)	54.5 (52.5-58.2)	0,07
IgG4 Sialylation	14.1 (11.3-14.9)	13.6 (12.4-14.7)	0,85
IgG4 Sialylation per galactose	26.5 (25.2-28.6)	24.9 (23-26.7)	0,15
	4 to 18 years old		

	Healthy	Patients	
	Median % (IQR)	**Median % (IQR)**	***p*-value**
IgG1 Hybrid-type	0.3 (0.3-0.4)	0.3 (0.3-0.4)	0,84
IgG1 Bisection	10.5 (9.2-11.9)	11.8 (10-13.1)	0,16
IgG1 Fucosylation	97 (96.1-97.5)	95.9 (95.5-97.4)	0,1
IgG1 Galactosylation	59.7 (55.2-64)	58.4 (56.8-62.5)	0,65
IgG1 Sialylation	9.8 (8.6-10.8)	9.5 (8.4-11)	0,99
IgG1 Sialylation per galactose	16.2 (15.1-17.4)	16.5 (15.1-17.8)	0,68
IgG2/3 Hybrid-type	0.3 (0.3-0.4)	0.3 (0.3-0.4)	0,88
IgG2/3 Bisection	9.5 (8.5-10.8)	9.6 (8.4-10.2)	0,86
IgG2/3 Fucosylation	98 (97.7-98.3)	98 (97.5-98.2)	0,67
IgG2/3 Galactosylation	52.5 (48.9-54.8)	54.5 (49.3-56.8)	0,31
IgG2/3 Sialylation	10.1 (9.2-11.4)	10.8 (9.5-12.8)	0,46
IgG2/3 Sialylation per galactose	20.4 (18.8-21.5)	20.2 (19.7-21.8)	0,63
IgG4 Bisection	12.7 (10.6-15.3)	12 (11.4-16.2)	0,88
IgG4 Galactosylation	53.5 (48.2-58.4)	54.1 (49.8-57.7)	1
IgG4 Sialylation	13.7 (11.8-14.7)	12.9 (11.1-14.7)	0,65
IgG4 Sialylation per galactose	24.6 (23.4-25.9)	24.6 (22.3-25.7)	0,67
α	0,0027		

Table S5. Correlation between IgG Fc glycosylation and clinical variables in the pediatric meningococcal patients.

Spearman's correlation tests were performed, after multiple testing correction, p-values below 2.7x10-3 were considered statistical significant (indicated in bold).

https://mbio.asm.org/content/9/3/e00546-18/figures-only

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Chapter 4

Hemostatic response to sepsis

Chapter 4.1

Gene Variations in the Protein C and Fibrinolytic Pathway: Relevance for Severity and Outcome in Pediatric Sepsis

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Abstract

The host response to infection involves complex interplays between inflammation, coagulation, and fibrinolysis. Deregulation of hemostasis and fibrinolysis are major causes of critical illness and important determinants of outcome in severe sepsis. The hemostatic responses to infection vary widely between individuals, and are in part explained by polymorphisms in genes responsible for the protein C and fibrinolytic pathway. This review gives an overview of genetic polymorphisms in the protein C and fibrinolytic pathway associated with susceptibility and severity of pediatric sepsis. In addition, genetic polymorphisms associated with adult sepsis and other pediatric thromboembolic disorders are discussed, as these polymorphisms might be candidates for future molecular genetic research in pediatric sepsis.

Introduction

Severe sepsis is a major clinical problem, being associated with high morbidity and mortality.[1] The overwhelming proinflammatory host response in severe sepsis damages the microvascular endothelium, resulting in activation of the coagulation cascade, decreased activity of natural anticoagulant mechanisms, and impairment of fibrinolytic system.[2] This may result in widespread deposition of fibrin throughout the microcirculation, manifesting as disseminated intravascular coagulation (DIC), ultimately contributing to multiple organ dysfunction and death.[3]

In sepsis, hemostasis and fibrinolysis are deregulated. Both alterations of the protein C (PC) pathway, one of the natural anticoagulant mechanisms, and impairment of the fibrinolytic pathway have been described.[4] Moreover, these pathways have been implicated in patient outcome. More specifically, decreased levels of PC[5,6] and increased levels of plasminogen activator-inhibitor-1 (PAI-1)[7-9] are associated with a negative outcome in sepsis.

Responses to infection vary widely between individuals, both clinically and registered by results of laboratory tests. Host susceptibility and severity of sepsis are in part explained by polymorphisms in genes responsible for the PC and fibrinolytic pathway. This seems plausible, as these polymorphisms are reported to regulate both the amount and functional quality of these proteins.[10,11]

This review aims to give an overview of genetic polymorphisms in PC and fibrinolytic pathway potentially affecting host susceptibility and severity of pediatric sepsis. In addition, genetic polymorphisms associated with adult sepsis and other pediatric thromboembolic disorders are discussed, as these polymorphisms might be candidates for future molecular genetic research in pediatric sepsis. Increased insight into hemostatic mechanisms associated with morbidity and mortality is essential to improve patient outcome in pediatric sepsis.

The Protein C Pathway

The main functions of the PC pathway are to control coagulation by inactivation of activated (a) factor V (cofactor of factor Xa) and factor VIIIa (cofactor of factor IX), subsequently preventing thrombin generation, and to neutralize PAI-1, concomitantly increasing fibrinolytic capacity. Other effects of the PC pathway-on inflammation, apoptosis, and barrier stabilization-are beyond the scope of this review and have been discussed elsewhere.[12]

The five main players in the PC pathway are (pro)thrombin, thrombomodulin (TM), endothelial cell protein C receptor (EPCR), PC, and protein S (PS).[13] TM, an endothelial cell surface glycoprotein, binds circulating thrombin and forms a TM-thrombin-complex (Fig. 1). This complex rapidly activates PC bound to EPCR. Activated PC (aPC) then dissociates from the EPCR, binds to PS, and forms a complex that inactivates factor Va and factor VIIIa.

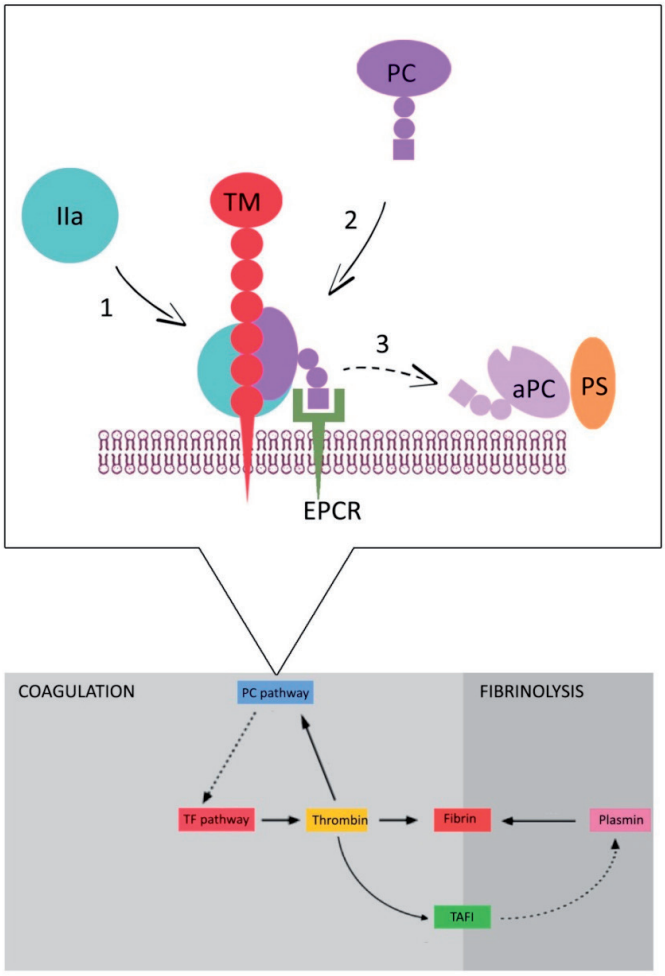

Fig. 1 The protein C pathway.

IIa, thrombin; TM, thrombomodulin; EPCR, endothelial cell protein C receptor; (a)PC, (activated) protein C; PS, protein S. TM binds circulating thrombin (1) and forms a TM-thrombin-complex, which activates PC bound to EPCR into aPC (2). APC then dissociates from the EPCR, binds to PS, and forms a complex that inactivates factor Va and factor VIIIa (3).

Thrombin and Prothrombin

Thrombin (activated blood-coagulation factor II) results from cleavage of prothrombin (coagulation factor II) and is a key player in the activation of the PC pathway. Although thrombin promotes its own formation by activating factors V and XI, thrombin also inactivates factor Va using the slower PC pathway.

Prothrombin is encoded by the *F2* gene. The common G20210A mutation in the *F2* gene (rs1799963) is a G-to-A gain-of-function transition causing higher levels of prothrombin and thrombin, which may lead to increase of fibrin clot formation and, potentially, to enhanced thrombotic risk.[14-16] This mutation has not yet been studied in severe sepsis, in which it may affect the severity and outcome by aggravating the procoagulant state in these patients.

Literature review of thromboembolic disorders in children shows that studies have focused on patients with symptomatic thrombosis; carriers of the 20210A allele have been proven to be more susceptible to venous and cerebral thrombosis[17-22], with often thrombosis at a young age.[23]

If the 20210A allele is present in combination with the *Protein C* (*PROC)* promotor CG haplotype in cerebral venous thrombosis[20], or in combination with the factor V Leiden allele in venous thromboembolism, the risk of thrombosis increases.[24]

Another polymorphism in the *F2* gene, the A19911G polymorphism (rs3136516), is in complete linkage disequilibrium (LD) with the *F2* G20210A polymorphism, and is also known to modulate plasma prothrombin levels. This leads to the conclusion that three possible haplotypes are associated with these two polymorphisms, concomitantly correlating with prothrombin levels in plasma, with the highest levels in AG haplotype.[25,26] Reports show that *F2* 19911AG or 19911GG genotypes are associated with a slightly increased risk for venous thrombosis (odds ratio [OR]: 1.5).[27]

The G20210A and the A19911G polymorphisms in the *F2* gene have not been studied in adult or pediatric sepsis. However, they may also likely contribute to the procoagulant state in sepsis, solely or in combination with other hemostatic gene polymorphisms.

The table in the Supplementary Appendix 1 gives an overview of studies on polymorphisms in the *F2* gene, and polymorphisms in other genes involved in the PC and fibrinolytic pathway, studied in pediatric sepsis and thromboembolic disorders in children. The Supplementary Appendix 1 also includes detailed methods on literature search and how studies were selected for this review.

Table 1 Overview of studies on genetic polymorphisms in the PC and fibrinolytic pathway in pediatric sepsis

Abbreviations: CI, confidence interval; DIC, disseminated intravascular coagulation; *PROCR*, endothelial cell protein C receptor; *PROC*, protein C; SNP, single nucleotide polymorphism; *SERPINE1*, serine proteinase inhibitor E1 (PAI-1); *CPB2*, carboxypeptidase B2 (TAFI).

[a]Relative risk.

[b]Hazard ratio.

Ref.	*Gene* - SNP	Disease	No. patients (age)	No. controls (age)	Origin of patients	Association with disease	Odds ratio (95% CI)	Comments
[41]	*PROCR* - 23 bp insert	Severe sepsis	45 (0-15 y)	160 (unknown)	Turkey	None	5.3 (0.9-32.9)	
[50]	*PROC* promotor - C1654T and A1641G	Meningococcal disease	288 (1 wk-18 y)	309 (newborns)	Central Europe	None	-	In patients <1 year (n=70), CG allele frequency was significantly higher than in controls. Severity was also higher in CG carriers (OR: 3.4, 95% CI: 1.1-11.2). Patients with CG-CG genotype needed more adrenergic support than other genotypes (OR: 6.6, 95% CI 1.3-34.1)
7	*SERPINE1* - -675 4G/5G	Meningococcal disease	175 (3 mo-18 y)	226 (unknown)	UK and The Nether-lands	Severity	2.0[a] (1.0-4.0)	
95	*SERPINE1* - -675 4G/5G	DIC in meningococcal disease	86 (median 68 mo)	152 (median 67 mo)	Europe	Severity	1.6[b] (1.0-4.9)	
96	*SERPINE1* - -675 4G/5G	Meningococcal disease	330 (1 mo-27 y)	316 (newborns)	Europe	Severity	2.3 (1.0-5.1)	
97	*SERPINE1* - -675 4G/5G	Meningococcal disease	510 (2 mo-67 y)	155 (2-68 y)	UK	Severity	0.1 (0.0-0.4)	Protective effect of 5G/5G genotype for mortality
[41]	*SERPINE1* - -675 4G/5G	Severe sepsis	42 (0-15 y)	113(unknown)	Turkey	Susceptibility	2.2 (1.1-4.2)	
[127]	*CPB2* - Thr325Ile	Meningococcal sepsis	60 (1 mo-16 y)	52 (1 mo-16 y)	The Nether-lands	Severity	13.7 (1.5-123)	In patients with DIC on admission, 325Ile/Ile genotype was overrepresented
[127]	*CPB2* - Ala147Thr	Meningococcal sepsis	60 (1 mo-16 y)	52 (1 mo-16 y)	The Nether-lands	None	-	
[127]	*CPB2* – G438A	Meningococcal sepsis	60 (1 mo-16 y)	52 (1 mo-16 y)	The Nether-lands	None	-	

Thrombomodulin

TM-which is encoded by *THBD*-is expressed by endothelial cells and binds thrombin. The normally procoagulant function of thrombin then alters to an anticoagulant function. In addition, TM inhibits the procoagulant function of thrombin by promoting enhanced activity of antithrombin. The TM protein contains six epidermal growth factor (EGF)-like repeats, of which the last two are required for thrombin binding.[28]

Many interactions exist between TM and inflammation. Proinflammatory cytokines increase the activity of proteolytic enzymes, which result in increased cleavage of endothelial TM. Interestingly, cleaved fragments in plasma, soluble TM (sTM), are a sensitive marker for endothelial damage.[29,30] In sepsis, sTM levels are higher in nonsurvivors than in survivors.[31] Endothelial TM expression, on the other hand, is decreased in proinflammatory states.[32] In observational case-control and cohort studies, recombinant human sTM (rhTM) was proven to potentially improve mortality with a positive effect on DIC.[33] However, three randomized controlled trials failed to demonstrate any significant improvement for survival in the rhTM group.[34] Hence, the implications for clinical practice are not clear and need to be clarified in future studies.

Genetic polymorphisms in *THBD* have not been studied in severe sepsis. The only available data, from animal studies in mice with a point mutation in the *THBD* gene, showed an impaired capacity to activate PC when compared with wild type mice. Upon infection with gram-negative bacteria, mutant mice had an increased activation of the coagulation cascade and displayed a decreased survival compared with wild-type mice.[35]

In several other thromboembolic conditions, the *THBD* Ala455Val polymorphism (rs1042579)-which alters the sixth EGF, thrombin-binding region-has been studied, with conflicting results. The Ala455/Ala455 genotype was associated with increased susceptibility to ischemic stroke.[36] However, in two other large studies, this polymorphism was not associated with susceptibility to venous thrombosis[37] or susceptibility to atypical hemolytic uremic syndrome (HUS)[38]. The *THBD* Ala455Val polymorphism was found to have no influence on sTM level.[39]

Fan et al sequenced the *THBD* gene in 10 Japanese atypical HUS pediatric patients and identified the *THBD* c.1499C > T polymorphism as a potentially causative mutation for HUS. This finding was based on prediction programs, search of the literature, and position of the missense mutation in a three-dimensional structure.[40]

In conclusion, sTM may be an important player in sepsis, and *THBD* polymorphisms could theoretically influence sTM levels and endothelial TM expression.

Protein C Receptor

The EPCR, encoded by *PROCR*, binds the TM-thrombin complex and amplifies the activation of PC into aPC.

A 23 base pair (bp) insertion between intron II and exon III of *PROCR*-which leads to inability to sustain PC activation-has been studied in a small cohort of 45 children with severe sepsis (29 survivors, 16 nonsurvivors). Three (6.7%) of these patients were carriers of this polymorphism, which was not found to be significantly more frequent than in the control group (1.25%). Additionally, the frequency of the 23 bp insertion was not increased in nonsurvivors compared with survivors. Thus, the influence of the 23 bp insertion on susceptibility or severity of pediatric sepsis needs evaluation in a larger cohort of patients to draw definite conclusions.[41] Table 1 gives an overview of studies on genetic polymorphisms in the PC and fibrinolytic pathway, studied in children with sepsis.

The H1 haplotype includes 6333C, and the H3 haplotype includes 1651G and 6936G alleles. In patients with ischemic stroke, carriers of the *PROCR* 1651G allele (rs867186) had higher soluble EPCR levels in plasma, competing for PC with membrane-associated EPCR. This theoretically results in inefficient activation of PC and, therefore, in higher risk for ischemic stroke.[42] In adults with severe sepsis and/or septic shock, simultaneous carriage of alleles belonging to the H1 and H3 haplotypes was associated with a protective effect on the risk for developing severe sepsis or septic shock (adjusted OR: 0.34).[43]

Evaluation of *PROCR* haplotypes in pediatric sepsis has not yet been performed.

Protein C

PC, encoded by *PROC*, is cleaved to aPC by the TM-thrombin complex. The majority of sepsis patients acquire PC deficiency, regardless of the causative organism, presumably due to increased consumption, degradation, and/or decreased synthesis of PC.[5,6,44] Because decreased PC levels are associated with sepsis morbidity and mortality, therapeutic studies were directed at replacement of naturally occurring anticoagulant PC,[45] using also a recombinant form of aPC.[46] However, outcome did not improve. On the contrary, treatment with aPC seemed to be associated with a higher risk of bleeding.[47]

The *PROC* promotor contains three polymorphisms-C1654T, A1641G, and A1476T-that have a high degree of LD. Three haplotypes (CGT, TAA, and CAA) account for 88% of the observed haplotypes in healthy individuals.[48] These genetic variations are associated with variations in plasma PC levels, also after correction for environmental factors. The homozygous CGT genotype is associated with the lowest PC activity, while the homozygous TAA genotype is associated with the highest PC activity, which may result in different host responses in sepsis-associated coagulopathy or thromboembolic

disorders.[49] Polymorphisms in the promotor region of *PROC* have been studied individually and combined, as haplotype, in pediatric and adult sepsis and in other thromboembolic disorders in children.

