Perinatal *Staphylococcus epidermidis* infection and the immature brain: a neuroinflammatory link

Giacomo Gravina

Department of Physiology
Institute of Neuroscience and Physiology
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2022

Cover illustration: z-stack projection of hippocampal microglia cell (yellow) surrounded by astrocytes (red). Cell nuclei are shown in cyan.

Original imaging: Tetyana Chumak, MD, PhD

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Giacomo.gravina@gu.se

ISBN 978-91-8009-823-6 (PRINT)
ISBN 978-91-8009-824-3 (PDF)

Printed in Borås, Sweden 2022
Printed by Stema Specialtryck AB

To my dear family

“The real voyage of discovery consists not in seeking new landscapes, but in having new eyes”

Marcel Proust

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Sahlgrenska Academy, University of Gothenburg
Gothenburg, Sweden

ABSTRACT

Preterm birth and its associated complications are among the most serious global health issues that modern society faces. Due to the prolonged medical care and immature immune system, preterm infants have a higher susceptibility to infections, which puts them at a higher risk of developing neurological impairments as well as neurodevelopmental diseases. *S. epidermidis* is one of the most common nosocomial infections in preterm infants. Despite being considered a harmless commensal for a long time, *S. epidermidis* has emerged as the predominant pathogen of neonatal sepsis, leading to inflammation-related morbidities. Moreover, the incomplete maturation of the preterm infants´ organs might lead to an increased risk of episodes of hypoxia and it is believed that the ongoing infection can worsen the effects of cerebral hypoxia-ischemia (HI), further increasing the risk for perinatal brain injury.

The hypothesis of this doctoral thesis is that systemic inflammation induced by *S. epidermidis* infection leads to immune reactions in the periphery and the brain, which increases vulnerability to brain injury, leading to neurological impairments. Thus, the overall aim of this thesis was to explore the different aspects of the pathogenesis associated with *S. epidermidis* infection, ranging from its sensitizing effects to the neuroinflammatory responses.

Using our established animal model for *S. epidermidis* infection and Hypoxia-ischemia (HI), in **Paper I** we induced HI 24 hours or 5 days after *S. epidermidis* infection, demonstrating a sex-dependent sensitization 24 hours after infection in male, but not female mice. We also found a dramatic upregulation of peripheral cytokines, brain Chemokine ligand 2 (CCL2) together with decreased plasma levels of Complement protein 5a. As neuroinflammation contributes to perinatal brain injury, in **Paper II** we analyzed hippocampal

microglia activation both at morphological and transcriptional levels. We found that *S. epidermidis* induced significant changes in microglial morphology as well as in their transcriptional programs. We also found that microglial inflammasome activation might act in synergy with blood-brain barrier alterations as well as leukocyte infiltration into the brain. To further characterize the neuroinflammatory response in the hippocampus of *S. epidermidis* infected mice, in **Paper III** we carried out hippocampal global protein expression analysis, revealing astrocytic activation as well as vascular changes. These alterations were associated with increased lipocalin 2 levels in both plasma and brain. We also demonstrated that a similar pattern of events might occur in a cohort of preterm infants with signs of infection by analyzing plasma levels of lipocalin 2.

To conclude, this thesis clearly highlights a previously unrecognized and important contribution of *S. epidermidis* in triggering neuroinflammation in the developing brain. Overall, the findings in this thesis shed new light on how *S. epidermidis* affects the immature brain, representing a suitable platform for the development of novel treatment or preventative strategies in babies who experience *S. epidermidis* infection.

Keywords: *S. epidermidis*, neonatal brain injury, hypoxia-ischemia, microglia, neuroinflammation

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SAMMANFATTNING PÅ SVENSKA

Perinatal *Staphylococcus epidermidis*-infektion och den omogna hjärnan: en neuroinflammatorisk länk

För tidig födsel (prematurbörd) och tillhörande komplikationer är bland de svåraste globala hälsoproblemen som det moderna samhället står inför. Prematurbörd kan innebära långvarig medicinsk vård för barnet och tillsammans med ett omoget immunförsvar är dessa barn mer mottagliga för infektioner, vilket i sin tur ger högre risk att störa hjärnutveckling och ge långsiktiga neurologiska problem. *S. epidermidis* är en av de vanligaste nosokomiala infektionerna (vårdrelaterade infektioner) hos för tidigt födda barn. Trots att *S. epidermidis* under lång tid har ansetts vara en ofarlig bakterie, har den framträtt som den dominerande patogenen för neonatal sepsis, vilket leder till inflammationsrelaterade sjukdomar. Vidare kan ofullständigt utvecklade organ hos barnet leda till ökad risk för episoder av hypoxi (syrebrist) och man tror att en pågående infektion kan förvärra effekterna av cerebral hypoxi-ischemi (HI), vilket tillsammans ökar risken för perinatal hjärnskada.

Hypotesen för denna doktorsavhandling är att systemisk inflammation framkallad av *S. epidermidis*-infektion leder till immunreaktioner i kroppen och hjärnan samt ökad risk för hjärnskada och neurologiska problem. Det övergripande syftet med denna avhandling var att utforska de olika aspekterna av patogenesen associerad med *S. epidermidis*-infektion, allt från sensibiliserande effekter till neuroinflammatorisk respons.

Genom att använda våra etablerade djurmodeller för *S. epidermidis*-infektion och HI, inducerade vi HI 24 timmar eller 5 dagar efter *S. epidermidis*-infektion, vilket visade en könsberoende sensibilisering av hjärnskada vid 24 timmar efter infektion hos han-möss men inte hon-möss (**Artikel I**). Vi fann också en stark uppreglering av cytokiner i blod, kemokinen CCL2 (Chemokine ligand 2) i hjärna tillsammans med minskade plasmanivåer av komplementprotein 5a. Eftersom neuroinflammation bidrar till perinatal hjärnskada, analyserade vi i **Artikel II** aktivering av mikroglia i hippocampus både genom morfologiska och transkriptionella metoder. Vi fann att *S. epidermidis* inducerar morfologiska förändringar i mikroglia samt i deras transkriptom. Vi visade också att aktivering av inflammasome-komplexet i mikroglia kan verka i synergism med förändring av blod-hjärnbarriären samt infiltration av vita blodkroppar till hjärnan. För att bättre karakterisera det neuroinflammatoriska svaret i hippocampus hos *S. epidermidis*-infekterade möss, analyserade vi

globalt hippocampus proteinuttryck i **Artikel III**, som visade aktivering av astrocyter såväl som vaskulära förändringar. Dessa förändringar var associerade med ökning av proteinet lipokalin 2 i både plasma och hjärna. Vi demonstrerade också att liknande mönster av processer kan inträffa i en kohort av för tidigt födda barn med tecken på infektion genom att analysera plasmanivåer av lipokalin 2.

För att summera, denna avhandling demonstrerar tydligt tidigare okända och betydelsefulla sätt för *S. epidermidis* att framkalla neuroinflammation i den omogna hjärnan. Sammantaget, resultaten i denna avhandling kastar nytt ljus över hur *S. epidermidis* påverkar den omogna hjärnan, vilket skapar en viktig plattform för att utveckla nya behandlingsstrategier för att minska effekterna av *S. epidermidis*-infektion hos spädbarn.

Nyckelord: *S. epidermidis*, neonatal hjärnskada, hypoxi-ischemi, mikroglia, neuroinflammation

ABSTRACT IN ITALIANO

Infezione perinatale da *Staphylococcus epidermidis* e il cervello immaturo: un legame neuroinfiammatorio

La nascita pretermine e le sue complicanze sono tra i problemi sanitari globali più gravi che la società moderna stia affrontando. A causa dell'assistenza medica prolungata e dell'immaturità del loro sistema immunitario, i neonati prematuri mostrano una maggiore suscettibilità alle infezioni, il che li espone ad un maggior rischio di sviluppare disturbi neurologici e malattie del neurosviluppo. Le infezioni nosocomiali da *S. epidermidis* sono tra quelle più comuni nei neonati pretermine. Nonostante per molto tempo sia stato considerato un batterio commensale innocuo, *S. epidermidis* sta emergendo come l'agente patogeno predominante nelle sepsi neonatali. Inoltre, la maturazione incompleta degli organi dei neonati prematuri potrebbe correlarsi ad un aumentato rischio di episodi di ipossia, e si ritiene che le infezioni possano peggiorare gli effetti dei danni cerebrali ipossico-ischemici (HI), aumentando così la probabilità complessiva di danno cerebrale perinatale. L'ipotesi di questa tesi di dottorato è che l'infiammazione sistemica indotta dall'infezione da *S. epidermidis* possa determinare reazioni immunitarie periferiche e cerebrali, e quindi una maggiore vulnerabilità agli insulti cerebrali secondari, contribuendo così al peggioramento del quadro clinico. Pertanto, l'obiettivo di questa tesi è quello di esplorare i diversi aspetti della patogenesi associata all'infezione da *S. epidermidis*, partendo dalla maggiore sensibilità cerebrale fino alla risposta neuroinfiammatoria.

Grazie all'utilizzo di modelli animali consolidati nello studio dell'infezione da *S. epidermidis* e/o ipossia-ischemia, nell'**Articolo I** abbiamo indotto l'insulto ipossico-ischemico 24 ore o 5 giorni dopo l'infezione da *S. epidermidis*, dimostrando una maggiore sensibilizzazione cerebrale 24 ore dopo l'infezione solo nei topi maschi, ma non nelle femmine. Abbiamo anche riscontrato una notevole sovra-regolazione delle citochine, delle chemochine periferiche e dei livelli di CCL2 (Chemokine ligand 2) nell'encefalo, insieme ad una diminuzione dei livelli plasmatici della proteina del complemento 5a. Poiché la neuroinfiammazione contribuisce al danno cerebrale perinatale, nell'**Articolo II** abbiamo analizzato l'attivazione della microglia ippocampale sia a livello morfologico che trascrizionale. I risultati mostrano che *S. epidermidis* induca cambiamenti a livello trascrizionale e morfologico nella microglia. Abbiamo anche scoperto che l'attivazione dell'inflammasoma nella microglia potrebbe agire in sinergia con l'alterazione della barriera ematoencefalica e con l'infiltrazione leucocitaria. Per meglio caratterizzare la

risposta neuroinfiammatoria nell'ippocampo di topi infetti da *S. epidermidis*, nell'**Articolo III** abbiamo condotto un'analisi proteomica globale a livello dell'ippocampo, dimostrandone l'attivazione astrocitaria e cambiamenti del profilo vascolare. Queste alterazioni sono state associate ad un aumento dei livelli di lipocalina 2 sia plasmatici che nel cervello. Alti livelli plasmatici di lipocalina 2 sono stati osservati anche in una coorte di neonati pretermine con segni da infezione. In conclusione, nella presente tesi abbiamo evidenziato l'importante e sconosciuto contributo delle infezioni da *S. epidermidis* nell'indurre una robusta risposta neuroinfiammatoria nel cervello in via di sviluppo. Complessivamente, i risultati di questa tesi contribuiscono a chiarire come *S. epidermidis* influisca sulla maturazione cerebrale. Questi risultati potrebbero contribuire allo sviluppo di nuove strategie farmacologiche atte a trattare o prevenire i danni cerebrali associati alle infezioni da *S. Epidermidis* nei neonati prematuri

Keywords: *S. epidermidis*, insulti cerebrali, ipossia-ischemia, microglia, neuroinfiammazion

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I

***"Staphylococcus epidermidis* Sensitizes Perinatal Hypoxic-Ischemic Brain Injury in Male but Not Female Mice"**

Gravina G, Svedin P, Ardalan M, Levy O, Ek CJ, Mallard C and Lai JCY. Front Immunol. 2020 Apr 21;11:516. DOI: 10.3389/fimmu.2020.00516

II

"**Transcriptome network analysis link perinatal *Staphylococcus epidermidis* infection to microglia reprogramming in the immature hippocampus**"

Gravina G, Ardalan M, Chumak T, Rydbeck H, Xiaoyang Wang, Ek CJ, Mallard C. *Submitted*

III

"**Hippocampal proteomics analysis links lipocalin2 with astrocyte reactivity and vascular alteration following perinatal *Staphylococcus epidermidis* infection.**"

Gravina G, Ardalan M, Chumak T, Nilsson A, EK CJ, Danielsson H. Pekny M, Pekna M, Sävman K, Hellström A, Mallard C. *In Manuscript*.

