Unraveling the immune response in sepsis and meningitis. Diagnostic and therapeutic approaches.

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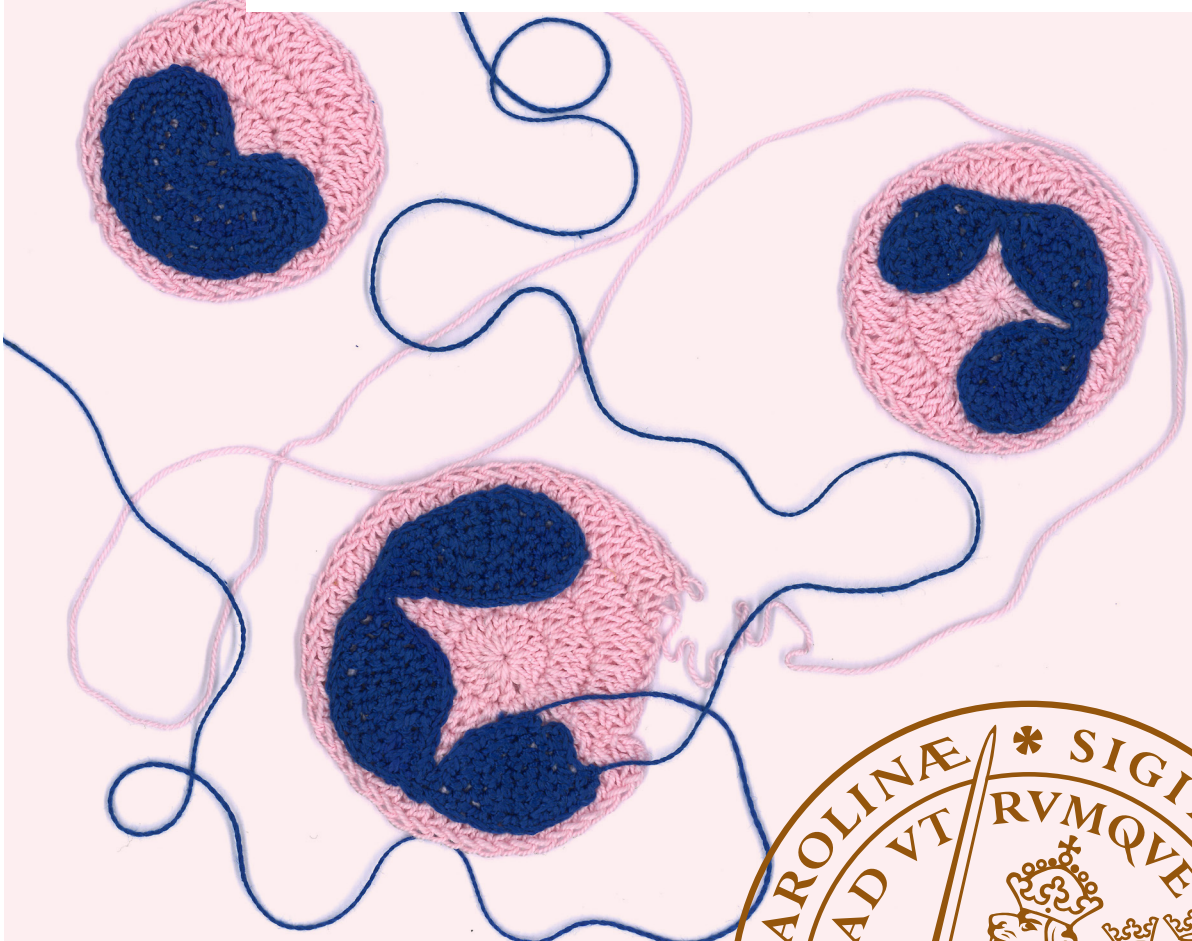

Unraveling the immune response in sepsis and meningitis

Diagnostic and therapeutic approaches

Jane Fisher

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Severe infections continue to constitute a large burden, with high mortality and risk of sequelae. Sepsis is a dysregulated host response to an infection that causes life threatening organ damage. Meningitis is a severe infection of the brain that often leads to sepsis and death or lasting neurological damage. The host response is often responsible for more damage than the pathogen itself. This thesis focuses on two components of the host response. The first is the endothelial glycocalyx, a complex matrix of glycans and proteins that modulates blood vessel function. The second are neutrophil extracellular traps (NETs), which are large structures composed of DNA and granule proteins that are released from neutrophils in response to bacteria. The aim of this thesis was to explore diagnostic and therapeutic aspects of these components of the host immune response during sepsis and bacterial meningitis.

Translational research methods were used to find solutions to this clinical problem. An observational cohort study revealed that plasma glypicans, a component of the glycocalyx, are elevated in sepsis before the onset of organ dysfunction. A small cohort study also revealed that cerebrospinal fluid (CSF) NETs are elevated in bacterial meningitis. CSF NETs were also present to a great extent in a cohort of patients neurosurgically treated with external ventricular drains, but were not significantly elevated in those who developed infections as a result of the procedure. NETs were also present in the CSF in a rat model of bacterial meningitis and their removal using DNase increased bacterial killing. Glymphatic fluid distribution in the brain was disrupted rats with bacterial meningitis and partially restored after treatment with DNase. A second rat model was used to test and validate a new scoring system to quantify neurologic outcomes in experimental meningitis.

In this thesis work, glypicans were identified as a marker of endothelial damage in sepsis and CSF NETs were identified as a potential biomarker and therapeutic target in bacterial meningitis. Disruption of NETs by DNase should be explored further as a therapeutic in bacterial meningitis. A neurologic scoring system was established for testing of novel adjuvant therapies, such as DNase, in rat models of bacterial meningitis in the future.

Key words: Sepsis; bacterial meningitis; NETs; DNase; glymphatic; neurological sequelae

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Such gardens are not made by singing 'Oh, how beautiful!' and sitting in the shade

-Rudyard Kipling

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Abstract

Severe infections continue to constitute a large burden, with high mortality and risk of sequelae. Sepsis is a dysregulated host response to an infection that causes life threatening organ damage. Meningitis is a severe infection of the brain that often leads to sepsis and death or lasting neurological damage. The host response is often responsible for more damage than the pathogen itself. This thesis focuses on two components of the host response. The first is the endothelial glycocalyx, a complex matrix of glycans and proteins that modulates blood vessel function. The second are neutrophil extracellular traps (NETs), which are large structures composed of DNA and granule proteins that are released from neutrophils in response to bacteria. The aim of this thesis was to explore diagnostic and therapeutic aspects of these components of the host immune response during sepsis and bacterial meningitis.

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In this thesis work, glypicans were identified as a marker of endothelial damage in sepsis and CSF NETs were identified as a potential biomarker and therapeutic target in bacterial meningitis. Disruption of NETs by DNase should be explored further as a therapeutic in bacterial meningitis. A neurological outcome scoring system was established for testing clinically relevant effects of novel adjuvant therapies, such as DNase, in rat models of bacterial meningitis in the future.

Popular science summary

Severe infections have never been in the spotlight more than they are now during the COVID-19 crisis. However severe infections, such as sepsis and meningitis, have been a major cause of death and disability long before the year 2020. Sepsis, a severe reaction to infections that leads to life threatening organ failure, is the cause of one in five deaths worldwide, a number that is increasing as bacteria gain resistance to antibiotics. Bacterial meningitis, a devastating infection of the protective membranes in the brain, often leads to sepsis, death, and neurological disability in survivors. Often the pathogen itself is not the main cause of damage in these infections. Instead the body and immune system often react so strongly to the pathogen that they cause collateral damage to the patient's own organs.This thesis focuses on two parts of this so-called "host response" to the infection: the endothelium and neutrophils.

The endothelium is a tight layer of cells that lines all blood vessels in the body and keeps components of the blood from leaking out. The glycocalyx is a protective layer of sugars and proteins that helps the endothelium fulfil its function. This thesis work showed that glypicans, an often ignored component of the glycocalyx, are elevated in patients with sepsis before they develop organ dysfunction.

Neutrophils are the most abundant white blood cells in the body and the first to respond to an infection. They carry an arsenal of tools to kill bacteria, but these tools can often backfire and damage healthy cells. One such tool is called "neutrophil extracellular traps" or NETs, in which neutrophils send out a sticky web of DNA coated with antimicrobial proteins to trap bacteria.

The work in this thesis found NETs in the brains of patients with bacterial meningitis, and also in patients receiving a brain surgery procedure, known as a ventriculostomy, that has a high risk of developing bacterial meningitis. Bacteria can hide in these NETs to avoid being killed by the immune system. Using a drug called DNase to dissolve NETs in the brains of rats with bacterial meningitis enhanced normal neutrophil killing mechanisms, killing of most of the bacteria. The large sticky NETs may also clog the brain's waste disposal system, known as the glymphatic system, leading to dangerous fluid build-up in the brain. Lastly, a rat model of bacterial meningitis was optimized for testing drugs such as DNase in the future.

Take home message: This thesis work identified two new biomarkers in infections: glypicans and NETs. It also suggests that removing NETs using DNase might help clear the infection and reduce dangerous fluid build-up during bacterial meningitis. The therapeutic potential of DNase should be explored further in animal models and, eventually, in human trials.

Studies included in this thesis

Study I: Elevated plasma glypicans are associated with organ failure in patients with infection

Jane Fisher, Adam Linder, Peter Bentzer

Intensive Care Medicine Experimental. 2019; 7: 2.

Study II: Neutrophil extracellular traps in the central nervous system hinder bacterial clearance during pneumococcal meningitis

Tirthankar Mohanty, Jane Fisher, Anahita Bakochi, Ariane Neumann, José Francisco Pereira Cardoso, Christofer A. Q. Karlsson, Chiara Pavan, Iben Lundgaard, Bo Nilson, Peter Reinstrup, Johan Bonnevier, David Cederberg, Johan Malmström, Peter Bentzer, Adam Linder

Nature Communications. 2019; 10(1):1667.

Study III: Acute S. pneumoniae meningitis disrupts CNS fluid transport by the glymphatic system

Chiara Pavan, Jane Fisher, Helen Axelberg, Anna Lenice Ribeiro Xavier, Marta Ramos Adam Linder, Peter Bentzer, Iben Lundgaard, Maiken Nedergaard

(Manuscript)

Study IV: Neutrophil extracellular traps in ventriculostomy-related infections – interim analysis of a prospective observational cohort study

Jane Fisher, Johan Widén, Mathilda Hultén, Luisa Ohlmeier, Carolin Ketteler, Špela Lemež, José Francisco Pereira Cardoso, Adam Linder

(Manuscript)

Study V: A functional observational battery for evaluation of neurological outcomes in a rat model of acute bacterial meningitis

Jane Fisher, Chiara Pavan, Luisa S. Ohlmeier, Bo Nilson, Iben Lundgaard, Adam Linder, Peter Bentzer

Intensive Care Medicine Experimental. 2020; article in press

Other publications

Non-corticosteroid adjuvant therapies for acute bacterial meningitis. Jane Fisher, Adam Linder, Maria Grazia Calevo, Peter Bentzer. 2019; Cochrane Database of Systematic Reviews 9: CD013437

Albumin infusion rate and plasma volume expansion- a randomized clinical trial in postoperative patients after major surgery. Svajunas Statkevicius, Johan Bonnevier, Jane Fisher, Björn P Bark, Erik Larsson, Carl M Öberg, Päivi Kannisto, Bobby Tingstedt, Peter Bentzer. *Critical care. 2019; 23(1):191.*

Is Heparin-Binding Protein Inhibition a Mechanism of Albumin's Efficacy in Human Septic Shock?. Jane Fisher, Adam Linder, Peter Bentzer, John Boyd, Hyejin Kong, Terry Lee, Keith Walley, James Russell. *Critical Care Medicine. 2018; 46(5):e364-e374.*

Heparin-Binding Protein: A Key Player in the Pathophysiology of Organ Dysfunction in Sepsis. Jane Fisher, Adam Linder. *2017; Journal of Internal Medicine 281(6):562-574.*

Heparin-Binding Protein (HBP) Improves Prediction of Sepsis-Related Acute Kidney Injury. Jonas Tverring, Suvi T. Vaara, Jane Fisher, Meri Poukkanen, Ville Pettilä, Adam Linder, and the FINNAKI Study Group. *2017; Annals of Intensive Care 7(1):105.*

Heparin-Binding Protein (HBP): A Causative Marker and Potential Target for Heparin Treatment of Human Sepsis-Induced Acute Kidney Injury. Jane Fisher, James A. Russell, Peter Bentzer, Devyn Parsons, Stefano Secchia, Matthias Mörgelin, Keith Walley, John Boyd, Adam Linder. 2017; *Shock 48(3):313–20.*

Heparin-Binding Protein Is Important for Vascular Leak in Sepsis. Peter Bentzer, Jane Fisher, HyeJin Julia Kong, Mattias Mörgelin, John H. Boyd, Keith R. Walley, James A. Russell, Adam Linder. *2016; Intensive Care Medicine Experimental 4(1):33.*

Elevated Plasma Angiopoietin-2 Levels Are Associated With Fluid Overload, Organ Dysfunction, and Mortality in Human Septic Shock. Jane Fisher, James J. Douglas, Adam Linder, John H Boyd, Keith R Walley, James A Russell. *2016; Critical Care Medicine 44(11):2018–27.*

Short-Term Organ Dysfunction Is Associated With Long-Term (10-Yr) Mortality of Septic Shock. Adam Linder, Terry Lee, Jane Fisher, Joel Singer, John Boyd, Keith Walley, James Russell. *2016; Critical Care Medicine 44(8):e728-36.*

Abbreviations

AMP = antimicrobial peptide

AQP-4 = aquaporin-4

CNS = central nervous system

CoNS = coagulase negative staphylococci

CSF = cerebrospinal fluid

DIC = disseminated intravascular coagulation

DNase = deoxyribonuclease

EDTA = ethylenediaminetetraacetic acid

EVD = external ventricular drain

FOB = functional observational battery

GAG = glycosaminoglycan

GPI = glycosylphosphatidylinositol

HBP = heparin binding protein

IL-6 = interleukin-6

IP = intraperitoneal

MPO = myeloperoxidase

NADPH = nicotinamide adenine dinucleotide phosphate

NETs = neutrophil extracellular traps

PCR = polymerase chain reaction

PMA = phorbol 12-myristate 13-acetate

ROS = reactive oxygen species

SIRS = systemic inflammatory response syndrome

VRI = ventriculostomy-related infection

Introduction

Under normal circumstances, the innate and adaptive immune responses are able to neutralize invading pathogens before they are able to cause a serious infection. In the case of bacterial infections, antibiotics can kill the bacteria and help to clear the infection. However in some cases the infection becomes severe, requiring hospitalization and intensive care support. The body's own response to the infection plays a major role, often having a greater impact than the pathogen itself.