The allele distributions of the *PROC* C2405T (C1654T, rs1799808) and A2418G (A1641G, rs1799809) promotor polymorphisms did not differ significantly between children with meningococcal infection (n = 288) and healthy newborns (n = 309). However, the CG haplotype was significantly more frequent in meningococcal disease patients younger than 1 year (n = 70) (52.1% vs. 42.7% in controls). In addition, patients carrying the CG haplotype were more likely to develop sepsis (86% vs. 64% in non-sepsis patients), and patients with the CG-CG genotype had lower systolic blood pressure during pediatric intensive care unit (PICU) admission needing more adrenergic support than patients with other genotypes. Thus, in young children with meningococcal infection, the CG allele might have influenced susceptibility and severity.[50]

These findings are different from findings of a prospective gene-association study in adult patients with severe sepsis. In a small discovery cohort and a large replication cohort, the 1641 AA genotype was associated with greater systemic inflammatory response, more organ dysfunction and higher mortality. There was no association of C1654T polymorphism with severity of sepsis. Analysis based on haplotypes revealed that the higher mortality associated with the 1641A allele was completely confined to the CA haplotype. Survival of patients carrying the TA haplotype was not different from survival of patients carrying the 1641G allele.[51] In a study of 240 severe sepsis patients from the Chinese Han population, the 1654C/1641A haplotype was also associated with organ dysfunction and was observed to be an independent risk factor for fatal outcome of severe sepsis.[52] These findings were confirmed by results from a population of East Asian ancestry in North America, where investigators studied the *PROC* T673C (rs2069912) polymorphism, which is known to be in LD with the CA haplotype.[53]

Apart from sepsis, the *PROC* C1654T and A1641G promotor polymorphisms have been studied in pulmonary thromboembolism. Patients were more likely to carry the TA haplotype than controls. The TA-TA genotype did not differ between patients and controls.[54]

Thus, *PROC* promotor polymorphisms are associated with PC level and the outcome of pediatric and adult sepsis. Studies of haplotypes combining three SNPs in the *PROC* promotor region in sepsis have not yet been published.

Protein S

PS, encoded by *PROS1*, is a plasma protein that serves as a cofactor for aPC and inhibits blood coagulation.

PS deficiency is associated with an increased risk of thrombosis.[55] It is well known that acquired PS deficiency occurs secondary to sepsis.[56] Whether *PROS1* polymorphisms affect the susceptibility and severity of sepsis is still to be evaluated.

In Asian patients, *PROS1* K196E polymorphism (rs121918474) was found to be a significant risk factor associated with development of deep vein thrombosis (DVT).[57,58] This mutation seems specific for the Japanese population.[59]

The Fibrinolytic Pathway

The fibrinolytic pathway actively degrades existing fibrin clots. Effects of the fibrinolytic pathway on wound healing, malignancies, and the central nervous system are not in the scope of this review, and have been discussed elsewhere.[60]

Plasmin is the major fibrinolytic protease and degrades fibrin into soluble fibrin degradation products (FDPs) (Fig. 2). Plasminogen (Plg) is cleaved into plasmin by Plg activators. Inhibition of fibrinolysis occurs on several levels: by Plg activator inhibitors (PAI), by thrombin-activatable fibrinolysis inhibitor (TAFI), and by other plasmin inhibitors such as α2-antiplasmin and α2-macroglobulin. In addition, factor XIII stabilizes fibrin, thereby making the fibrin clot more resistant to fibrinolysis.[61,62]

The PC pathway interacts with the fibrinolytic pathway by way of three crosslinks. The aPC forms a complex with PAI-1, rendering PAI-1 unable to inhibit fibrinolysis.[63] The second and third crosslinks are via thrombin. Thrombin activates TAFI and TAFIa binds to Plg,[64] thereby attenuating the binding of Plg to fibrin. Thrombin also activates factor XIII.

The fibrinolytic pathway is impaired in sepsis, mainly due to fulminant increase of PAI-1 levels.[65] In meningococcal sepsis, it was clearly shown that higher PAI-1 levels are associated with negative outcome, and that genetic polymorphisms in the host influence PAI-1 levels.[7,8] Moreover, persistently high plasma PAI-1 levels are associated with severity of disease and mortality.[9]

Plasmin(ogen)

Plg, encoded by *PLG*, is a circulating zymogen primarily synthesized by the liver,[66] and is cleaved into its active form plasmin by Plg activators.[67] Binding of both Plg and Plg activators to fibrin clots ensures local fibrinolytic activity.

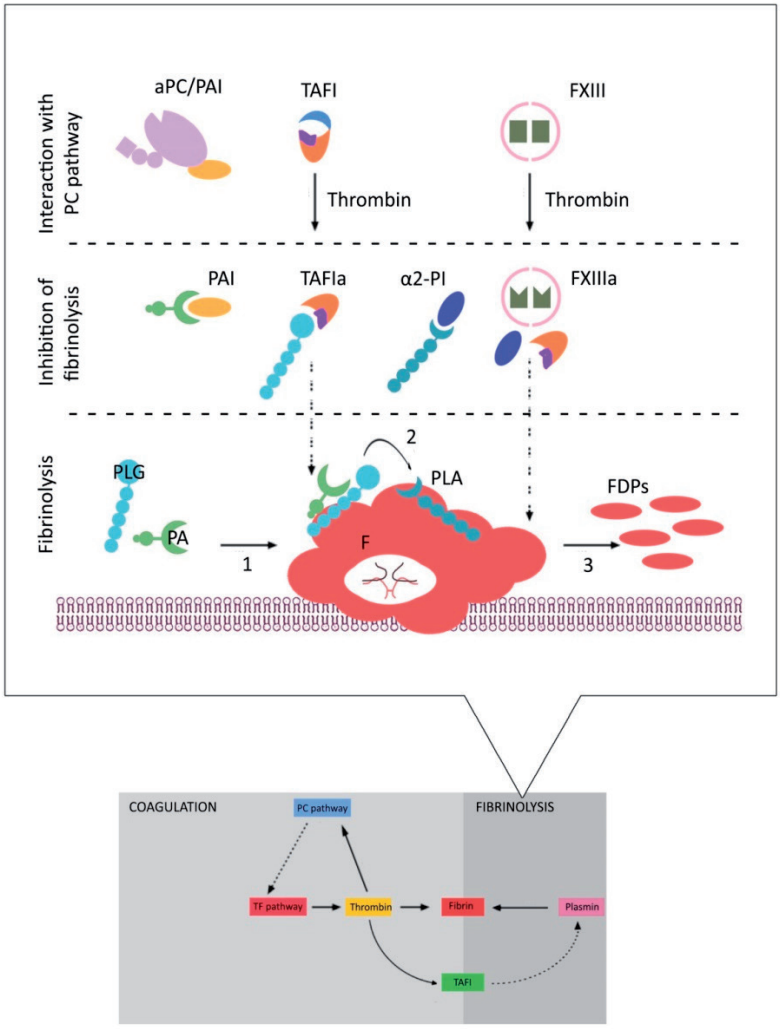

Fig. 2 The fibrinolytic pathway.

Plg, plasminogen; PA, plasminogen activators; Pla, plasmin; F, fibrin; FDPs, fibrin degradation products; PAI, plasminogen activator inhibitors; TAFI(a), (activated) thrombin-activatable fibrinolysis inhibitor; α2-PI, α2-plasmin inhibitor; FXIII(a), (activated) factor XIII; aPC, activated protein C. Fibrinolysis occurs after both PLG and PA bind to F (1). The conversion of Plg to Pla by PA (2) results in degradation of F into FDPs (3). Fibrinolysis is inhibited (A) by PAI, which binds to PA; (B) by Plg-bound TAFIa, which attenuates the binding of Plg and PA to F; (C) by α2-PI, which binds to free circulating Pla; and (D) by FXIIIa which stabilizes fibrin by incorporation of α2-PI and TAFI, making the fibrin clot more resistant to fibrinolysis. PC pathway and fibrinolytic pathway interact via aPC-PAI complex, and via thrombin activating TAFI and FXIII.

Initially, it has been suggested that low levels of Plg may increase the risk of venous thrombosis.[68-70] However, thrombosis in family members with Plg deficiency was not statistically different from thrombosis in family members without Plg deficiency.[71] In addition, dysplasminogenemia was also not found to be a risk for thrombosis.[72]

Plasmin induces proinflammatory effects by stimulating the production of cytokines, reactive oxygen species, and other mediators.[73] Although several genetic polymorphisms in *PLG* gene have been associated with protein activity,[74-76] studies associating *PLG* genetic polymorphisms with sepsis in humans have not yet been published. In murine staphylococcal sepsis, inhibition of Plg activation by tranexamic acid and subsequent downregulation of plasmin resulted in a significant increase in severity and mortality.[77]

Plasminogen Activators

Two types of Plg activators exist: tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). T-PA, encoded by *PLAT*, is synthesized in endothelial cells of smaller vessels.[78] The rate of tPA-catalyzed Plg activation in the absence of fibrin is low, but in the presence of fibrin the reaction rate increases up to 1,000-fold.[79,80] U-PA, encoded by *PLAU*, is likewise produced by endothelial cells, and also by monocytes and macrophages. In contrast to tPA, uPA has substantial Plg activator activity in the absence of fibrin.

Activation of Plg by tPA is the major pathway that leads to fibrin clot lysis, whereas activation of Plg by uPA is in association with cell surfaces.[81] Once formed, plasmin cleaves tPA and uPA into two-chain proteases, which exhibit higher proteolytic activity, implying a positive feedback on the fibrinolytic cascade.[61] The ability to cleave fibrin polymers has made Plg activators efficient therapeutic tools for both arterial and venous thrombosis. [82,83]

The uPA system and the effects on proinflammatory signaling, leukocytes, and breach of the blood-brain barrier have been studied in the cerebrospinal fluid and serum of patients with bacterial meningitis. Elevated uPA levels in blood samples of bacterial meningitis patients correlate with adverse clinical outcome.[84] In sepsis, uPA levels were shown to be increased as well; uPA levels in plasma of nonsurvivors and in sepsis patients were higher compared with uPA levels in survivors and non-sepsis patients with severe infectious disease. Therefore, it has been suggested that uPA may be of prognostic value in patients admitted to the medical intensive care unit for severe infectious disease.[85,86]

Studies on genetic polymorphisms in *PLAT* and *PLAU* are limited. The Alu insertion polymorphism, which consists of the presence or absence of a 311 bp Alu in *PLAT*, is associated with tPA release rate from vascular endothelial cells. Alu/Alu genotypes release more tPA than other genotypes.[87] In adult bacterial osteomyelitis, the Alu/Alu genotype and the Alu allele were significantly more frequent in patients than in controls.[88]

Thus, an activated fibrinolytic system might contribute to bone tissue destruction and development of osteomyelitis.[88]

The *PLAT* C7351T polymorphism (rs2020918) is also strongly correlated with endothelial tPA release[89], but was not associated with susceptibility to pediatric chronic thromboembolic pulmonary hypertension (CTEPH) or pulmonary embolism (PE).[90]

Plasminogen Activator Inhibitors

The activity of both Plg activators is regulated by specific PAIs. Three types of PAIs have been described so far: PAI-1 produced by endothelial cells,[91] PAI-2 by human placenta,[92] and protease nexin-1 (PN-1) by human fibroblasts[93].

PAI-1, encoded by *SERPINE1*, is the most important fibrinolytic inhibitor in vivo, and the -675 4G/5G insertion/deletion polymorphism within the promotor region (rs1799889) has been extensively studied. In meningococcal disease, it has clearly been shown that higher levels of PAI-1, as seen in 4G/4G homozygotes, are associated with more severe disease, that is, higher median pediatric risk of mortality (PRISM) score,[94] more DIC, more vascular complications, and higher mortality.[7,95-97] None of these studies showed an association with susceptibility to disease. In only one study including 42 children, sepsis susceptibility seemed significantly associated with the 4G/4G genotype (54.8% patients had a 4G/4G genotype compared with 24.8% controls). However, the latter study was limited by a small sample size and variation in disease-causing pathogens.[41]

This association between the 4G/5G polymorphism and sepsis was further supported by a meta-analysis of 12 case-control studies and three cohort studies, including both pediatric and adult sepsis cases. The 4G homozygotes were found to be more susceptible to sepsis than other genotypes (OR: 1.3, 95% confidence interval [CI]: 1.1-1.6). In addition, there was a significant association with sepsis-related mortality (OR: 1.7, 95% CI: 1.3-2.3).[8] In adult patients with severe pneumonia, the 4G allele has also been associated with increased susceptibility to community-acquired pneumonia, increased mortality, and fewer ventilator-free days.[98,99]

The *SERPINE1* 4G/5G polymorphism has already been extensively studied in venous thrombosis[18,90,100,101] and ischemic stroke.[102-106] Most studies, including both small and large groups of patients, failed to associate this polymorphism with disease. Nevertheless, the selection of patients, with variety in clinical phenotype within each study, may partly explain negative results.

Close to and highly in LD with the *SERPINE1* -675 4G/5G promotor polymorphism is the *SERPINE1* -844 A/G polymorphism (rs2227631).[107] This polymorphism has been studied

in Puumala hantavirus (PUUV) infection, also known as nephropathia epidemica, which is the most common cause of hemorrhagic fever accompanied by renal syndrome in Europe. Carriers of the -844G allele suffered from more severe renal impairment than noncarriers. The platelet count nadir of the -844G allele carriers was higher than that of noncarriers, and was concordant with the observation that the severity of renal impairment does not associate with the depth of thrombocytopenia in acute PUUV infection.[108]

The role of PN-1, encoded by *SERPINE2*, in sepsis should be elucidated. PN-1 has a large inhibitory spectrum: it is the most efficient tissue inhibitor of thrombin, but is also a powerful inhibitor of Plg activators and plasmin. In the last decade, PN-1 research has made significant progress and has revealed its contribution to regulation of coagulation and fibrinolysis.[109] Injury-related cytokines have been proven to induce an increase in PN-1 expression in different cell types.[110,111] In addition, clot lysis is accelerated in the absence of PN-1.[109] Thus, PN-1 might be an underestimated regulator of fibrinolysis.

So far, *SERPINE1* polymorphisms are among the most well studied in pediatric sepsis and are associated with severity and outcome. In contrast, not much is yet known about *SERPINE2*, which might be a promising gene for further evaluation. Also, evaluation of PAI-2, encoded by *SERPINB2,* in pediatric sepsis has not yet been performed.

Plasmin Inhibitors

Plasmin is inhibited by α2-plasmin inhibitor (α2-PI, α2-antiplasmin), α2-macroglobulin (α2-MG) and PN-1. α2-PI, encoded by *SERPINF2*, is the primary fast-reacting physiological inhibitor of plasmin, and inhibits fibrinolysis by direct binding to free circulating plasmin.[112]

In melioidosis, a common form of community-acquired *Burkholderia pseudomallei* sepsis in Southeast Asia and Northern Australia, patients have elevated α2-PI levels.[113] Likewise, α2-PI levels in plasma and lung homogenates were elevated in mice infected with *B. pseudomallei*. During experimental melioidosis in α2-PI-deficient mice, a strongly disturbed host response was found, as reflected by enhanced bacterial growth at the primary site of infection accompanied by increased dissemination to distant organs. Furthermore, α2-PI deficiency and profibrinolytic state subsequently resulted in enhanced activation of coagulation with exaggerated systemic inflammation, increased distant organ injury, and more lethality. Thus, α2-PI seems to be a protective mediator during gram-negative sepsis.[113]

α2-MG, encoded by *A2M*, is a plasma protein that inhibits a wide variety of proteolytic enzymes, including plasmin, trypsin, thrombin, kallikrein, and chymotrypsin. Plasma α2-MG values in patients with sepsis are decreased, possibly by formation of complexes with several proteases, and may be associated with fatal outcome.[114,115]

No studies on polymorphisms in any genes encoding for plasmin inhibitors in sepsis and thromboembolic disorders were found, but previously described functional studies show an important role for proteins, especially in gram negative sepsis.

Fibrin(ogen)

Thrombin converts fibrinogen into fibrin, so that fibrin monomers can polymerize into a fibrin clot. Fibrin facilitates two opposing processes, that is, binding and activation of profibrinolytic enzymes-Plg and tPA-leading to degradation of fibrin into soluble FDPs, and binding of antifibrinolytic enzymes to generate stable clots-factor XIIIa binds α2-PI to fibrin-bound plasmin.

Fibrin is composed of two sets of three structurally different chains-α, β, and γ-which are encoded by *FGA*, *FGB*, and *FGG*, respectively.

The *FGB* G455A polymorphism (rs1800790) is associated with plasma fibrinogen concentration, and may therefore increase cardiovascular risk in adults.[116,117] Associations in pediatric sepsis are unknown, but in adult sepsis the haplotype *FGB* −854G/ −455A/ +9006A was found to be associated with lower mortality and a trend towards lower organ dysfunction.[118]

In children, this polymorphism was not associated with susceptibility to ischemic stroke [103,119] or venous thrombosis.[90,100,120] It has been suggested that high values of plasma fibrinogen may be associated with thromboembolic disorders mainly in elderly, and that elevated fibrinogen values reflect accumulated age-associated risk factors for cardiovascular disease.[121]

The *FGA* A312G (Thr312Ala) polymorphism (rs6050) is associated with fibrin fibers thickness and α-chain cross-linking, hence difference in resistance to fibrinolysis.[90,122] Patients with CTEPH had significantly higher AA genotype and A allele frequencies than PE patients and controls.[90]

The *FGB* -148T allele (rs1800787) is associated with lower TNFalpha release in response to systemic LPS infusion in humans than the -148C allele.[123] This polymorphism was not associated with susceptibility to ischemic stroke[124] and CTEPH/PE[90].

So far, the impact of polymorphisms in *FGA*, *FGB*, and *FGG* on pediatric sepsis needs to be established.