CONTENT

ABBREVIATIONS

ADHD	Attention deficit hyperactivity disorder
BBB	Blood-brain barrier
BCA	Bicinchonic acid assay
C	Complement
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CLR	C-type lectin receptors
DAMPs	Damage-associated molecular pattern
E	Embryonic
ELISA	Enzyme-linked immunosorbent assay
$Gram^-$	Gram-negative
$Gram^+$	Gram-positive
HI	Hypoxia-ischemia
IFN	Interferon
IL	Interleukin
Lcn2	Lipocalin 2
MAP-2	Microtubule-associated protein 2
MBP	Myelin basic protein
NEC	Necrotizing enterocolitis
NLR	NOD-like receptors

NLRP3	NLR Pyrin domain 3
NOD	Nucleotide-binding and oligomerization domain
PAMPs	Pathogen-associated molecular pattern
PNAG	Poly-N-acetyl-glucosamine
PND	Post-natal day
PRRs	Pattern recognition receptors
RLR	Retinoic acid-inducible gene 1-like receptors
RNAseq	RNA sequencing
ROS	Reactive oxygen species
S.	Staphylococcus
TLRs	Toll-like Receptors

1 INTRODUCTION

"You start a question, and it's like starting a stone. You sit quietly on the top of a hill; and away the stone goes, starting others" Robert Louis Stevenson

Inflammation has emerged as a common driving force in the development of brain injury, particularly in preterm new-born infants, a condition strongly associated with developmental impairment and permanent neurological deficits, such as cognitive, motor, and sensory disabilities. Due to the long period of hospitalization and exposure to medical devices as well as an immaturity of the immune system, preterm infants are at high risk of biofilm-related infections. The most common infection in late-onset neonatal sepsis is coagulase-negative staphylococci, such as *Staphylococcus (S.) epidermidis*. These bacterial infections are involved in peripheral immune cell responses to infection or tissue injury but their role in the pathogenesis of inflammation-induced brain injury remains largely unknown. The present thesis aims to explore the mechanisms that drive inflammation into the immature brain and thereby gives insights into the pathogenesis of neonatal brain damage and provides a platform for the future development of novel preventative or therapeutic interventions in a targeted manner.

1.1.1 Prematurity: a global health problem

Preterm birth and its associated complications are among the most serious global health issues that modern society faces[1]. Epidemiological studies show that globally around 11% of all live births are preterm (defined as infants born before 37 weeks of gestation)[2,3]. In Sweden, the preterm birth rate is 5.8%[4]. Prematurity can further be subdivided based on the week of gestation and specifically: extremely preterm (<28 weeks), very preterm (28-32< weeks) and moderate preterm (32-37 weeks)[3]. Most of the preterm births are moderate (~84%), followed by very preterm (~10%) and extremely preterm (~5%)[3]. The low incidence of extremely preterm births might be explained by the reduced survival rate, which negatively correlates with the week of gestation[3]. However, when considering the incidence of prematurity, disparity in the economic status of the different countries has to be considered. Thus, high-income countries have more than a 90% survival rate of extremely preterm infants while survival in low-income countries is only 10% of the survival rate[3]. Another difference to be considered is the higher incidence of boys born preterm compared to the girls[5].

1.1.2 Causes of preterm birth

Around 40-45% of preterm labor occurs spontaneously with intact membranes, while 30–35% of the cases are medically indicated and the remaining 25–30% might occur in case of preterm premature rupture of the membranes[6]. Multiple pathological processes might cause preterm labor[7]. Although the exact etiology is often not known, emerging evidence supports a central role of infection/inflammation in the cause of prematurity[8]. Accordingly, proinflammatory cytokines have been associated with uterine activation, premature rupture of the membranes and premature delivery [9-11]. However, infection/inflammation is not the only cause of preterm labor. Other processes such as hemorrhage and vascular disease, decidual senescence, disruption of maternal-fetal tolerance, decrease in progesterone and stress are well known factors that can induce preterm labor[7], suggesting that multiple etiologies are responsible for preterm labor. Although many mechanisms are still unclear, it is becoming evident that other factors, such as obesity, smoking, nutrition, depression, drug and alcohol use, might also contribute to preterm labor[6] (Fig 1).

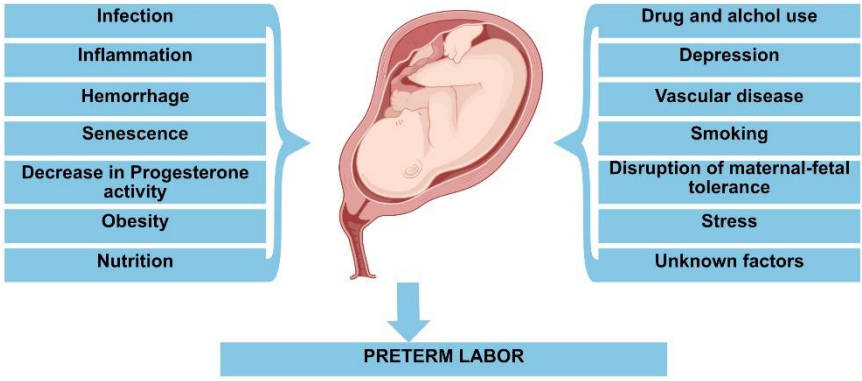

Figure 1. Causes of premature birth

1.1.3 Comorbidities associated with prematurity

With improvements in the health care systems, the global incidence of neonatal disorders associated with prematurity became relatively stable in the period ranging from 1990 until 2019, which was further associated with social and economic development status[12]. Complications of preterm birth can be the direct cause of neonatal deaths and are responsible for 35% of deaths in the general neonatal population[13]. As the organ development of preterm infants is not fully completed at birth, several complications may arise (Fig 2). For

example, as nephrogenesis ends at 36 weeks of gestation[14], the number of glomeruli is significantly lower in preterm infants[15], increasing the risk of chronic kidney diseases[14]. In the lungs, the full maturation of alveoli occurs ~36 weeks of gestation ending approximately when the infant is 3 years old[16] and its incomplete maturation might lead to an increased risk of episodes of hypoxia and lung diseases, such as bronchopulmonary dysplasia, wheezing, and asthma[17]. Furthermore, prematurity is associated with decreased vascularity and higher blood and pulmonary pressures in adult life [18]. Another co-morbidity associated with prematurity is necrotizing enterocolitis (NEC), a devastating disease in neonates[19]. The intestinal immaturity together with abnormal microbial colonization and an episode of hypoxia-ischemia might contribute to the development of NEC[19]. Further, functional disability and neurological impairments are more common in preterm infants[2]. Prematurity has been associated with several neurodevelopmental conditions, such as cerebral palsy (with higher incidence in boys than in girls[20]), deficits in cognitive performance, delayed language skills, visual-spatial or perceptual problems, behavioral and learning difficulties at school age[20,21]. Interestingly, cognitive scores in preterm infants were directly proportional to their birth weight and gestational age, showing more than twice the relative risk of developing attention deficit hyperactivity disorder (ADHD) in preterm infants compared to the infants born at term[22]. Importantly, as preterm infants require prolonged medical care and do not have a fully developed immune system, preterm infants have a higher susceptibility to infections[13].

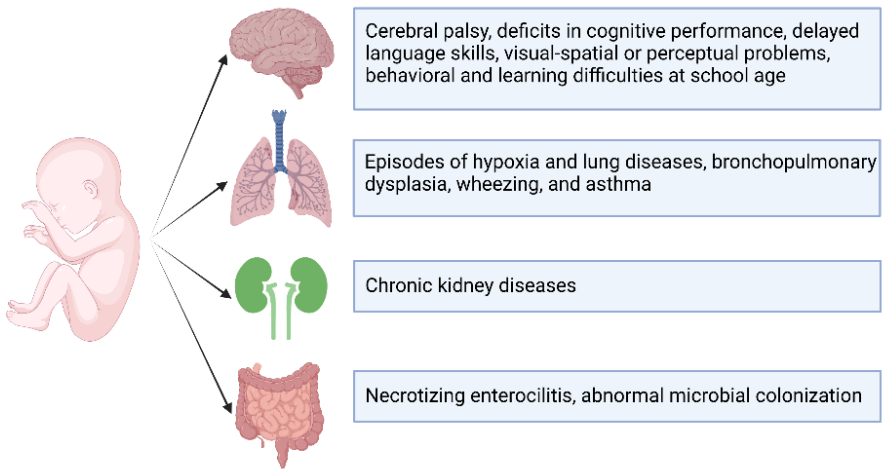

Figure 2. Risks associated with prematurity

1.1.4 Infection in preterm

While term infants most commonly acquire infections during delivery, preterm infants are at increased risk of early-onset sepsis (associated with intrauterine infection) as well as secondary sepsis (defined as late-onset sepsis; >72 h after birth). The most common early-onset sepsis infections are due to Group B streptococci, Escherichia coli and other Gram-positive bacteria. However, in most cases, preterm infants acquire infection during hospitalization following birth[23]. Therefore, late-onset sepsis is the most common infectious condition in preterm infants. Gram$^+$ cocci, such as *S. epidermidis*, is the most common cause of late-onset sepsis in neonatal intensive care units worldwide and disproportionately affects preterm infants[24]. As skin bacteria, *S. epidermidis* may contaminate samples that have been taken inappropriately. Thus, the diagnosis of infection might be strengthened by including apnea and the need for increased respiratory support together with an abnormal white cell count[23]. However, due to the high risk of developing infection, most preterm infants admitted to neonatal intensive care units receive antibiotic treatments, which enhance the intrinsic antibiotic resistance of staphylococci strains, increasing the risk of morbidity in preterm-infected infants[25,26].

1.2 *S. epidermidis* infection

S. epidermidis is the most common nosocomial pathogen in neonatal intensive care units and is responsible for up to 50% of all cases of late-onset neonatal septicemia[27]. *S. epidermidis* is identified as coagulase-negative staphylococcus by its inability to produce coagulase, an enzyme that enables the conversion of fibrinogen to fibrin[28]. *S. epidermidis* is a Gram$^+$ bacterium that colonizes the skin and mucous membrane of the human body[29]. The incidence of coagulase-negative staphylococci infections inversely associates with neonatal maturity, becoming higher in very low birth weight infants[30]. Due to the lack of pyrogenic events and the reduced arsenal of toxins released by *S. epidermidis*, the clinical signs of *S. epidermidis* infections are usually subacute or chronic and nonspecific[28], making it difficult to diagnose. In many cases, *S. epidermidis* has a mutually beneficial role within the host organism[31]. *S. epidermidis* functions as a probiotic agent and prevents the colonization of other pathogens such as *S. aureus*, by producing factors that inhibit their growth[32-34]. Furthermore, the presence of *S. epidermidis* in human skin stimulates keratinocytes to produce antimicrobial peptides, preventing S. *aureus* colonization[31]. Recently, it has been shown that *S. epidermidis* exerts a crucial role in maintaining skin integrity. Thus, the study from Zheng et al demonstrates that *S. epidermidis* is an important skin bacterium that increases levels of ceramides in the host skin, preventing skin dehydration and maintaining the skin's protective barrier[35]. However, in contrast to its

commensal role, *S. epidermidis* might cause invasive infections. Therefore, an important question, that partly remains unanswered, is to understand when and how *S. epidermidis* becomes a pathogen, especially in preterm infants.

1.2.1 *S. epidermidis*: virulent factors, biofilm formation and its resistance to the immune system

The most known virulent factor of *S. epidermidis* is associated with its ability to form biofilms[36]. A biofilm is defined as a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface[37]. Inside the biofilm, bacteria produce several proteins, which function as channels to ensure the circulation of nutrients and other materials to support the bacteria[37]. Further, inside the biofilm bacteria is generally more resistant to antimicrobial treatment than the planktonic counterpart[38]. Biofilm formation in *S. epidermidis*, as well as in other bacteria types, is a multistage process, with an initial attachment of the bacteria to the surface and subsequent formation and maturation of micro- and macro-colonies[39]. Inside this complex structure, *S. epidermidis* may down-regulate its metabolic activity[40], being protected against antibiotics and immune attacks, such as complement deposition, leukocyte phagocytosis, opsonization and the activity of antimicrobial proteins[41]. However, under certain conditions, such as changes in pH, nutrient depletion, and waste accumulation within the biofilm, *S. epidermidis* can detach and disseminate itself in the bloodstream causing sepsis[27]. Apart from the biofilm-related genes, other virulence factors produced by *S. epidermidis* have been described[29] such as the cytolytic phenol-soluble modulin, a hemolytic toxin associated with necrotizing enterocolitis, and the mecA gene, which confers methicillin resistance to *S. epidermidis*[42]

1.2.2 *S. epidermidis* in the neonatal care unit

The need for intensive care in the first weeks of life together with an immature immune system pose preterm infants at a higher risk of developing staphylococcal infections. Indeed, the need for intravascular catheters, nutrition tract tubes as well as prolonged total parenteral nutrition increase colonization and infection rates[43]. Interestingly, molecular analysis of *S. epidermidis* strains from infants' and nurses' hands showed that some *S epidermidis* isolates were shared[44], suggesting that some of the infections might be avoided by improving hygienic procedures.

Despite *S. epidermidis* being considered a harmless commensal for a long time, it has recently emerged as the predominant pathogen of neonatal sepsis[27], leading to inflammation-related morbidities[45]. The mortality rate due to *S. epidermidis* sepsis ranges from 1.9% to 4.8% in the general neonatal

population, tending to increase up to 9% in very low birth weight infants[27]. Diagnosis of *S. epidermidis* might be challenging due to the non-specific symptoms[43]. Currently, the diagnosis largely depends on microbial blood cultures (which are limited in preterm infants due to the low collectible amount of blood) and positive cultures might be due to sample contamination[43]. In 38% of meconium samples from preterm infants, *S. epidermidis* was detected, and its presence dramatically increased up to ~90% in feces collected 7 days after birth[46]. In a study at neonatal intensive care units in Brazil, *S. epidermidis* was found in 48% of infected neonates, of which 87% were mecA positive[47]. Similarly, in a randomized multicentric clinical trial conducted in Estonia, Soeorg et al aimed to molecularly identify the different strains of staphylococcus infection. They found a high prevalence of *S. epidermidis* that was resistant to methicillin and also carried the mecA locus (91.4% of the total cases) in neonates[48].