Neutrophils

One cell type that plays a major role in the host response to an infection is the neutrophil. Neutrophils are the most abundant immune cell in humans, making up about 70% of the total white blood cells[1,2]. They are also among the first cells to respond during an infection and play a crucial role in killing bacteria[1,2]. Neutrophils have several different weapons at their disposal that they use to protect us from infections. Within their granules and vesicles, neutrophils store over 300 different proteins that they use for bacterial killing and other functions[3].

Neutrophil granule proteins include antimicrobial proteins, such as heparin binding protein (HBP) and other serprocidins, that can kill bacteria directly by disrupting the bacterial cell membranes[4–6], or can weaken them by degrading or inhibiting surface virulence factors[6–8]. Other enzymes such as myeloperoxidase (MPO)[9] and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex[10] generate reactive oxygen species (ROS) that can kill bacteria. Some neutrophil proteases can release antimicrobial peptides (AMPs) when they degrade other proteins, for example proteinase-3 mediated cleavage of cathelicidin to generate LL-37[11], or neutrophil elastase mediated cleavage of thrombin to generate several different AMPs[12]. Neutrophils granule proteins and ROS can cause damage and induce inflammation in the host tissues, contributing to the pathophysiology of several diseases[13–17].

Bacterial killing by neutrophils can occur intracellularly or extracellularly[6,18] (**Figure 1**). Intracellular killing occurs through phagocytosis: the bacteria are engulfed by the neutrophil and then exposed to the various antimicrobial proteins and chemicals inside the granules[19]. Neutrophils can also release their granule contents outside of the cell, in a process called degranulation, where they can kill bacteria extracellularly[20].

Neutrophils also release chemotactic factors that recruit other immune cells to the infection site to aid with bacterial killing[21–23].

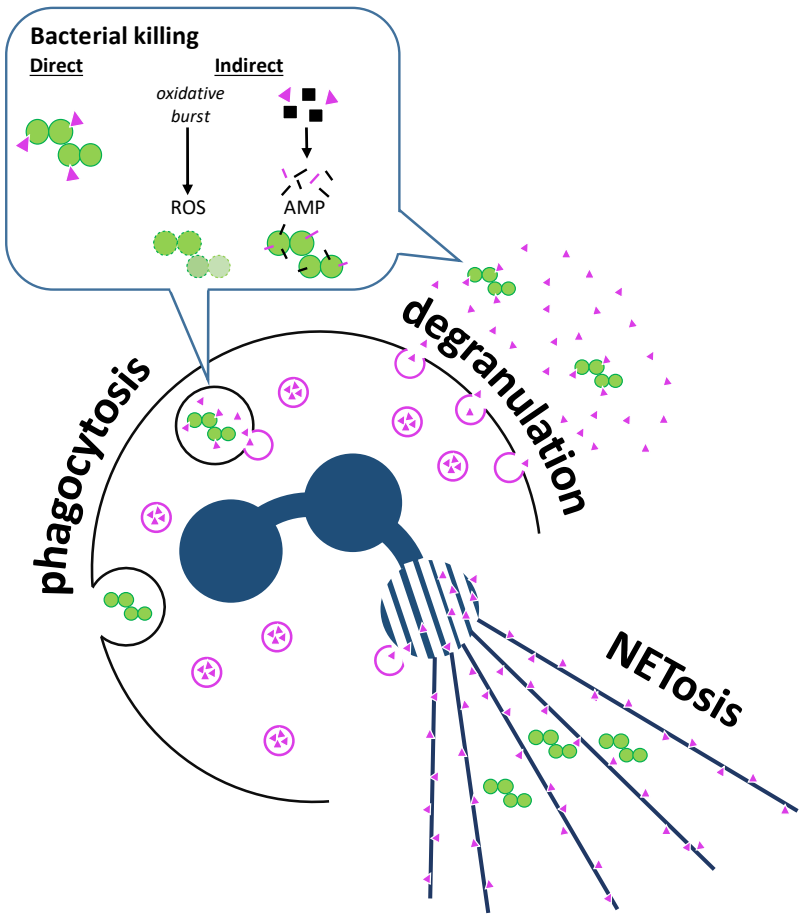

Figure 1. Killing mechanisms of neutrophils include phagocytosis, degranulation and NETosis. Bacteria (green circles) can be killed by antibacterial granule proteins (pink triangles) when they are released to the extracellular environment (degranulation) or when bacteria are taken up by phagocytosis and exposed to the granules fuse with the phagosome. Some granule proteins can kill bacteria directly by disrupting the bacterial membrane, while others can kill bacteria indirectly by carrying out an oxidative burst reaction that creates reactive oxygen species (ROS), or by enzymatically degrading other proteins and thus creating new antimicrobial peptides (AMP). Lastly DNA (blue) can be coated with antimicrobial proteins and expelled in a process called NETosis.

Neutrophil extracellular traps (NETs)

Another mechanism of extracellular killing is the formation of neutrophil extracellular traps (NETs) in a complex process called NETosis[24]. NETs are formed when the proteins from the granules mix with the DNA from the nucleus, and then are expelled from the cells[24]. These complexes of DNA and granule proteins form large, sticky structures[24]. NETs are often referred to as "double-edged swords" because they have been documented to have both beneficial and harmful effects for the host[25]. NETs are relevant players in non-infectious diseases, including autoimmune diseases and cancer, in normal functions such as pregnancy, and in infections by a variety of pathogens[25–28]. NETs can be formed in response to bacteria, viruses, fungi, and even parasites[26]. Different bacterial species can vary in their ability to induce NET formation[29].

The web-like structures of NETs can trap and immobilize bacteria while the coating of antimicrobial proteins and peptides can kill them[24]. The DNA backbone of NETs and DNA fragments can also kill bacteria[30,31]. However bacteria are experts at evading the components of the immune system, and NETs are no exception[32]. Streptococcal species express endonucleases, which can degrade the DNA backbone of NETs and allow the bacteria to escape the trap[33,34]. Some bacteria can even incorporate NET components into biofilms, thus encouraging biofilm formation[35–37].

NETs can also bind and degrade pro-inflammatory cytokines, thus dampening excessive inflammatory responses, as was shown in animal models of gout[38]. Whether such a mechanism can also dampen the systemic inflammation during severe infections such as sepsis and meningitis is unknown. However any anti-inflammatory effects of NETs may be counteracted by their pro-inflammatory and cytotoxic effects for the host's cells[39–41].

Lastly NETs induce thrombus formation[42,43]. The presence of NETs results in a more rigid clot that is resistant to fibrinolysis[44]. Formation of a clot around a NET that has entrapped bacteria can be beneficial as it helps stabilize the NET and prevent bacterial escape[45,46]. However, excessive thrombus formation due to dysregulated NET formation could be a factor in the coagulopathies often observed during severe infections. Disseminated intravascular coagulation (DIC) is a major problem during sepsis as it blocks local blood flow and the resulting hypoxia causes tissue damage and death[47]. Thromboses can also occur in the blood vessels of the brain as a devastating complication of bacterial meningitis[48].

Sepsis

Yearly, sepsis affects about 48 million people and causes 20% of all deaths worldwide[49]. Sepsis is a dangerous condition that occurs in response to an infection and leads to organ dysfunction, and often death.

According to the society of Critical Care Medicine and the European Society of Intensive Care Medicine task force[50]:

> Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection.

This definition underscores the fact that most of the damage in sepsis is caused not by the pathogen, but rather by the body's own response to the infection. One particularly important aspect of the host response in sepsis is that of the endothelium.

The vascular endothelium in sepsis

The vascular endothelium lines all the blood vessels in the body, and separates the blood from the tissues. The endothelium is held together by tight junctions between the endothelial cells[51]. This creates a semi-permeable barrier that prevents excess fluid from leaking out from the blood. Endothelial dysfunction is central to sepsis pathophysiology, contributing to hemostatic disturbances, inflammation, edema, and low blood pressure[52,53]. Many of the functions of the endothelium are modulated by the endothelial glycocalyx.

The endothelial glycocalyx

The glycocalyx is a complex extracellular gel of membrane-associated proteins and glycosaminoglycans (GAGs)[54]. Proteoglycans are proteins with attached GAG side chains that form the foundation of the glycocalyx. Two main types of proteoglycans on the cell surface are syndecans and glypicans[55]. Syndecans have a transmembrane domain that attaches them to the cell surface. Glypicans on the other hand are attached to the phospholipids of the cell membrane by a single bond known as a glycosylphosphatidylinositol (GPI) anchor.

The glycocalyx has several functions (**Figure 2**). First, it modulates coagulation on the endothelial surface, as the GAG chains of proteoglycans are similar in structure to the anticoagulant heparin, and thus create an antithrombotic surface[56,57]. The glycocalyx also fine-tunes cell signaling. On one hand the dense layer of long GAG chains can obscure some receptors, preventing some proteins from binding[52], or it can act as a

co-receptor, increasing some protein interactions[58]. The intracellular domain of syndecans can also directly activate certain signaling pathways[59,60]. The glycocalyx is also a major contributor to the barrier function of the endothelium, repelling white blood cells and negatively charged proteins[61]. The GAG chains also bind plasma proteins, such as albumin, contributing to an osmotic gradient that ultimately reduces the passage of water and proteins across the endothelium[62,63].

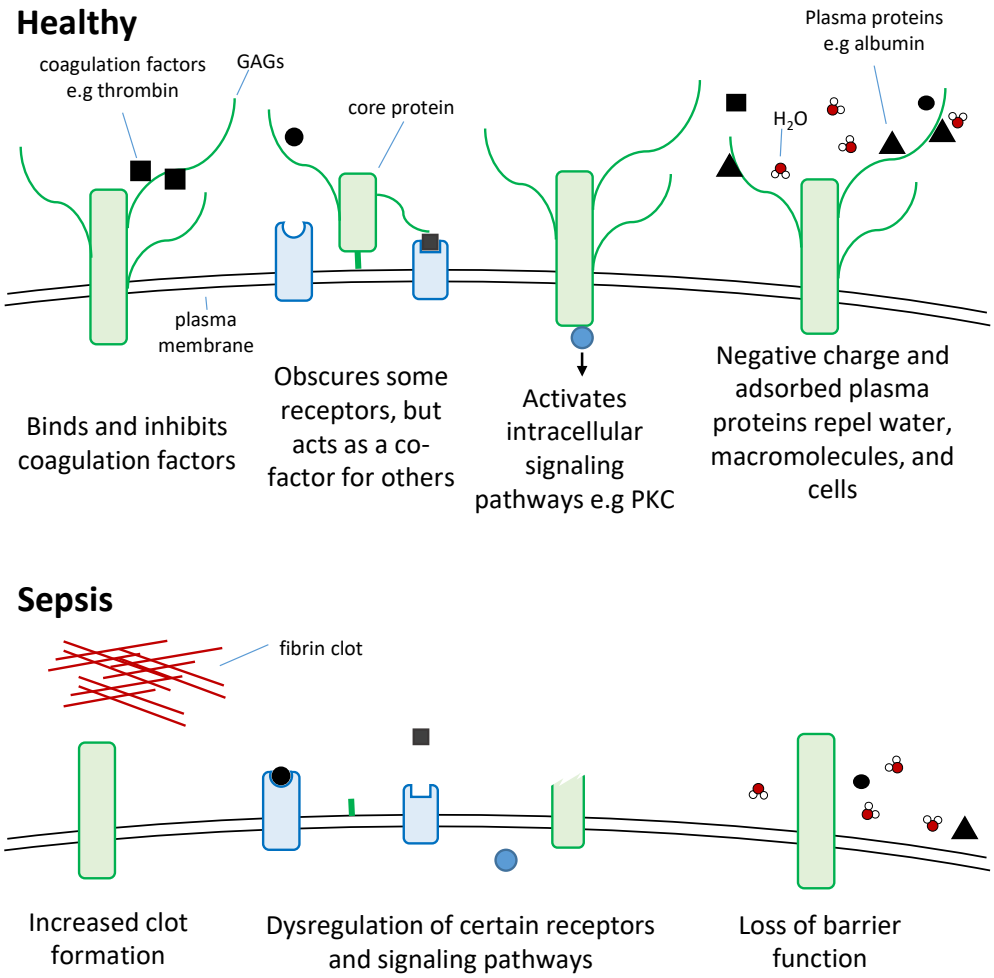

Figure 2. Some functions of the glycocalyx and consequences of its disruption during sepsis.