Thrombin-Activatable Fibrinolysis Inhibitor

TAFI, encoded by the gene *CPB2*, circulates in plasma as a Plg-bound zymogen. TAFI links coagulation to fibrinolysis. Once TAFI is activated by thrombin or hyper-activated by the thrombin/TM complex, TAFIa attenuates fibrinolysis by inhibiting the binding of Plg and tPA to the fibrin clot.[125]

TAFI levels vary widely between individuals and levels are largely regulated by genotype.[126] In sepsis, TAFI levels are decreased, and several polymorphisms have been investigated.[127,128] The *CPB2* 325Ile/Ile genotype (rs1926447) was found to be overrepresented in pediatric meningococcal sepsis patients with DIC on admission compared with the 325Thr/Thr genotype.[127] In adult meningococcal sepsis, the *CPB2* 325Ile/Ile genotype was slightly, but not significantly, more common among parents of patients with meningococcal disease than in controls. In terms of severity, patients whose parents were carriers of the *CPB2* 325Ile/Ile genotype had an increased risk of dying from infection compared with all other genotypes.[129]

The Ala147Thr polymorphism (rs3742264) in pediatric meningococcal disease was not found to be associated with DIC on admission.[127] This polymorphism, however, was associated with susceptibility to ischemic stroke; the 147Thr allele frequency was higher in patients than in controls.[119]

Thus, *CPB2* Thr325Ile polymorphism has an effect on severity of pediatric sepsis, but effects on mortality have been reported only for adult sepsis patients.

Factor XIII

Factor XIII (FXIII), composed of two A subunits and two B subunits and encoded by F13A and F13B, respectively, stabilizes the fibrin clot and has also an antifibrinolytic function. Fibrin monomers are cross-linked by FXIII to form a blood clot. Thrombin-activated FXIII (FXIIIa) then stabilizes fibrin further by incorporation of the fibrinolysis inhibitors α2-PI and TAFI, making the fibrin clot more resistant to fibrinolysis.[130-132]

Several FXIII polymorphisms have been identified in both subunits, of which the association of Val34Leu (rs5985) has been studied extensively.[133] This polymorphism is associated with the rate of FXIII activation by thrombin; the 34Leu variant is cleaved more rapidly than the 34Val variant.[134] Functional studies found that plasma FXIII levels are influenced by the Val34Leu polymorphism upon endotoxin stimulation[135], and plasma FXIII levels are associated with severity of sepsis.[136-138] Susceptibility to sepsis has been studied in a group of 1,008 prospectively enrolled very low-birth-weight infants. The carriers of the *F13A* 34Leu allele were more susceptible to develop sepsis and had a longer period of hospital care compared with noncarriers.[139]

In other pediatric thromboembolic disorders, conflicting results have been published. Although higher FXIII activity was found for 34Leu carriers,[140] the Val34Leu polymorphism was not associated with susceptibility to venous thrombosis or ischemic stroke[100,103,140,141]. In two large studies, Leu/Leu genotype[142] and 34Leu allele[19] revealed a protective effect on venous thrombosis.

The role of *F13A* Val34Leu polymorphism on susceptibility to sepsis has been shown for premature neonates, but needs to be elucidated in future research in older children with sepsis.

Conclusions and Future Directions

Host response to infection involves complex and multifaceted interplays between inflammation, coagulation, and fibrinolysis. The challenge in sepsis is to balance between a proinflammatory and procoagulant response to provide bacterial clearance, but to avoid an excessive response which leads to endothelial damage. In sepsis, decreased activity of the PC pathway and inhibition of fibrinolysis overall results in a procoagulant state. Host genetic polymorphisms in these pathways are of great interest, as they may elucidate the hypotheses on genetic predisposition for sepsis and adverse outcomes. Some polymorphisms in the PC and fibrinolytic pathway have been proven to be associated with host susceptibility and severity of pediatric sepsis. It has been hypothesized that gene polymorphisms associated with adult sepsis or other thromboembolic disorders in children may also be candidates for future genetic research in pediatric sepsis.

In pediatric sepsis, the high PAI-1 producer *SERPINE1* 4G/4G genotype is most significantly associated with sepsis mortality. Also, the *CPB2* Thr325Ile polymorphism seems predictive of DIC. However, effects on mortality have been reported for adult sepsis patients only. Studies associating protein plasma levels with genetic polymorphisms or with outcome of sepsis, for instance TM, PC, FXIII, and Plg activators, may direct future molecular genetic studies to genes encoding these proteins. Moreover, we believe that the association of *PROCR* 23 bp insertion should be evaluated in a larger group of patients.

As other studies in pediatric thromboembolic disorders have shown associations with a prothrombotic state, we hypothesize that *F2* G20210A or A19911G polymorphisms, which both increase prothrombin concentration, may contribute to adverse outcome in pediatric sepsis. Also interesting to study in pediatric sepsis are haplotypes in *PROCR* leading to higher sEPCR, and for PN-1 encoding *SERPINE2* gene, which might be an underestimated regulator of fibrinolysis.

Previous findings in literature have been mainly based on candidate-gene studies in relatively small patient cohorts. Genome-wide association studies (GWAS) are able to reduce this biased approach. First GWAS results in meningococcal disease patients have identified polymorphisms in the *CFH* region, which play a role in complement activation, and therefore may be associated with sepsis susceptibility.[143] In adults with sepsis due to pneumonia, a GWAS revealed *FER*, which encodes a cytosolic non-receptor tyrosine kinase that influences neutrophil chemotaxis and endothelial permeability, to be associated with a reduced risk of death.[144]

Future studies should include large-scale cohorts of homogeneous patients, without variation in causal pathogen, preferably without underlying disease, and minimal environmental influence on phenotype. Newly identified polymorphisms or combination of polymorphisms in hemostatic genes may aid in uncovering the intriguing interplay of coagulation and fibrinolysis in sepsis, eventually leading towards steps in individualization of treatment in fulminant sepsis and possibly achieving a decrease in morbidity and mortality of this life-shortening complication.

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Chapter 4.2

ADAMTS-1 and ADAMTS-18 levels in meningococcal sepsis

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Introduction

The disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family includes 19 proteases which play various roles in for example coagulation and inflammation.(1-3)

ADAMTS-13, the von-Willebrand factor (vWF)-cleaving protease processing large multimeric vWF into an optimal size for normal coagulation(4), has been the most extensively studied ADAMTS-protein in sepsis. Previous studies demonstrated that decreased ADAMTS-13 levels, presumably leading to increased formation of thrombi, are associated with more severe disease and poor outcome.(5, 6)

In contrast, other ADAMTS-proteins have not been studied in sepsis yet, despite animal studies hinting towards an important role in inflammation or sepsis. ADAMTS-1 levels increase during systemic inflammation(7, 8) and ADAMTS-18 inhibits platelet activation.(9-11)

We studied ADAMTS-1 and ADAMTS-18 protein levels in pediatric meningococcal sepsis, and studied the association with mortality, coagulation markers, and inflammatory markers.

Methods

This is a retrospective laboratory study in remaining samples of prospectively conducted meningococcal sepsis studies.(12-14) Children with meningococcal sepsis presenting to the pediatric intensive care unit (PICU) between October 1991 and February 2000 for whom samples were available, were included. All patients fulfilled internationally agreed criteria for sepsis.(15) Blood samples were collected on admission to PICU, at 24 hours, and at 1 month after PICU admission. Samples were processed on ice and stored at -80°C until analysis, without freeze thaw cycles in between.

ADAMTS1 and ADAMTS18 levels were measured in serum and EDTA plasma, respectively, via commercially available human ELISA kits as described by the manufacturer. The lowest level of detection of our ADAMTS-1 assay was 1.6 ng/mL. All individual meningococcal studies as well as the current laboratory study were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines, and were approved by the ethical committee of Erasmus MC (MEC-2015-497). Written informed consent was obtained from parents or legal guardians.

Clinical data had been collected prospectively. We indicated illness severity by the Pediatric Risk of Mortality (PRISM) score(16), predicted death based on the base excess and platelet count at presentation (BEP) score(17), predicted death based on the Rotterdam score (12), and disseminated intravascular coagulation (DIC) score (18). Patients were classified as *death* if death occurred during the meningococcal sepsis admission. Coagulation and inflammation markers were measured for clinical reasons or were obtained in several meningococcal sepsis studies.(12-14)

Categorical variables are presented as counts (percentages) and continuous variables are presented as medians [interquartile ranges, IQR]. Friedman tests were used to compare ADAMTS levels between the three time points. To compare ADAMTS level between survivors and non-survivors, we used Mann-Whitney U tests. Correlations between ADAMTS level and illness severity (PRISM score, BEP score, Rotterdam score, Lactate, DIC score), coagulation markers (Platelets, Fibrinogen, Prothrombin time international normalized ratio (PT INR), Protein C, Activated protein C, Plasminogen-activator inhibitor-1 (PAI-1), Ionized calcium), and inflammatory markers (C-reactive protein (CRP), Tumor necrosis factor (TNF), Interleukin (IL)-1 beta (IL-1B), IL-6, IL-8, IL-10) on admission to PICU were assessed by Spearman's rank correlation. Statistical analyses were performed with SPSS version 21 (Armonk, USA). Graphs were created with GraphPad Prism 5.00. A p-value of less than 0.05 was considered statistically significant.

Results

We included 70 children admitted to PICU with meningococcal sepsis, of which 61 patients and 37 patients had samples available for ADAMTS-1 and ADAMTS-18 measurements, respectively. Patient characteristics are shown in table 1.

Median ADAMTS-1 level at admission to PICU did not differ from ADAMTS-1 level at t=24 hours or at t=1 month (PICU admission, n=35, 1.6 ng/mL (IQR 1.6-2.0); t=24 hours, n=42, 1.6 ng/mL (IQR 1.6-1.6); t=1 month, n=35, 1.6 ng/mL (IQR 1.6-1.6); Friedman test p=0.37).(Figure 1A) Likewise, ADAMTS-18 level did not differ between the three examined time points (PICU admission, n=37, 17.7 ng/mL (IQR 10.7-29.2); t=24 hours, n=28, 22.4 ng/mL (IQR 17.5-27.1); t=1 month, n=21, 21.8 ng/mL (IQR 18.8-27.4); Friedman test p=0.70). (Figure 1B)

At admission to PICU, ADAMTS-1 level in non-survivors was significantly higher than in survivors (2.0 ng/mL (IQR 1.6-3.1, n=11) compared to 1.6 ng/mL (IQR 1.6-1.6, n=23), p=0.02). After 24 hours, there still was a trend for higher ADAMTS-1 levels in non-survivors compared to survivors (2.1 ng/mL (IQR 1.6-3.3, n=3) compared to 1.6 ng/mL

(IQR 1.6-1.6, n=37), p=0.09). (Figure 2A) ADAMTS18 levels did not differ significantly between non-survivors and survivors at all three time points (PICU admission: non-survivors 12.5 ng/mL (IQR 9.6-25.7, n=9) compared to survivors 18.4 (IQR 13.4-30.5, n=28), p=0.36; t=24 hours: non-survivors 22.7 ng/mL (n=1) compared to survivors 22.1 (IQR 17.0-27.6, n=27), p=1.0). (Figure 2B)

Table 1: patient characteristics

	ADAMTS-1 cohort (n=61)	ADAMTS-18 cohort (n=37)
Sex (male)[1]	35 (59%)	21 (57%)
Age (years)[1]	3.0 [1.8-9.7]	2.2 [1.1-8.4]
Illness severity at PICU admission		
PRISM score[2]	20 [14-26]	24 [18-32]
P(death BEP)[3]	6.1 [3.4-19.8]	9.7 [4.3-25.0]
P(death Rotterdam)[4]	12.2 [1.6-77.0]	12.5 [4.4-88.6]
Lactate (mmol/L)[5]	4.5 [3.3-6.5]	4.7 [3.6-6.4]
DIC score[6]	5 [4-7]	5 [4-7]
Mortality[1]	11 (19%)	9 (24%)

Categorical variables are presented as counts (%), continuous variables as median [IQR]. Abbreviations: PRISM=Pediatric Risk of Mortality(16); *P*(death BEP)=predicted death rate based on the BEP score(17); *P*(death Rotterdam)=predicted death rate based on the Rotterdam score(12); DIC=Disseminated intravascular coagulation(18).

[1] Data was available for 59/61 ADAMTS-1 patients, and for all ADAMTS-18 patients.

[2] PRISM score was available for 50/61 ADAMTS1 patients, and for all ADAMTS-18 patients.

[3] *P*(death BEP) was available for 52/61 ADAMTS1 patients, and for all ADAMTS-18 patients.

[4] *P*(death Rotterdam) was available for 52/61 ADAMTS1 patients, and for 36/37 ADAMTS18 patients.

[5] Lactate was available for 52/61 ADAMTS1 patients, and for all ADAMTS-18 patients.

[6] DIC score was available for 52/61 ADAMTS1 patients, and for 35/37 ADAMTS18 patients.

In table 2, we present correlations between ADAMTS-1 and ADAMTS-18 levels and illness severity, coagulation markers, and inflammatory markers. ADAMTS-1 was significantly correlated to predicted death based on the BEP score (r=0.513, p<0.01) and predicted death based on the Rotterdam score (r=0.50, p<0.01). ADAMTS-1 was also significantly correlated to platelets (r=-0.41, p<0.05), activated protein C (r=0.523, p<0.05), IL-1B (r=0.55, p<0.05), and IL-8 (r=0.49, p<0.05). For ADAMTS-18, we did not observe significant correlations.

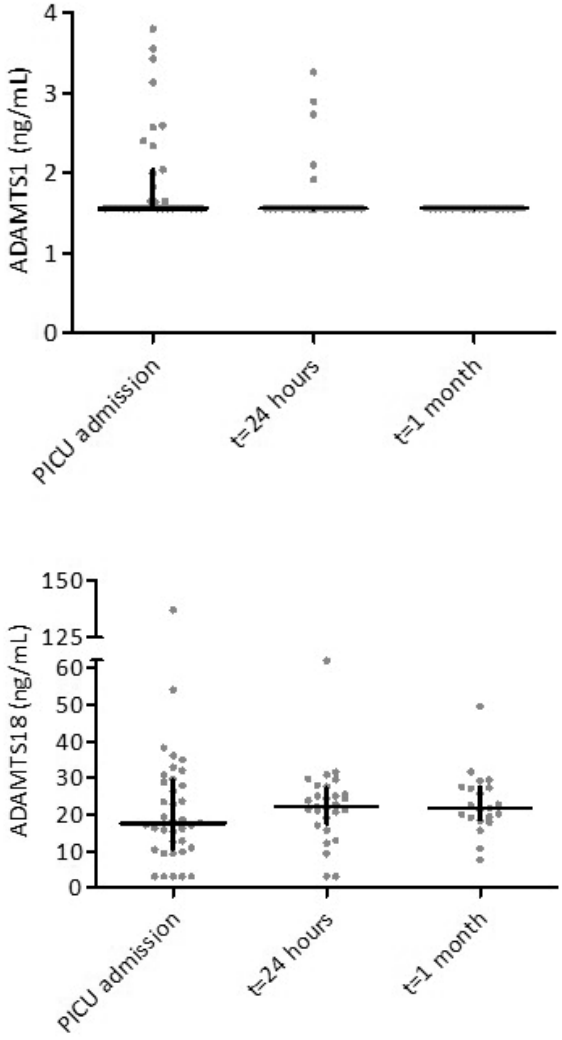

Figure 1: ADAMTS-1 and ADAMTS-18 levels during PICU stay.

ADAMTS-1 (panel A) and ADAMTS-18 (panel B) levels at admission to PICU, at 24 hours, and at 1 month. Horizontal lines indicate the median value, vertical lines represent IQR.

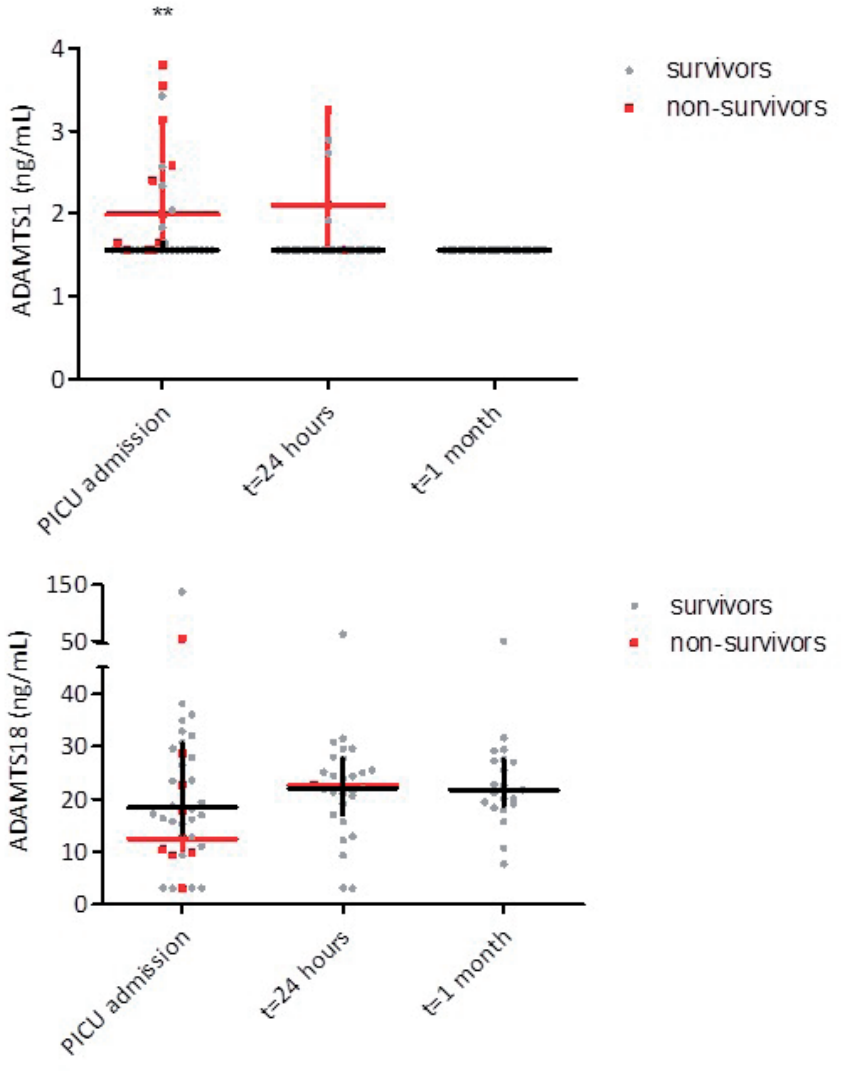

Figure 2: ADAMTS-1 and ADAMTS-18 levels in association with mortality.

ADAMTS-1 (panel A) and ADAMTS-18 (panel B) levels in survivors and non-survivors at admission to PICU, at 24 hours, and at 1 month. Horizontal lines indicate the median value, vertical lines represent IQR. ** $p<0.05$.