1.3 Perinatal infection and neurological conditions

S. epidermidis may exacerbate other co-morbidities, such as bronchopulmonary dysplasia, white matter injury, and retinopathy of prematurity[49]. Epidemiological studies have shown that children who have experienced late-onset neonatal sepsis have lower total and verbal intelligence quotients as well as memory and attention impairments. Furthermore, preterm infants exposed to late-onset sepsis showed an increased risk of abnormal motor outcome at school age[50,51] and neurodevelopmental diseases, such as ADHD[51]. In other studies, preterm infants with late-onset bacteremia/sepsis performed worse on tests of general cognitive ability, language, academic achievement, executive function[52] and had a higher risk of developing cerebral palsy[53] compared to the preterm infants without bacteremia. White matter and cortical injuries, spastic triplegia or quadriplegia, have also been seen in term infants with proven neonatal infection[54], pointing out the harmful long-term effects of neonatal infections. Similarly, mice exposed to infection after birth display behavioral deficits together with hypomyelination and a reduction of oligodendrocytes[55]. However, the role of *S. epidermidis* in brain injury remains largely unclear.

1.4 Hypoxia-ischemia

Epidemiological studies suggest that an ongoing infection can worsen the effects of cerebral hypoxia-ischemia (HI), increasing the risk for perinatal brain injury[56]. However, little is known about the effects of the combination of exposure to *S. epidermidis* and HI on the developing brain. HI can be a life-threatening event in infants and is estimated to account for a million neonatal deaths per year worldwide, as well as long-term disability[57,58]. Hence, HI,

being the fifth leading cause of death in infants, is a recognized contributing factor to neonatal encephalopathy in full-term infants, and is often associated with persistent motor, sensory, and cognitive impairments[59]. However, the incidence and outcome of HI in preterm infants are less well understood, due to heterogeneous and variable inclusion criteria and outcomes in studies[59,60].

Cerebral HI results in neuronal excitotoxicity, cellular apoptosis, and microglial activation[61]. Neuronal death following HI occurs through mechanisms that involve energy deprivation which often occur in subsequent, but overlapping, phases[62]. In the primary phase, oxygen deprivation during HI forces mitochondria to produce energy in the absence of oxygen, switching to anaerobic metabolism, reducing energy production, and increasing lactate production paired with an increase in reactive oxygen species (ROS). This phase is characterized by the failure of energy-dependent cell membrane ion channels, which triggers an acute intracellular influx of calcium and sodium and cell membrane depolarization, as well as accumulation of extracellular glutamate. This in turn triggers the second phase which is characterized by acute inflammatory responses, excitotoxicity and oxidative stress[63]. Furthermore, as reviewed by Fleiss et al, a tertiary phase might occur which is associated with more persistent inflammation and epigenetic changes[64]. Experimental and clinical evidence has shown that infections increase the vulnerability of the perinatal brain, enhancing the detrimental effects of HI. The combined effects of HI and inflammation/infection greatly contribute to neonatal encephalopathy[65]. Specifically, several animal models provide evidence to show that administration of purified or synthetic compounds that mimic Gram$^{+/-}$ bacterial infections increases the vulnerability to subsequent HI events[66-68]. Mechanisms underlying the sensitizing effects of *S. epidermidis* infection on HI remain unclear.

1.5 Immune responses to infection

To fight infections, mammalian hosts have developed an immune defense system against different types of invading pathogens, such as bacteria, viruses, protozoa etc. Infectious agents are recognized by the host immune system, which mounts a cascade of events that lead to the activation and recruitment of immune cells with consequent clearance of the pathogen. The immune system consists of two components, the innate and the adaptive immune systems, which cooperate to protect the host organism against microbial infections[69]. The innate immune system is the first line of defense, which occurs within minutes and lasts for a few days. The cells in the immune system such as macrophages, neutrophils, and dendritic cells, detect the microbial intruders and mount an appropriate proinflammatory response. The initial sensing of the infection is largely mediated by pattern recognition receptors

(PRRs)[70]. In contrast, the adaptive immune response produces a long-lasting effect, including immunological memory. The adaptive immune events occur over weeks to years and the main cells constituting the adaptive immune system are B- and T lymphocytes[71].

1.5.1 Toll-like receptors

Toll-like receptors (TLRs) are a class of PRRs that recognize and bind to molecules with specific structural patterns on pathogens or damaged cells. Such molecules are known as pathogen/damage-associated molecular patterns (PAMPs or DAMPs). Often, the PAMPs are components of the bacterial wall such as lipopolysaccharide (in $Gram^-$ bacteria), peptidoglycan and lipoteichoic acids (in $Gram^+$ bacteria)[69]. The recognized molecular pattern by TLRs varies based on the nature of the pathogen. The large family of TLRs comprises 13 members with different recognition targets. For example, TLR2 is mainly involved in the recognition of $Gram^+$ bacteria, including *S. epidermidis*, TLR4 recognizes $Gram^-$ bacteria, while TLR3 recognizes viruses etc[72]. These receptors are crucial for triggering an inflammatory response. One of the first events of the inflammatory response is the activation of TLRs in circulating immune cells or tissue-resident macrophages, which produces an array of chemokines and cytokines, crucial to coordinate the inflammatory response. In the circulation, the production of cytokines activates a cascade of events that induce vasodilation and increased blood vessel permeability, allowing immune cells to migrate. Furthermore, formation of blood clots is often observed in response to bacterial infection which prevents the bacteria from disseminating into other parts of the body[69]. However this event might have serious and deleterious consequences on the health, including thrombosis of small vessels and impaired organ perfusion[73]. Structurally, TLRs are type I integral membrane receptors, with an N-terminal ligand exposed to the extracellular space, which functions as a recognition domain, a single transmembrane helix, and a C-terminal cytoplasmic signalling domain (also known as Toll/IL-1 Receptor (TIR)[74]. After recognition, dimerization of TLRs occurs, triggering the activation of signalling pathways through the TIR domain. This later includes interaction with the adaptor molecule MyD88, which induces the generation of inflammatory cytokines[72]. In the MyD88-dependent pathway, MyD88 recruits IRAK-4 to TLRs with consequent phosphorylation and activation of IRAK-1. This will activate MAP kinase phosphorylation cascades and AP-1 transcription factors. However, MyD88 might also induce the activation of the TAK1/TAB complex, which culminates in the degradation of IκB and nuclear translocation of the transcription factor, NF-κB. Similarly, the MyD88-independent pathway culminates in the translocation of the transcription factor NF-κB and the production of cytokines. However, the initial activation step is dependent on TIR domain-containing adaptor (TRIF).

TRIF interacts with TRAF6 and TRAF3. In the TRAF6 pathway, recruitment of the kinase RIP-1 activates the TAK1 complex, which leads to NF-κB activation and translocation. However, when TRAF3 is activated, the IKK-related kinases TBK1 and IKKi are recruited, which lead to IRF3 dimerization and translocation into the nucleus and induction of the expression of type I interferon (IFN) genes[72,75]. Thus, TLR activation leads to both cytokine and type I IFN production, which are central mediators of the inflammatory response (Fig 3). Importantly, TLR activation also mediates phagocytosis-mediated antigen presentation, which represents a bridge between the innate and adaptive immune systems[71]. As the adaptive immune system is out of the scope of this thesis, I direct the reader to an excellent review for further exploration[76].

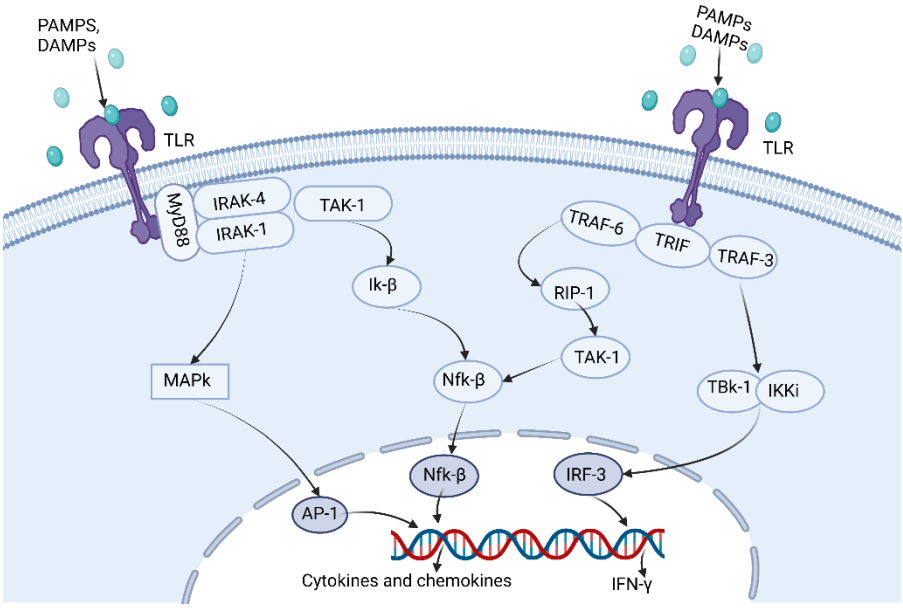

Figure 3. Toll-like Receptors signalling

1.5.2 Nucleotide-binding and Oligomerization Domain (NOD)-like receptors in infection

Proteins in this family contain a NOD domain and are intracellular receptors that recognize PAMPs. The NOD-like receptor (NLR) family comprises 22 members in humans and more than 30 members in mice; among them, NOD1 and NOD2 exert crucial roles in pathogen recognition and activation of the host immune response[77]. Structurally, NLRs are formed by three domains: (i) N terminal domain, also known as the nucleotide-binding domain, contains an

N-terminal Caspase Recruitment Domain that is shared by all members of the NLR family and acts as a downstream signalling molecule, (ii) central nucleotide-binding oligomerization domain and (iii) C-terminal domain which is used to identify the ligand and functions as a PAMP sensor[77,78]. It is unclear if NLRs recognize the bacterial component directly or are activated by other PRRs like TLRs. However, it has been demonstrated that NLRs can be activated by distinct substructures from bacterial peptidoglycan. Specifically, NOD2 is activated by muramyl dipeptide, found in both $Gram^-$ and $Gram^+$ bacteria, while NOD1 senses peptidoglycan containing *meso*-diaminopimelic acid, commonly found in $Gram^-$ bacteria. In the cytosol, NOD1 and NOD2 are present as monomeric molecules and upon activation NOD1 and NOD2 self-oligomerize and undergo a conformational change. Activated NOD1 and NOD2 recruit kinase proteins through the CARD domain and subsequently trigger NF-κB or MAPK pathways, which together stimulate the expression of inflammatory and anti-microbial peptide genes (Fig 4)[79].

1.5.3 NLRP3 and inflammasome

Another member of the NLR family of receptors is NLR Family Pyrin Domain Containing 3 (NLRP3). Distinct from the NOD1 and NOD2 proteins, NLRP3 activates the caspase-1 inflammasome and the production of interleukin 1β (IL-1β) and IL-18. Like all the NOD receptors, NLRP3 contains a sensor, an adaptor (called PYCARD) and an effector (caspase-1). Once activated, oligomerization of NLRP3 recruits ASC proteins, which oligomerize into multiple ASC copies. This recruits caspase-1, which is self-cleaved into the active form of caspase-1 [80], that in turn cleaves pro-IL-1β and pro-IL-18 into the active forms, which consequently results in a proinflammatory response[81]. As an important immune mechanism, inflammasome activation is tightly regulated. Indeed, in the first step of activation, the inflammasome is primed by recognition of PAMPs directly by TLRs (through the NF-κB mechanism (described in section 1.5.1) or NOD proteins, with consequent upregulation of the inflammasome genes, such as NLRP3, caspase-1 and pro-IL-1β. Secondly, once transcribed, NLRP3 oligomerizes to form the active inflammasome (Fig 4)[80]. Interestingly, apart from peripheral immune cells, inflammasome genes have been found in several cell types in the brain[82], such as neurons[83], astrocytes, microglia[84] and oligodendrocytes[85]. Therefore, as it is widely expressed in the brain, it has been implicated in several neurological conditions, such as Alzheimer´s disease, Parkinson's disease, stroke, encephalitis etc[82], suggesting that this mechanism might be a future target for preventing/treating neurological conditions.