During sepsis, the endothelial glycocalyx is severely diminished[64,65]. The loss of the antithrombotic surface properties of the glycocalyx during sepsis may promote coagulopathies, which are a major problem during sepsis[47]. Loss of the glycocalyx can also increase endothelial cell sensitivity to some factors[66,67] while inhibiting others[58,68]. Increases in vascular endothelial permeability during sepsis may also be exacerbated by the loss of the permeability-modulating properties of the endothelial glycocalyx[62]. In these ways, loss of the glycocalyx could contribute to the general endothelial dysfunction that occurs in sepsis[52].

Shedding of the glycocalyx can occur through several mechanisms[69] (**Figure 3**). GAG chains can be removed from the protein core by enzymes known as GAGases. The protein core can also be removed from the membrane by various mechanisms. Because syndecans have a transmembrane domain, they must be shed by proteases that cut the protein core[70]. The shedding mechanisms of glypicans are less studied and more complex. The GPI anchor can be cleaved by enzymes known as phospholipases[71]. The protein core can also be cleaved by furin-like convertases, but glypicans contain several disulphide bonds that keep the two parts of the protein core attached to each other unless they are exposed to a reducing environment[72]. Because the shedding mechanisms of glypicans are distinct from those of syndecans and GAGs, measurement of glypicans in plasma could give insight into the factors that drive glycocalyx shedding in sepsis.

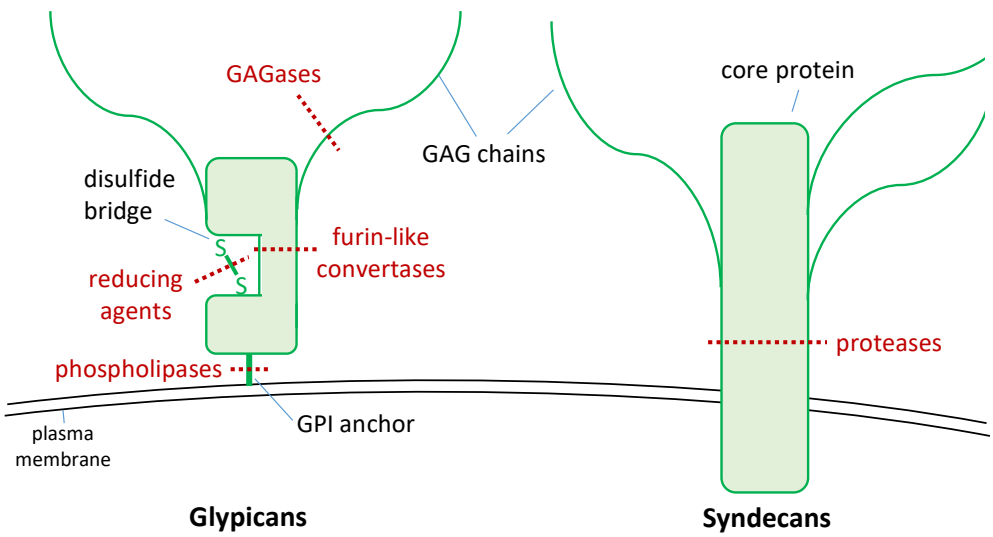

Figure 3. Schematic depiction of the possible mechanisms of syndecan, glypican, and GAG chain shedding from the glycocalyx.
Red dashed lines indicate possible cleavage sites. The responsible enzyme or agent for each site is indicated in red text.

Bacterial meningitis

Pathophysiology

When an infection is present in the meninges of the brain, this is known as meningitis. The meninges are a set of three membranes that surround the brain and the spinal cord[73,74] (**Figure 4**). The dura is a thick membrane that lies just below the skull and is closely associated with the arachnoid membrane. The pia membrane is closely associated with the parenchyma of the brain and spinal cord. Between the pia membrane and the arachnoid membrane is the subarachnoid space, which is filled with cerebrospinal fluid (CSF). The CSF originates in the ventricles, and then flows into the subarachnoid space[75].

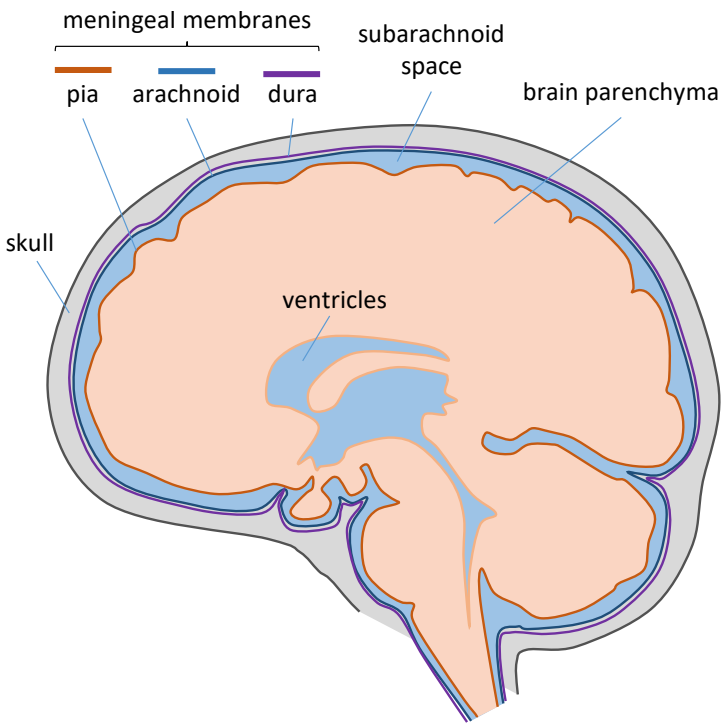

Figure 4. A schematic depiction of the brain and the meninges.

Meningitis can be community-acquired or hospital-associated (nosocomial). Bacteria can enter the CSF through several routes. Most commonly, bacteria enter the blood from another infection site and then cross over the blood-brain barrier and enter the CSF[76]. Bacteria can also enter the CSF by extension of a nearby infection site in the skull, for example from an inner ear infection[77]. Some pathogens can also transmigrate into the olfactory nerves and into the central nervous system[78]. Lastly bacteria can enter directly through a breach in the skull or spinal cord, either from a traumatic injury or a hospital procedure such as a surgery or lumbar puncture[79,80].

Infiltration of large numbers of neutrophils into the CSF, known as pleiocytosis, is a hallmark of bacterial meningitis[81]. Normally the brain does not come in contact with bacteria or neutrophils because it is protected by the blood-brain barrier[82], which is composed of specialized endothelial cells that form a tight and highly selective barrier. In the rare cases when bacteria and neutrophils enter the central nervous system, the results can be devastating to the sensitive tissue of the brain.

Mortality of bacterial meningitis is high at 10-30% in high-income countries[83–87], reaching up to 50% in low-income countries[88–90]. This high mortality in spite of adequate antibiotic treatment suggests that damage to the brain tissue and subsequent death is largely caused by the host response. A major cause of damage are the inflammatory processes that are triggered by bacterial products[91,92]. Blood clots can also form in the vessels in the brain leading to local ischemia and tissue death[48]. Another major cause of tissue damage in bacterial meningitis is the accumulation of excess fluid in the brain, which leads to edema and increased intracranial pressure[92]. Accumulation of fluid in the brain is particularly dangerous because the brain is encased inside the skull, which does not allow the tissue to expand very much. Even small increases in intracranial pressure can be damaging[93].

Fluid accumulation in the brain can be caused by increased permeability of the blood brain barrier, which lets excess fluid cross into the CSF from the blood, resulting in vasogenic edema[93–96]. Inflammatory responses that alter the cell membrane and increase intracellular water content can lead to cytotoxic edema[96,97]. Another conceivable cause of increased intracranial pressure in the brain is the impairment of normal fluid clearance pathways of the brain, known as the glymphatic system[98].

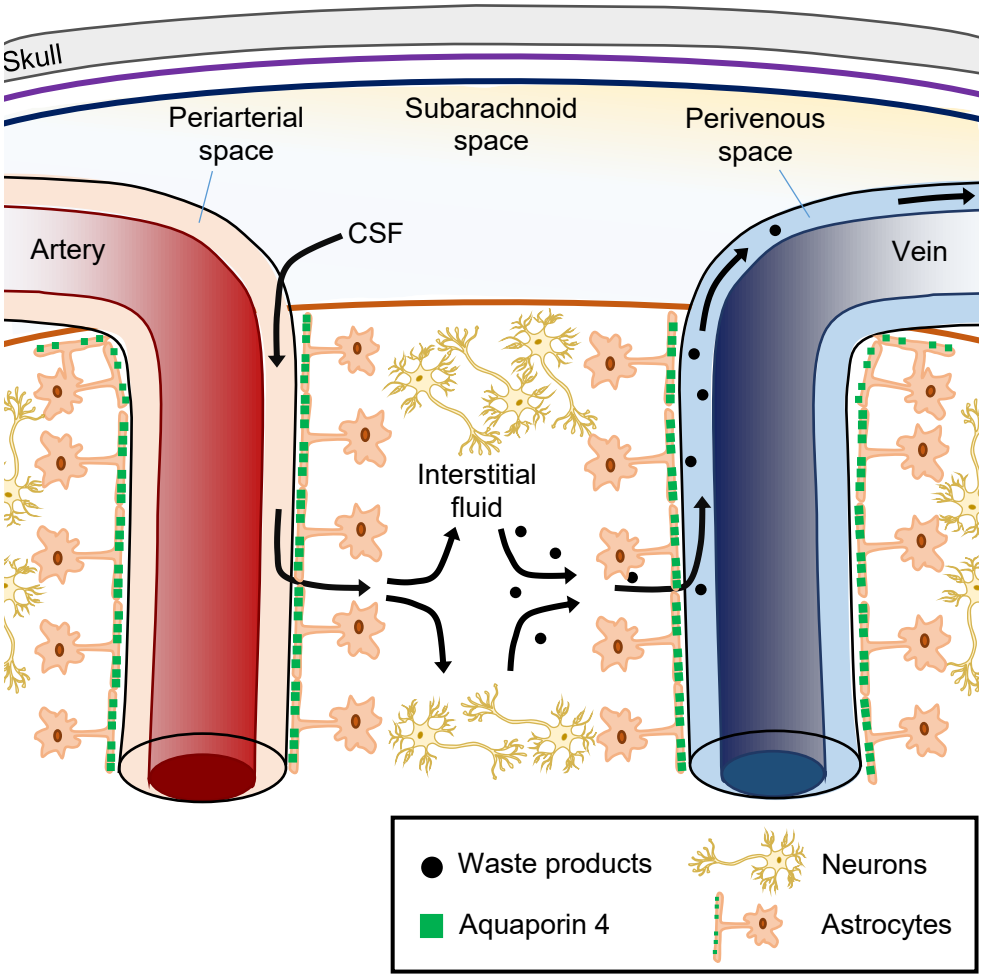

Figure 5. The glymphatic flux of fluid in and out of the brain.

The glymphatic system

Most of the brain lacks the lymphatic vessels that are found elsewhere in the body, with the exception of the meningeal lymphatic vessels present in the dura membrane[99]. Waste removal and fluid circulation in the brain was recently discovered to be mediated by the glymphatic system[100] (**Figure 5**). Cerebrospinal fluid circulates into the peri-vascular spaces surrounding the blood vessels and then into the interstitial space of the brain parenchyma. Solutes and waste products from the interstitial fluid then are circulated back into these peri-vascular spaces, and this fluid

then drains into the cervical lymph nodes. Together this CSF clearance process is called the glymphatic system[100].

Astrocytes are non-neuronal cells that are present throughout the brain and play a variety of roles in normal brain function[101]. Astrocytes have many branching protrusions from the main cell body. The ends of these structures, known as endfeet, surround the perivascular spaces around the blood vessels in the brain. Astrocytes express aquaporin (AQP)-4 water channels in a highly polarized manner, with the highest expression found closest to blood vessels[102]. Knockout of AQP-4 disrupts glymphatic fluid flow, indicating that AQP-4 plays an important role in mediating fluid exchange between the interstitial fluid and perivascular spaces[100]. Disruptions of glymphatic flux have been implicated during stroke [103] and in neurological diseases such as Alzheimer's disease[104–106]. At the start of this thesis work, it was unknown whether the glymphatic pathways are affected during bacterial meningitis.

Neurological symptoms and sequelae

The disease presentation and clinical course of bacterial meningitis is heterogeneous. Most patients with meningitis present with at least two of four symptoms: fever, headache, neck stiffness, and altered consciousness[107]. As the disease progresses, several different neurological symptoms may appear[108]. In some cases they resolve before or shortly after the patient is discharged from the hospital, but some sequelae can remain for years. The presence of lasting neurological sequelae in survivors and the associated spillover effects on their family members and caregivers mean that the disease burden of bacterial meningitis goes far beyond hospital costs and deaths[109].

Hearing loss

Hearing loss is the most common neurological deficit that occurs in survivors of bacterial meningitis[108]. It usually develops early in the disease course and is likely due to damage caused by the presence of bacteria or inflammatory products in the inner ear[110–112]. Hearing loss can in some cases be permanent or can resolve over time[108]. Hearing loss affects quality of life and mental health, and is particularly damaging in children because it hinders language and social development, which has lasting consequences into adulthood[113–115].