Table 2: Spearman correlation coefficients between ADAMTS levels and illness severity, coagulation markers, and inflammatory markers at admission to PICU.

	ADAMTS1		ADAMTS18	
	Spearman's rho	**p**	**Spearman's rho**	**p**
Illness severity				
PRISM score	0.214 (27)	0.28	0.054 (37)	0.75
P(death BEP)	0.513 (29)	**<0.01**	-0.096 (37)	0.57
P(death Rotterdam)	0.504 (27)	**<0.01**	-0.179 (36)	0.30
Lactate	0.158 (29)	0.41	-0.048 (37)	0.78
DIC score	0.390 (18)	0.11	0.044 (35)	0.80
Coagulation markers				
Platelets	-0.409 (29)	**<0.05**	0.169 (37)	0.32
Fibrinogen	-0.250 (25)	0.23	0.271 (34)	0.12
PT INR	-0.121 (14)	0.68	-0.086 (30)	0.65
Protein C	0.117 (17)	0.66	0.270 (37)	0.11
Activated protein C	0.523 (17)	**<0.05**	-0.060 (37)	0.73
PAI-1	0.427 (17)	0.09	-0.098 (35)	0.58
Ionized calcium	0.063 (20)	0.79	-0.088 (35)	0.61
Inflammatory markers				
CRP	-0.353 (28)	0.07	0.273 (36)	0.11
TNF	0.364 (23)	0.09	0.049 (35)	0.78
IL-1B	0.551 (17)	**<0.05**	-0.021 (37)	0.90
IL-6	0.479 (17)	0.05	0.070 (35)	0.69
IL-8	0.490 (17)	**<0.05**	-0.046 (35)	0.79
IL-10	0.322 (17)	0.21	-0.140 (36)	0.42

Numbers of patients in whom both ADAMTS level and the marker were known, thus whereupon the correlation has been based, are indicated between brackets. Abbreviations: PRISM=Pediatric Risk of Mortality(16); *P*(death BEP)=predicted death rate based on the BEP score(17); *P*(death Rotterdam)=predicted death rate based on the Rotterdam score(12); DIC=Disseminated intravascular coagulation(18); PT INR=prothrombin time international normalized ratio; PAI-1=Plasminogen activator inhibitor-1; CRP=C-reactive protein; TNF=Tumor necrosis factor; IL=interleukin.

Discussion

This study in children admitted to PICU with meningococcal sepsis shows that ADAMTS-1 levels in non-survivors are significantly higher than in survivors. In addition, high ADAMTS-1 levels are correlated to pro-inflammatory markers and illness severity, measured by the BEP score and Rotterdam score. These findings correspond to findings from animal studies: plasma ADAMTS-1 levels were remarkably higher in rats after

injection with *Escherichia coli* lipopolysaccharides compared to rats from the control group.(7) Also, ADAMTS-1 was positively correlated to IL-1beta in experimental studies. (19) Overall, our findings indicate that ADAMTS-1 levels could be of prognostic value in pediatric meningococcal sepsis.

The mechanism of ADAMTS-1 in meningococcal sepsis is a matter of debate. A relevant interaction between ADAMTS-1 and vascular endothelial growth factor (VEGF) might be present. In severe sepsis, VEGF levels are increased and mortality is associated with low VEGF levels.(20-22) ADAMTS-1 binds VEGF(23), thereby suppressing VEGF signaling which could inhibit endothelial survival and increase apoptosis, which in turn may contribute to the development of sepsis-induced organ dysfunction.(21)

ADAMTS-18 levels did not differ significantly between the three time points. Also, ADAMTS-18 level was not associated with illness severity, coagulation markers, or inflammation markers. This could partly be explained by a small number of samples.

Our study is limited by the use of remaining samples from historic meningococcal sepsis studies. The stability of ADAMTS-1 and ADAMTS-18 proteins in stored samples is unknown. However, if samples are affected, we assume that all samples would be affected equally. Secondly, the ELISA kit for ADAMTS-1 had a detection range of 1.56 ng/mL. In more than half of our samples, ADAMTS-1 level was below the detection range. Future studies should utilize more sensitive assays to measure ADAMTS-1 levels. Lastly, we did not include a control group. We considered convalescent samples (taken at 1 month after PICU admission) as controls, but the ADAMTS-1 course after critical illness is unknown. Normal values in healthy woman range between 0.50 and 56.6 ng/ml.(24) Normal values in children need to be determined in future studies. Nevertheless, we are the first to study ADAMTS-1 and ADAMTS-18 levels in an homogeneous group of sepsis patients. Also, because of previous laboratory studies from our research group, we were able to correlate ADAMTS-1 and ADAMTS-18 levels to coagulation markers and inflammation markers, and thereby strengthening our findings.

In conclusion, ADAMTS-1 is associated with illness severity in meningococcal sepsis patients, with higher ADAMTS-1 levels in non-survivors than in survivors. ADAMTS-18 levels are not associated with survival. Future studies should confirm the prognostic value of ADAMTS-1 in sepsis and should study possible pathophysiologic mechanisms to identify potential therapeutic targets.

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Chapter 4.3

Circadian Variation of Plasminogen-Activator-Inhibitor-1 Levels in Children with Meningococcal Sepsis

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Abstract

Objective: To study whether the circadian variation of plasminogen-activator-inhibitor-1 (PAI-1) levels, with high morning levels, is associated with poor outcome of children with meningococcal sepsis presenting in the morning hours.

Design: Retrospective analysis of prospectively collected clinical and laboratory data.

Setting: Single center study at Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands.

Subjects: 184 patients aged 3 weeks to 18 years with meningococcal sepsis. In 36 of these children, PAI-1 levels at admission to the PICU were measured in plasma by ELISA.

Interventions: None.

Measurements and main results: Circadian variation was studied by dividing one day in blocks of 6 hours. Patients admitted between 6:00 am and 12:00 am had increased illness severity scores and higher PAI-1 levels (n=9, median 6912 ng/mL, IQR 5808-15600) compared to patients admitted at night (*P*=0.019, n=9, median 3546 ng/mL, IQR 1668-6118) or in the afternoon (*P*=0.007, n=7, median 4224 ng/mL, IQR 1804-5790). In 184 patients, analysis of circadian variation in relation to outcome showed more deaths, amputations and need for skin grafts in patients admitted to the PICU between 6:00 am and 12:00 am than patients admitted during the rest of the day (*P*=0.009).

Conclusions: Circadian variation of PAI-1 levels is present in children with meningococcal sepsis and is associated with illness severity, with a peak level in the morning. Whether circadian variation is an independent risk factor for morbidity and mortality in meningococcal sepsis needs to be explored in future studies.

Introduction

Meningococcal endotoxins and the subsequent inflammatory host response induce excessive coagulation and downregulation of fibrinolysis in meningococcal sepsis. Hence, the delicate balance between coagulation and anti-coagulation shifts towards thrombosis and widespread deposition of fibrin throughout the microcirculation, with thromboembolism contributing to the need to amputate extremities, multiple organ dysfunction, and eventually death.[1]

In meningococcal sepsis, plasminogen-activator-inhibitor-1 (PAI-1) levels are increased and result in inhibited fibrinolysis and impaired anticoagulant mechanism, since PAI-1 neutralizes activated protein C[2], leading to severe disseminated intravascular coagulation (DIC).[3, 4] The *PAI1* 4G/5G polymorphism is associated with PAI-1 levels and with outcome. The highest risk for mortality is present in 4G/4G homozygous individuals, who produce the highest levels of PAI-1.[3]

The genotype is one of the multiple mechanisms which influences PAI-1 levels. Physiologically, levels are also subject to circadian variation, causing a PAI-1 peak in the morning.[5, 6] In meningococcal sepsis, clustering of fatal cases in the morning hours has been reported repeatedly. These findings are generally interpreted as the result of delayed detection of symptoms and subsequent health care seeking behavior, as signs of severe illness may easily be overlooked in the night or early morning hours.[7, 8] We hypothesize that the circadian variation of PAI-1, with high morning levels, is associated with excess mortality of cases presenting in the morning hours, possibly due to aggravation of multiple organ failure secondary to more severe DIC.

Here, we aim to study the circadian variation of PAI-1 levels in children with meningococcal sepsis in relation to outcome.

Materials and Methods

We retrospectively analyzed clinical and laboratory data of a cohort of 184 patients aged 3 weeks to 18 years with meningococcal sepsis, who were enrolled in Rotterdam based meningococcal studies from 1988 to 2005.[3, 9] All patients fulfilled internationally agreed criteria for sepsis.[10] Meningococcal sepsis was diagnosed clinically (n = 28) and/or by positive culture or PCR from sterile sites (n = 156). In 36 of these children, PAI-1 levels at admission to the PICU were measured in plasma by ELISA as described before.[3, 9] Blood samples were taken on admission to the PICU, processed on ice and stored at -80 degree Celsius until analysis.[11] All studies were approved by the ethical committee of Erasmus MC, and written informed consent was obtained from parents or legal guardians.

Circadian variation was studied by dividing one day in blocks of 6 hours. Illness severity was indicated by the probability of death based on the BEP score (P (death BEP))[12], DIC score[13], and Pediatric Risk of Mortality (PRISM)[14]. Quantitative variables are presented either as mean (±SD) when normally distributed or as median (IQR). For normally distributed variables, t-tests were used to compare two groups, while for non-normal variables the Mann-Whitney U test was used. To compare PAI-1 levels and illness severity between four time periods, we used a One-Way ANOVA when the depending variable was normally distributed and the Kruskal-Wallis test when the depending variable was non-normally distributed. The correlation between PAI-1 and illness severity was studied by Pearson's (for normally distributed variables) or Spearman's (for non-normally distributed variables) correlation. Chi squared tests or Fisher's exact tests-in case of small sample size-were used to assess the association between two categorical variables. Data were analyzed using SPSS version 21.

Results

Baseline characteristics of the total cohort of 184 patients and the 36 children in whom PAI-1 levels were measured (PAI-1 cohort) are presented in Table 1. Both groups had similar demographics, but illness severity in the PAI-1 cohort was higher as reflected by higher P (death BEP) and higher DIC score at admission.

Patients admitted between 6:00 am and 12:00 am had higher PAI-1 levels (n=9, median 6912 ng/mL, IQR 5808-15600) than patients admitted at night (P=0.019, n=9, median 3546 ng/mL, IQR 1668-6118) or in the afternoon (P=0.007, n=7, median 4224 ng/mL, IQR 1804-5790).(Fig 1A) The distribution of *PAI1* 4G/5G genotype (P=0.71) and allele frequency (P=0.72) did not differ between four time periods.

In concordance with the variation in PAI-1 levels, patients admitted between 6:00 am and 12:00 am had an increased illness severity based on the BEP score (median P (death BEP) 0.26, IQR 0.16-0.44) than patients admitted at night (P=0.009, median P (death BEP) 0.05, IQR 0.02-0.15), patients admitted in the afternoon (P=0.001, median P (death BEP) 0.05, IQR 0.04-0.05), or patients admitted in the evening (P=0.037, median P (death BEP) 0.10, IQR 0.04-0.24). Illness severity reflected by the DIC score did not differ between four time periods (P=0.13). Moreover, illness severity correlated to PAI-1 levels (correlation between BEP score and PAI-1: r=0.77, n=36, P<0.001; correlation between DIC score and PAI-1: r=0.67, n=34, P<0.001).

The morning PAI-1 peak level and the morning peak in illness severity in 36 children was not associated with poor outcome, as defined by deaths, amputations and/or need for skin grafts (*P=0*.24). To increase power, we analyzed circadian variation in the total group of 184 patients, and found a worse outcome in patients admitted to the PICU between 6:00 am and 12:00 am than patients admitted during the rest of the day (*P*=0.009). Of the 184 patients, 49 patients were admitted between 6:00 am and 12:00 am, of whom 12 patients (7%) eventually had a poor outcome (6 deaths, 4 amputations and 2 skin grafts). This in contrast to 135 patients who were admitted during the rest of the day, of whom 13 patients had a poor outcome (00-06h: 2 deaths and 1 amputation (2%); 12-18h: 2 amputations (1%); 18-00h: 4 deaths, 3 amputations and 1 skin graft (4%)). (Fig 1B) Patients admitted between 6:00 am and 12:00 am showed a trend for higher PRISM, BEP score, and DIC score compared to patients admitted during the rest of the day. (PRISM 21.3 (±11.0) vs 16.1 (±10.0), *P*=0.06; P (death BEP) 0.06 (IQR 0.02-0.20) vs 0.04 (IQR 0.03-0.10), *P*=0.16; DIC 5.2 (±1.6) vs 4.5 (±2.1), *P*=0.09)

Table 1: Baseline characteristics of all patients (n=184) and the PAI-1 cohort (n=36).

	All patients (n=184)	PAI-1 cohort (n=36)	*P*
Age (median, IQR)	3y (18m-8y)	2y (12m-9y)	ns
Sex (% male)	59%	56%	ns
P (death BEP) (median, IQR)	0.05 (0.03-0.12)	0.09 (0.04-0.23)	<0.01
DIC score at admission (mean, ±SD)	4.7 (1.9)	5.6 (2.0)	<0.05

Abbreviations: P (death BEP)=Probability of death based on the BEP score, DIC=Disseminated intravascular coagulation, IQR=Interquartile range, SD=Standard deviation, m=month(s), y=year(s).

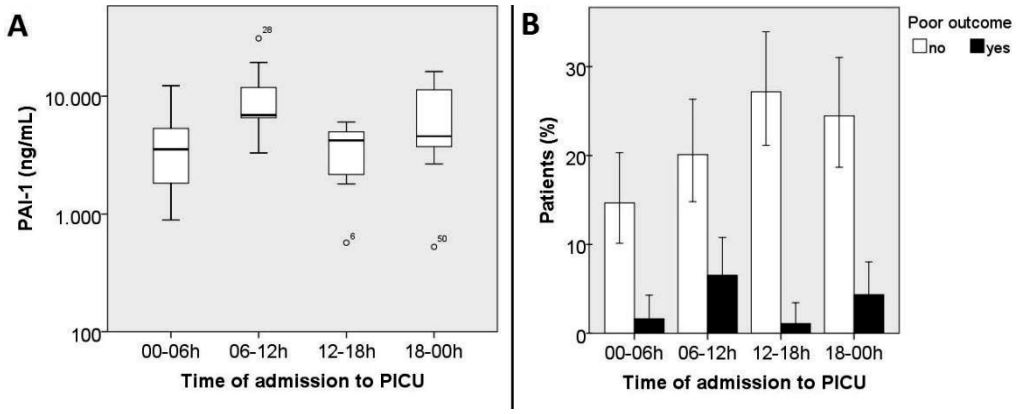

Fig 1. Time of admission to PICU in relation to plasminogen-activator-inhibitor-1 levels and outcome.

(A) Patients admitted between 6:00 am and 12:00 am had higher plasminogen-activator-inhibitor-1 levels than patients admitted at night (*P*=0.019) or in the afternoon (*P*=0.007). (PAI-1 level on y-axis in logarithmic scale. Bar within box represents median, box represents Q1-Q3, whiskers represent 1.5*IQR, dots are outliers) (B) Outcome in patients admitted to the PICU between 6:00 am and 12:00 am was worse than patients admitted during the rest of the day (*P*=0.009). (Error bars represent 95% CI)

Discussion

This study provides insight into the circadian variation of PAI-1 levels in children with meningococcal sepsis, and shows a significant PAI-1 peak level in the morning. Because the distribution of *PAI1* 4G/5G genotype and allele frequency, known to be associated with PAI-1 levels[3], did not differ between time periods, it is likely that PAI-1 levels in meningococcal sepsis are associated with circadian variation. However, numbers are limited and we cannot exclude other factors also influencing PAI-1 levels in these patients.[15]

Illness severity is one of the main factors associated with PAI-1 levels and multiple studies have associated high PAI-1 levels with increased illness severity.[3, 16-19] Also in our cohort, we found a strong correlation between PAI-1 levels and illness severity. Thus, given the correlation between PAI-1 and illness severity, PAI-1 could either be a marker for illness severity or a contributor to illness severity.[20] Future studies should specify the role of circadian variation of PAI-1 levels in meningococcal sepsis severity.

Our results show that patients admitted to the PICU in the morning have a worse outcome than patients admitted during the rest of the day. These results of outcome are in line with a retrospective epidemiological study from Western Norway, where meningococcal sepsis patients, both adults and children, hospitalized between 7:00 am and 11:00 am had a poorer prognosis than those admitted during other hours of the day.[8] Although multiple factors could have influenced increased morning severity, especially a possible delay in detection of symptoms during the night and early morning, in our opinion, morning PAI-1 peak levels might have contributed to this effect.

Scheer and Shea[5] reported a true endogenous circadian rhythm in circulating PAI-1 independent of behavioral and environmental factors. Absolute values of healthy adults had a peak-to-trough amplitude of 1.24 ng/mL, corresponding with an increase from trough to peak of 124%. In our cohort, the lowest median PAI-1 value of a time period was 3546 ng/mL, which increased to a peak value of 6912 ng/mL in the morning, corresponding with an increase of 95%. The extremely high PAI-1 level in meningococcal sepsis compared to patients with meningitis alone or healthy controls has been described before.[3] However, this is the first report describing that circadian variation of PAI-1 levels - and associated circadian variation of illness severity - is also present in meningococcal sepsis patients with extremely high PAI-1 levels.

In conclusion, our data demonstrate an association between circadian variation of PAI-1 levels and illness severity in pediatric meningococcal sepsis patients, with a significant peak level in the morning. Future study in a larger cohort of patients should focus on the question whether the morning PAI-1 peak is an independent risk factor for morbidity and mortality in meningococcal sepsis.

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Chapter 5

General discussion

Introduction

The aim of this thesis was to study determinants of pediatric sepsis outcome and thereby identifying prognostic markers which are able to detect patients at risk for an adverse outcome at an early stage. First, we characterized children with severe bacterial infections across Europe, and studied risk factors for mortality and disability. Secondly, we studied markers in inflammation and hemostasis in response to sepsis, to study inter-individual variation and associations with outcome. Thirdly, we studied genetic polymorphisms associated with severity of sepsis and used preliminary genetic findings for a functional study. Lastly, we explored whether circadian variation could be a relevant determinant in meningococcal sepsis.

Sepsis in children

What is sepsis?