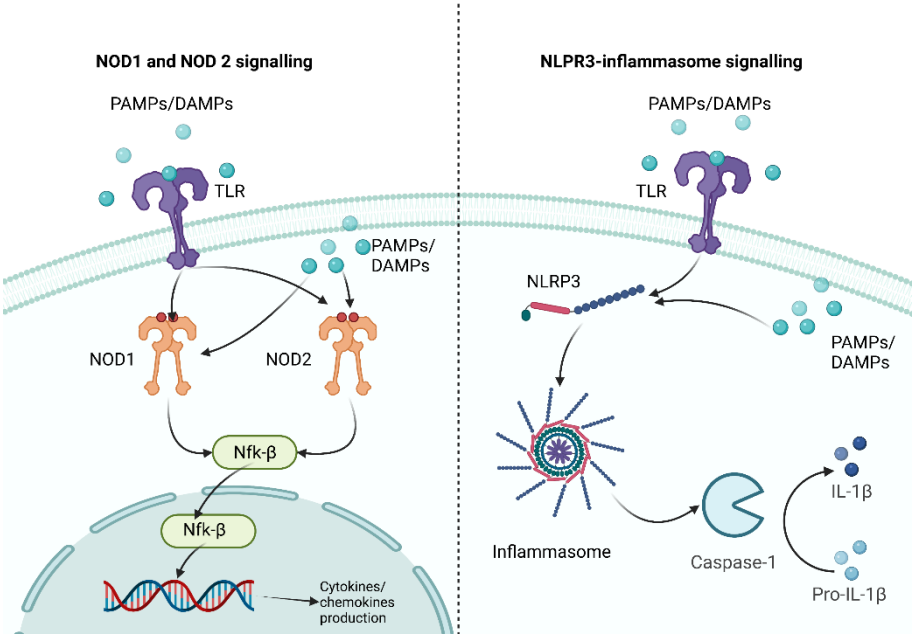

Figure 4. NOD signalling pathway and NLRP3 inflammasome pathway.

1.5.4 Complement proteins

In addition to what has been discussed above, the innate immune system also relies on a complex protein network named complement proteins. The complement system is present in blood and body fluids as inactive precursors, which become activated in the presence of a pathogen and rapidly, within minutes, kills the bacteria through an activation cascade. Complement activation is a multi-step process in which each event is indispensable for the activation of the next. In the classical pathway, complement activation starts with the recognition and binding of the foreign molecules on the surface of the bacteria through the C1 complex. This binding leads to activation of C1r and C1s, which cleave C2 and C4, which then trigger the formation of a C3 convertase that converts C3 into C3a and C3b. C3b, at high levels, binds C3 convertase, which forms another complex called C5 convertase that in turn produces C5a and C5b. The presence of C5b on the surface of bacteria attracts other complement components such as C6, C7, and C8, and multiple copies of C9 to form the complement membrane attack complex (MAC). MAC is a cytolytic effector of innate immunity that forms pores in the plasma membrane of pathogens, leading to changes in osmosis of the bacteria and its death[86-88]. Apart from the classical pathway, other activation pathways have been described. These three pathways have the same outcome. Particularly, the

lectin pathway is activated when mannose-binding lectin encounters a carbohydrate motif on the surface of the pathogen, which leads to the cleavage of C4 and C2 and the activation of the cascade[87]. In addition, the alternative pathway, using complement factor B and factor D, might amplify the number of C3b molecules deposited via the classical and lectin pathways. However, these pathways might also be activated by the spontaneous hydrolysis of C3 into C3H2O, which has a similar structure to C3b. The hydrolyzed C3 subsequently reacts with factor B and factor D and triggers the complement cascade[89]. However, complement proteins are not merely involved in the direct killing of bacteria through the formation of the MAC pore. During the formation of the MAC complex, C3a and C5a are cleaved, released and can act as a chemoattractant that induces a proinflammatory response. Particularly, C3a and C5a bind their respective G-protein–coupled receptors (C3aR and C5aR) on neutrophils, monocytes and macrophages, which guide them toward sites of complement activation. During an infection, C3a and C5a are constantly released into the bloodstream to amplify the inflammatory response and enhance the phagocytic activity of innate immune cells[90].

1.5.5 Mechanism of immune evasion in *S. epidermidis*-biofilm infection

The primary innate immune response against bacterial infections involves an array of immune effectors such as recognition of PAMPs, complement activation, recruitment of phagocytes and production of cytokines, which help to clear the pathogens. However, some bacteria, including *S. epidermidis*, have developed mechanisms that elude the host immune system. One of them is biofilm formation, which acts as a shield by providing mechanical protection from immune cells, complement proteins as well as antimicrobial proteins[91]. The immune response against *S. epidermidis*-biofilm infection is less pronounced than the planktonic counterpart[41]. Interestingly, it has been shown that biofilm-embedded *S. epidermidis* was (i) killed less efficiently by neutrophils, (ii) included diminished deposition of C3a and (iii) IgG on the bacteria surface compared to planktonic bacteria[92]. An exemption is N-acetylglucosamine, a component of the biofilm, which induces a strong activation of C5a and C3a[93]. Therefore, the high activation of complement proteins against N-acetylglucosamine, but not *S. epidermidis*, might be a decoy for the actual target[91,92]. Due to the reduced efficacy of the immune system against biofilms, treatment that includes antibiotic alone is often inadequate to clear the infection. Therefore, often the removal of the infected indwelling device is the first step in the eradication of biofilm infections, followed by an antibiotic treatment[94].

1.6 Differences in the immune system between newborn and adult

The neonatal immune system is often defined as "immature" due to impairment in the B and T cells' responses, which are unable to produce adequate adaptive immunity[95]. At birth, the maturity of the adaptive immune system relies mainly on the passive immunity transferred by the mother such as IgG antibodies. Similarly, the reduced capacity of presenting the antigen to T-cells limits the ability to mount an adaptive immune response in the newborn. Therefore, in the neonatal period, immunity mainly relies on innate immunity in fighting infection or other insults[96]. As organ development in newborns is still incomplete at the time of delivery, several differences between newborns and adults have to be considered when studying the immune system, including both the innate and adaptive immune systems. Generally, term babies have higher white blood cell counts than adults[96,97], including neutrophils[98,99], basophils[100], eosinophils[100], dendritic cells[101], natural killer cytotoxic cells[102] as well as monocytes[100]. Besides the higher number of these cells in the blood of newborn infants, many differences are linked to their reactivity toward stimuli. For example, neutrophils, although more abundant than in adults, have reduced adherence, chemotactic and migration capacity, as well as bactericidal activity[103-106]. Similarly, neonatal monocytes display impaired chemotactic activity and altered phagocytosis[107,108]. Natural killer cytotoxic cells display reduced capacity to lyse target cells, probably due to the decreased number of cytoplasmic granules and poor degranulation ability[102,109,110]. Furthermore, the reduced amount of complement proteins in the serum of newborns[97] might also contribute to the higher susceptibility to infection. Most of the reduction in complement proteins can be attributed to C8 and C9 proteins[111]. In addition, impairment in the opsonization mechanism has been described[112].

The development of the immune system is related to gestational age at birth, which might be an underlying cause of increased comorbidities observed in preterm compared to term infants[113]. Firstly, as IgG antibodies are mainly transferred across the placenta after the 32nd week of gestation, preterm infants, especially the very preterm, have lower levels of IgG, which results in an impaired opsonisation[113,114]. Similarly, the amount of complement proteins is further reduced in preterm infants[115] by lack of production of C1, C4 and factor B[116]. Furthermore, preterm infants have a reduced pool of neutrophils and monocytes, affecting the infants' ability to promptly respond to infection[117,118]. The higher susceptibility to infection in preterm infants is often enhanced by the impairment of neutrophils as they have reduced phagocytosis and motility, affecting the migration towards the site of infection[119-121].

1.7 Neuroinflammation

The central nervous system has long been considered an immunological quiescent organ compared to other peripheral tissues[122]. However, it is now well established that neuroinflammation has both physiological and pathological roles in the brain (Fig 5). Upon insults such as infection, neuroinflammation plays important beneficial roles by eliminating cell debris and misfolded proteins as well as tissue repair and stress[123]. Understanding the positive aspects of neuroinflammation might help to understand and prevent neurological disease onset and progression[124]. These include, for example, its involvement in long-term potentiation and learning, in which activation of a cytokine network, such as IL-6, IL-1β, IL-18 and TNF occur[125]. Similarly, the propagation of cytokines from the periphery to the brain has been implicated in sickness behavior[126], which is induced by infections or immune trauma and is indispensable for enhancing recovery by conserving energy[127]. Of note, beneficial effects of cytokine exposure and activation of brain immune cells (microglia and astrocytes) have generally a short duration to ensure its safety[128].

To date, many studies have focused on the pathological roles of neuroinflammation and showed that the cerebral immune response is a key factor underlying several neurological diseases[122]. In response to insults, neuroinflammation can cause tissue damage, which results in the subsequent onset of brain injury. Further, peripheral inflammation might propagate into the brain with the consequent alteration of glial cells (mainly microglia and astrocytes), neurons and blood-brain barrier[129], resulting in synaptic impairment, neuronal death and an exacerbation of several pathologies within the brain[130-132]. A growing body of evidence suggests that neuroinflammation induces neurodegeneration[133]. At the same time, inflammatory responses are also triggered by tissue loss and neurodegeneration[134], suggesting that both events are tightly connected. However, the exact link between neuroinflammation and neurodegeneration is still unclear. Understanding the context and timing of the event might be crucial in the development of new therapeutic opportunities, especially in preterm infants.

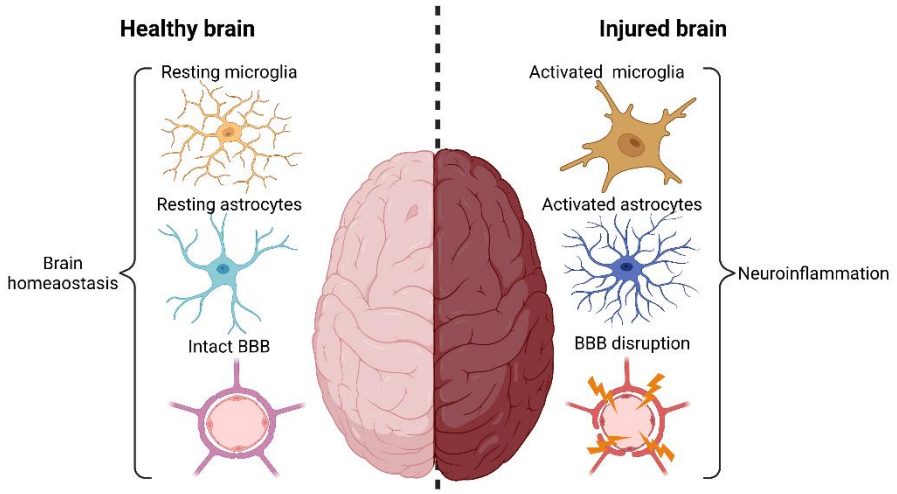

Figure 5. Representation of the healthy and injured brain

1.7.1 Microglia

Microglia play a prominent role in neuroinflammation and are well recognized critical contributors to the detrimental outcome after immature brain injury[135]. They are often defined as the brain macrophages by their ability to induce an inflammatory response. Under resting conditions, microglia display a highly branched and ramified morphology whereas upon activation they assume an amoeboid shape together with retraction of microglia branches[136]. In the brain, microglia are broadly distributed in every area and account for ~10% of the total number of cells[137]. Historically, microglia have been thought to exist in different forms and with difference in their reactivity and polarization towards either the M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotype. However, recent evidence indicates microglia are more dynamic and heterogeneous than previously thought[138].

Although the origin of microglia has long been a matter of debate, evidence now converges into a mesodermic origin[139]. In mice, microglia migration from the yolk-sac into the brain starts at embryonic day 8.5–10. After colonization, the fetal microglia that have high proliferative potential expand in the fetal brain until peaking in number on postnatal day (PND) 14 in rodents[140]. In humans, microglia infiltrate the brain through the choroid plexus, meninges and ventricles at around 4.5 gestational weeks[141].

Microglia have been considered the first line of defense in the brain, with crucial functions in immune surveillance[142]. Under physiological conditions, microglia exert a variety of fundamental processes, necessary for ensuring

brain homeostasis[143]. These processes include embryonic neurogenesis[144], neuronal differentiation[145], synaptic pruning[146] and elimination[147], removal of apoptotic neurons[148], and learning-dependent synapse formation[149]. They are involved in vasculogenesis and vascular sprouting in the embryonic brain[150,151]. Moreover, inflammatory mediators, such as IL-1β, IL-6, TNF-α, and IFN-γ, produced by microglia cells enhance neurogenesis and oligodendrogenesis[152]. In response to acute injury, microglia phagocytosis ability is mediated by surface receptors (as described in section 1.5) that respond to the "eat me"/"don't eat me" signals, after pathogen or danger-molecule recognition[153]. Those signals are crucial to initiate the transcription of pro-inflammatory mediators[153]. Activation of microglia is primed by TLR signalling. Indeed, it is believed that $Gram^+$ bacteria, such as *S. epidermidis*, act via TLR2 signalling. However, a study from our group demonstrated that *S. epidermidis* induced brain damage in both TLR2 dependent and independent fashions, suggesting that other PRRs are also involved in the inflammatory response in the brain[154]. Additionally, microglia are NLRP3 inflammasome-competent cells[155], being able to produce IL-1β and IL-18, which have been implicated in developmental brain injury[156,157] as well as in stroke[158]. Hence, disruption in microglia homeostasis as well as an excessive inflammatory response have been implicated in numerous neurodevelopmental conditions such as autism[159]. However, it is still unclear how brain inflammation and microglia activation arise in the brain[160].