Focal neurological deficits

Focal neurological deficits are caused by damage to a specific site of the central nervous system, such as a specific part of the brain or a specific nerve. They can range from small localized deficits, such as loss of control of specific facial muscles, to large

deficits that affect movement of an entire limb[116]. Focal neurological deficits are most often caused by tissue damage due to disruptions to the cerebral blood supply by intracerebral clots or bleeding, but can also be caused by formation of abscesses or the presence of bacteria in the subdural space (subdural empyema)[48,117–119]. Focal neurological deficits that persist can interfere with daily life or the ability to work, so rehabilitation therapy is recommended for survivors of bacterial meningitis[108].

Seizures

Seizures are relatively common before and during admission for bacterial meningitis[108]. They are caused by damage to the cerebral cortex as a result of inflammation or cerebrovascular events. The heightened inflammatory state during bacterial meningitis is thought to lead to neuron hyperexcitability, resulting in a low seizure threshold[120]. Seizures can also appear months or years after the infection is resolved in survivors[108]. Seizures developing after bacterial meningitis are often resistant to antiepileptic drugs, making them particularly difficult to control[120]. Seizure disorders often result in reduced quality of life for the patient and increased burdens for caregivers[121–123].

Cognitive deficits

Cognitive deficits are impairments in normal cognitive processes such as learning and intellectual disabilities or deficits in attention, memory, or executive functioning. They can result from damage to various regions of the brain, especially in subcortical structures such as the hippocampus[124,125]. Cognitive deficits in children can have lifelong consequences – children who survived bacterial meningitis are more likely to underachieve at school[126,127], have lower IQ scores[128], and are 10% less likely to complete high school[129]. In adults, cognitive impairments tend to be mild memory impairments and general mental slowness, which could interfere with the ability to work or to conduct daily activities[108]. Unlike physical impairments, cognitive deficits after bacterial meningitis are unlikely to improve over time[130].

Therapeutics for bacterial meningitis

Antibiotics

The first most obvious treatment for bacterial meningitis is the use of antibiotics to kill the invading pathogen. However the specific choice of antibiotic is limited by the need to be able to cross the blood brain barrier at sufficiently high doses, by the

identity of the pathogen, and by local patterns of antibiotic resistance[81]. The identity of the infecting pathogen is determined by CSF culture, which takes 1-3 days, and so empiric antibiotic treatment is initiated while awaiting the culture results. Antibiotics that are usually recommended as empirical treatment are the cephalosporins cefotaxime and ceftriaxone, and possibly the carbapenem meropenem if *Listeria monocytogenes* infection is suspected[81,131]. The exact antibiotics are then adjusted once the pathogen is identified. Antibiotics are typically administered for a long duration from 7 to 14 days depending on the pathogen[81]. However, even in spite of sufficient antibiotic treatment, the mortality of bacterial meningitis remains high[83–90].

Corticosteroids

Adjuvant therapies, administered in addition to antibiotics, could be used to reduce mortality and neurological sequelae of bacterial meningitis. Currently the only adjuvant therapy recommended for bacterial meningitis is corticosteroids. Corticosteroids are strong anti-inflammatory drugs that are thought to limit damage caused by the inflammatory response to the pathogen. Some studies suggest that use of corticosteroids may reduce passage of some antibiotics over the blood brain barrier, rendering antibiotic treatment less effective or requiring higher doses of antibiotics[132–135]. Nonetheless, corticosteroids were found to reduce mortality from 20% to 18% in a systematic review, although they did not have a significant effect in low-resource countries[136], and are thus recommended for use in bacterial meningitis[81]. However, even after the regular use of corticosteroids was adopted, the rate of unfavorable outcome (death or debilitating sequelae) remains high at 38%, suggesting that other adjuvant therapies should be explored[86].

Non-corticosteroid adjuvant therapies

To determine whether there is evidence for the effectiveness of any adjuvant pharmacological therapies, other than corticosteroids, we carried out a systematic literature review according to a Cochrane systematic review protocol created by our group. Randomized controlled trials in humans are the highest level of evidence, short of systematic reviews, and are typically required before a new therapy is applied in routine clinical practice[137], so we limited our search these types of studies. We found that randomized controlled trials have been carried out or registered for five different pharmacological adjuvant therapies: paracetamol (3 studies)[138–140], immunoglobulins (3 studies)[141–143], heparin (1 study)[144], pentoxyfilline (1 study)[145], a mixture of various vitamins and cofactors (Cytoflavin; 1 study) [146], and phenytoin (1 study)[147].

Overall we found that only studies of immunoglobulins and Cytoflavin reported an overall positive effect on either mortality or neurological sequelae, but these studies are small and have a high risk of bias. The rest of the studies indicated no effect or a negative effect on mortality and neurological sequelae in bacterial meningitis. This indicates that there is no high quality evidence for the effectiveness of any adjuvant pharmacological therapies, other than corticosteroids, suggesting that studies of new adjuvant therapies are needed. The various included studies are detailed below.

Paracetamol

Paracetamol is an analgesic that may have mild anti-inflammatory properties[148,149]. Three clinical trials have tested paracetamol in bacterial meningitis in children. The first found no effect on neurologic sequelae and no overall reduction in mortality, although a post-hoc analysis indicated a possible reduction in short term (3-day) mortality[138]. A second study found no effect on either mortality or neurologic sequelae[139]. A third, larger study, has not yet been published, however the results have been posted to clinicaltrials.gov (trial NCT01540838)[140]. The results of this study indicate only very small differences between the treatment groups, trending toward reduced mortality but increased neurological sequelae in the paracetamol group.

Immunoglobulins

Immunoglobulins taken from healthy blood are most often administered intravenously and may help to neutralize bacteria and interfere with cytokine effects, although their benefit in infections is unclear [150,151]. Three randomized controlled trials administered immunoglobulins in bacterial meningitis. One study found a significant reduction in mortality in patients receiving intrathecal immunoglobulins[141]. However the sample size was small, at only 20 patients in total. Another study was slightly larger and reported fewer deaths and a significant reduction in neuropsychological defects and paralyses in the immunoglobulin treated group[142]. Both studies did not report quality control measures such as allocation concealment and blinding, indicating a high risk of bias. One study did not report mortality or incidence of neurological sequelae[143], even though they claim to have been measured, indicating selective outcome reporting bias.

Heparin

Heparin is an anticoagulant that prevents blood clot formation, although it may have other beneficial effects[152], and was therefore hypothesized to prevent cerebrovascular events in bacterial meningitis. One clinical trial in a small group of 15 patients with bacterial meningitis found increased mortality and increased focal neurological signs in the group receiving heparin treatment[144]. This study also did not report quality

control measures such as allocation concealment and blinding, indicating a high risk of bias.

Pentoxifylline

Pentoxyfilline is a vasoactive drug that is used to treat vascular disorders of the extremities and may prevent clot formation [153,154]. One study administered pentoxyfilline to patients with bacterial meningitis and found no effect on mortality or severe neurological sequelae. This study reported that blinding and allocation concealment were not carried out, indicating a high risk of bias.

Vitamins and cofactors

One randomized controlled trial studied the administration of a mixture of four vitamins and cofactors (Cytoflavin; a mix of succinic acid, riboxin, nicotinamide, and riboflavin) in children with bacterial meningitis[146]. This study did not report mortality, but reported a shorter duration of neurological symptoms in the treated group. This study also did not report quality control measures and therefore has a high risk of bias.

Phenytoin

Phenytoin is used to treat seizures[155], which are a major neurological symptom of bacterial meningitis[108]. One study has been registered to clinicaltrials.gov (NCT01478035)[147] for the use of phenytoin in adults with pneumococcal meningitis, but the results have not been reported in any database or publication.

DNase

In 1959, professor Tillett's group at Bellevue Hospital, New York City, presented data indicating that administration of bovine DNase as an adjuvant to penicillin reduced mortality in bacterial meningitis from 30% to 12%[156]. This was not a randomized controlled trial, but it bears mention because of its particular relevance to this thesis. DNase is an enzyme that degrades extracellular DNA, which is the major structural component of NETs. Although DNase was never explored further as a therapeutic in bacterial meningitis after Tillett's study, aerosolized DNase has since been approved for human use as a therapeutic for cystic fibrosis (brand name Pulmozyme). DNase has also been injected safely into healthy humans and patients with systemic lupus erythematosus[157]. Therefore, the safety of DNase administration in humans is well studied, and if future studies indicate that DNase may be effective

in bacterial meningitis it would be relatively quick to bring it to clinical practice compared to other new investigational compounds.

Pneumococcal meningitis – a special case

Streptococcus pneumoniae (pneumococci) are the most common and most deadly pathogen causing bacterial meningitis [86,158–160]. Pneumococcal meningitis also carries the highest risk for most neurologic sequelae[108]. Pneumococci are a capsulated gram positive bacteria[161]. Their prevalence in bacterial meningitis cases can be explained by their widespread carriage in the healthy population. Up to 10% of healthy adults and 40% of healthy children are colonized by pneumococci in the nasopharyngeal tract[161]. This frequent colonization is due to a host of virulence factors that allow pneumococci to evade the immune system[162,163].

The high rate of death and neurological sequelae of pneumococcal meningitis can be attributed to ability of pneumococci to induce a strong and persistent inflammation even after their death. When the bacteria are lysed, they release cell wall components such as peptidoglycans, wall teichoic acid and lipoteichoic acids, which activate toll-like receptors and induce inflammatory responses[164]. Pneumolysin is a cytoplasmic protein that is also released from pneumococci following their lysis. It creates pores in the membranes of host cells, causing them to lyse and die[165]. In addition to its cytotoxic properties, it can also interfere with or amplify various immune functions[166–169].

Lysis of pneumococci that results in release of pneumolysin and cell wall components can be triggered in several ways. Pneumococci are generally resistant to lysis by normal innate immune components such as the complement system due to their protective capsule[170]. However, pneumococci express an enzyme called autolysin that triggers self-lysis in some situations[163]. They can also be lysed by antibiotics that attack the bacterial cell wall, termed "lytic antibiotics"[171]. These include beta-lactam antibiotics such as cefotaxime and ceftriaxone, which are recommended for empirical treatment of bacterial meningitis[81,171]. Therefore antibiotic therapy itself may have harmful secondary effects due to the release of inflammatory and cytotoxic factors from pneumococci and could explain why the mortality and neurologic sequelae of pneumococcal meningitis remain high in spite of sufficient antibiotic treatment. Non-lytic antibiotics that do not attack the cell wall may reduce this damage[172,173], but there is currently a lack of clinical evidence to support their use in bacterial menignitis.

Ventriculostomy-related infections

Nosocomial infections of the brain can occur in the hospital following a procedure or surgery that exposes the brain to bacteria. External ventricular drains (EVDs), also known as ventriculostomy catheters, are lifesaving devices that involve insertion of a catheter into the ventricles of the brain. They are used to monitor and regulate intracranial pressure in conditions such as subarachnoid hemorrhage and traumatic brain injuries[174]. This breach of the sterile brain environment sometimes leads to ventriculostomy-related bacterial infections (VRI)[175]. The infection can occur in the ventricles where the catheter is inserted (ventriculitis), and can spread to the meninges, which are connected to the ventricles, resulting in meningitis. The two infection loci are not typically distinguished when diagnosing and treating VRI[175]. The infecting organisms are most often skin colonizers, including coagulase negative staphylococci (CoNS; e.g *Staphylococcus epidermidis*), *Staphylococcus aureus*, and *Cutibacterium acnes*[175].

Diagnostic considerations

Diagnosis of VRI is complex. Patients treated with an EVD are often sedated or unconscious, so self-reporting of symptoms is difficult. The underlying conditions that are typically treated with an EVD often result in symptoms that mimic ventriculitis and meningitis: neck stiffness, headaches, fever, and lowered consciousness[176]. CSF markers that are typically altered in ventriculitis and meningitis, including leukocytes, glucose, lactate and albumin, can also be affected by the underlying condition[176]. Additionally, several of the bacteria that often cause VRI, especially CoNS and *C. acnes*, cause only minimal inflammation[175]. Therefore typical CSF markers of infections may be unreliable for diagnosis of VRI[176].

Detecting the presence of bacteria via CSF culture or polymerase chain reaction (PCR) is considered the gold standard for diagnosis of VRI. However, the sensitivity of CSF cultures is variable and often result in false negatives, especially if the patient is treated with antibiotics for another infection[177]. Their specificity may also be unreliable: the sample can be easily contaminated or the EVD colonized by skin bacteria, leading to false positives. Even the definition of what constitutes a VRI is variable between institutions and individual studies, resulting in huge variation in the reported incidence of VRI from 0 to 45%[178–181]. Some studies indicate that a VRI diagnosis does not lead to increased mortality, although it is associated by increased length of hospitalization[181,182]. This lack of impact on mortality may simply be

because treatment is initiated very early in the disease course, or it could indicate that VRI diagnosis does not necessarily correspond to the presence of severe infection.

Therapeutic considerations

Given the uncertainty of VRI diagnosis techniques and the severe nature of meningitis and ventriculitis, the threshold for initiating treatment is very low. The main treatment for VRI is a high dose of broad spectrum antibiotics, in Sweden typically cefotaxime, meropenem, or vancomycin[131]. However with the increasing risk of antibiotic resistance[183,184] and unpleasant side effects of antibiotics[185], it is desirable to reduce the amount of antibiotic treatment that is given. In one study, only about one in eight patients who received empirical treatment for VRI actually had a final VRI diagnosis, suggesting that patients might frequently be over-treated[179]. Better techniques for diagnosis of VRI could be the key to reducing unnecessary antibiotic treatment in these patients.