To date, the 2005 international consensus conference definitions for pediatric sepsis and organ dysfunction are commonly used.[1] (Figure 7) However, SIRS criteria are easily met in patients with mild-infectious or even non-infectious diseases.[2, 3] On the other hand, a relatively large proportion (approximately 12%) of infected patients with organ dysfunction does not meet SIRS criteria.[4] Thus, SIRS criteria seem to be limited in their capacity to identify sepsis patients at-risk for organ dysfunction and subsequently mortality.

Therefore, new sepsis criteria in adults have been established recently, named *Sepsis-3*. [5] The main difference with previous criteria is that a dysregulated host response to infection "needs to" cause life-threatening organ dysfunction in order to be classified as *sepsis*. Infection with organ dysfunction (i.e. sepsis according to Sepsis-3 criteria) is associated with an in-hospital mortality greater than 10%.

The Sequential (Sepsis-related) Organ Failure Assessment (SOFA) score grades organ dysfunction and is the cornerstone of Sepsis-3 criteria.[5, 6] Unfortunately, these criteria are not applicable to children - since the SOFA score has not been validated in the pediatric population - resulting in an urge to translate Sepsis-3 criteria to pediatric sepsis.[7, 8] Recent attempts to apply Sepsis-3 criteria in children focuses on how to grade pediatric organ dysfunction; both pediatric SOFA score and Pediatric Logistic Organ Dysfunction-2 (PELOD-2) score seem feasible and show promising results in detecting sepsis patients and subsequently predicting mortality in patients admitted to the Pediatric Intensive Care Unit (PICU).[9-11] In the near future, pediatric sepsis researchers need to establish and validate new pediatric sepsis criteria.[12] It is important that (a derivative from) pediatric Sepsis-3 criteria are also applicable to emergency department patients. Apart

from uniformity for research and administrative purposes, proper criteria will aid in early recognition, early treatment, and a better outcome.[13] Notably, the 2016 NICE guideline on sepsis[14] relies on physiological parameters, thus potentially suitable for emergency department patients, but seems overly sensitive and would result in overtreatment.[15]

SIRS[a]

The presence of at least two of the following four criteria, **one of which must be abnormal temperature or leukocyte count:**

- Core[b] temperature of >38.5°C or <36°C.
- Tachycardia, defined as a mean heart rate >2 SD above normal for age in the absence of external stimulus, chronic drugs, or painful stimuli; or otherwise unexplained persistent elevation over a 0.5- to 4-hr time period **OR for children <1 yr old: bradycardia, defined as a mean heart rate <10th percentile for age in the absence of external vagal stimulus, β-blocker drugs, or congenital heart disease; or otherwise unexplained persistent depression over a 0.5-hr time period.**
- Mean respiratory rate >2 SD above normal for age or mechanical ventilation for an acute process not related to underlying neuromuscular disease or the receipt of general anesthesia.
- Leukocyte count elevated or depressed for age (not secondary to chemotherapy-induced leukopenia) or >10% immature neutrophils.

Infection

A suspected or proven (by positive culture, tissue stain, or polymerase chain reaction test) infection caused by any pathogen OR a clinical syndrome associated with a high probability of infection. Evidence of infection includes positive findings on clinical exam, imaging, or laboratory tests (e.g., white blood cells in a normally sterile body fluid, perforated viscus, chest radiograph consistent with pneumonia, petechial or purpuric rash, or purpura fulminans)

Sepsis

SIRS in the presence of or as a result of suspected or proven infection.

Severe sepsis

Sepsis plus one of the following: cardiovascular organ dysfunction OR acute respiratory distress syndrome OR two or more other organ dysfunctions. Organ dysfunctions are defined in Table 4.

Septic shock

Sepsis and cardiovascular organ dysfunction as defined in Table 4.

Modifications from the adult definitions are highlighted in boldface.

[a]See Table 3 for age-specific ranges for physiologic and laboratory variables; [b]core temperature must be measured by rectal, bladder, oral, or central catheter probe.

Table 3. Age-specific vital signs and laboratory variables (lower values for heart rate, leukocyte count, and systolic blood pressure are for the 5th and upper values for heart rate, respiration rate, or leukocyte count for the 95th percentile)

Age Group[a]	Heart Rate, Beats/Min[b,c]		Respiratory Rate, Breaths/Min[d]	Leukocyte Count, Leukocytes × 10^3/mm^3[b,c]	Systolic Blood Pressure, mm Hg[b,c,e,f]
	Tachycardia	Bradycardia			
0 days to 1 wk	>180	<100	>50	>34	<65
1 wk to 1 mo	>180	<100	>40	>19.5 or <5	<75
1 mo to 1 yr	>180	<90	>34	>17.5 or <5	<100
2–5 yrs	>140	NA	>22	>15.5 or <6	<94
6–12 yrs	>130	NA	>18	>13.5 or <4.5	<105
13 to <18 yrs	>110	NA	>14	>11 or <4.5	<117

Figure 7: 2005 international consensus conference definitions for pediatric sepsis and organ dysfunction.

(Reprint with permission from [1])

Sepsis and severe focal infections in Europe

In this thesis, we defined sepsis according to the 2005 international consensus conference definitions.[1]

An overview of sepsis and severe focal infections in Europe has been provided by the EUCLIDS consortium; of 2844 hospitalized children with life-threatening infections, 1229 children (43.2%) had sepsis and 1615 children (56.8%) had severe focal infections.[16] Pneumonia, central nervous system infection, and skin and soft tissue infection were the most common infectious syndromes, respectively. A causative organism was identified in only half of cases, which is comparable to pathogen detection rates from 30% to 65%

in other studies.[17-20] Pathogen detection by blood culture is complicated by early administration of antibiotics and by low circulating microbial loads.[21] Therefore, new diagnostics to improve pathogen detection and optimal antimicrobial therapy are needed.[22] Although the mortality rate of 2.2% in our cohort was relatively low, approximately a third of children required PICU admission.

We focused on 795 children admitted with community-acquired sepsis to European PICUs and studied risk factors for mortality and disability. Our main finding was that, although the relatively low mortality (6%) persisted in this PICU cohort, almost one third of sepsis survivors (31%) were discharged with disability, ranging from mild to severe.[23] Notably, of previously healthy children who survived sepsis, 24% survived with disability. Mortality and disability were independently associated with *Streptococcus pneumoniae* infections and illness severity.

The incidence in disability we found, was similar to incidence reported in two other studies; a decline in functional status was observed in 28% to 34% of pediatric sepsis survivors.[18, 24] Although one study reported impaired neuropsychological performance and impaired educational functioning post sepsis[25], the nature of disabilities has not been specified yet and remains a gap in knowledge.

Mortality due to sepsis decreased over the past decades, but this trend has stagnated in recent years.[17, 19] In the absence of therapeutic possibilities to control the dysregulated host response to sepsis, future efforts should focus on prevention and early recognition of sepsis. Early recognition could be achieved by identification of reliable biomarkers. The PERFORM study, successor of EUCLIDS, will study biomarkers which could be useful in early discrimination between viral and bacterial causes of infection, when patients present to the emergency department (ED) and treatment decisions are being made. A first set of two biomarkers resulting in a signature distinguishing viral versus bacterial infection in patients admitted with suspected severe infection was recently published.[22] Further results targeting patients at presentation in ED are expected in the following years. Prevention could be improved by continuously updating current immunization programs. In our PICU study, a bacterial infection was confirmed in 428 (54%) of all patients admitted with sepsis. *Neisseria meningitidis* was the most commonly identified pathogen, of which serogroup B was most prevalent, followed by *Streptococcus pneumoniae* of which serotypes 3 and 10A were most commonly identified. Recently, a menB vaccine (Bexsero®) has been licensed for active immunization against menB and this vaccine has been implemented in the Austrian, Czech Republic, Lithuanian and UK routine immunization schedules.[26] It had been anticipated that this vaccine would cover approximately 70% to 80% of MenB strains, depending on geographical region and age.[27, 28] Preliminary data report 93% vaccine uptake for two doses by 12 months of age.[29] Future studies should determine the impact

on disease and herd protection, but a significant drop is expected, as seen previously after introduction of MenC immunization, and other countries should include MenB immunization in their immunization schedules as well.[30, 31] Microbiological trends need to be monitored closely to keep immunization programs up to date. Because of increasing incidence of MenW cases (from 0,02 cases before 2015 to 0,5 cases per 100.000 in 2017, predominantly MenW belonging to clonal complex 11), a quadrivalent conjugate vaccination (MenACWY) has been implemented in The Netherlands in May 2018.[32] Four other European countries (Austria, Greece, Italy and UK) had already included MenACWY vaccination as part of the routine immunization schedule.[26] Monitoring of trends in pneumococcal disease is also warranted. After current immunization for pneumococcal disease has been proven effective in the decline of invasive pneumococcal infections, the incidence of non-vaccine serotypes has increased, suggesting serotype replacement.[33] Immunization against serotype 3, most commonly identified in our cohort, is included in Pneumococcal Conjugate Vaccine (PCV) 13, but not in PCV7 or PCV10. Protection against serotype 10A is possible via Pneumococcal Polysaccharide Vaccine (PPSV) 23, but this vaccine will not result in an immune response in children younger than 2 years of age.[34, 35]

We assessed short-term outcome of children with sepsis: mortality at PICU discharge and disability by the pediatric overall performance scale at discharge.[36] However, data from adults show also adverse outcome years after sepsis. One-year, 2-year and 5-year all-cause mortality among adults ≥45 years of age with sepsis were 23%, 28.8% and 43.8%, respectively, compared with death rates of 1%, 2.6% and 8.3% among participants who did not develop sepsis.[37] This association persisted even after adjustment for confounders, e.g. comorbidities. Also, patients with severe sepsis (community-acquired and hospital-acquired) had significantly poorer 1- to 10-year mortality rates (30.5%) compared with patients with non-septic critical illness (22.1%) and a general population (11.8%).[38] This finding persisted in patients without underlying comorbidities; mortality rates in severe sepsis patients without underlying comorbidities was higher compared with the other groups. Cohorts of severely ill children, e.g. caused by (meningococcal) septic shock, showed impaired long-term neuropsychological outcome and adverse physical outcomes (e.g. scars and orthopedic sequelae).[39-41] We hypothesize that long-term disability is also substantial in less severe cases of sepsis. Altogether, these findings highlight the need to include short-term as well as long-term outcome measures in future pediatric sepsis trials to identify potential interventional targets.[42]

Speculating on potential interventional targets, the focus should also include post-discharge management of sepsis survivors. For example, these children need to be checked up regularly at primary healthcare facilities. Healthcare use is elevated after severe sepsis[43], thus regular checkups aimed at physical and neuropsychological functioning could detect problems at an early stage. Also, rehabilitation programs could

improve (long-term) disability. Rehabilitation improves ICU-acquired weakness, that is characterized by impaired muscle strength and causes functional disability, which is also observed in sepsis survivors.[44]

Sepsis from a global perspective

We have reported data from high-income countries, but the burden of pediatric sepsis is global. Of the 6.3 million children who died in their first 5 years of life in 2013, more than half died due to infections. Pneumonia, diarrhoea, and malaria were the leading causes. [45]

The Sepsis Prevalence, Outcomes, and Therapies (SPROUT) study attempted to picture global epidemiology of severe sepsis.[18] Despite skewed enrollment of 83% of patients at sites in developed regions, this is an important study showing striking differences in severe sepsis point prevalence and mortality across regions, in favor of high-income countries (Europe: prevalence 6.2%, mortality 29%; Asia: prevalence 15.3%, mortality 40%; South America: prevalence 16.3%, mortality 11%; Africa: prevalence 23.1%, mortality 40%).

These data urges scientists and policy makers to collaborate in order to equalize pediatric sepsis outcome worldwide, as also highlighted by the WHO recently.[46] Prevention, in terms of optimal hygiene and immunization, and early recognition of sepsis are key elements for optimization.

Conclusions and recommendations for future studies:

1. The concept of sepsis shifts from "systemic inflammatory response syndrome in the presence of infection" to "a life-threatening condition that arises when the body's response to an infection injures its own tissues and organs".(Sepsis-3) These criteria need to be translated for use in children and should be validated and implemented in both PICU patients and emergency department patients.

2. Although mortality is relatively low, community-acquired pediatric sepsis continues to cause a devastating effect in high-income countries by high incidence of disability. Future pediatric sepsis trials need to include disability as outcome measure in order to delineate the nature of the disabilities and to study the add-on effect of sepsis when underlying conditions are already present. Furthermore, research efforts should focus on early recognition of sepsis.

3. The most common community-acquired invasive pathogens were meningococci -especially serogroup B (menB) - and pneumococci - especially serotypes 3 and 10A. MenB immunization should be added to current immunization programs of The Netherlands

and other countries, and pneumococcal immunizations should be updated to PCV13 to improve prevention.

4. In addition to short-term outcome, long-term outcome needs to be included in future pediatric sepsis trials to identify potential interventional targets and to shape post-discharge management.

5. Worldwide differences in severe sepsis prevalence and outcome need to be addressed. Prevention and early recognition of sepsis need to be optimized globally.

Host inflammatory response to infection

We studied neutrophil extracellular traps (NETs), human leukocyte antigen-DR (HLA-DR) expression on monocyte subsets, and IgG Fc glycosylation to explore the host inflammatory response to sepsis, to assess inter-individual variation, and to establish the prognostic value of these factors.

Neutrophils - neutrophil extracellular traps

We measured levels of NETs, using myeloperoxidase (MPO)-DNA, in 60 children with meningococcal sepsis, and studied the association with outcome.[47] We found that NETs levels were higher in the acute phase of disease, i.e. at admission to PICU and at 24 hours after admission, compared to 1 month after admission. We did not find an association between NETs, severity parameters, and outcome. Our findings indicate that the positive and negative effects of NETs, respectively containing meningococcal infection and inducing tissue damage and deregulate coagulation, might be balanced in our cohort exclusively including patients with central venous access in PICU. Thus, our data suggest that NETs could not be used as prognostic marker for outcome. However, the study group was relatively small and may have been underpowered to detect these effects.

The currently available literature on NETs or components of NETs in sepsis origin from adult studies or animal studies. Several adult studies demonstrated increased (derivatives of) NETs during infectious conditions: increased neutrophil elastase-DNA in patients with pneumonia and non-pulmonary sepsis compared with critically ill controls [48], increased plasma histone and cf-DNA levels in 17 sepsis patients compared to nonseptic critically ill patients [49], elevated cell free (cf)-DNA and MPO-DNA in patients with influenza A virus infection [50], and higher serum cf-DNA/NETs levels in 31 sepsis patients compared to healthy controls [51]. Most of these studies reported an association between NETs levels and illness severity or mortality.[48, 50, 51] Thus, in the early phase of infection in adults or animals, NETs are increased and, in contrary to findings from our study, seem to be associated with illness severity.

However, a major strength of our cohort is that our group is homogenous regarding pathogen, whereas other sepsis studies included a heterogeneous mixture of causative micro-organisms. Since the induction of NETs vary per pathogen, depending on size, expression of virulence factors, and evading mechanisms[52, 53], it is essential that future larger studies explore the prognostic value of NETs in homogenous cohorts like we did.

Neutrophil extracellular traps as crosslink between inflammation and coagulation

NETs are a key link between inflammation and coagulation; in a process named immunothrombosis.[54] During systemic inflammation in sepsis, NETs or their components may damage tissue and endothelia, which initiate the coagulation cascade. In addition, NETs stimulate platelet adhesion, which may account partly for the thrombocytopenia observed in sepsis.[55] NETs also promote various pro-coagulant and anti-fibrinolytic processes, such as fibrin clot formation, factor XII activation, and via histones, interaction with thrombomodulin-dependent protein C leading to increased thrombin generation.[53] Ideally, a balance in NETs is needed to prevent excess thrombin generation, while preserving adaptive hemostasis. Although NETs levels in our small cohort were not associated with hemostatic factors, future studies should focus on identifying patients at risk for excess thrombin and fibrin generation.[56] Once adequately identified, these high-risk patients may benefit from therapeutic approaches aiming to degrade NETs.[57, 58]

Monocytes - human leukocyte antigen-DR expression

In our study, we measured mHLA-DR on monocyte subsets by flow cytometry in 37 healthy children and 37 critically ill children (12 sepsis, 11 post-surgery, 10 trauma, and 4 admitted for other reasons) - participating in a randomized controlled trial on early versus late initiation of parenteral nutrition[59] - upon admission and on day 2, 3, and 4 of PICU stay.[60] Our study revealed that the percentage of HLA-DR expressing cells as well as the HLA-DR mean fluorescence intensity within all monocyte subsets in critically ill children was lower compared to controls, and decreased further during PICU stay. These findings were most pronounced for classical monocytes and in patients admitted for sepsis. In addition, low HLA-DR on classical monocytes on day 2 and day 3 after PICU admission was significantly associated with the occurrence of nosocomial infections and with mortality, respectively. Our study confirms previous findings of decreased mHLA-DR after critical illness, and that decreased mHLA-DR is associated with nosocomial infections and/or death.[61-63]

The reason why HLA-DR depressed patients are more susceptible to adverse events, is generally attributed to monocyte unresponsiveness after an initial pro-inflammatory phase.[64] Low mHLA-DR in patients with sepsis results in poor TNF-α response to LPS, and consequently the inability to induce an adequate pro-inflammatory response when necessary.[65] Thus, mHLA-DR seems a suitable marker for immunosuppression.

Several opportunities arise to apply this information in practice.[66] Apart from predicting mortality and secondary infections on the short term, as mentioned earlier, mHLA-DR could be used as predictor for long-term outcome. We only examined four time points and detected lowest mHLA-DR expression on day 4. But the declining trend

was still ongoing and the question when mHLA-DR restores remains unanswered. A study in 28 children undergoing elective cardiac surgery hinted at a restoration time of 7 days.[67] Nevertheless, long-term complications reported months to years after sepsis should be studied in relation to mHLA-DR. The attempts done so far were in small adult studies which did not find an association between long-term clinical complications and mHLA-DR alterations. A study in 8 long-term sepsis survivors reported recurrent infections for months to years after surviving sepsis and also infections caused by opportunistic infections.(37) However, laboratory analysis in that study showed no substantial differences in mHLA-DR expression between sepsis survivors and controls. Another study sampled 15 septic shock survivors 6 months after ICU discharge; restored and normal values of mHLA-DR were found for almost all patients.[68] At the PICU of Erasmus MC-Sophia, we currently undertake an observational study to describe mHLA-DR course in children suspected for sepsis, with a follow up of 28 days after admission to PICU.