1.7.2 Astrocytes

Astrocytes are the most abundant cell type in the mammalian brain, accounting for ~ 20-40% of all glia[161,162]. In rodents, astrogenesis starts ~E18 and lasts until PND7[163]. In the embryonic neuronal tube, a pool of naïve neural precursors differentiates into the radial glia which further differentiates into astrocytes[163]. In resting conditions, astrocytes in humans and rodents display differences in morphology and function, which have to be taken into account when studying astrocytes in animal models[164]. For example, human astrocytes are more complex than rodent astrocytes. Astrocyte soma is larger and more complex in humans than their rodent counterparts[165]. Moreover, the astrocyte/neuron ratio in the human cortex is 1.65, while in rodents, it is approximately 0.35[166,167]. Despite the differences in morphology, astrocytes exert numerous functions that are similar in both humans and rodents, such as maintaining BBB, neurotransmission and neuroinflammation[162]. Astrocytes are also involved in the maintenance of brain homeostasis, supporting neurons and regulating neuronal activity. The highly complex morphology of these cells sustains numerous contacts with different cell types, enabling both contacts with neuronal synapses as well as forming end-feet wrapping around blood vessels[168]. Indeed, due to the close contact with neurons, astrocytes can

sense neuronal changes[164]. Moreover, contact with endothelial cells enables astrocytes to regulate cerebral vascular tone and blood flow[169]. Thus, by regulating the astrocytic end feet Ca^{2+}content, astrocytes may trigger arteriolar dilation and constriction[169]. Specifically, moderate Ca^{2+} levels induce vasodilation whereas elevated concentration induces vasoconstriction. Similarly, by sensing extracellular K^{+} ions, astrocytes can induce both vasodilation and vasoconstriction[169]. Therefore, due to the polyfunctional activity of astrocytes, disruptions in any of these mechanisms affects brain development and may lead to perturbations of CNS and neurological diseases[170]. Astrocyte pathological activation has been observed in several neurodevelopmental diseases[171], such as elevated expression of GFAP in the brain and cerebrospinal fluid in autistic patients[172,173]. Additionally, astrocytes may participate in the innate immune responses against infection[174]. Bacterial products may activate astrocytes, which become reactive and release cytokines and chemokines, enhancing neuroinflammation[174]. The prompt response of astrocyte to infection is possible due to the expression of TLR2, TLR4, TLR5 and TLR9 that recognize bacterial ligands[175,176].

1.7.3 The blood-brain barrier (BBB)

The BBB is a term used to indicate the complex microvascular network in the brain, which separates neural tissue from circulating blood[177]. The BBB acts as a gateway that regulates molecular transport from the periphery to the brain, and vice versa. The BBB is formed by endothelial cells, pericytes, astrocyte end-feet and microglia[178]. Endothelial cells are the main restrictive unit of the cerebral vasculature forming the BBB. Pericytes, with their processes, surround endothelial cells, serving as regulators of capillary diameter and cerebral blood flow[179]. The unique properties of the BBB are given by the specialized junctions (tight junctions) between endothelial cells, which prevent the free passage of certain molecules. Further, the presence of transporters in the endothelial cells regulate the influx and efflux of molecules in and out of the brain[177]. Due to the high metabolic demand of the brain, the BBB has to ensure the entry of molecules and nutrients into the brain. Generally, there are four routes of transport through the BBB, such as passive diffusion, carrier-mediated transport, selective transport via ATP-binding cassette transporters and transcytosis[177]. The development of the BBB starts in humans at ~12 weeks of gestation[180] while in rodents it is formed and functional at ~E15.5[181]. In the developing brain, angiogenesis plays an important role in forming new vessels. During this process, glial cells are in close contact with the vessels. Microglia, for example, have been suggested to contribute to vessel formation and their depletion results in a sparser brain vascular network. Similarly, astrocytes have been implicated in the development of brain vessels. Indeed inhibition of astrogenesis resulted in a reduction of blood vessels[163]. Subchapter heading

2 AIM

The central hypothesis of this PhD thesis is that systemic inflammation induced by *S. epidermidis* leads to immune reactions in both the periphery and the brain, which increases vulnerability to secondary brain injury, leading to neurological impairments. However, the mechanism of *S. epidermidis*-induced brain damage remains unknown. In the three projects presented in this thesis, we explored different aspects of the pathogenesis associated with *S. epidermidis*, ranging from the sensitization effects to the neuroinflammatory response.

Specifically, the PhD projects aim to investigate:

• The potentiating effects of *S. epidermidis* on neonatal cerebral hypoxia-ischemia (**Paper I**)

• Molecular and morphological alteration of hippocampal microglia cells after *S. epidermidis* infection (**Paper II**)

• The proteomic changes associated with *S. epidermidis* infection on the immature hippocampus (**Paper III**)

• Translational opportunity of the experimental findings (**Paper III**)

3 MATERIAL AND METHODS

"The true method of knowledge is experiment"
William Blake

This chapter describes the technology and the methods used in the different projects of this thesis, highlighting both the benefits and the limitations of the employed techniques. The specific information on the material and methods used can be found in each **Paper**/manuscript.

3.1 Animal experiments

The primary goal of clinically-related neuroscience research is to understand the function and dysfunction of the human brain. However, answering research questions regarding cellular and molecular mechanisms are often restricted when using human material. Therefore, animal models might be suitable for addressing research questions[182]. The findings in this thesis (**Paper I-III**) are based mainly on animal experiments to study infection and cerebral hypoxia-ischemia. All experiments are approved by the local Gothenburg animal ethical Committee (Dnr 5.8.18-02238/2017 and 5.8.18-11818/2019). All personnel working with animals are highly trained and has competence according to Swedish law (L 150, SJVFS 2019:9). All animal-related research was planned with the 3Rs principle (reduce, refine, replace) in mind and procedures were performed and reported according to the ARRIVE and PREPARE international guidelines[183]. All data collection and analysis were performed by a blinded person and animals have been randomized before the experiment. The animal strains used in this thesis are listed in Table 1.

Table 1. Animal strains used in this thesis

Strain	Referred	Phenotype	Paper
C56Bl/6J	C56Bl/6J	Wild type mice	I, II, III
Lysozyme M- enhanced green fluorescent protein mice	LysM-EGFP	Knock-in mice with EGFP inserted in the lysozyme M to assess infiltration of peripheral myeloid cells[184]	II
Glial Fibrillary Acidic Protein$^{-/-}$, Vimentin$^{-/-}$	GFAP$^{-/-}$ Vim$^{-/-}$	Mice with impaired astrocytes reactivity by lacking GFAP and Vimentin[185]. These mice were created on C57BL/129 mixed genetic background	III
C57BL/129 mixed genetic background	WT	Wild type mice	III

3.1.1 An overview of animal experiments

Animal models for infectious diseases have largely been used to study the host-pathogen interaction as well as numerous pathological conditions and their consequences. They usually provide a better understanding of the temporal and cellular/molecular events that occur between the infection/pathology and the potential harmful outcome. The need for animal models to study infections becomes relevant when considering that many infectious diseases are potentially lethal or disabling and thereby difficult to study directly in humans[186]. Similarly, when studying hypoxia-ischemia in term/preterm infants, the need for using animal models becomes crucial to identify potential novel mechanisms and molecular targets, which can then be further validated in humans. The use of mice models has numerous benefits such as a short gestational period (21 days in contrast to 9 months in humans), a relatively large number of pups per gestation and easy handling. Moreover, the conserved core mechanisms for gene regulation and homology in neural networks[182], as well as the key stages of brain development[193], help neuroscientists to study molecular mechanisms underlying brain injury in humans. However, differences between humans and mice have to be taken into consideration, especially in the neuroscience field. Firstly, the human brain is gyrencephalic while mice have lissencephalic brains. The presence of gyri and sulci in the human brain is crucial for higher efficiency of neural processing, speeding cell communication and increasing cell contacts[187]. Further, considering the timing of brain development, there are numerous differences between humans and mice, which have to be taken into consideration when planning experiments in neonatal mice. This includes white and grey matter ratio to body size, white matter development, oligodendrocyte maturation, axonal growth, BBB establishment and immune system development that in human occurs between 23–32 week of gestation while in mice between PND0-5[187-189]. Thus, in all of the **Papers,** we used PND4-5 mice, which resemble the brain developmental stage in preterm infants born between 23 and 32 weeks of gestation.

3.2 *S. epidermidis* bacterial growth

The strain *S. epidermidis* 1457, obtained from an adult patient with a central venous catheter infection, was used in all **Papers** presented in this thesis. Bacterial growth depends on several factors both intrinsic to the bacteria (lifespan, size, etc) or external (nutrition availability, temperature, etc). The typical bacterial growth curve consists of 4 phases (lag, exponential, stationary and death phase).

- The **lag phase**, also known as steady-state growth, occurs immediately after the inoculation and the bacterial population remains temporarily unchanged[190]
- In the **exponential phase**, the bacteria divide at a constant rate doubling over time its number. The rate of division largely depends on the composition of the media[190]
- The **stationary phase,** also known as the linear phase, occurs when the nutrients are scares and bacterial metabolites start to accumulate in the media. In this phase, bacteria start to die due to the limited amount of nutritional factors[190]
- The **death phase** occurs when there is a continuation of the bacterial incubation without media change. In this phase, due to lack of nutritional factors as well as secondary metabolites accumulation, bacteria die, reducing their number[190]

The process of bacterial culture used in this thesis is illustrated in Figure 6. On day 0, bacterial stock was inoculated in a tube containing culture media and incubated overnight in a shaker at 37 °C. The day after, a small amount (between 10-20 µl) of the overnight culture was inoculated in a new flask (previously kept at 37 °C) and incubated in a shaker at 37 °C for ~5 hours. To ensure that bacteria used for injection were viable, we checked the optical density (OD) of the culture. OD between 0.3 and 0.4 represents the exponential phase, in which bacteria are most viable. When the OD was between 0.3 and 0.4, the culture (12 ml) was centrifuged at 3,000 × g for 5 min at 4°C and then resuspended in endotoxin-free saline to obtain a final concentration of 3.5×10^7 colony forming units (CFU)

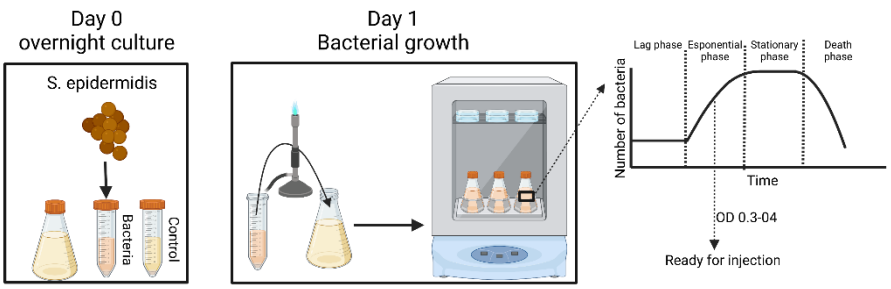

Figure 6. Representation of bacteria culture and growth curve

3.3 *S. epidermidis* animal model

There are only a few animal models to study *S. epidermidis* infections in brain. One group using adult rats injected heat-inactivated *S. epidermidis*

intracerebrally and found early microglia and astrocyte responses, opening of the BBB and subsequent infiltration of $CD8a^+$, $CD4^+$ and $CD20^+$ lymphocytes[191]. To my knowledge, the only large animal model of *S. epidermidis* infection that has been reported is in piglets. Using piglets, the researchers injected live bacteria intra-arterially[192,193] and found that *S. epidermidis*-infected pigs had several signs of sepsis, such as hypotension, respiratory acidosis, and internal organ hemorrhages, BBB disruption, and neuroinflammation. Large animal models have several advantages such as high similarity with the human brain, not only structurally but also in neuronal complexity[194]. However, often mice models are preferred due to the ease of maintenance, short life cycle, and abundant genetic resources[195].

We and others have developed models in newborn mice (PND0-5) that mimic the clinical *S. epidermidis* infection in preterm infants[154,196,197]. In these models, either intravenous or intraperitoneal injection of *S. epidermidis* results in bacteria in the blood and spleen and the infection induced the expression of several cytokines and chemokines in blood and a mortality rate of 6% (similar to *S. epidermidis* infected preterm infants), highlighting the clinical relevance of the model used in this thesis.