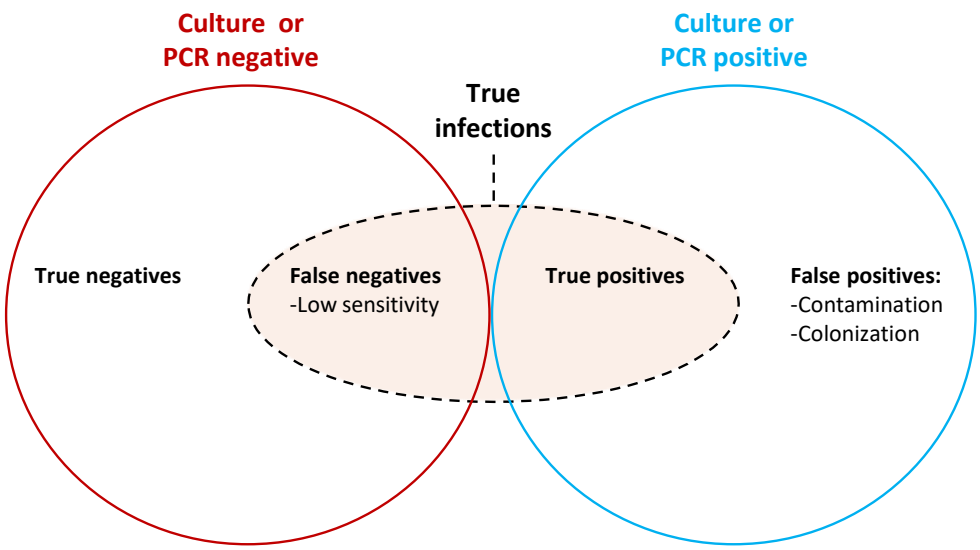

Figure 6. A depiction of possible diagnostic outcomes in a population of culture- or PCR-negative and positive patients.

Aims

The overarching aim of this thesis was to explore the diagnostic and therapeutic potential of endothelial and neutrophil responses in sepsis and bacterial meningitis.

Specific aims

Specific aims of this thesis were:

1. To determine whether glypicans are shed from the endothelium during sepsis, and determine whether elevated plasma glypicans are associated with development of organ dysfunction.

2. To determine whether NETs are present in the CSF during acute bacterial meningitis and whether their removal using DNase affects bacterial killing in the brain.

3. To determine whether acute bacterial meningitis reduces glymphatic flux in the brain and whether removal of NETs using DNase affects glymphatic flux.

4. To determine whether NETs are present in the CSF during suspected nosocomial meningitis and ventriculitis in patients treated with an EVD.

5. To design and test a scoring system for detection of clinically relevant neurological symptoms in a rat model of bacterial meningitis.

Methods

Methods in translational research

Translational research bridges the gap between clinical and preclinical research. It starts from a clinical question and brings it to the laboratory bench, and works toward answers that can eventually change clinical practice. Usually a combination of interdisciplinary methods are required to answer the complex questions posed by the many problems facing human health. From our experiences, different methods have different strengths and limitations, which are briefly discussed below.

Clinical studies

Clinical trials, in which patients are randomized to an intervention group or a control group, are considered a top tier of evidence to test the effectiveness of a new diagnostic or therapeutic method[137]. However clinical trials are expensive, often take years of work, and require much safety data and strict regulatory control[186]. For these reasons clinical trials may not always be feasible. Instead, other evidence is first collected to determine whether a new intervention is promising enough to warrant the arduous process of testing it in clinical trials.

Observational cohort studies are non-interventional clinical studies that can be used to gain insight into new biomarkers or the effectiveness of certain routine interventions[187]. Observational cohort studies can be retrospective or prospective. Certain markers can be measured in stored samples and applied retrospectively to the cohort to determine whether their levels can predict outcomes, giving insight into their potential as prognostic biomarkers or therapeutic targets. If some patients received an intervention as part of routine hospital care, the outcomes in these patients can be compared to those who did not receive the intervention, although the lack of randomization limits the conclusions that can be drawn from these types of studies.

Observational cohort studies are useful because they involve human subjects and are therefore highly relevant to the human disease, but they also have many limitations. The main limitation, especially in sepsis and meningitis, is that the disease can be highly heterogeneous: it can be caused by many different pathogens and can have varying levels of severity, with patients being admitted to the hospital during different

stages of the disease[188]. Patients also receive many concomitant treatments that can confound the results[188]. Observational studies are also limited by the lack of ability to apply randomize subjects to intervention and control groups, thereby limiting their value for testing novel interventions.

Animal models

The strengths and limitations of animal models make them a good complement to observational cohort studies. Animals can all be infected by a single pathogen at a specific dose, and treated at the same time relative to the start of the infection. Concomitant treatments can be limited or removed entirely. This can result in far less heterogeneity than is found in the human disease. This can be both a strength and a weakness – conclusions about effectiveness can be achieved more easily in a homogenous cohort, but they may lose their applicability to the heterogeneous conditions found in human disease[188].

An advantage of animal models is that outcomes can be measured in detail in ways that would not be possible in human studies. Animals can be sacrificed at specific times and their organs removed to visualize, in detail, changes to cells and tissues. This allows researchers to elucidate disease mechanisms and molecular effects of interventions. On the other hand, because animals cannot talk, certain outcomes such as pain, depression, or cognitive defects are not easily assessed, requiring surrogate measures.

The main limitation of animal models is the fact that they simply are not humans. Although researches should strive to choose animals in which the disease course resembles the relevant human disease as closely as possible[189], this is often not possible due to ethics, funding, or simply inconvenience. These differences are not trivial – many interventions that show promise in animal models of sepsis and meningitis eventually fail in clinical trials[188,190].

Ex vivo and *in vitro* models

Experiments can also be done on human cells and tissues that have been removed from the body (*ex vivo*) or cells that have been modified to replicate in culture (*in vitro*). These types of experiments remove the complexity of the disease condition and the multitude of interacting cells and signals that are found in a whole organism. This allows for examination of very specific molecular mechanisms. The effect of a drug on a specific receptor or signaling pathway can easily be elucidated, and many different drugs can be screened in high-throughput assays. *Ex vivo* and *in vitro* models can't

answer whether an intervention is clinically effective, but they can be used to complement animal and clinical studies, allowing researchers to elucidate specific biological mechanisms that are relevant to the disease.

Methods used in this thesis

The limitations and strengths of the different research methods discussed above were considered before they were applied to this thesis work. To increase its translational value, this work used clinical studies, animal models, and ex vivo and in vitro models to answer relevant clinical questions. Details of all methods used in this thesis are described in the included papers. Below is a brief discussion and rationale of the main methods.

Clinical studies

Retrospective observational cohort study

A retrospective observational cohort study was the main methodology in **study I**. This study design was chosen because the clinical question was largely exploratory – we simply wanted to know whether plasma glypicans are elevated during sepsis. Plasma samples were available from 184 patients from a previously collected prospective nonconsecutive convenience sample study. The study had included adult patients with fever and clinically suspected infection who were admitted to the Clinic for Infectious Diseases at Skåne University Hospital March 2006 to April 2008.

Because this cohort was enrolled before the new Sepsis-3 definition was adopted, they were originally classified based on systemic inflammatory response syndrome (SIRS) criteria, organ failure, and final diagnosis, according to the sepsis criteria proposed by the American College of Chest Physicians/Society of Critical Care Medicine[191]. To ensure that our findings would be relevant to the current sepsis definition, we retrospectively re-classified the patients to fit the new Sepsis-3 criteria, which emphasize organ dysfunction. Therefore patients were separated into two groups: infection with organ failure (sepsis; 64 patients) and infection without organ failure (120 patients). Patients with organ failure were further subdivided into two groups: those that already had organ failure at the time of enrollment and sample collection (37 patients) and those that developed organ failure after enrollment and sample collection (27 patients).

Prospective observational cohort studies

Two cohorts of patients were prospectively enrolled in studies II and IV, where we quantified NETs in CSF of patients with various neurological conditions. At the time of starting this thesis work, NETs had never been measured CSF of in humans in any condition. Analysis of NETs requires fresh CSF samples, so previously collected samples could not be used and patients had to be enrolled prospectively. Prospective studies are considered advantageous because they require definition of a study question and enrolment criteria before the study begins, ensuring the use of a relevant cohort[187]. However prospective enrolment can take a lot of time to reach the desired number of patients, especially when a condition is not very common.

In **study II**, we enrolled 6 patients with bacterial meningitis, 4 with viral meningitis, 3 with neuroborreliosis, and 3 with subarachnoid haemorrhage. CSF samples were collected when patients underwent a lumbar puncture for suspicion of either condition. The cohort was small, mainly because bacterial meningitis is fairly uncommon in Sweden, typically with less than 10 cases per year admitted to Skåne University hospital in Lund, where we enrolled patients.

In **study IV**, we enrolled 30 patients who were treated with an EVD at the neurointensive care unit at Skåne University hospital in Lund and had a CSF sample collected for clinical chemistry analysis. To determine whether NETs are elevated in patients with a suspected ventriculostomy-related infection, we measured CSF NETs by immunofluorescence analysis and collected clinical data from the patients' charts. This cohort was smaller and had less samples from patients with suspected infections than we had anticipated for several reasons. We relied on the clinical chemistry department to save samples that were left over from other analyses. If there was insufficient volume left over or if the technician did not remember to save the sample then we were unable to analyze it. Although we enrolled several patients with a suspected infection, we often did not receive samples taken on or near the day that the infection was suspected. A possible explanation for this is that often samples from patients with a suspected infection were used for more analyses, and therefore there was less sample left over. The small cohort size made it difficult to statistically explore the many possible factors leading to NET formation in this cohort, as small sample sizes can lead to inaccuracies in many statistical tests[192].

Rat model I

The most common animals used for meningitis models are mice, rabbits, and rats[193]. While mice are the least expensive, their small size does not allow for repeated sampling of large volumes of CSF, which we required for analysis of NETs. Rabbit

models are expensive and hindered by ethical restrictions[194] which limits the number of animals that can be used, increasing the risk of conducting an under-powered study[193]. Rat models can use infant or adult rats. Infant rats (typically 6-12 days old) exhibit both cortical necrosis and hippocampal apoptosis, similar to human meningitis, while adult rats mainly exhibit cortical damage[193]. However in rats the peak rate of brain growth and gliogenesis occurs at post-natal day 7-10, and they only reach adult levels of neurotransmitters and synaptic density after they are 60 days old[195]. These factors could reduce the translatability of infant rat models to adult meningitis. Therefore we chose to develop an adult rat model of bacterial meningitis. We used this first rat model in **studies II and III**.

Infection and timeline

We infected rats with *S. pneumoniae* because it is the most common pathogen causing bacterial meningitis. We used a pneumococcal strain isolated from one of the patients with bacterial meningitis that was included in the observational cohort in **study II**, in order to increase the clinical relevance of the model. Bacteria were introduced into the meninges by cutting a window from the skull bone, carefully puncturing the thin membranes with a needle and placing a catheter inside. A suspension of bacteria in saline solution was infused slowly over 10 minutes to allow the pressure to normalize and reduce spillage out from the puncture. A timeline is present in **Figure** 7.

Most models of bacterial meningitis described in the literature introduce bacteria by injection into the cisterna magna[196–199], while our method of introducing bacteria via a craniotomy is not described (**Figure 8**). One reason we chose this method is because the group had previous experience with using a craniotomy to introduce traumatic brain injury and this was easily adapted for injection of bacteria. In **study III** this method was necessary because a tracer solution was later injected via the cisterna magna. A previous puncture of the cisterna magna would have led to tracer leakage and inconsistent results, so it was necessary for the bacteria and tracer injection sites to be distinct.

Treatment and evaluation

To determine what roles NETs play in bacterial meningitis, we dissolved NETs in infected rats using DNase, an enzyme that breaks down the extracellular DNA backbone of NETs. We administered the drug either intrathecally (directly into the CSF) or intravenously. Intrathecal administration ensured that the drug would reach the CSF immediately and at a high concentration, without having to cross the blood brain barrier. We administered DNase intrathecally at the same time as the infection to test the effect of degrading NETs in an early stage of the infection. To evaluate the

effect of DNase in a more clinically relevant time frame (after the infection is established) we also administered DNase intrathecally, 10 hours after the infection.

Clinically, intravenous administration is a more convenient and safer route than intrathecal injection. However, because the drug must cross the blood brain barrier to reach the CSF, large amounts of the drug may be required to have an effect on CSF NETs. Therefore we also administered DNase intravenously to determine whether it is able to cross the blood brain barrier at sufficient concentrations to dissolve NETs. We chose to administer a bolus at 6 hours after the infection, to ensure that the infection was established at time of administration, followed by a continuous infusion over the next 18 hours. The continuous infusion ensured that there would be a constant supply of DNase present in the blood, since intravenously administered DNase has a half-life of 3-4 hours[157].

Rats were evaluated for all outcomes at 24 hours after the infection. In **study IV**, a fluorescent tracer was injected and allowed to distribute in the brain for an additional 30 minutes before samples were collected.

Confirmation of bacterial meningitis

We confirmed that the rats displayed symptoms and features consistent with bacterial meningitis. We confirmed the presence of viable bacteria in the brain homogenate of infected rats and no bacteria in control rats. In **study III**, the whole brains were used for immunofluorescence analysis and therefore viable bacterial counts could not be determined. As a surrogate measure, we stained the brain sections using an anti-pneumococcal antibody to confirm the presence of bacteria in the brains of the infected rats.