In addition to a prognostic role, mHLA-DR could be used as stratification marker for immunomodulatory studies. The lack of stratification systems in previous sepsis studies resulted in heterogeneous groups of patients and is the main reason why numerous sepsis studies have negative results.[69] In the light of a two phased inflammatory response, i.e. inflammation or suppression, mHLA-DR could reflect an immunosuppressive state and could guide immune stimulatory therapy. In one adult study, mHLA-DR has been used to identify septic patients eligible for immunostimulatory therapy and to monitor the response of treatment. IFN-gamma was administered to septic patients with low mHLA-DR. These patients experienced restoration of the deficient mHLA-DR. Additionally, in-vitro study demonstrated that TNF-alpha secretion after LPS induction restored.[70] Another study demonstrated reversed monocyte deactivation via granulocyte–macrophage colony-stimulating factor (GM-CSF). Patients with sepsis-induced immunosuppression (n=38), defined by a cut-off value of mHLA-DR, were treated with either GM-CSF or placebo. Both randomization groups showed comparable baseline mHLA-DR levels, which significantly increased within 24 hours in the GM-CSF group. After GM-CSF treatment, mHLA-DR was normalized in 19/19 treated patients, whereas this occurred in only 3/19 control subjects. The use of GM-CSF may shorten the time of mechanical ventilation and hospital/intensive care unit stay, but was not associated with mortality in this small cohort.[71] Further immunomodulatory trials in adult sepsis are still ongoing, e.g. with interleukin-7, and results are soon to be expected.[72] Studies in children have been restricted to case-reports only.[73]

We also analyzed blood monocyte subset distribution in children with sepsis, post-surgery, and trauma, and we described a shift towards increased intermediate monocytes in sepsis patients compared to patients from the other two diagnostic categories. Expansion of intermediate and/or non-classical monocytes in infection or inflammatory conditions has

been described previously, corresponding to the assumption that these subsets are pro-inflammatory because of their ability to produce high amounts of TNF-α and IL-1β.[74] The shift towards CD16+ monocytes would then result in a pro-inflammatory response to contain the infection. These data suggest that shifts in monocyte subsets could help identifying sepsis patients at an early stage. However, since sepsis patients may develop symptoms days before admission to PICU, the shifts we reported may reflect a progressed course of disease and thus more differentiated monocytes compared to patients post-surgery or after trauma, who usually are admitted to PICU at short notice.

Additional alterations in the host inflammatory response to infection

Although mHLA-DR has been most extensively studied[75], sepsis-induced immunosuppression is reflected by a variety of markers in both innate and adaptive immune system.[64] (Figure 8)These markers are detectable early, within 48 hours of sepsis onset[76], and could be used to identify immunosuppressed patients who might benefit from immunomodulatory therapies. The role of programmed cell death-1 (PD-1) could be an interesting target for future studies. PD-1 is a key marker for T-cell exhaustion, reflecting restriction of activation, proliferation, and effector functions of immune cells. Excessive PD-1 expression has been observed in patients with sepsis, but this need to be validated in further studies.[64]

The host inflammatory response is also connected to the microbiota composition. Several environmental factors, such as feeding type, antibiotic exposure, and crowding conditions affect the respiratory microbiome composition, potentially affecting the risk, frequency and severity of subsequent respiratory infections.[77-79] Future studies are needed to specify the microbiota and to identify patients at risk.

B cells - Immunoglobulin G N-glycosylation

The property of glycans to modify immunoglobulin's structure and effector function, raises the question whether differences in IgG Fc glycosylation are present in meningococcal sepsis, and whether specific patterns are associated with outcome of disease. We found IgG1 Fc fucosylation to be lower in patients as compared to age-matched healthy controls. [80] Since young age is an important risk factor for disease susceptibility and severity, presumably due to an immature immune system, we compared young children to older children. [81, 82] A cut off age of 4 years was used for young children because IgG levels increase significantly by approximately 20% per year in the first years of life, whereas the increase is limited (approximately 2% per year) thereafter.[83] In young children, the IgG1 Fc fucosylation decrease was more pronounced. In addition, in patients below the age of 4 years, IgG1 Fc bisection was higher as compared to controls. An association with poor outcome, defined by death or need for amputation, was found in patients below the age of 4 years: patients with poor outcome had lower levels of IgG1 hybrid-type glycans

and IgG2/3 sialylation per galactose when admitted to the PICU, suggesting that these features could be a predictor for meningococcal sepsis outcome.

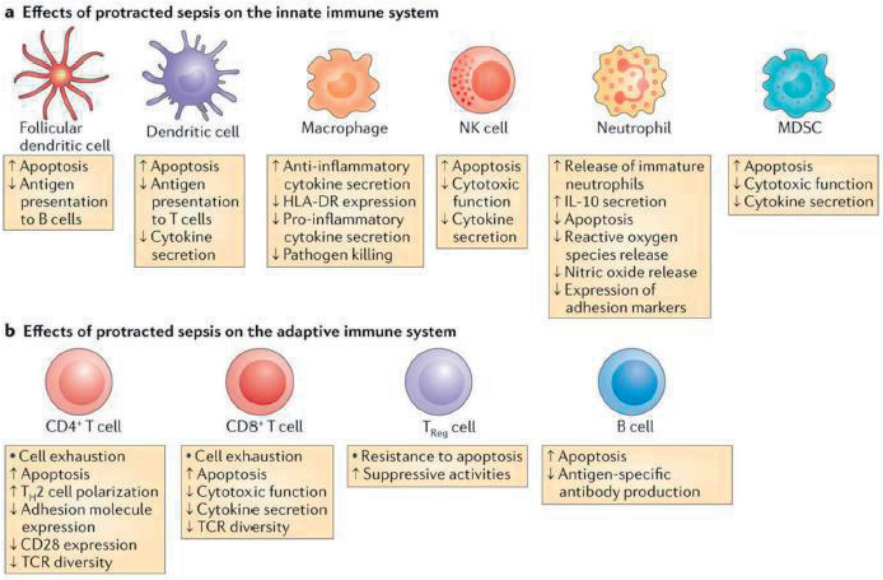

Figure 8: Sepsis-induced suppression of innate and adaptive immunity.
(Reprint with permission from [64])

a) Sepsis rapidly triggers extensive apoptosis in dendritic cells, monocytes and immature macrophages, NK cells, and MDSCs. Conversely, sepsis delays neutrophil apoptosis, a result thought to be secondary to the mechanisms of neutrophil activation. After initial mobilization and activation of neutrophils, subsequent neutrophils that are released from bone marrow have lower bactericidal functions and decreased cytokine production. Recent data show that a subset of neutrophils release large amounts of the immunosuppressive cytokine IL-10.
b) Sepsis causes massive loss of CD4+ and CD8+ T cells as well as B cells. TReg cells are more resistant to sepsis-induced apoptosis and consequently, there is an increased percentage of TReg cells in the circulation relative to the other lymphocyte subsets. This contributes on a more immunosuppressive phenotype. Surviving CD4+ and CD8+ T cells have either a shift from a pro-inflammatory Th1 cell to an anti-inflammatory Th2 cell phenotype or develop an "exhaustive" phenotype characterized by increased programmed cell death-1 expression and reduced cytokine secretion. CD4+ T cells have decreased expression of CD28 and reduced TCR diversity, which both likely contributing to the impaired anti-microbial response to invading pathogens.

Abbreviations: IL-10 = interleukin-10, MDSCs = myeloid derived suppressor cells, NK = natural killer, TCR = T cell receptor, TReg = T regulatory.

The deviations we found, may be induced by the meningococcal infection itself or were already present in the children before the meningococcal infection occurred. We speculate that the low IgG fucosylation observed in young meningococcal patients may reflect a history of (viral) infections, or in a broader sense antigenic stimuli, resulting in the build-up of low fucosylation IgG responses against the respective antigens which manifests itself in a shift of the total IgG pool towards lower fucosylation.[84] Hence, the low fucosylation may be a marker of a short-term history of infections rather than a result from meningococcal infection. However, in both situations, the IgG Fc glycosylation features have the potential to be used to predict meningococcal sepsis outcome in very young patients, which should be validated in larger study populations.

Antigen-specific IgG Fc glycosylation was previously shown to differ substantially from the glycosylation of the total pool of IgG.[85] Although one study described changes of glycans during sepsis[86], the role of glycans on IgG specifically has not been studied in sepsis. Other studies in inflammatory conditions show that IgG glycosylation is able to change fast as shown in patients experiencing acute systemic inflammation after cardiac surgery, where part of the patients showed an increased antibody galactosylation the first day after surgery.[87] These findings support our hypothesis that the features in meningococcal sepsis may be induced by the meningococcal infection. For specific IgG to meningococcal outer membrane vesicle (OMV) antigens, obtained after OMV vaccination, no change in fucosylation was seen over time.[88] However, no comparison was made between total IgG before and after vaccination and glycosylation changes in antigen-specific IgG might be substantially different between vaccinated and naturally infected individuals. Furthermore, low IgG1 Fc fucosylation enhances binding of IgG to FcγRIIIa and FcγRIIIb, thereby increasing antibody-dependent cellular cytotoxicity (ADCC).[89, 90]

Conclusions and recommendations for future studies:

1. Children admitted to PICU for meningococcal sepsis have higher NETs levels in the acute phase of infection compared to controls, but NETs levels are not associated with outcome. Future, larger, well powered studies in other homogenous cohorts could study associations with inflammatory and hemostatic factors, and might elucidate the role of NETs as prognostic marker.

2. In critically ill children, HLA-DR expression on all monocyte subsets decreased the first four days of PICU stay and was lower compared to controls on all examined time points, especially on classical monocytes and in children admitted for sepsis. Low HLA-DR expression on classical monocytes was associated with nosocomial infections and death. We are currently validating these findings in a larger cohort of children, including

subgroups of different clinical infectious syndromes and different pathogens. Also, mHLA-DR expression should be monitored for a longer period of time to detect restoration.

3. Monocyte subsets might have specific functions and alterations in monocyte subset distribution could be disease-specific. Although we observed an increase in intermediate monocytes in sepsis patients, it remains unclear whether we could use this shift in early detection of sepsis. This finding could be a reflection of a more progressed course of disease rather than a direct effect of a septic state.

4. Monocytic HLA-DR potentially is an adequate marker for sepsis-induced immunosuppression, and should be used as inclusion marker for immunomodulatory trials and could be included in routine clinical measurements.

5. In meningococcal sepsis, IgG1 low fucosylation and high bisection were found, especially below the age of four years. Further research is needed to determine whether the observed features are a result of the meningococcal infection itself or rather a marker of a short-term history of viral infections.

6. IgG Fc glycosylation changes (decreased levels of IgG1 hybrid-type glycans and IgG2/3 sialylation) associated with illness severity and have the potential to be used as outcome predictors, which should be validated in larger study populations.

Hemostatic response to infection

We reviewed genetic polymorphisms in the protein C and fibrinolytic pathways associated with severity of sepsis. Also, based on the association between outcome and polymorphisms in genes encoding for A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS)-1 and ADAMTS-18, we studied the association between protein levels and outcome. Lastly, we explored whether circadian variation could be a relevant determinant of pediatric sepsis outcome.

Genetic polymorphisms in sepsis

Genetic polymorphisms partly determine inter-individual variety in the host response to infection and have been associated with susceptibility and severity of sepsis.[91, 92] The EUCLIDS consortium genome-wide association study (GWAS), including approximately 1500 meningococcal disease patients, reported an association between polymorphisms in the *CFH* region, which play a role in complement activation, and susceptibility.[93] In adults with sepsis due to pneumonia, a GWAS revealed *FER*, which encodes a cytosolic non-receptor tyrosine kinase that influences neutrophil chemotaxis and endothelial permeability, to be associated with a reduced risk of death.[94] Although genetic variations have the potential to affect the host response to an infectious challenge, genetic findings have not been translated to clinical practice. Future research efforts should confirm the relevance of genetic variations in functional studies and study their potential as prognostic marker or response to specific therapies. Theoretically, the genetic profile of each person could be known, which could be helpful in estimating the probability of the development of sepsis and subsequently in early identification of patients.

Genetic polymorphisms in the protein C and fibrinolytic pathway

We reviewed gene variations in the PC and fibrinolytic pathway, which have been associated with severity of pediatric sepsis. The high PAI-1 producer *SERPINE1* 4G/4G genotype is most significantly associated with sepsis mortality. Also, the *CPB2* Thr325Ile polymorphism seems predictive of disseminated intravascular coagulation. However, effects of the *CPB2* Thr325Ile polymorphism on mortality have been reported for adult sepsis patients only. Several studies associated protein plasma levels with genetic polymorphisms or with outcome of sepsis (for instance Thrombomodulin, PC, FXIII, and Plasminogen activators), but future GWAS should reveal whether genes encoding these proteins are relevant in the context of severity of sepsis.

Based on adult studies and other studies in pediatric thromboembolic disorders, we hypothesize that *F2* G20210A or A19911G polymorphisms, which both increase prothrombin concentration, may contribute to adverse outcome in pediatric sepsis. Also interesting to study in pediatric sepsis are haplotypes in *PROCR* leading to higher sEPCR,

and for PN-1 encoding *SERPINE2* gene, which might be an underestimated regulator of fibrinolysis.

Thus, genetic polymorphisms or combination of polymorphisms in hemostatic genes may increase patients risk for a more fulminant course of sepsis. Identifying these polymorphisms, especially when translated protein levels are associated with outcome, may result in closer monitoring and alternative treatment modalities for sepsis (e.g. direct intervention on altered protein levels). Additionally, future studies should take possible variations between different pathogens into account.

Genetic polymorphisms in A Disintegrin and metalloproteinase with thrombospondin motifs

Unpublished data from severity analysis of the EUCLIDS consortium identified a SNP in ADAMTS-1 (rs9975310, uncorrected p-value 0.0003) to be associated with skin graft or amputation, and a SNP in ADAMTS-18 (rs149698955, uncorrected p-value 0.0006) with death. Based on these preliminary genetic findings, we measured ADAMTS-1 and ADAMTS-18 protein levels at 3 time points (at admission to PICU, after 24 hours and after 1 month) in a retrospective cohort of meningococcal sepsis patients. Samples for ADAMTS-1 and ADAMTS-18 measurement was available for 61 patients and 37 patients respectively. We found that high ADAMTS-1 levels were correlated with pro-inflammatory markers and illness severity, and ADAMTS-1 levels in non-survivors were significantly higher than in survivors.

These findings correspond to findings from animal studies: plasma ADAMTS-1 levels were remarkably higher in rats after injection with *Escherichia coli* lipopolysaccharides compared to rats from the control group.[95] Also, ADAMTS-1 was positively correlated to IL-1beta in experimental studies.[96] Overall, our findings indicate that ADAMTS-1 levels could be of prognostic value in pediatric meningococcal sepsis. The mechanism of ADAMTS-1 elevation in meningococcal sepsis is a matter of debate. A relevant interaction between ADAMTS-1 and vascular endothelial growth factor (VEGF) might be present. In severe sepsis, VEGF levels are increased and mortality is associated with low VEGF levels.[97-100] ADAMTS-1 binds VEGF[101], thereby suppressing VEGF signaling which could inhibit endothelial survival and increase apoptosis, which in turn may contribute to the development of sepsis-induced organ dysfunction.[98]

ADAMTS-18 inhibits platelet activation.[102] In mice, antibodies against ADAMTS-18 resulted in a reduced bleeding time in the tail vein. In addition, ADAMTS-18 knock-out mice have a lower blood flow and a faster thrombus formation.[103, 104] ADAMTS-18 levels did not differ significantly between the three time points in our cohort of meningococcal sepsis. Also, ADAMTS-18 level was not associated with illness severity,

coagulation markers, or inflammation markers. This could partly be explained by a small number of samples and needs to be explored in future studies.

The identification of genetic polymorphisms associated with severity, from GWAS studies in large cohorts, should be followed by protein measurements in order to study the association between protein level and outcome. Currently, our consortium undertakes a study to measure a set of proteins/markers (including ADAMTS-1 and ADAMTS-18, but also ADAMTS-13, vWF, Fibronectin, PAI-1, protein C, protein S, Thrombomodulin, and soluble PD-1) based on the association between genetic polymorphisms in encoding genes and severity of disease. The results are soon to be expected and will report associations with illness severity, differences between pathogens, and provide more insight into the pathophysiology of sepsis.

Plasminogen activator inhibitor-1 circadian variation as determinant in sepsis outcome

Clustering of fatal cases in the morning hours has been reported repeatedly.[105, 106] These findings are generally interpreted as the result of delayed detection of symptoms and subsequent health care seeking behavior, as signs of severe illness may easily be overlooked in the night or early morning hours. However, we explored whether circadian variation could be a determinant in outcome of sepsis.

This exploration is based on studies showing that circadian variation in PAI-1 level leads to a PAI-1 peak in the morning.[107, 108] Since increased PAI-1 levels are associated with poor outcome[109, 110], we hypothesized that circadian variation of PAI-1 is associated with excess mortality of cases presenting in the morning hours, possibly due to aggravation of multiple organ failure secondary to more severe DIC.

Indeed, our results in children with meningococcal sepsis show that patients admitted to the PICU in the morning have higher PAI-1 levels on admission compared to patients admitted at night or in the afternoon.[111] Also, patients admitted to the PICU in the morning have a worse outcome than patients admitted during the rest of the day. However, it remains undetermined whether PAI-1 is a marker for illness severity[109, 112-115] or a contributor to illness severity.[116]

Although multiple factors could have influenced increased morning severity, especially a possible delay in detection of symptoms during the night and early morning, morning PAI-1 peak levels might have contributed to this effect. Because coagulation disturbances in both gram-negative and gram-positive infections have been described, we would expect similar findings in sepsis caused by other micro-organisms.[117] Also, circadian variation in the inflammatory response has been described in experimental studies, suggesting

that the quality of inflammatory response differs during the day.[118, 119] Thus, the timing of the day may be an underestimated factor in the host response to infection which requires further study.

Conclusions and recommendations for future studies:

1. Future research efforts should focus on translating genetic findings into functional studies, in order to use these data for the prediction of disease outcome and to guide the development of novel therapies.