3.4 Hypoxia-ischemia animal model

Perinatal brain injury in humans is seen in the cerebral white and grey matters, with a shift towards the white matter in preterm infants. To study the complex events that occur during and after HI, several animal models have been developed. The HI animal model used in our experiments (**Paper I and III**) is based on the modified Levine preparation. The original Levine model was developed in 1960 for studying hypoxic-ischemic conditions in adult rats[198]. This model consists of unilateral ligation of one common carotid artery combined with whole-body hypoxia, which results in ischemic conditions in the ipsilateral forebrain[198]. In 1981 this model was modified to study HI in PND7 rats, which resulted in neuronal destruction in the ipsilateral forebrain as in adult animals, but also increased necrosis of white matter. This model is also known as Rice-Vannucci HI model[199] and was later adjusted to PND7-9 neonatal mice[200,201]. The HI model has more recently also been applied to younger neonatal mice at PND5 to better reflect brain injury that develops in preterm infants[202]. Importantly, to ensure a similar overall degree of injury at different ages the duration of the hypoxia needs to be adjusted in PND5 and PND9 mice because younger mice are more resistant to hypoxia exposure. Thus, in the HI model used in this thesis, PND5 mice were exposed to 60 min of hypoxia (10% O2) and PND9 mice were exposed to 50 min hypoxia (10% O2) (**Paper I**).

3.5 Patients

To seek translational opportunity, in **Paper III**, we validated our results using blood samples from preterm babies[203]. Details of the number of patients as well as the patient characteristics can be found in **Paper III**. A total of 183 preterm infants were used. Data on birth weight, gestational age and sex were prospectively recorded accordingly to the study protocol. The Mega Donna Mega study protocol was approved by the Regional Ethics Review Board in Gothenburg (MEGADONNAMEGA 16-7) and the proteomic analysis was approved by the Swedish Ethical Review Authority (Dnr 303-11, T570-15). Written informed consent to participate was provided by the parents or legal guardians of all included infants. ClinicalTrials.gov Identifier: NCT03201588

3.6 Immunoassay

3.6.1 Protein quantification and concentration by Enzyme-linked immunosorbent assay (ELISA) and Bicinchoninic Acid Assay (BCA).

An immunoassay technique used in all the **Papers** in this thesis is ELISA. ELISA was first invented by Eva Engvall and Peter Perlmann in 1971[204] and has now become one of the most common techniques used to detect and quantify the concentration of a particular protein in biological samples. The first step of the "sandwich ELISA" is the coating of the wells using capture antibodies. These antibodies have a high affinity for the target molecule in the sample and when the plate is washed, no-bound proteins will be removed. After, a secondary antibody, with high affinity for the primary antibody, called detection antibody, was added. To be detectable and quantifiable, another group of antibodies conjugated with biotin were added to the solution, which will bind to the detection antibody. In the last step, another chemical, such as TMB substrate, was added to catalyze an enzymatic color reaction, allowing the antibody complex to be determined and quantified. Based on the timing of the stop solution added to the plate, the ELISA results may vary from plate to plate. Therefore, in our experiments to avoid any possible bias, all the measurements for the same analytic molecule were performed on the same plate.

Using ELISA, we were able to detect several proteins in different biological specimens. In **Paper I**, we quantified Complement protein 5a in the blood, brain and liver. Similarly, in **Paper II and III**, using this technique, we measured the concentration of Caspase-1 and LCN2.

To relate the specific molecular quantification by ELISA to total protein concentration in each specimen such as the brain (in **Paper I-III**) and liver

(**Paper I**), ELISA was complemented with another immunoassay (BCA). Similar to ELISA, BCA results in a shift of color under alkanic conditions, which can be measured. BCA relies on the presence of peptide bindings in proteins and therefore the reductions of Cu2+ to Cu1+ are proportionate to the total protein[205]. Therefore, in all experiments in the thesis, the target protein concentration identified by the ELISA was divided by the total protein concentration to obtain a reliable result in relation to the overall protein content in the sample.

3.6.2 Cytokines and chemokine assay

In **Paper I**, we employed an immune assay technique to determine and quantify multiple cytokines and chemokines. We determined the peripheral immune response by measuring 23 cytokines and chemokines in the blood of *S. epidermidis* infected and saline-injected mice. To do so, we used the Bio-Plex ProTM Mouse Cytokine 23plex Assay (Bio-Rad). This multiplex array is an evolution of the ELISA method that allows simultaneous measurement of multiple molecules in a single sample. The multiplex assay employs bead sets, where each bead set is coated with a specific capture antibody that binds the target molecule. Therefore, fluorescence or streptavidin-labeled detection antibodies bind the target molecule[206], similar to the ELISA. Each bead/complex has a fluorescent dye with a distinct emission wavelength that aids to discriminate the different beads. This method applies the same principle as in ELISA, but the main difference is the location of the reaction. Thus, the capture antibodies are bound directly to the plate in the ELISA assay while in the multiplex the capture antibodies are bound to the surface of the beads. Multiplex array presents the great advantage of evaluating multiple cytokines/chemokines at the same time, reducing an enormous amount of time compared to the traditional ELISA and also reduces number of animals required. However, it requires specialized equipment as well as a higher money investment compared to the ELISA.

3.7 Immunohistochemistry and immunofluorescence

Immunohistochemistry (IHC) and immunofluorescence (IF/IHC) are antibody-based techniques, which were used in all **Papers** presented in this thesis. These techniques allow us to detect one or more proteins in biological tissues, giving us information about their expression as well as their spatial location. Immunostaining techniques use a primary antibody that first detects the antigen in the tissue. The primary signal is amplified by using a secondary antibody conjugated to biotin or a fluorophore that may differ based on the technique employed (IHC or IF/IHC). To visualize the results of staining, a microscope is often used. Bright field microscopes are often used to visualize

the IHC slides while fluorescence microscopes, such as confocal microscopes, are often used for IF/IHC. For both IHC and IF/IHC techniques, it is important to maintain tissue integrity. In all the experiments (**Paper I-III**), protein integrity was achieved by whole-body fixation through transcardial perfusion with 6% buffered formaldehyde followed by tissue immersion-fixation in the same solution. After fixation, the brain tissue was sectioned using either a microtome (**Paper I**) or a cryostat (**Paper II-III**). Sections that were cut on the microtome (**Paper I**) had a thickness of 7µm whereas sections were cut on the cryostat (**Paper II and III**) were 40µm thick. The thicker sections allowed the collection of 3-D spatial information of analyzed cells. Two approaches were used to handle brain sections during the staining procedure. The classical approach is to mount the cut section directly onto a slide, which will then be subjected to immunostaining. Using the second approach, free-floating sections (usually thicker) are stained suspended in a solution before mounting on the glass slides[207].

Based on the target that to analyze and the commercial availability, different antibodies can be employed to assess cell/tissue morphology or protein location. The antibodies used in this thesis together with the target cell/molecules are listed in Table 2. In **Paper I**, antibodies against microtubule-associated protein-2 (MAP-2) and myelin basic protein (MBP) were used to study grey and white matter loss respectively. In particular, MAP-2 staining is commonly used to evaluate infarct area after injury, as it labels neurons and dendrites and thereby reflects the grey matter integrity in the brain. MBP labels myelin present in the brain, which gives information about the white matter injury after the HI event. Both MAP-2 and MBP staining allowed us to understand the effect of *S. epidermidis* combined with HI injury on several regions of the brain.

To study microglia morphology in **Paper II**, we stained the 40µm thick sections with Ionized calcium-binding adapter molecule-1 (Iba-1), which is a marker of microglia cells expressed in both resting and activated microglia. To gain spatial information on the microglia morphology, we used thicker sections, performing the free-floating staining. This latter allowed us to have a better antibody penetration and stained cells were suitable for 3-D cell reconstruction[207]. The free-floating staining was also used in other experiments in both **Paper II&III** to study astrocytes (GFAP), immune cell infiltration (GFP) as well as changes in the cerebral vascular bed (CD31) and astrocyte endfeet (AQP4). The antibodies and staining approaches are listed in Table 2.

Table 2. Antibodies employed in this thesis. Approach A indicates the classical staining with section mounted directly on the glass while approach B indicates the free-floating staining.

Antibody	Cell target	Staining approach	Paper
Microtubule-associated protein-2 (MAP-2)	neurons and dendrites	A	I
Myelin basic protein (MBP)	Myelin	A	I
Ionized calcium-binding adapter molecule-1 (Iba-1)	Microglia	B	II
Glial fibrillary acidic protein (GFAP)	Astrocytes	B	III
Cluster of differentiation 31 (CD31)	Blood vessels	B	II&III
Green fluorescent protein (GFP) in LysM-EGFP-ki pups	Infiltrating leukocytes	B	II
Aquaporin 4 (AQP4)	Astrocytes endfeet	B	III

In Paper II, IF-IHC staining of sections from LysM-EGFP-ki mice was performed to detect GFP positive leukocytes (anti-GFP antibody). The sections were also stained with anti-CD31 and DAPI to visualize vessels and cell nuclei, respectively. This triple staining allowed us to discriminate infiltrating cells in the brain parenchyma from the leukocytes inside blood vessels.

3.8 Microscopy and Image analysis

Immunostained (IHC and IF-IHC) brain sections were imaged and analyzed by using different microscopy techniques. The type of employed microscope largely depends on the type of sections used as well as the type of analysis to be performed. In **Paper I and III**, images from MAP-2 (grey matter) and MBP (white matter) stained sections were captured using a 4× objective lens on Olympus BX60 light microscope. Afterward, images were processed and analyzed using Fiji-build[208] of ImageJ[209] software. The region of interest was manually outlined (hippocampus, cortex, thalamus and striatum) and the area loss was calculated as follows: [(contralateral side – ipsilateral side)/contralateral side × 100%]. The mean of the percentage of tissue loss for all levels was compared among animals (**Papers I**).

In **Paper II and III,** immunostained sections (Iba-1, GFAP, CD31 and AQP4) were imaged and analyzed using a light microscope which is equipped with newCAST software (Visiopharm, Hørsholm, Denmark) modified for stereology with a digital camera (Leica DFC 295, Germany) and a motorized stage (Ludl Mac 5000, US). First, the molecular layer of dentate gyrus (MDG) and CA1 stratum radiatum (CA1.SR) were outlined manually under a 5 × objective lens and then using a 100× oil-immersion objective lens, the volume of Iba-1$^+$ microglia and GFAP$^+$ astrocytes were estimated using 3D nucleator by assuming a rotational symmetry of microglia and astrocytes. The number

of half-lines was set at 6, giving a reliable measurement of the cell soma. For each animal, 50-80 cell somas were analyzed using an optical disector probe with a height of 15µm. Measurements of the length density (Lv) of $CD31^+$ and $AQP4^+$ capillaries were performed by using the Global Spatial Sampling method [210] under 63× objective lens by counting the number of intersections between isotropic virtual planes and capillaries as previously described [211]. The capillary diameter was measured in two hippocampal subregions (CA1.SR and MDG) using a 100× oil-immersion objective lens. This measurement was performed on 60-80 capillaries per region per animal which were sampled unbiased and randomly using an optical disector probe with a height of 25 µm. The selection criteria were the in-focused outer wall of the capillary and being entirely or partially inside the unbiased counting frame and not crossing the forbidden lines of the frame.

For 3-D reconstruction of microglia (microglia activation) in **Paper II** was done by using Filament Tracers algorithm in the Imaris software (v8.4) by tracing the length, number and complexity of microglia branches as described before [212].

Additionally, immunofluorescence images in **Paper II and III** were captured using a laser scanning confocal microscope Zeiss (LSM 800). Z-stacks of images were captured from brain sections with z-plane step size of 4.0 µm using 10× air objective lens, or 1.0 µm using 40× oil-immersion objective lens. Images were processed using Fiji software version 1.53c and maximum projection of z-stacks were made to present the results [208]. Large field images covering the whole hippocampus area were created from low magnification images using Fiji stitching plug-in [213]. These images were used to reveal immune cell infiltration

3.9 Polymerase chain reaction (PCR)

In all presented **Papers** in this thesis, PCR was used to assess the presence of a specific gene or its expression level. PCR was first invented in 1983 by Kary Mullis and Michael Smith[214] and since then, PCR has become a fundamental method in genetic and molecular biology research. The PCR method is based on enzymatic/temperature reactions, which include three fundamental steps:

- Denaturation, in which the double helix of DNA temporarily separates into the single strands
- Annealing, in which the primers can bind to the single strand of DNA. The primers have to be specific for the target region of DNA

- Extension of DNA strands. In this last step, the presence of DNA polymerase can replicate the DNA target region[215]

By repeating these steps, the amount of DNA that can be detected will increase. There are several types of PCR methods that share the same principles and steps. The differences are mainly due to the detection method. The qualitative PCR method detects the presence of a specific gene and the results are visualized in a gel. This method was used in **Paper II** to genotype the LyzM-GFP ki mice. An evolution of this technique is the real-time PCR (RT-qPCR or simply qPCR) that uses a specific fluorescent dye (such as SYBR-green), which is intercalated in the double-stranded DNA. The fluorescent signal is directly in proportion with DNA product and therefore this method is useful and suitable for the quantification of DNA. qPCR results are expressed as number of threshold cycles (CT), which are specific for each gene. Changes in CT mean changes in the expression of those genes. Thus, high CT is equivalent to less gene product and vice versa. One of the critical steps of qPCR is the normalization of the CT. Thus, the expression of the target gene is often normalized with genes that do not change with the experimental conditions, often referred to as housekeeping genes[216]. However, in our experiments, we were unable to find housekeeping genes that were stable under all conditions, perhaps due to the developmental period. Thus, we used quantification of total cDNA to normalize the target gene expression in the PCR analysis. Therefore, the value of each gene was calculated as follows: ([$cDNA_{targetgene}$/average of $cDNA_{total}$ in all samples]/total $cDNA_{targetgene}$)[217]. RT-qPCR was used in **Paper I and III** to reveal the difference in the gene expression of complement protein 5 (C5a) and its receptors (C5aR1 and C5aR2) (**Paper I**) and lipocalin 2 (**Paper III**).