Bacterial meningitis is known to increase neutrophil infiltration[81], blood brain barrier permeability[94], and brain tissue edema[200]. Brain sections were stained for DNA and MPO and a clear increase in invading neutrophils was seen in infected animals. To measure blood brain barrier permeability, we intravenously injected two tracers (radiolabelled ^{51}Cr- Ethylenediaminetetraacetic acid (EDTA) and ^{125}I-albumin). We confirmed that the blood-to-brain transfer constant was increased in infected rats, indicating increased permeability consistent with bacterial meningitis. To measure brain tissue edema, brains were weighed immediately after removal from the body. Brains from infected rats were significantly heavier, most likely due to increased water content. Lastly, infected rats lost significantly more weight and had significantly higher plasma interleukin-6 (IL-6) than saline control rats, confirming that rats displayed systemic effects consistent with severe infections such as bacterial meningitis.

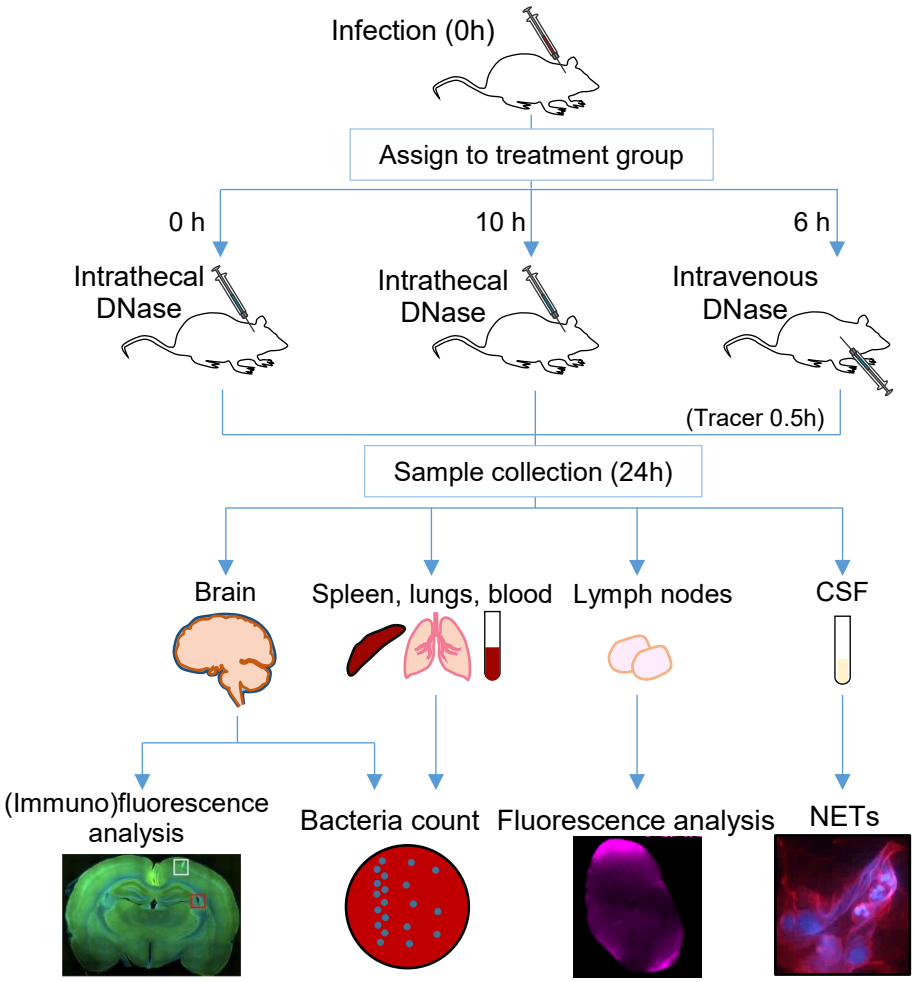

Figure 7. Schematic depiction of the timeline, sample collection, and analyses in rat model I, used in studies II and III.

Rats were infected at 0h and treated with either intravenously or intrathecally with DNase or saline control solution at the indicated time points. In study III, some rats were also treated with intravenous antibiotics at the 6 hour time point (not shown). Samples were collected for further analysis 24 hours after the infection. In study III, rats also received an intracisternal injection of a fluorescent tracer at 24 hours, which was allowed to distribute for 0.5 hours. Samples were collected 24 hours after the infection and analyzed by various methods.

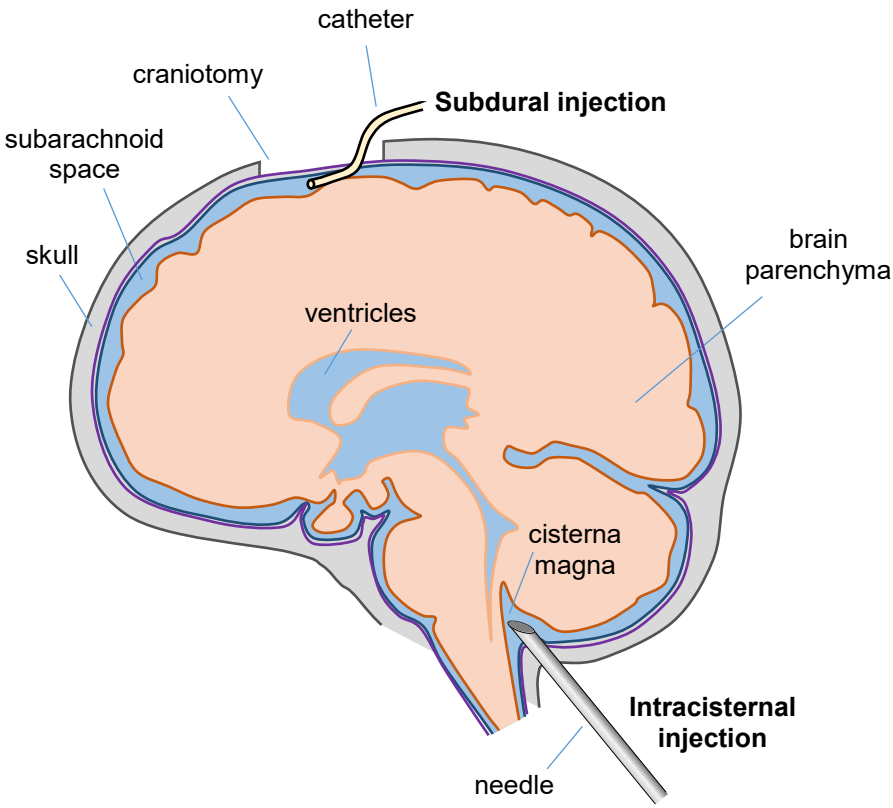

Figure 8. Subdural and intracisternal infection routes used in rat model I and II respectively

Rat model II

In order to test the effect of novel therapeutics, it is important to evaluate clinically relevant and patient-important outcomes. In **study V**, we chose to develop a different rat model of bacterial meningitis that could be used to quantify neurological symptoms and sequelae, as these are a major consequence of bacterial meningitis in survivors and severely impact patient quality of life[108].

Infection and timeline

In order to allow rats to develop more neurological symptoms than they did in the previous model, we evaluated the rats for 6 days after the infection (**Figure 9**). We infected rats with a clinical isolate of pneumococci had intermediate sensitivity to penicillin, ensuring that some level of infection would be present in spite of antibiotic treatment. We administered bacteria or saline solution via the cisterna magna to be consistent with other meningitis models in the literature[196–199] (**Figure 8**). Rats were evaluated at 24 hours after the infection for CSF bacteria and neurological symptoms to confirm the presence of bacterial meningitis, and then a standard treatment was administered until the end of the study. The standard treatment included antibiotics and corticosteroids, which is the recommended treatment for human bacterial meningitis[81,201]. Rats were evaluated again at 48 hours and 6 days after infection. Rats were evaluated at each time point by measuring viable bacterial counts in the CSF, and by a functional observational battery to evaluate neurological symptoms. Additionally at 6 days after the infection, brains were collected for immunofluorescence analyses.

Neurological evaluation

Because the neurological symptoms of bacterial meningitis are highly varied [107,108], we wanted to be able to evaluate a wide range of symptoms. However we found very few studies in the literature that attempt to quantify the wide range of possible neurological symptoms in rat models of meningitis. Therefore, we looked to the field of neurotoxicology where battery tests are frequently used to test neurologic effects of new therapeutics[202–204]. The functional observational battery is a well-accepted method for identifying neurological symptoms in rats[205]. We therefore modified the functional observational battery to include symptoms that are relevant for bacterial meningitis. Details of the choice of symptoms and method for evaluation of each symptom are found in **study V**.

Symptoms were divided into clinical signs, which are general signs of illness that are not specific to bacterial meningitis, and neurological signs that indicate damage to the brain and nerves. Neurological signs were subdivided into four groups: gait and

posture, involuntary motor movements, focal neurological signs, and neuromotor tests. Each symptom within each group was scored on a scale from zero (normal or absent) to 3 (severe). The scores were summed to obtain a score for each group of symptoms and a total combined score.

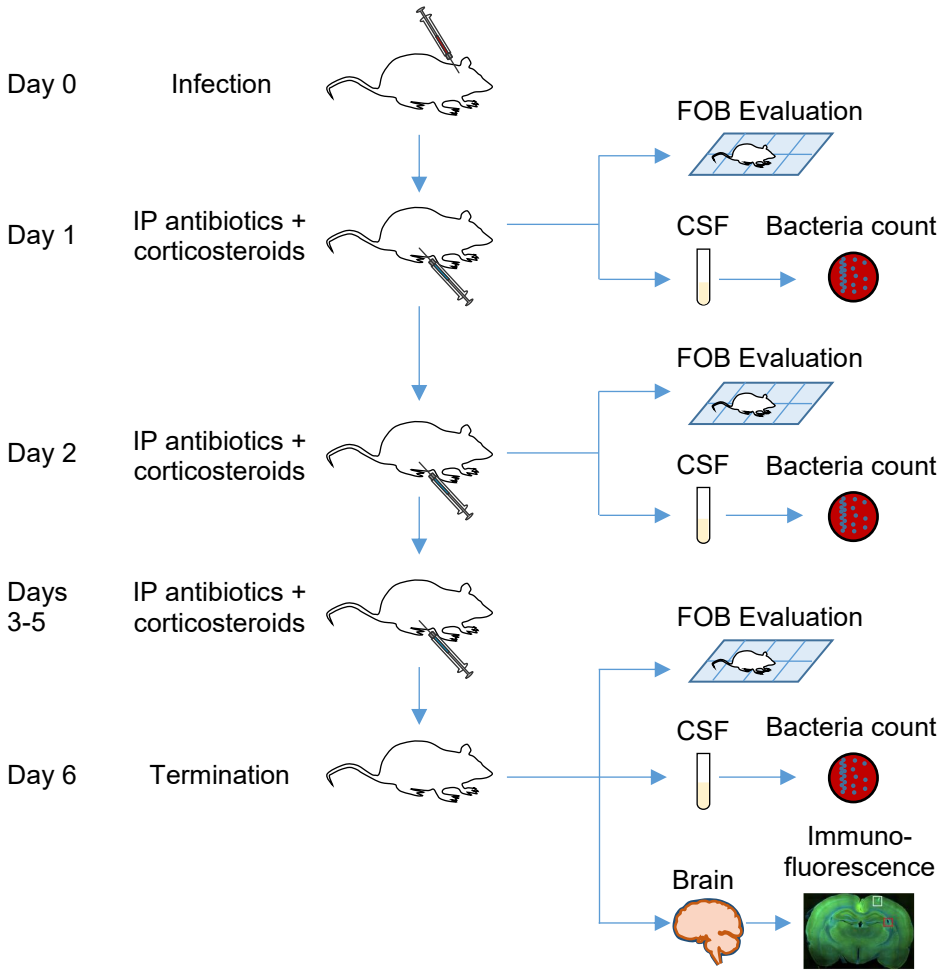

Figure 9.Schematic depiction of the timeline, sample collection, and analyses in rat model II, used in study V.
Rats were infected at 0h and then evaluated 24 hours after the infection by the functional observational battery (FOB) and by analysis of CSF bacterial counts. Standard treatment of antibiotics and corticosteroids given intraperitoneally (IP) was initiated after this evaluation and continued until the end of the experiment. Rats were also evaluated on day 2 and 6 after the infection. Additionally on day 6, when rats were terminated, brains were collected for immunofluorescence analysis.

Table 1. Symptoms evaluated in the modified functional observational battery.

Clinical signs	Gait and posture	Involuntary motor movements	Focal neurological signs	Neuromotor tests
Breathing difficulty	Ataxia	Tremors	Lacrimation	Righting reaction
Movement	Hindlimb movement/ positioning	Jerks and spasms	Salivation	Negative geotaxis response
Grooming	Forelimb movement/ positioning	Tonic movements	Eyelid drooping or closure	
Vocalizing	Body positioning	Stereotypy	Whisker whisking	
Porphyrin accumulation	Spine curvature	Bizarre behaviours	Pupil reaction	
Cloudy eyes			Blink reflex	
Bulging eyes			Pinna (ear) reflex	
			Hearing loss	

Ex vivo and in vitro studies

In **study II**, we used isolated human and rat neutrophils to explore mechanisms of bacterial killing. Neutrophils were isolated using a polymorphprep gradient. In our experience, this isolation method induces some neutrophil activation. The alternative of stimulating whole blood was unfeasible because of high background from red blood cell autofluorescence, and the inability to control the neutrophil number between donors. Therefore we always used included a negative control to account for background levels of activation.