2. The hemostatic responses to infection vary widely between individuals, and are in part explained by genetic polymorphisms in hemostatic genes. Identifying these polymorphisms and their association with outcome, may allow detection of patients at risk for poor outcome. Also, possible variations between different pathogens need to be taken into account.

3. ADAMTS-1 is associated with illness severity in meningococcal sepsis patients, with higher ADAMTS-1 levels in non-survivors than in survivors. ADAMTS-18 levels are not associated with survival. Future studies should confirm the prognostic value of high ADAMTS-1 in sepsis and the associated pathophysiologic mechanisms.

4. We described an association between circadian variation of PAI-1 levels and illness severity in pediatric meningococcal sepsis patients, with a significant peak level in the morning. Future study in a larger cohort of patients should focus on the question whether the morning PAI-1 peak is an independent risk factor for morbidity and mortality in meningococcal sepsis.

Strength-weakness analysis of this thesis and suggestions for future studies

Of the studies included in this thesis, the studies on behalf of the EUCLIDS consortium (chapter 2) are prospective, multicenter, cohort studies. The main strength is that we were able to describe the largest prospectively enrolled cohort of children with community-acquired infections in high-income countries so far. In addition, because we only enrolled children with community-acquired infections, in contrary to previous studies which included health care-associated infections as well, our results have implications for policy makers in public health, e.g. to optimize prevention strategies. Lastly, we included disability as outcome measure, whereas previous studies usually only reported mortality. Therefore, our study depicts a more complete picture of the impact of pediatric sepsis. However, there might be an enrollment bias in our cohort of community-acquired sepsis in favor of invasive meningococcal, pneumococcal, staphylococcal, and group A streptococcal infections, because the primary aim of our consortium was to identify genes associated with susceptibility and severity of infections caused by these organisms. Subsequently, due to the genetic basis of this study, a bias towards enrollment of previously healthy children might have occurred. Finally, consistent with an international consortium, multiple centers from multiple countries are involved, representing different epidemiological context, healthcare structures, and case-mix.

Four studies from this thesis were based on data and samples from Erasmus MC-Sophia Children's Hospital based historic meningococcal sepsis cohorts. We studied markers or proteins in a - for meningococcal sepsis large - group of children. Furthermore, we had a homogenous group of patients which is, especially in the context of a complex host response to sepsis, important in reducing variation between study subjects. Because we had samples from multiple time points, we could study alterations during PICU stay. Another advantage of this historic cohort was that multiple clinical and laboratory data have been collected during the years, allowing us to study the relation to other coagulation markers and inflammation markers, and thereby reporting a more complete picture of the host response. Limitations of this cohort are that the samples used are relatively old (since 1988), and there is an unknown effect of time on the stored samples. However, if samples are affected, we assume that all samples would be affected equally. The ELISA kit to measure ADAMTS-1 lacked sensitivity. The kit had a detection range of 1.56 ng/mL and in more than half of our samples, ADAMTS-1 level was below the detection range.

Our HLA-DR study is the first study that examined HLA-DR expression on all three major blood monocyte subsets in a population of critically ill children, allowing us to compare children admitted for sepsis, post-surgery, and after trauma. Limitations from this study are that we included a small number of patients and controls. Therefore we were unable

to statistically analyze alterations during PICU stay. We could not observe restoration of mHLA-DR because we only examined four time points. Lastly, we determined the proportion of HLA-DR expressing monocytes - which has a potential of inter-observer variability - as well as the HLA-DR MFI on monocytes - that may depend on the type of flow cytometer and instrument settings used - making comparison between different laboratories troublesome. This highlights the need for more standardized measurements.

Following this strength-weakness analysis, future studies in pediatric sepsis trials should ideally be designed as prospective, multicenter study, including centers from resource-limited countries. The aim should be to recruit a large cohort of children, resulting in large cohorts homogenous in clinical presentations and pathogens. Future collaboration with the EUCLIDS consortium could be interesting since this consortium includes many study centers and has already collected many samples eligible to address (a part of) new objectives. Before inclusion of patients, the definition of sepsis needs to be clarified (sepsis-3 criteria should be translated into children). Eligible patients should be children admitted to PICU with sepsis, both community-acquired and hospital-acquired. Exclusion of patients with underlying conditions would not be proposed, since increasing proportions of affected patients have underlying conditions. However, analysis should include adjustment for underlying conditions. An adequately sized healthy age-sex-matched control population needs to be formed.

The search for inflammatory and hemostatic prognostic markers reliably reflecting the host response to sepsis needs to continue. Blood for research purposes need to be drawn as soon as possible after admission to PICU. If specific markers could be measured in urine or saliva, this would be preferable because of the less invasive procedure, which could be helpful especially in young children. Time points should be defined and sampling and processing of samples should be done according to Good Clinical Laboratory Practice. The time to freezer should be restricted as much as possible. Sample handling will be a major challenge in multicenter studies, but SOPs should ensure uniformity between centers. For measurements, collaboration with specialists in the field is needed to make use of the newest laboratory techniques. For example, quantitative measurement of HLA-DR (via anti-HLA-DR antibodies) will reduce inter-observer variability. Also, by using multiplex assays for protein measurements, limited blood volume is needed to analyze multiple proteins/markers in immunology and hemostasis. Studying *delta* levels of these markers could reduce the bias of pre-existing differences between individuals and could better reflect the impact of the respective marker in the host response to sepsis.

Regarding outcome measures: both short-term and long-term outcome (e.g. physical condition, neuropsychological functioning, quality of life, patient-reported outcome) should be included. *Delta* disability should be included as outcome measure of pediatric sepsis in an attempt to reduce bias due to pre-existing illness. Disability at study enrollment needs to be assessed and also at visits to the outpatient clinic (e.g. 6 and 12 months after admission to PICU). Blood samples at the outpatient clinic, if consent obtained, could relate laboratory markers to long-term outcome.

Final conclusions

Community-acquired pediatric sepsis continues to cause a devastating effect in high-income countries, mainly by high incidence of disability. The nature of disabilities needs to be delineated and should help in shaping post-discharge programs. Also, long-term outcome studies need to be undertaken to measure the full impact of sepsis; not only in the most severely ill patients, but in all pediatric sepsis survivors.

This thesis focused on determinants of pediatric sepsis outcome. We studied whether specific inflammatory, hemostatic, genetic, and environmental factors are associated with the severity of sepsis. We aimed to identify reliable prognostic markers in order to detect patients at risk of poor outcome at an early stage.

Based on the studies in this thesis, mHLA-DR, IgG Fc glycosylation changes, and ADAMTS-1 have the potential to serve as prognostic marker. NETs would not be suitable as prognostic marker. The importance of multiple factors, including genetic variation and circadian variation, contributing to the outcome of sepsis are emphasized. Eventually, a combined set of markers, each of them reflecting different parts of the host response to sepsis, and each of them easily available in blood, urine or saliva, would be ideally to depict the real-time state of the host response and to indicate outcome.

For the near future, early recognition, antibiotic treatment, and supportive care will remain the cornerstone of sepsis treatment. However, the complex host response to infection depending on host-pathogen-environmental factors, and involving interplays between inflammatory and hemostatic cascades, makes a successful *one size fits all* therapeutic approach unlikely. Exciting developments may arise in the next decade, for example immunomodulatory therapy (guided by mHLA-DR) or novel therapies based on unbiased approaches to genetic data (as shown by our study on ADAMTS-1).

Altogether, understanding, monitoring and controlling of the host response to sepsis should result in individualization of sepsis treatment and subsequently improved outcome.

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Appendices

SUMMARY

Bacterial infections are one of the leading causes of death and disability among children. The host response to infection involves complex interplays between inflammation, coagulation, and fibrinolysis. In meningococcal sepsis, the inflammatory response induce excessive coagulation and downregulation of fibrinolysis, contributing to the need to amputate extremities, multiple organ dysfunction, and eventually death. In this thesis, we characterized children with severe bacterial infections and we studied the following six determinants in relation to outcome: neutrophil extracellular traps (NETs), human leukocyte antigen-DR (HLA-DR) expression on monocyte subsets, glycosylation of the fragment crystallizable (Fc) region of immunoglobulin G (IgG), A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS)-1, ADAMTS-18, and plasminogen-activator-inhibitor-1 (PAI-1). In addition, we explored the role of genetic variation within individuals in the host response to infection.

Chapter 2 characterizes children with life-threatening infections, recruited through the multinational prospective European Childhood Life-threatening Infectious Disease Study (EUCLIDS).

Chapter 2.1 aimed to understand the burden of disease and outcomes in children with infections in Europe. A total of 2844 patients were recruited and analyzed. The disease burden is mainly in children younger than 5 years of age and is largely due to vaccine-preventable meningococcal and pneumococcal infections. Interestingly, the causative organism remained unidentified in approximately 50% of patients. Mortality in children admitted to European hospitals for sepsis or severe focal infections is low: 2.2% deaths occurred. Almost 40% of patients required admission to a pediatric intensive care unit (PICU).

Chapter 2.2 focused on 795 children admitted to European PICUs with community-acquired sepsis, and studied risk factors for mortality and disability. The three most common clinical presentations were sepsis without a source, meningitis/encephalitis, and pneumonia. Of patients with confirmed bacterial infection, *Neisseria meningitidis* and *Streptococcus pneumoniae* were the main pathogens. We showed that invasive bacterial disease remains responsible for ongoing mortality and morbidity in high-income countries. Almost one third of sepsis survivors were discharged with disability, including a large proportion of previously healthy children. Independent risk factors for mortality and disability were *Streptococcus pneumoniae* infections and illness severity.

Chapter 3 studies the immune response to critical illness, with emphasis on the inflammatory response in children with sepsis.

Chapter 3.1 investigates the role of NETs in pediatric meningococcal sepsis. We measured myeloperoxidase (MPO)-DNA, a marker for NETs, in serum of meningococcal sepsis patients upon admission to PICU, at 24 hours, and at 1 month and studied the association with clinical outcome. We found that children admitted to PICU for meningococcal sepsis have higher NETs levels at admission and at 24 hours than at 1 month. By confocal microscopy live imaging, we found that *Neisseria meningitidis* were fast and strong inducers of NETosis. NETs levels were not associated with outcome, cell-free DNA or other inflammatory markers.

Chapter 3.2 measured blood monocyte subset distribution and HLA-DR expression on monocyte subsets in children with sepsis, post-surgery, and trauma in relation to nosocomial infections and mortality. Upon PICU admission, critically ill children had a higher proportion of classical monocytes (CD14++CD16-) than healthy children. Monocytic HLA-DR expression decreased during PICU stay and was lower compared to controls on all examined time points, especially on classical monocytes and in children admitted for sepsis. Patients who developed nosocomial infections or died had lower HLA-DR expression on classical monocytes at day 2 and day 3, respectively.

Chapter 3.3 explored whether the glycan structure attached to the IgG Fc region, which influences the effector functions, might be indicative for susceptibility and/or severity to meningococcal sepsis. We studied differences in IgG Fc glycosylation between 60 pediatric meningococcal sepsis patients admitted to PICU and 46 age-matched healthy controls, employing liquid chromatography with mass spectrometric detection of tryptic IgG glycopeptides. Meningococcal sepsis patients under the age of 4 years showed lower IgG1 fucosylation and higher IgG1 bisection than age-matched healthy controls. This might be a direct effect of the disease; however, it could also be a reflection of previous immunologic challenges and/or a higher susceptibility of these children to develop meningococcal sepsis. Within the young patient group, levels of IgG1 hybrid-type glycans and IgG2/3 sialylation per galactose were associated with illness severity and severe outcome, suggesting that these glycosylation features might have the potential to serve as a disease outcome predictor.

Chapter 4 focuses on the deregulation of coagulation and fibrinolysis in children with sepsis.

Chapter 4.1 elaborates on the variation between individuals in hemostatic responses to infection, which are in part explained by genetic polymorphisms. Our review gave an overview of genetic polymorphisms in the protein C and fibrinolytic pathway associated

with susceptibility and severity of pediatric sepsis. In addition, genetic polymorphisms associated with adult sepsis and other pediatric thromboembolic disorders were discussed, as these polymorphisms might be candidates for future molecular genetic research in pediatric sepsis.

Chapter 4.2 translates genetic findings into a functional study. Genetic studies from our consortium identified SNPs in ADAMTS-1 and ADAMTS-18 to be associated with outcome. We studied ADAMTS-1 and ADAMTS-18 protein levels in pediatric meningococcal sepsis, and studied the association with mortality. We found that serum ADAMTS-1 expression is associated with death in meningococcal sepsis patients, with higher ADAMTS-1 levels in non-survivors than in survivors. ADAMTS-18 protein levels were not associated with survival.

Chapter 4.3 studies the circadian variation of PAI-1 levels in children with meningococcal sepsis. Our retrospective analysis revealed that patients admitted between 6:00 am and 12:00 am had increased illness severity scores and higher PAI-1 levels compared to patients admitted at night or in the afternoon. In a larger group, analysis of circadian variation in relation to outcome showed more deaths, amputations and need for skin grafts in patients admitted to the PICU between 6:00 am and 12:00 am than patients admitted during the rest of the day. Thus, circadian variation of PAI-1 levels was present in children with meningococcal sepsis and was associated with illness severity, with a PAI-1 peak level in the morning.

Chapter 5 provides an overview of our main findings, a general interpretation of these findings, and the implications of our results. In addition, we discuss strategies for future research. This thesis shows that community-acquired sepsis continues to cause a devastating effect in high-income countries, mainly by high incidence of disability. Furthermore, this thesis provides considerable insight into the host response to sepsis by studying specific inflammatory, hemostatic, genetic, and environmental factors in relation to outcome. These determinants could help to detect patients at risk for poor outcome at an early stage, and have given us new directions to individualize sepsis treatment.

DUTCH SUMMARY (NEDERLANDSE SAMENVATTING)

Bacteriële infecties zijn één van de belangrijkste oorzaken van sterfte en invaliditeit bij kinderen. De reactie van het lichaam op infectie bestaat uit een complex samenspel tussen inflammatie, coagulatie en fibrinolyse. In het geval van meningococcen sepsis kan de inflammatoire reactie leiden tot buitensporige coagulatie en remming van fibrinolyse, hetgeen bijdraagt aan de noodzaak tot amputaties, multi-orgaanfalen en sterfte. In dit proefschrift hebben we kinderen met ernstige bacteriële infecties nader omschreven en hebben we de volgende zes determinanten bestudeerd in relatie tot uitkomst: *neutrophil extracellular traps (NETs), human leukocyte antigen-DR (HLA-DR)* expressie op subsets van monocyten, glycosylering van de *fragment crystallizable (Fc)* regio van immunoglobuline G (IgG)*, A Disintegrin and Metalloproteinase with Thrombospondin motifs* (ADAMTS)-1, ADAMTS-18 en *plasminogen-activator-inhibitor-1* (PAI-1). Daarnaast hebben we genetische variatie in de afweer tegen infecties onderzocht.

Hoofdstuk 2 beschrijft kinderen met ernstige bacteriële infecties, geïncludeerd via de prospectieve, internationale *European Childhood Life-threatening Infectious Disease Study (EUCLIDS).*

Hoofdstuk 2.1 had als doel om meer inzicht te krijgen in ernstige bacteriële infecties in Europa. In totaal werden 2844 kinderen geïncludeerd en geanalyseerd. De ziektelast ligt voornamelijk bij kinderen jonger dan 5 jaar en is grotendeels te wijten aan door vaccins te voorkomen meningokokken- en pneumokokkeninfecties. Echter, bij ongeveer 50% van de patiënten kon geen verwekker worden geïdentificeerd. Sterfte onder kinderen die in Europese ziekenhuizen worden opgenomen wegens sepsis of ernstige focale infecties is laag, namelijk 2.2%. Bijna 40% van de patiënten hadden behandeling nodig op een kinder intensive care (kinder-IC).

Hoofdstuk 2.2 richtte zich op 795 kinderen die op de kinder-IC waren opgenomen vanwege community-acquired sepsis en bestudeerde risicofactoren voor sterfte en invaliditeit. De drie meest voorkomende ziektebeelden waren sepsis zonder focus, meningitis/encefalitis en pneumonie. Van de patiënten met bewezen bacteriële infectie waren *Neisseria meningitidis* en *Streptococcus pneumoniae* de vaakst voorkomende pathogenen. We hebben aangetoond dat invasieve bacteriële infecties in Westerse landen nog steeds zorgen voor sterfte en invaliditeit. Bijna een derde van de kinderen die sepsis overleefden werd ontslagen met invaliditeit, waaronder een groot deel van kinderen die voorheen gezond waren. Risicofactoren voor sterfte en invaliditeit waren *Streptococcus pneumoniae* infecties en de ernst van de ziekte.

Hoofdstuk 3 bestudeert de immuunrespons tijdens kritiek zieke toestand, met nadruk op de inflammatoire respons bij kinderen met sepsis.

Hoofdstuk 3.1 onderzoekt de rol van NETs in meningococcen sepsis. We hebben myeloperoxidase (MPO)-DNA, een marker voor NETs, gemeten in serum van meningococcen sepsis patiënten bij opname op de kinder-IC, na 24 uur en na 1 maand en bestudeerden de associatie met uitkomst. We vonden dat kinderen bij opname op de kinder-IC en na 24 uur hogere NETs waarden hadden dan na 1 maand. Met behulp van confocale microscopie zagen we dat *Neisseria meningitidis* snelle en sterke inducers van NETosis waren. De hoeveelheid NETs was niet geassocieerd met uitkomst of andere inflammatoire markers.

In **hoofdstuk 3.2** werd de verdeling van monocyten subsets en HLA-DR expressie op deze subsets bestudeerd in kritiek zieke kinderen (kinderen met sepsis, kinderen na operatie en na trauma). Deze data werd gerelateerd tot het ontstaan van nosocomiale infecties en sterfte. Bij opname op de kinder-IC hadden kritiek zieke kinderen een hoger percentage klassieke monocyten (CD14 ++ CD16-) dan gezonde kinderen. HLA-DR expressie nam tijdens opname op de kinder-IC af en was op alle onderzochte tijdstippen lager dan bij controle patiënten, maar vooral op klassieke monocyten en in kinderen met sepsis. Patiënten die nosocomiale infecties ontwikkelden of overleden hadden op dag 2 en dag 3 na opname op de kinder-IC een lagere HLA-DR expressie op klassieke monocyten dan kinderen die geen infectie ontwikkelden of de ziekte overleefden.