However, as RT-qPCR replicates DNA and not RNA, one step prior to the qPCR is the conversion of RNA into DNA. This method is also called Retro transcription PCR (RT-PCR) and was used in **Papers I-III**.

3.10 Blood-Brain Barrier (BBB) permeability

To study alteration in BBB permeability after *S. epidermidis* infection in **Paper II**, we used ^{14}C-sucrose permeability assay. Using this assay, we revealed increased permeability of BBB following infection. The ^{14}C-sucrose permeability assay is based on the utilization of a radioactive molecule (^{14}C-sucrose, molecular weight 342.3 Da) that is not metabolized or transported in mammals, thus, is used as a passive permeability marker. By quantifying the extravasation of 14C-sucrose into the brain, it is possible to calculate alteration in the permeability of BBB. In our experiments, we injected ^{14}C-sucrose i.p.

30 min before the animal was sacrificed and then blood and brain tissue were collected in tubes for liquid scintillation counting (counts per minute/the weight). The results in **Paper II** are presented as brain tissue/plasma ratio after correction for residual vascular space. Although in **Paper II** we used ^{14}C-sucrose permeability assay to evaluate BBB permeability, it is worth mentioning the Evans blue method is widely used for the assessment of BBB. As injection of ^{14}C-sucrose, Evans blue dye binds albumin that in physiological BBB condition does not cross the BBB, and therefore can be detected in the brain following BBB disruption[218]. However, due to Evans blue´s ability to alter junctional morphology and endhotelium[219], its use has been replaced by other methods[220]. The radioactive label on the sucrose gives high accuracy and ease to measure trace amounts of compound and also avoids extraction problems that may be encountered with other labeling techniques.

3.11 Magnetic-activated cell sorting (MACS) isolation

Magnetic-activated cell sorting (MACS) is a method used in **Paper II** to isolate microglia cells from the hippocampus of saline and *S. epidermidis* infected mice. It is a suitable method to separate different cell populations based on the specific cell surface markers. This method was first invented in 1990 by Miltenyi and colleagues[221] and now is widely used in different research areas such as immunology, cancer, neuroscience, etc. The utilization of magnetism coupled with a specific antigen allows the target cell to attach to the specific nanoparticle while eluting the rest of the cells.

In the protocol used in **Paper II**, the first step was to dissociate the neural tissue by using enzymes. This step is crucial to break down cell-cell interaction, generating a single cell suspension. Based on the experiment, the single-cell suspension can be directly used for further application, such as RNA-seq, in vitro study, etc. However, as the main focus of **Paper II** was to understand the effect of *S. epidermidis* on microglia cells, we further processed the single-cell suspension to specifically isolate microglia, using CD11b$^+$ beads. Thus, the cell suspension together with the CD11b$^+$ beads was loaded into the MACS column and placed in a magnetic field. This allowed us to retain the CD11b$^+$ cells, and elute CD11b$^-$ cells, which could be used for further applications, in our case RNA-sequencing.

3.12 RNA sequencing

In 1977 a method to sequence DNA was developed for the first time [222] by Sanger, Nicjlen and Coulson. Using this method, after 13 years of effort, in 2003 the human genome project was completed with an accuracy of 99.99%[223],

opening new possibilities to study gene function and dysfunction in complex organisms. Since then, the further development of sequencing techniques has significantly progressed over the years, being now widely used for DNA and transcriptome profiling[224]. The Next Generation Sequencing (NGS) technology generates, in a single run, hundreds of gigabases of nucleotide-sequence output. The generation of an enormous amount of data by this high-throughput RNA-seq approach and its analysis now represents a major challenge for research projects. The sequencing data presented in **Paper II** was performed by Novogene Co., LTD (Cambridge, UK) and was generated using Illumina technology. This technology uses a flow cell consisting of an optically transparent slide to which oligonucleotide anchors are bound. This allows the DNA target, complemented with adapter sequences, to bind the anchor and start the sequencing reaction[225].

In **Paper II**, we investigated the transcriptome profile of microglia cells, previously sorted using MACS technology, from the neonatal hippocampus after *S. epidermidis* infection/saline. Depending on the technology used for the sequencing, the initial concentration of the cDNA is the limiting factor of the reaction and may vary from experiment to experiment. Therefore, to reach the desired concentration of cDNA (150 ng/µl) for the method we used, for each sample we pooled hippocampi from 8 mice. The various steps of the isolation and sequencing are illustrated in Figure 7. In the first step, hippocampi were isolated as described in section 3.11, and microglia were isolated. Next, RNA was isolated from the microglia cell pellet and reverse transcribed into cDNA. This was further used for library preparation, which consists of rRNA removal, the addition of the adaptor, DNA fragmentation etc. After, the DNA library was used for the numerous cycle of reactions, which include the incorporation of four fluorescent dye terminators (modified nucleotide), recording the termination of the reaction and the start of the new cycle[225]. The raw data was then mapped to Mus-musculus mm10 genome using STAR software[226] and used for bioinformatics analysis.

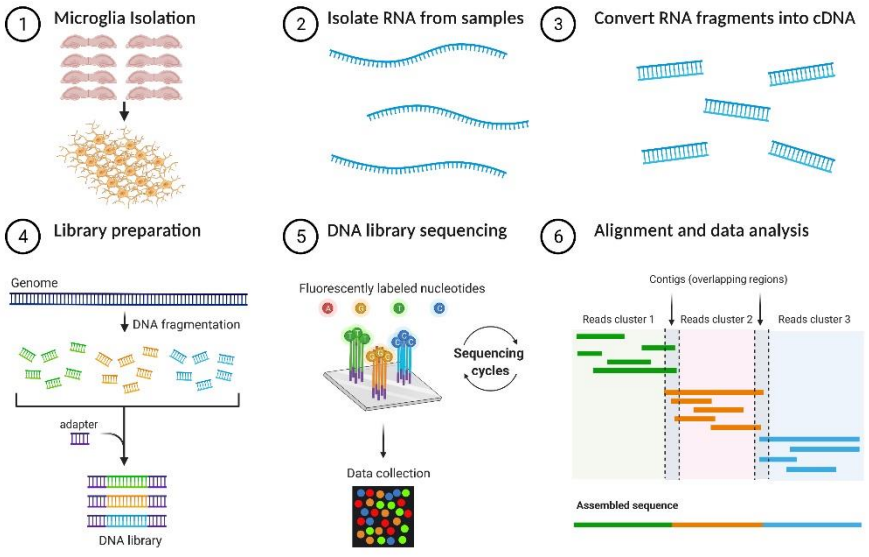

Figure 7. RNA sequencing steps. Adapted from "RNA sequencing" and "Next Generation Sequencing (Illumina)" templates by BioRender.com 2022. Retrieved from https://app.biorender.com/biorender-templates

3.13 Proteomics

With the advent of omics technology, it is possible now to quantify with high accuracy transcriptomic and proteomic profiles of a specific biological specimen[227]. The development of proteomic methodologies has allowed a deeper understanding of the protein interaction and alteration in physiological and pathological conditions[227]. In neuroscience, for example, proteomic analysis helps to understand protein-protein interaction networks and signalling in the brain function and dysfunction[228]. Although transcriptomic analysis offers a suitable approach to reveal changes in the mRNA expression, correlation between mRNA and protein expressions often represents a challenge, mainly due to the numerous post-transcriptional modifications[227,229]. Therefore, proteomic analysis has the direct potential to identify disease markers and/or progression[230]. To understand the protein expression changes in the hippocampus of infected mice, in **Paper III** we employed global proteomic analysis to characterize the protein expression in the immature hippocampus. To do so, hippocampi from *S. epidermidis* and saline mice were collected and analyzed by the Proteomic Core Facility at the University of Gothenburg using Tandem Mass Tag spectrometry. The workflow of the TMT spectrometry is illustrated in Figure 8. After tissue collection, proteins were extracted, denatured, alkylated and digested into the peptide samples. These

were then labeled with TMT (a chemical label that facilitates sample multiplexing) and pooled into one sample. This was then subjected to Liquid chromatography-mass spectrometry[231]. The raw data were then used for quantification and annotation.

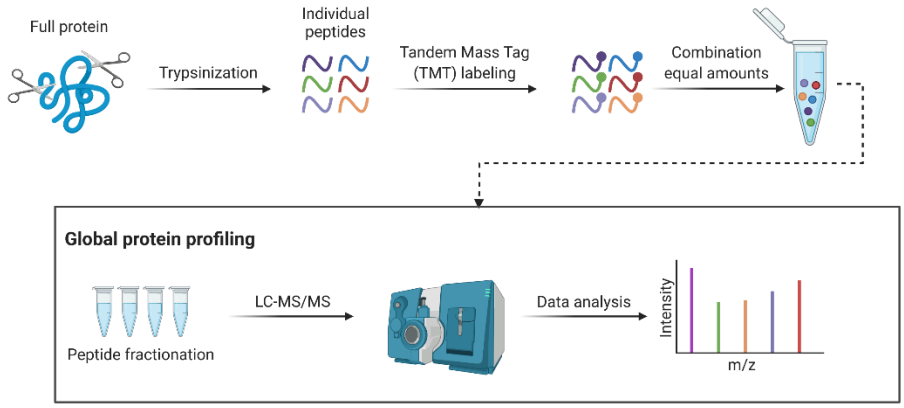

Figure 8. Workflow of Tandem Mass Tag spectrometry

3.14 Differential expression analysis

One of the big challenges in high-throughput omics is the data analyses and thereby bioinformatics methods have dramatically evolved to handle large data sets, which also help in data interpretation. Programming languages, such as R[232], are often used in bioinformatics. The benefits of using R are that statistical analyses are reproducible, free-cost and support a variety of statistical tools together with high and rich quality graphs.

RNA-seq and proteomics results are most often employed to analyze differential expression of genes or proteins (DEGs and DEPs respectively). Differential expression has been widely used in an array of organisms and biological conditions to reveal potential molecular mechanisms underlying diseases and/or physiological processes[233]. In **Paper II**, DEGs analysis was performed in R using "DESeq2" package. DESeq2 is an evolution of the previous DESeq package, which integrates and facilitates quantitative analysis of comparative RNA-seq data using shrinkage (reduction in the effects of sampling variation) estimators for fold change. In **Paper III**, DEPs analysis was performed using Perseus software[234], which provides robust and powerful computational tools to analyze mass spectrometry-based proteomics[235].

3.15 Weighted correlation network analysis

Weighted correlation network analysis (WGCNA) is a method to analyze omics results from a network perspective. WGCNA is increasingly used in bioinformatics, being applied in many different biological contexts such as cancer research, neuroscience and genetics[236]. The WGCNA method was first developed in 2005 by Steve Horvath and collaborators[237] and further implemented in 2008[236]. This method assumes that all systems are scale-free networks. Scale-free networks rely on the existence of a few highly connected/correlated genes/protein that influences a higher number of other genes/proteins. Additionally, the key concept of WGCNA is the "connectivity" parameter. This variable is calculated based on the correlation between genes and its strength is based on the correlation value[238]. Therefore, the identification of "hub" genes is crucial to identify possible targets and can reveal new molecular mechanisms underlying certain biological processes, which might help to develop new pharmacological targets[238]. Thus, WGCNA is an unsupervised method, which helps to identify hidden patterns and exclude subjectivity. Additionally, it is a good method to find clusters of highly correlated genes. These clusters are then further correlated with a specific sample trait such as treatment, sex, etc. In the thesis, the workflow of the WGCNA analysis included:

1. The construction of a gene co-expression network based on connectivity measure
2. Module identification. These are defined as clusters of densely interconnected genes created by using a hierarchical clustering
3. Module correlation with external sample traits (sample features i.e. sex, age, treatment, disease status)
4. Extrapolation of the interest module highly correlated with an interest trait and hub gene identification, which might be the key driver in the network[236]

WGCNA analysis was performed in R language using the package "WGCNA".