We induced NETs using either bacteria or phorbol 12-myristate 13-acetate (PMA), a powerful synthetic inducer of NETs. We measured bacterial viability, phagocytosis, and myeloperoxidase activity following treatment with various inhibitors of neutrophil killing and DNase. Additionally, we added supernatant from PMA-induced NETs after treatment with and without DNase to live bacteria and measured the level of killing by extracellular factors.

Ethical considerations

All studies using human or animal subjects were approved by the local ethics committee. Specific application numbers for each study are described in the respective paper.

In the rat models, a particularly important consideration was the use of ethical endpoints[206]. It was important to identify symptoms and features that indicate that a rat is in great pain and terminally ill. In these cases the rat would be terminated to reduce the length of its suffering as much as possible. In our studies, we used a lack of movement, severe difficulty breathing, and severe cramping as signs that a rat was in pain and terminally ill. A lack of grooming was an indication to evaluate rats more frequently for signs of terminal illness.

An important ethical aspect of clinical studies is the obtaining of informed consent. In patients such as those included in this thesis work, including those with sepsis, meningitis, or those treated with an EVD, an important consideration is the fact that often the patients have altered or diminished consciousness due to the disease condition. In Sweden, according to the Medicinal Products Act, SFS 2015:315[207], such patients require the consent of a legally assigned custodian. In those cases it can be particularly difficult to gain informed consent during the illness. In the included studies, because all samples were collected as part of hospital routines, we were able to use an "opt-out" method of consent, where samples are collected and used and the patient is given the chance at a later time to refuse their participation in research studies. If future clinical trials are to be carried out in similar cohorts of patients, these trials may not be possible to carry out in Sweden as consent is required prior to administration of the intervention and therefore impossible to get from unconscious patients because of Medicinal Products Act, SFS 2015:315[207].

Results

A very brief summary of the main results of each paper is presented herein. Readers are encouraged to read the papers at the end of this thesis for a detailed presentation of all results.

Study I

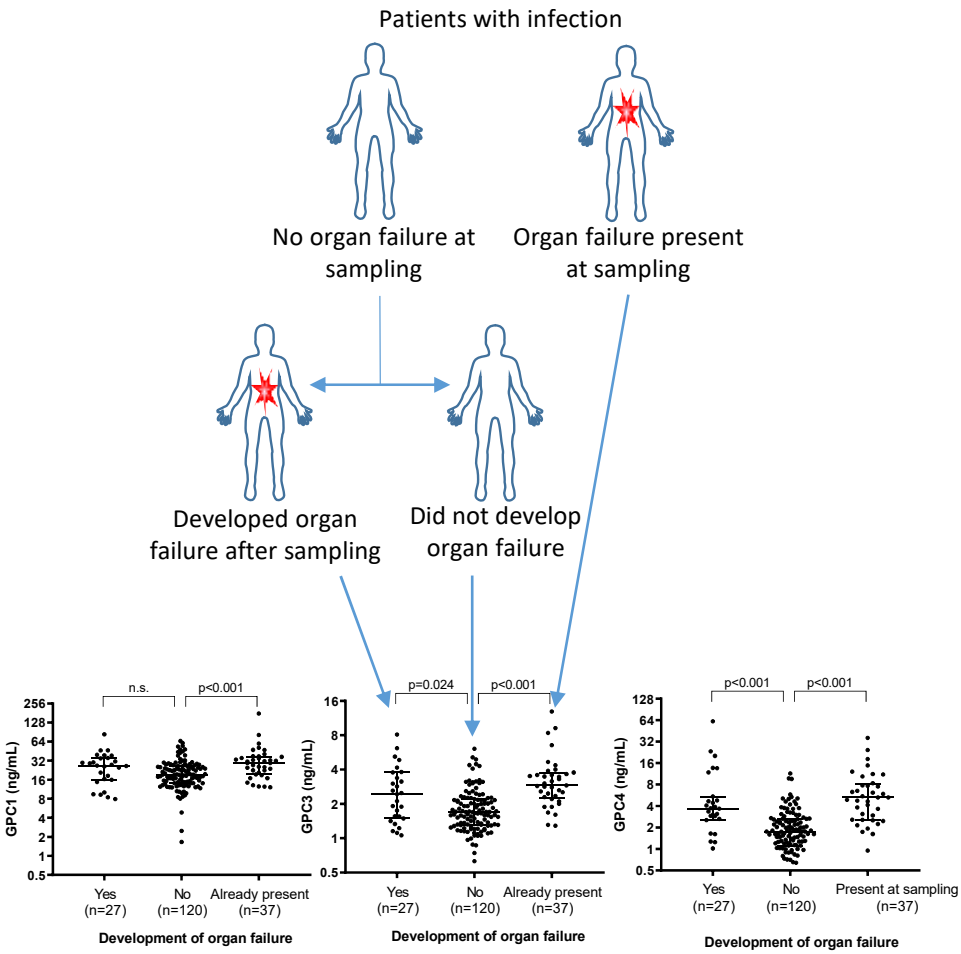

Figure 10. Summary of the main results of Study I.
Plasma was collected from patients with infection who either had organ failure at the time of sampling, or did not. Some patients who did not have organ failure later developed it. Glypicans 1, 3 and 4 were elevated in patients who had organ failure at the time of sampling and in those who later developed it, compared to those who never developed organ failure.

In this study, we found that glypicans 1, 3, and 4 were elevated in the plasma of patients with sepsis, while glypicans 2, 5 and 6 were not detectable. In a cohort of patients with infections, glypicans 1, 3, and 4 were elevated in patients who had organ failure at the time of sampling, and in those patients who developed organ failure after the sample was taken, compared to patients who did not develop organ failure at any time. Glypican levels were significantly correlated with various clinical markers of inflammation and disease severity, including C-reactive protein, lactate, and procalcitonin. All three glypicans were associated with organ failure even when adjusted for potential confounding variables in a multivariate logistic regression model.

Study II

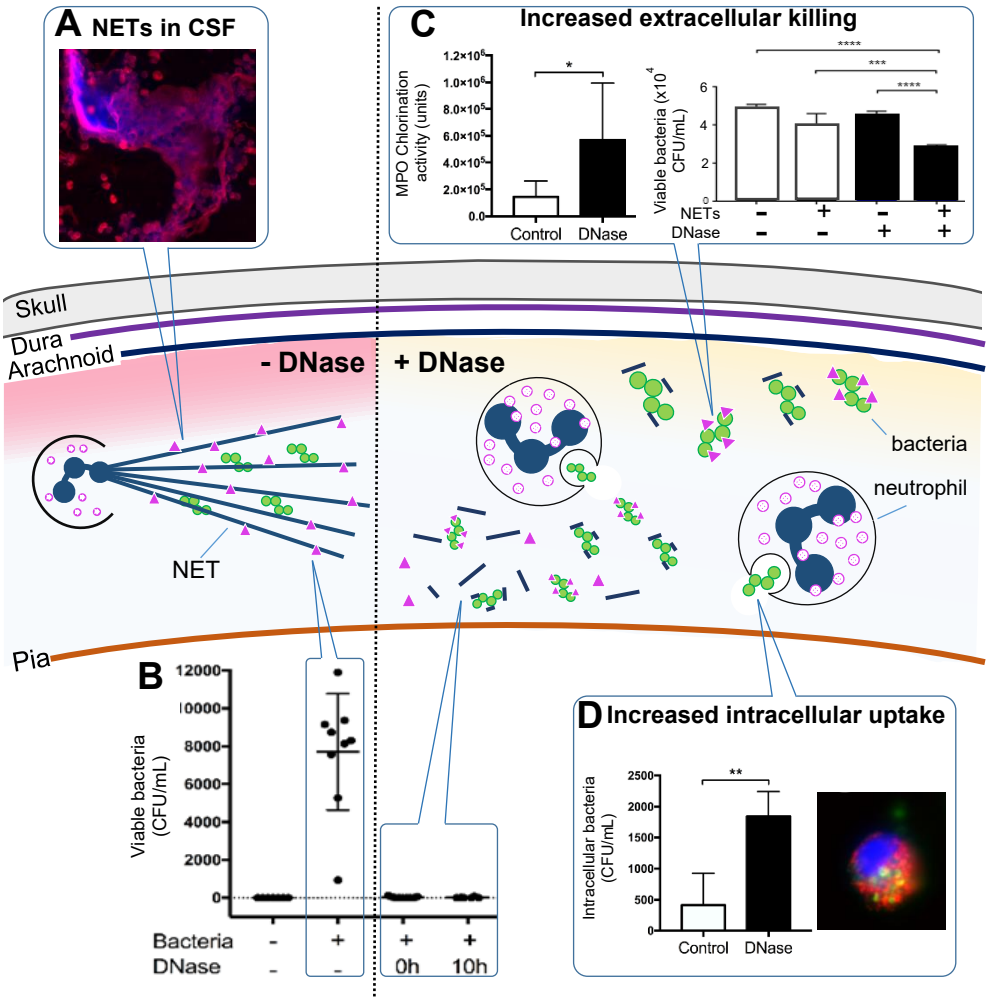

Figure 11. Summary of the main results of Study II.
A) NETs were found in the CSF of patients with acute bacterial meningitis and in a rat model of pneumococcal meningitis. B) Treatment of infected rats with DNase, an enzyme that dissolves the DNA backbone of NETs, resulted in reduced numbers of bacteria in the brain, blood, lungs, and spleen indicating that removal of NETs improves bacterial killing. C) To determine the mechanisms by which NETs increase bacterial killing, we exposed isolated human neutrophils to bacteria and treated them with DNase. We found that DNase-treated samples had more myeloperoxidase activity in the supernatant. Additionally when NETs were chemically induced and the supernatant applied to live bacteria, the supernatant of NETs treated with DNase killed more bacteria then that of untreated NETs, indicating that DNase treatment increased extracellular killing by neutrophils. D) Neutrophils exposed to bacteria and treated with DNase were found to have more intracellular bacteria than neutrophils not treated with DNase, indicating that DNase treatment increased intracellular uptake of bacteria.

In this study we showed, for the first time, that NETs are present in the CSF of patients with bacterial meningitis. NETs were low or undetectable in the CSF of other neuroinflammatory conditions, including neuroborreliosis, subarachnoid hemorrhage, and viral meningitis. To examine the role of NETs in acute bacterial meningitis, we administered DNase to rats with pneumococcal meningitis in order to remove NETs. We found that there were significantly fewer bacteria in the brain and other organs in DNase-treated rats, indicating that removal of NETs improved bacterial killing. To determine the mechanisms of bacterial killing after DNase treatment, we exposed isolated neutrophils to bacteria, with or without DNase. We found that DNase-treated samples had higher numbers of intracellular bacteria than did controls indicating increased intracellular uptake when NETs are removed. The supernatant of DNase-treated NETs also had increased MPO activity and led to increased bacterial killing, indicating that extracellular killing mechanisms are also increased after DNase treatment.

Study III

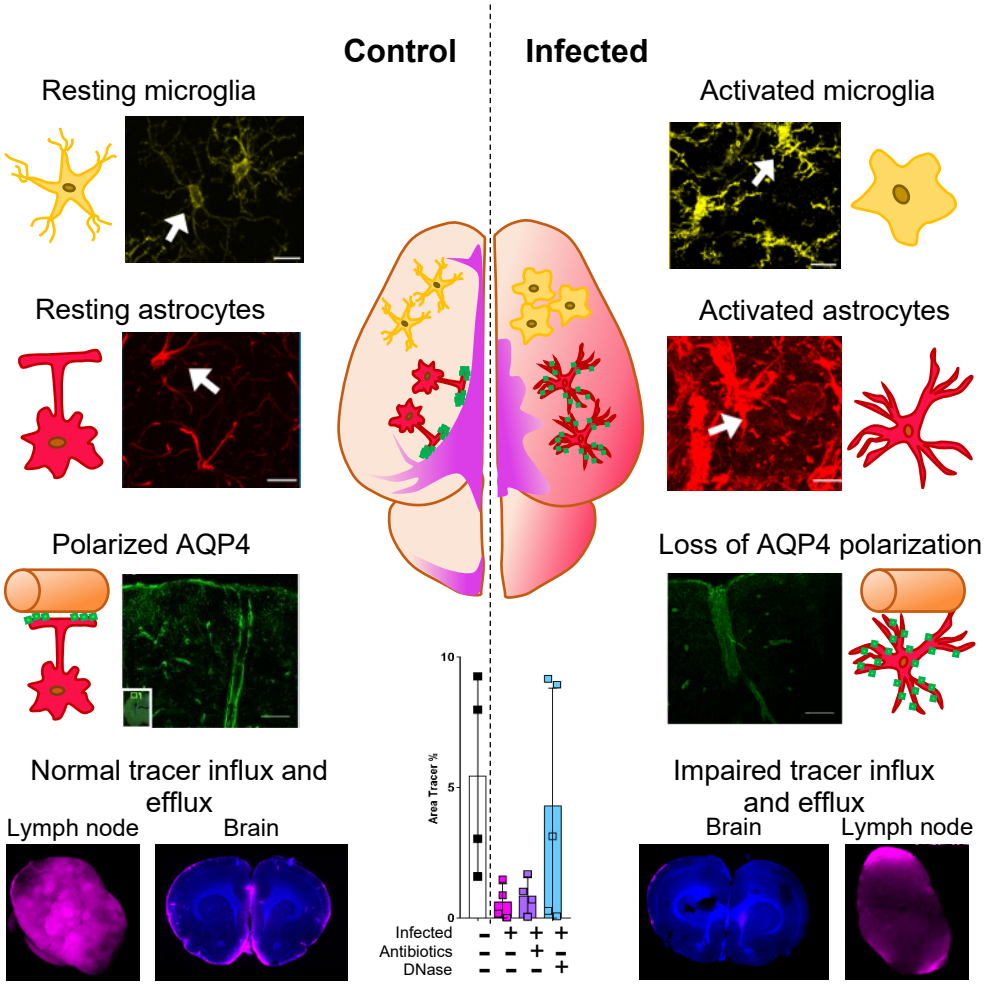

Figure 12. Summary of the main results of Study III.
Rats were infected with pneumococci or saline control, and after 24 hours a fluorescent tracer was injected and brains collected for analysis. In infected rats, microglia activation was increased, identified the presence of ionized calcium-binding adapter molecule 1 (Iba1+; yellow), by cell body hypertrophy, and by the presence of short, thick processes (arrows). Astrocyte activation was also increased, identified by increased expression of glial fibrillary acidic protein (GFAP; red), astrocytic hypertrophy and overlapping of the astrocytic domains (arrows). AQP-4 polarization around large vessels was reduced, with more AQP-4 found away from the endfeet of astrocytes. Tracer influx into the brain and efflux into the lymph nodes was severly reduced in infected rats. Administration of DNase restored the quantified influx of tracer (area tracer %) in some rats, while adminstration of antibiotics did not.