In **hoofdstuk 3.3** werd onderzocht of de glycaan structuur, welke verbonden is aan het Fc -gebied van IgG en effectorfuncties beïnvloedt, indicatief kan zijn voor vatbaarheid en/ of ernst van meningococcen sepsis. We bestudeerden verschillen in IgG Fc-glycosylering tussen 60 kinderen opgenomen op de kinder-IC met meningococcen sepsis en 46 gezonde controles. Hierbij werd gebruik gemaakt van massaspectrometrische detectie van IgG-glycopeptiden. Meningococcen sepsis patiënten jonger dan 4 jaar hadden lagere IgG1-fucosylering en hogere IgG1-bisectie dan controles. Dit zou een direct effect van de ziekte kunnen zijn, maar het zou ook een weerspiegeling kunnen zijn van eerdere immunologische prikkels of een teken van verhoogde vatbaarheid voor meningococcen sepsis. In kinderen jonger dan 4 jaar waren waarden van IgG1-hybride glycanen en IgG2/3-sialylatie per galactose geassocieerd met ernst van de ziekte en ernstige uitkomst, wat suggereert dat deze glycosylering kenmerken als mogelijke voorspellers voor uitkomst zouden kunnen dienen.

Hoofdstuk 4 richt zich op de ontregeling van coagulatie en fibrinolyse in kinderen met sepsis.

Hoofdstuk 4.1 gaat in op het verschil in hemostatische reactie tussen individuen, wat deels wordt verklaard door genetische polymorfismen. Onze review gaf een overzicht van genetische polymorfismen (in de proteïne C en fibrinolyse cascades) die geassocieerd zijn met vatbaarheid en ernst van sepsis. Bovendien werden genetische polymorfismen die zijn onderzocht in volwassenen met sepsis en in kinderen met trombo-embolische aandoeningen besproken, omdat deze polymorfismen mogelijke kandidaten zijn voor toekomstig genetisch onderzoek in kinderen met sepsis.

Hoofdstuk 4.2 vertaalt genetische bevindingen in een functioneel onderzoek. In genetische studies van ons consortium bleken SNP's in ADAMTS-1 en ADAMTS-18 geassocieerd met negatieve uitkomst. We hebben de hoeveelheid ADAMTS-1 en ADAMTS-18 eiwit gemeten in kinderen met meningococcen sepsis en bestudeerden de associatie met sterfte. We vonden dat ADAMTS-1 geassocieerd is met sterfte, waarbij hogere waarden van ADAMTS-1 gemeten werd in kinderen die stierven dan kinderen die de ziekte overleefden. ADAMTS-18 was niet geassocieerd met overleving.

Hoofdstuk 4.3 bestudeert de circadiane variatie van PAI-1 in kinderen met meningococcen sepsis. Onze retrospectieve analyse bracht aan het licht dat patiënten die tussen 6:00 en 12:00 uur werden opgenomen op de kinder-IC, ernstiger ziek waren en hogere PAI-1 waarden hadden in vergelijking met patiënten die 's nachts of 's middags werden opgenomen. In een grotere groep bleek een associatie tussen circadiane variatie en uitkomst te bestaan; onder kinderen die tussen 06:00 en 12:00 uur waren opgenomen op de kinder-IC kwamen meer sterfgevallen, amputaties en huidtransplantaties voor dan in patiënten die gedurende de rest van de dag werden opgenomen. Dus, een circadiane variatie van PAI-1 was aanwezig in kinderen met meningococcen sepsis (piek in de ochtend) en was geassocieerd met ernst van de ziekte.

Hoofdstuk 5 geeft een overzicht van onze belangrijkste bevindingen, een algemene interpretatie van deze bevindingen en de implicaties van onze resultaten. Daarnaast bespreken we aandachtspunten voor toekomstig onderzoek. Dit proefschrift laat zien dat *community-acquired* sepsis nog steeds ernstig verloopt in Westerse landen, voornamelijk door een hoge incidentie van invaliditeit. Bovendien biedt dit proefschrift meer inzicht in de respons van het lichaam op infectie door specifieke inflammatoire, hemostatische, genetische en omgevingsfactoren te bestuderen in relatie tot de uitkomst. Deze determinanten kunnen helpen om patiënten met risico op een slechte uitkomst in een vroeg stadium te detecteren en geven ons nieuwe aangrijpingspunten om de behandeling van sepsis te individualiseren.

LIST OF ABBREVIATIONS

α2-MG	α2-macroglobulin
α2-PI	α2-plasmin inhibitor
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin motifs
ADCC	Antibody-dependent cellular cytotoxicity
(a)PC	(activated) protein C
AUROC	Area under the receiver operating characteristic
BEP	Base excess and platelet count
CDC	Complement-dependent cytotoxicity
cfDNA	Cell free DNA
CI	Confidence interval
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CTEPH	Chronic thromboembolic pulmonary hypertension
CVC	Central venous catheter
DIC	Disseminated intravascular coagulation
DVT	Deep vein thrombosis
ED	Emergency department
EGF	Epidermal growth factor
EPCR	Endothelial cell protein C receptor
ER	Endoplasmic reticulum
ETT	Endotracheal tube
EUCLIDS	European Childhood Life-threatening Infectious Disease Study
Fc region	Fragment crystallizable region
FDPs	Fibrin degradation products
FP7	7th Framework Programme
GAS	*Group A streptococcus*
GM-CSF	Granulocyte–macrophage colony-stimulating factor
GWAS	Genome-wide association studies
HiB	*Haemophilus influenzae type B*
HLA-DR	Human leukocyte antigen-DR
HUS	Hemolytic uremic syndrome
IgG	Immunoglobulin G
IL	Interleukin
IQR	Interquartile range
LD	Linkage disequilibrium
LOS	Length of stay
MALDI-TOF-MS	Matrix assisted laser/desorption ionization time-of-flight mass spectrometric
MenACWY	Meningococcus serogroups ACWY
MenB	Meningococcus serogroup B
MenC	Meningococcus serogroup C
MenW	Meningococcus serogroup W
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MPO-DNA	Myeloperoxidase-DNA
MSS	Meningococcal septic shock
NETs	Neutrophil extracellular traps
NLR	Negative likelihood ratio
NPV	Negative predictive value
OR	Odds ratio
PAI-1	Plasminogen activator inhibitor, type 1
PCR	Polymerase chain reaction
PCV10	Pneumococcal Conjugate Vaccine 10, Synflorix
PCV13	Pneumococcal Conjugate Vaccine 13, Prevnar 13
PCV7	Pneumococcal Conjugate Vaccine 7, Prevnar
PD-1	Programmed cell death-1
PE	Pulmonary embolism
PELOD-2	Pediatric Logistic Organ Dysfunction-2
PICU	Pediatric Intensive Care Unit
PIM	Pediatric Index of Mortality
Pla	Plasmin
Plg	Plasminogen
PLR	Positive likelihood ratio
PN-1	Protease nexin-1
POPC	Pediatric Overall Performance Category
PPSV23	Pneumococcal Polysaccharide Vaccine 23
PPV	Positive predictive value
PRISM	Pediatric risk of mortality score
PS	Protein S
PT INR	Prothrombin time international normalized ratio
SD	Standard deviation
SE	Standard error
SFI	Severe focal infections
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential (Sepsis-related) Organ Failure Assessment
SOMVs	Small outer membrane vesicles
SPSS	Statistical Package for the Social Sciences
TAFI	Thrombin-activatable fibrinolysis inhibitor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
TNF	Tumor Necrosis Factor
tPA	Tissue-type plasminogen activator
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
vWF	von-Willebrand factor
WBC	White blood cell count

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PhD portfolio

Summary of PhD training and teaching activities

Name PhD student	Navin P. Boeddha
Departments	Intensive Care and Department of Pediatric Surgery Pediatrics, Division of Pediatric Infectious Diseases & Immunology
Research School	Molecular Medicine
PhD period	2013 - 2018
Promotors	Prof. dr. J.A. Hazelzet and Prof. dr. D. Tibboel
Co-promotors	Dr. M. Emonts and Dr. G.J. Driessen

	Year	ECTS
General academic and research skills		
Systematic literature search and Endnote (medical library)	2013	1.0
Research management for PhD students	2013	1.0
Basiskennis Regel en wetgeving Onderzoek (BROK-course), recertified in 2017	2013	1.0
Multiple statistical courses ("SPSS", "Course on R", "Survival analysis course")	2013-2016	2.0
Biomedical English Writing and Communication – by David Alexander	2014	3.0
CPO course: design, conductance, analysis and clinical implications	2014	0.3
Course "Scientific Integrity"	2014	0.3
Courses, workshops, and symposia		
Molecular Medicine - Workshop on Photoshop and Illustrator CS6	2013	0.3
Molecular medicine day, Rotterdam, The Netherlands	2013	0.3
EPAR PhD-day, Rotterdam, The Netherlands	2013	0.3
2nd Erasmus Critical Care Day, Rotterdam, The Netherlands	2013	0.3
2e Landelijke infectie- en afweerdag, Amersfoort, The Netherlands	2013	0.3
AAV Erasmus MC Wetenschapsmiddag	2013, 2015	0.6
10th SNP course: SNPs and Human diseases	2013	1.0
NIHES, Erasmus Summer Programme: Principles of genetic epidemiology, Genomics in molecular medicine, and Genome wide association analysis	2014	3.5
Seminar Research Impact and Relevance: How to publish a world-class paper, University Library - Erasmus University Rotterdam, The Netherlands	2014	0.3
Theme Sophia Research Day, Rotterdam, The Netherlands. *Oral presentations*	2014-2016	0.9
ESPID 7th Research Masterclass, Leipzig, Germany. *Co-chairing of a session*	2015	0.3
ESPID Online Antibiotic Management Course	2015	1.0
Molecular Medicine - Course Biomedical Research Techniques XV	2016	0.9
Course "A practical approach to serious infections", Reykjavik, Iceland	2016	1.0
ESPID Walter Marget Educational Workshop, Brighton, UK. *Case presentation*	2016	0.3

Conferences		
31st Annual Meeting of the ESPID, Milan, Italy	2013	1.0
Meningitis Research Foundation Conference, London, UK	2013	0.3
EUCLIDS annual meeting, Graz, Austria. *Oral presentation*	2013	1.0
32nd Annual Meeting of the ESPID, Dublin, Ireland	2014	1.0
33rd Annual Meeting of the ESPID, Leipzig, Germany. *Oral presentation*	2015	1.0
26th Annual Meeting of the ESPNIC, Vilnius, Lithuania. *Oral presentation*	2015	1.0
34th Annual Meeting of the ESPID, Brighton, UK. *Poster presentation*	2016	1.0
6th Congress of the European Academy of Paediatric Societies (EAPS), Geneva, Switzerland. *Oral presentation*	2016	1.0
35th Annual Meeting of the ESPID, Madrid, Spain. *Poster presentation*	2017	1.0
36th Annual Meeting of the ESPID, Malmo, Sweden. *Oral presentation*	2018	1.0
Teaching activities		
University Teaching Qualification (BKO)	2015	4.0
Co-lecturing "Pediatric infectious diseases in resource poor settings", Erasmus MC, minor Global Health	2015-2017	1.0
Supervising Master thesis of medical students (n=2)	2015-2016	2.0
Coaching medical students in Bachelor phase (n=6)	2016-2018	1.0
Supervising scientific research of medical student/senior author (n=1)	2018	1.0
Organisational activities		
AAV Erasmus MC - Board member (president from 2014-2016)	2013-2016	3.0
Molecular Medicine - Member of PhD committee	2014-2016	0.9
Memberships		
EUCLIDS consortium	2013-2017	
European Society for Pediatric Infectious Diseases (ESPID)	2013-2018	
European Society of Pediatric and Neonatal Intensive Care (ESPNIC)	2015-2018	
Honours and Awards		
Winner best case presentation, ESPID Walter Marget Educational Workshop, Brighton, UK	2016	
Runner-up ESPNIC Young Investigator Award, Geneva, Switzerland	2016	
Winner ESPID Young Investigator Award, Malmo, Sweden	2018	

ECTS = European Credit Transfer and Accumulation System (1 ECTS represents 28 hours work load)

List of publications

PhD thesis is based on:

1. Circadian Variation of Plasminogen-Activator-Inhibitor-1 Levels in Children with Meningococcal Sepsis
   Boeddha NP, Driessen GJ, Cnossen MH, Hazelzet JA, Emonts M.
   PLoS One. 2016 Nov 28;11(11):e0167004.

2. Gene Variations in the Protein C and Fibrinolytic Pathway: Relevance for Severity and Outcome in Pediatric Sepsis
   Boeddha NP, Emonts M, Cnossen MH, de Maat MP, Leebeek FW, Driessen GJ, Hazelzet JA.
   Semin Thromb Hemost. 2017 Feb;43(1):36-47.

3. Mortality and morbidity in community-acquired sepsis in European Pediatric Intensive Care Units: a prospective cohort study from the European Childhood Life-threatening Infectious Disease Study (EUCLIDS)
   Boeddha NP, Schlapbach LJ*, Driessen GJ, Herberg JA, Rivero-Calle I, Cebey-López M, Klobassa DS, Philipsen R, de Groot R, Inwald DP, Nadel S, Paulus S, Pinnock E, Secka F, Anderson ST, Agbeko RS, Berger C, Fink CG, Carrol ED, Zenz W, Levin M, van der Flier M, Martinón-Torres F, Hazelzet JA^, Emonts M^; EUCLIDS consortium. *^Contributed equally.*
   Crit Care. 2018 May 31;22(1):143.

4. Neutrophil Extracellular Traps in Children With Meningococcal Sepsis
   Hoppenbrouwers T, Boeddha NP*, Ekinci E, Emonts M, Hazelzet JA, Driessen GJ, de Maat MP. *Contributed equally.*
   Pediatr Crit Care Med. 2018 Jun;19(6):e286-e291.

5. Life-threatening infections in children in Europe (the EUCLIDS Project): a prospective cohort study
   Martinón-Torres F, Salas A*, Rivero-Calle I*, Cebey-López M*, Pardo-Seco J*, Herberg JA*, Boeddha NP, Klobassa DS, Secka F, Paulus S, de Groot R, Schlapbach LJ, Driessen GJ, Anderson ST, Emonts M, Zenz W, Carrol ED^, Van der Flier M, Levin M^; EUCLIDS Consortium. *^ Contributed equally.*
   Lancet Child Adolesc Health. 2018 Jun;2(6):404-414.

6. Differences in IgG Fc Glycosylation are Associated with Outcome of Pediatric Meningococcal Sepsis
de Haan N, Boeddha NP, Ekinci E, Reiding KR, Emonts M, Hazelzet JA, Wuhrer M, Driessen GJ.
MBio. 2018 Jun 19;9(3). pii: e00546-18.

7. HLA-DR Expression on Monocyte Subsets in Critically Ill Children
Boeddha NP, Kerklaan D, Dunbar A, van Puffelen E, Nagtzaam NMA, Vanhorebeek I, Van den Berghe G, Hazelzet JA, Joosten KF, Verbruggen SC, Dik WA, Driessen GJ.
Pediatr Infect Dis J. 2018 Oct;37(10):1034-1040.

8. ADAMTS-1 and ADAMTS-18 levels in meningococcal sepsis
Boeddha NP, Klobassa DS*, Driessen GJ, Ekinci E, Binder A, Hoggart CJ, Priem-Visser D, Leebeek FW, Zenz W, Hazelzet JA, de Maat MP^, Emonts M^. *^Contributed equally.*
Manuscript in preparation.

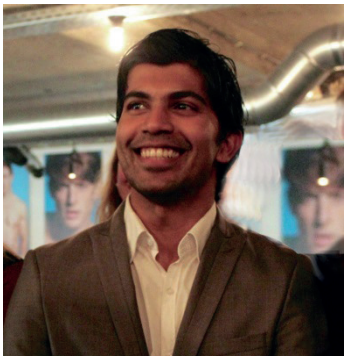
Other publications:

9. Translating Sepsis-3 Criteria in Children: Prognostic Accuracy of Age-Adjusted Quick SOFA Score in Children Visiting the Emergency Department With Suspected Bacterial Infection
van Nassau SC, van Beek RH, Driessen GJ, Hazelzet JA, van Wering HM, Boeddha NP
Front. Pediatr., 01 October 2018.

10. Target attainment of cefotaxime in critically ill children with meningococcal septic shock as a model for cefotaxime dosing in severe pediatric sepsis
Hartman SJ, Boeddha NP, Donders R, Ekinci E, Koch BC, Hazelzet JA, Driessen GJ, de Wildt SN.
Submitted.

11. Bacteremia in Childhood life-threatening Infections In Urban Gambia: EUCLIDS in West Africa
Secka F, Sarr I, Darboe S, Sey G, Wathuo M, Cebey-López M, Boeddha NP, Paulus S, van der Flier M, Klobassa DS, Emonts M, Zenz W, Carrol ED, de Groot R, Herberg J, Martinón-Torres F, Levin M, Bojang K, Anderson ST; EUCLIDS consortium.
Submitted.

ABOUT THE AUTHOR



Navin Prekash Boeddha was born in Suriname (South America) in August 1987. He is the fourth child of Cyrill and Santa Boeddha. At the age of 6 years, his family moved to The Netherlands, mainly for educational purposes. After Navin graduated from *Emmauscollege*, Rotterdam (2004), he attended medical school of Erasmus MC. During medical school, he fulfilled research electives in Paramaribo (Suriname, 2007, 2 months) and London (United Kingdom, 2010, 6 months). Additionally, he did a clinical elective in Kenya (2011, 2 months). After Navin obtained his medical degree (2011), he gained clinical experience in general pediatrics at Maasstad Hospital and in pediatric intensive care at Erasmus MC. In 2013, he commenced the work described in this thesis under supervision of prof. dr. J.A. Hazelzet and prof. dr. D. Tibboel, and he became member of the EUCLIDS consortium (prof. dr. M. Levin). Apart from research activities, he has been involved in teaching and organisational activities. Since 2017, Navin has been enrolled to the pediatrics residency programme of Erasmus MC-Sophia Children's Hospital (prof. dr. M. de Hoog). Currently, he is based at Amphia Hospital, Breda (dr. H.M. van Wering). In his spare time, Navin becomes happy by travelling and spending time with family and friends. He is married to Britt and they are blessed with their son Felan (2017). Also, he loves sports; he warns that starting a conversation on football will keep you busy for at least 20 minutes.

DANKWOORD

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