In this study we examined the effect of meningitis on glial cell responses and glymphatic flux. We found that microglia and astrocytes had structural changes indicating increased activation in rats with bacterial meningitis. Activated microglia were identified by the presence of short, thick cell processes in rats with bacterial meningitis, compared to control rats, in which microglia had long, branching processes and a small cell body consistent with resting microglia. Activated astrocytes were identified by increased expression of glial fibrillary acidic protein (GFAP) and increased and overlapping cell processes compared to resting astrocytes found in control rats.

Astrocytes had decreased polarization of AQP-4 around large vessels, indicating that AQP-4 was no longer primarily found in astrocyte endfeet, which could affect their ability to regulate fluid distribution. Distribution and drainage of a fluorescent tracer that was injected into the cisterna magna was impaired in rats with bacterial meningitis compared to controls. Together these data indicate that normal glymphatic system of fluid exchange between the CSF and interstitial fluid and its subsequent efflux are impaired during bacterial meningitis. Intravenous treatment with DNase restored normal tracer distribution in some rats, while antibiotic treatment did not, indicating that NETs may contribute to the disruption of glymphatic flow in bacterial meningitis.

Study IV

In this study we included 30 patients treated with an EVD, of which 11 were treated for a suspected VRI. Of these, only two had a positive CSF culture or PCR result. The majority (77%) of patients had at least 10% of cells forming NETs in the CSF in at least one sample, indicating that some level of NET formation is relatively common in patients treated with an EVD. In patients who had more than one sample, we found that the level of NETs varied considerably over time, suggesting that NETs are highly dynamic in these patients. The total number of NETs and percentage of NET forming cells were not significantly elevated in patients with a suspected infection compared to those without. NETs appear to have low value as a marker of infections, although this result was confounded by the lack of confirmed infections in the cohort.

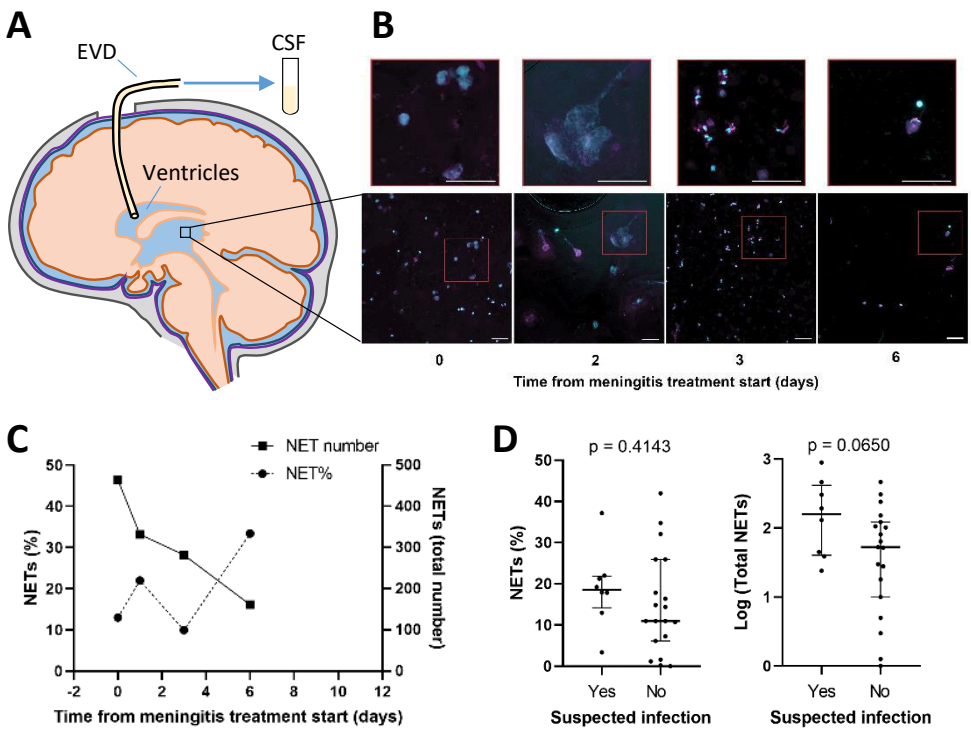

Figure 13. Summary of the main results of Study IV.
A) Schematic showing an EVD, and CSF sampling. B) Representative images and C) quanitification of CSF NETs from a patient with a suspected VRI over time relative to meningitis treatment start. D) NET percentage and total NETs in CSF of patients with an without a suspected infection.

Study V

In this study, we tested a functional observational battery score to quantify clinical and neurological symptoms in a rat model of bacterial meningitis. We found that the scoring system detected a broad range of relevant symptoms of bacterial meningitis. The most common symptoms by the 6th day of infection were hearing loss, involuntary motor movements and gait and posture abnormality. Infected rats had a higher combined score than control rats at all measured time points.

Table 2. Incidence of any neurological symptoms with score ≥2 at day 6.

	Infected (n=12)	Control (n=11)	P-value
Hearing loss; n (%)	9 (75%)	0 (0%)	0.0003
Focal neurological signs excluding hearing loss; n (%)	6 (50%)	0 (0%)	0.0137
Gait and posture abnormality; n (%)	8 (67%)	0 (0%)	0.0013
Involuntary motor movements; n (%)	9 (75%)	0 (0%)	0.0003
Neuromotor impairment; n (%)	2 (17%)	0 (0%)	0.4783

Discussion and future directions

Novel biomarkers

In this thesis work, we identified two new potential biomarkers: plasma glypicans in sepsis and CSF NETs in meningitis and ventriculitis.

In **study I**, we found that glypicans 1, 3 and 4 were elevated in the plasma of patients with infection who developed organ dysfunction (sepsis). Although the work did not indicate that plasma glypicans have a great diagnostic potential, they could be a useful addition to other markers of glycocalyx damage. Prior to this work, studies of glycocalyx shedding focused mainly on syndecans and GAGs[208,209]. Only one study had measured plasma glypican-3 levels in patients with lung cancer and lung infections[210]. Glypicans are shed by different mechanisms than are syndecans and GAGs[71,72]. Therefore, measurement of glypicans could provide insight into mechanisms of glycocalyx damage that measurement of syndecans and GAGs cannot.

In **study II**, we found that, in a small cohort of patients with various neuroinflammatory conditions, CSF NETs were present only in acute bacterial meningitis. This finding presented the tantalizing possibility that NETs could be a diagnostic biomarker for patients with bacterial meningitis. Such a biomarker would be particularly useful in patients treated with an EVD, where diagnosis of bacterial meningitis and ventriculitis is particularly difficult. Therefore, in **study IV**, we measured CSF NETs in a cohort of patients treated with an EVD, with and without a suspected VRI. Although the mean number of NETs was higher in patients with a suspected VRI, this difference was not significant. However the study was greatly confounded by the fact that very few patients had a confirmed infection by culture or PCR. Therefore future studies in a larger cohort of patients with more confirmed infections are needed to determine whether NETs can act as a biomarker for infections in patients with an EVD.

NETs as a therapeutic target

A major finding of this thesis work is that removal of NETs using DNase may have beneficial effects in experimental bacterial meningitis. In **study II**, we found that DNase treatment increased bacterial killing in rats with bacterial meningitis by

increasing innate neutrophil killing mechanisms. In an era of increasing antibiotic resistance, it is important to find new ways to kill bacteria during infections. The fact that DNase killed bacteria indirectly, through increased neutrophil killing, might mean that bacteria would be less likely to develop resistance to DNase treatment than they are to antibiotics. Also unlike antibiotics, it is unlikely that commensal flora would be killed by DNase treatment since it would primarily target bacteria that use NETs as an immune evasion strategy, while commensal bacteria normally employ many different means to evade killing by the immune system[32]. These results suggest that DNase should be explored in the future as an adjunct to antibiotic treatment in antibiotic resistant infections.

Because the NETs that we observed in bacterial meningitis were large and often associated with aggregates of cells, we hypothesized that NETs might block the normal pathways of CSF flow and clearance from the brain. In this way NETs could contribute to fluid build-up and increased intracranial pressure, which is a major problem in bacterial meningitis. In **study III**, we found that the normal glymphatic flow of fluid through the brain was disrupted in rats with bacterial meningitis. Treating the rats with DNase restored the normal glymphatic flow in some rats. This finding indicated that the possible effects of DNase treatment on intracranial pressure should also be explored in the future. In **study IV**, we found that 77% of patients treated with an EVD to regulate intracranial pressure had substantial NETs in the CSF. It is tempting to speculate that NETs may be involved in other diseases that lead to increased intracranial pressure, and that their potential as a therapeutic target in these conditions should be explored in the future.

Toward a clinically relevant model of bacterial meningitis

To provide evidence of the efficacy of novel therapeutics, such as DNase, it is important test their effect on clinically relevant and patient-important outcomes. In **paper V**, we developed a functional observational battery designed to measure the broad variety of neurological symptoms found in bacterial meningitis. Few reported rat models of bacterial meningitis quantify functional measures of a broad range of neurological symptoms. We suggest the meningitis-adapted functional observational battery is valuable because it measures wider range of relevant symptoms than other methods.

The rat model of pneumococcal meningitis in which we tested the functional observational battery presented with many relevant neurological symptoms at comparable levels to human pneumococcal meningitis. In our model, 75% of rats had

hearing loss and 50% of rats had focal neurological signs by day six. Hearing loss affects 22-69% of human adults with pneumococcal meningitis and focal neurological deficits occur in 11-36%[108]. In this model, we applied the recommended standard treatment for bacterial meningitis (antibiotics and corticosteroids) to all rats, to ensure that it would be relevant for testing therapies that are adjuvant to the standard treatment. We suggest that the rat model and neurological scoring system in this study are relevant tools for the evaluation novel therapeutics for bacterial meningitis, such as DNase, thus providing a foundation for future studies.

Limitations

As in all scientific studies, this work has several limitations. Most importantly, none of the studies used human subjects randomized to intervention and control groups, and therefore none of the diagnostic or therapeutic targets can be recommended for clinical use. However the studies in this thesis do provide a foundation for such work in the future. Additionally, each included study has several limitations that are described in detail in each paper, and briefly here.

In **study I**, a major limitation was that we did not directly measure vascular permeability in patients and therefore we do not know whether glypicans play a causative role in this important pathophysiological aspect of sepsis.

In **study II**, the major limitation is that we did not test DNase as an adjuvant to a standard meningitis treatment (antibiotics and corticosteroids), nor did we measure clinically relevant outcomes such as mortality or neurological symptoms. Therefore we do not know whether DNase may be beneficial in a more clinically relevant scenario. Such as study should be undertaken before DNase treatment can be explored in human trials.

The main limitation of **study III** is that we did not measure brain water content and we did not examine any clinically relevant outcomes after DNase treatment. Therefore we have only hinted at a new potential effect of targeting NETs in bacterial meningitis, but this must be characterized further in future studies before any conclusions can be made about its clinical application.

Study IV had several major limitations. One is that the cohort size was too small to be able to adjust for confounding factors that could lead to NET formation. Additionally, we included too few patients with confirmed infections, and relied on the clinician's suspicion of infection. This obviously leads to unreliable results as it is very likely that we unwittingly included patients who did not actually have an

infection in the "suspected infection" group. Although we could not determine whether NETs are a marker of infections, we did show for the first time that NETs are present in the CSF in patients with ventriculostomy and indicated that the consequences of their presence should be explored further.

The main limitation of **study V** was that our evaluation of the new scoring method was limited by the fact that we were unable to randomize rats on an individual level due to the risk of cross-infections. Randomization by cages would have made successful blinding difficult and therefore the evaluator was not blinded to the identity of the rats, which could have biased the results.

In spite of these limitations, this thesis provides a foundation for future studies of two potential new biomarkers and one new therapeutic target in sepsis and meningitis.

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