3.16 Transcriptome and proteomic analysis

As the output of omics technology is usually a large number of genes/proteins, Gene enrichment analysis has now become part of the analysis of such data. Using Gene Ontology (GO) analysis makes it possible to understand the molecular functions, cellular location and biological processes of a group of genes /proteins[239] identified by DE analysis or WGCNA. Similarly, Kyoto

Encyclopedia of Genes and Genomes (KEGG) is a useful database that integrates genomic, chemical and systemic functional information[240]. In **Paper II and III**, the enrichment analysis was performed using STRING plugin in Cytoscape. Cytoscape is a powerful software that integrates the protein network with the expression profile of a set of genes/proteins, helping the integration of molecular interaction network data[241]. Using the STRING database in Cytoscape helps to provide protein-protein associations and interactions. STRING predicts protein interactions based on co-expression analysis, evolutionary signals across genomes and automatic text-mining of the biomedical literature[242]. Additionally, we used other Cytoscape plugins that helped us to better understand the omic data. Specifically, in **Paper** II, we used cytohubba to identify potential hubs in the network[243] while in both **Paper II and III,** we used EnrichmentMap App to overcome gene-set redundancy and help the interpretation of large gene lists[244]. In both **Paper**s, GO enrichment terms with a false detection rate (FDR) < 0.05 were considered significant.

To further validate the results of the enrichment analysis in **Paper III**, we used Ingenuity Pathway Analysis (IPA). This is both a web-based and manually curated software that aids to provide a comprehensive interpretation of the omics results. Additionally, IPA uses algorithms that can reveal upstream regulators of protein networks through a set of direct or indirect relationships[245].

3.17 Combination of DEGs and WGCNA

Although DE and WGCNA analyze represent suitable methods for analyzing omics data, they also present several limitations that have to be taken into account. Often these limitations can be overcome by performing enrichment analysis. Thus, Pathway GO enrichment analysis provides the opportunity to remedy the multiple testing issue of classical gene-centric testing for differential expression. Grouping genes into the pathways and performing tests on the groups, reduce the number of tests needed. However, since pathway databases don't completely represent all truly occurring pathways, studies based on such databases introduce their ramifications. Only the pathways represented in the database can be found. There is an alternative approach to analyze genes as groups that circumvents the multiple testing issue while still doesn't limit the analysis to known pathways. It is based on constructing gene co-expression networks and is provided by the R package WGCNA[236]. However, the limitation of this method is the choice of parameter β based on scale-free network topology. As scale-free network constructions are based on a mathematical but not a biological concept, the correctness of assuming scale-free topology in biology/medicine has been discussed[246]. Additionally, the choice of signed or unsigned networks might differ in the module assignment

and therefore the analysis results. Therefore, to avoid any possible bias in the analysis of **Paper II**, we used both methods to identify potential mechanisms involved in the pathogenesis of *S. epidermidis* inducing brain injury. Thus, the overlap of the DEGs with the hub genes of the WGCNA modules allowed us to possibly overcome the limitations of both methods and gain meaningful and biologically relevant information. Using this approach, we were able to identify shared molecular and cellular mechanisms in both analyses, which strengthened our findings.

3.18 Statistical analysis and visualization

Statistical analyses presented in this thesis were performed using either GraphPad (GraphPad Software, CA, USA) or R software. In **Paper I,** all statistical tests were performed entirely in GraphPad Prism v.8. As the choice of a statistical test is largely dependent on the sample distribution, before a test the normal distribution was evaluated by generating a Q-Q plot. In the case of non-normal distribution, data were log-transformed before further statistical testing. In **Paper I**, as the variables to be considered were sex (male/female) and treatment (infection/saline), two-way analysis of variance (Two-way ANOVA) followed by Sidak's multiple comparisons posthoc test was performed in all the analyses. Further, data were presented as box plots with median and the whiskers at 10–90th percentile.

In **Paper II and III,** apart from the DEP analysis performed in Perseus software, all of the statistics were performed in R software (version 1.4.1106). The RNA-seq analysis was performed using DESeq2 v1.32.0 and WGCNA packages (as discussed in sections 3.14 and 3.15). Further, Two-way ANOVA with Tukey posthoc test was performed to analyze microglia morphology, BBB permeability and caspase-1 concentration in **Paper II** and LCN2 measurements in GFAP−/−vim−/− mice in **Paper III**, using the functions "aov" and "TukeyHSD". In contrast, in **Paper III**, as all the results derived from male samples with or without infection, comparisons only included two groups and we performed Student's t-test for astrocyte morphology, *lcn2* mRNA and CD31 measurements. Further, in **Paper III** correlation between LCN2 and cytokines and chemokines in a cohort of preterm infants was performed using either Spearman or Pearson correlations.

One of the advantages of using R and Cytoscape is the rich and high-quality graphs that can be generated. In **Paper II and III**, the GO biological processes were visualized using either Cytoscape or "GOplot" and "ggplot2" packages. Additionally, volcano plots and heatmaps were used to visualize the results of the DEGs/DEPs. Venn diagrams were used to visualize the overlap of biological processes and KEGG pathways between DEG analysis and

WGCNA. All IPA results were exported from the original software, except for the canonical pathway graph that was generated in GraphPadPrism. In addition, in **Paper II**, microglia sphericity was visualized using MATLAB (R2020b).

All the figures of this thesis were created with Biorender.com, including the summary figures of the results and conclusions.

4 RESULTS AND DISCUSSION

"There are two possible outcomes: if the result confirms the hypothesis, then you've made a measurement. If the result is contrary to the hypothesis, then you've made a discovery"- Enrico Fermi

This section of the thesis is a summary of the results obtained from each paper attached. The specific information of the results and discussion can be retrieved in the individual papers. Although three papers are presented as separate articles, they are indeed tightly linked. Thus, the hypothesis of **Paper II** is built on the results of **Paper I,** and **Paper III** is highly connected with both **Papers I and II**. However, the hypothesis of **Paper I** was generated based on two previous animal studies from our group focusing on *S. epidermidis* infection in neurodevelopment[154,247]. In 2015, a research article from our group was the first to demonstrate that *S. epidermidis* bacteremia was associated with impairments in neonatal brain development. However, the findings in that article derived from intravenous injection of the bacteria at PND1[154]. To better simulate a clinical situation, the age of the mice during bacterial injection was adapted in this thesis to study late-onset sepsis, in which *S. epidermidis* is the most common bacteremia. Therefore, *S. epidermidis* was injected at PND4, which resulted in sensitization of HI brain injury as well as alteration in the peripheral and brain inflammatory responses[247].

4.1 Sex-dependent sensitization following *S. epidermidis* infection

In 2001, Eklind et al examined the effects of bacterial endotoxin on HI brain injury, showing an increased sensitization 4 hours (h) after LPS injection in PND7 rats[67]. From 2001 onwards, several studies have focused on understanding the sensitization process in the immature brain[68,202,248-250]. Thus, numerous studies from our group and others have demonstrated that synthetic compounds that mimic aspects of $Gram^-$ and $Gram^+$ bacteria, as well as viral infection, dramatically potentiate the HI brain injury in several animal models, ranging from mice to rats and pigs at different neonatal ages. All the studies have used specific TLRs agonists to mimic bacterial or viral infections. Consistent with the previous findings, in 2020, Lai et al showed that using live *S. epidermidis* bacteria, a more severe brain injury was observed when *S. epidermidis* was injected 14h before HI compared to the saline-injected counterpart. However, as the time interval is known to play a crucial role in the HI sensitization[250], in **Paper I,** we aimed to understand the time window of vulnerability of the HI brain injury following *S. epidermidis* infection. By inducing HI either 24h or 5 days after *S. epidermidis* infection, we surprisingly found that only male mice were more vulnerable to HI 24h after infection,

suggesting that sex plays a crucial role in the increase in vulnerability. Specifically, we found increased injury in both grey and white matter overall as well as in single regions such as the cortex, hippocampus, striatum and thalamus. However, when the time interval between bacterial infection and HI was extended to 5 days, we did not observe any increase in the vulnerability[217], clearly highlighting the importance of timing in the sensitization. These results are consistent with the timing of the bacterial load in blood and spleen as previously showed by Lai et al[247], suggesting that it is particularly ongoing infections that increase the vulnerability of the immature brain.

4.2 Cytokines, chemokines and complement protein 5a (C5a) alterations in *S. epidermidis* infected mice

Sexual dimorphism has been observed in several neurodevelopmental disorders, in which males are more susceptible to develop neurological disorders such as autism[251] and ADHD[252,253]. Therefore, to reveal potential candidates that might explain the sexual dimorphism observed in the increased vulnerability to HI, in **Paper I,** we evaluated the inflammatory response by analysis of cytokines and chemokines in the blood. We did not find a dramatic difference between male and female mice. The only cytokines with differential production were IL-2, CCL5 and IL-17. IL-2 has previously been observed to be overproduced in males after bacterial infection[254] whereas, to our knowledge, there are no studies investing sexual dimorphism in the production of CCL5 or IL-17. Similarly, we did not observe sex differences in the level of CCL2 in the brain. To further explore the mechanism behind the sex-dependent sensitization, in **Paper I,** we studied complement protein 5 as an important contributor to the innate immune response. We analyzed the protein levels in the brain, blood and liver (main site of production). Interestingly, 24h after *S. epidermidis* infection (time of the HI sensitization), we found a reduced amount of C5a protein in the blood of infected male mice compared to male controls. In contrast, increased levels of plasma C5a were seen in both male and female infected mice 5 days after infection (when the HI sensitization does not occur). C5a level differences were observed only in blood but not in the brain or liver. To have a full picture of the C5a response, we also analyzed the mRNA levels of C5a as well as its receptors (C5aR1 and C5aR2). No significant differences were found in the mRNA levels, except for C5aR2 which was the only receptor upregulated in female mice in the brain 24h after infection. Thus, in **Paper I**, we argued for the importance of C5a deficiency in male mice in response to *S. epidermidis* infection, not excluding the possibility that in females, a similar significant reduction might be reached at another time point. Preterm infants have lower levels of complement proteins than term babies[97], which are further reduced following *S. epidermidis* infection, perhaps due to bacterial production of extracellular proteases[255,256]. These effects might

contribute to the underlying events that lead to sex-dependent sensitization. Therefore, the biphasic response of C5a in plasma may lead to a difference in downstream pathways, which might help to explain the mechanisms behind the sensitization.

4.3 *S. epidermidis* induces neuroinflammation in the immature hippocampus

Paper I suggested a strong involvement of neuroinflammation in HI sensitization. Thus, we decided to move forward, studying microglia activation following infection. In **Paper II**, we extensively analyzed microglia cells both at morphological and transcriptional levels. As microglia manifest phenotypical and molecular heterogeneity across different regions of the brain[257], we decided to focus on the hippocampus. Similarly, in **Paper III**, employing global proteomic analysis, we characterized the proteomic profile of the immature hippocampus following infection. Therefore, the aim of **Paper II and III** was to understand the molecular mechanisms underlying the increased sensitization to HI brain injury. Activation of the immune system in the immature brain might have a particular impact on neurodevelopment with serious long-lasting consequences for neurological and mental health[258]. Neuroinflammation is well recognized to be a critical contributor to perinatal brain injury[135]. Early inflammation has been linked to several neurodevelopmental disorders such as autism, cognitive impairment, cerebral palsy, epilepsy, and schizophrenia[259]. Neurodevelopmental disorders are usually characterized by aberrant and delayed development of the brain, with deleterious consequences on language, cognition and motor behavior[260]. In **Paper II**, we found enhanced neuroinflammatory responses in the immature hippocampus. As the main cell player of the brain immune response is microglia, we discovered that *S. epidermidis* has a strong impact on this immune cell. In **Paper III**, we discovered a prominent role for astrocytes and the production of *Lcn2* in the response to *S. epidermidis* infection. Astrocytes can contribute to neuroinflammation by e.g. production of pro-inflammatory cytokines and chemokines[174]. However, astrocytes perform numerous tasks, involving support for white matter development, synaptic plasticity and regulation of BBB and cerebrospinal fluid (CBF)[174]. Thus, our findings in **Paper III**, suggest that *S. epidermidis* infection has broader effects on the developing brain than just neuroinflammation.

4.4 Morphological microglia alteration following *S. epidermidis*

The first step of **Paper II** was the morphological characterization of hippocampal microglial cells. Microglia morphology provides a suitable understanding of its dynamic states. Thus, in resting conditions, microglia

display a highly branched and ramified morphology whereas upon activation they assume an amoeboid shape together with retraction of microglia branches[136]. In **Paper II**, we injected mice at PND4 with *S. epidermidis* or saline and collected the brain 24h later (a time point where we had observed HI brain sensitization in Paper I). We found that *S. epidermidis*, without crossing the BBB[154], altered microglia morphology. Specifically, we found that *S. epidermidis* infection induced an increase of the cell soma size together with retraction of its branches, being reduced in both number and length. This latter analysis further confirmed the activation of microglia cells 24h after *S. epidermidis* infection. We did not find a conclusive significant sex-based difference between male and female groups, suggesting that *S. epidermidis* activates microglia irrespective of sex.

4.5 Microglia transcriptome profile following *S. epidermidis* infection

As microglia alteration is often an underlying event of numerous neurodevelopmental disorders, leading to serious and detrimental consequences for the developing brain[160], identification of the potential mechanism of activation might lead to a better understanding of neurological conditions and/or therapeutic strategies. Therefore, in **Paper II**, we sought to characterize the molecular profile of hippocampal microglia. To do so, we isolated microglia cells using MACS technology and subjected the cells to RNA-seq. The large amount of data derived from omics technology might represent a challenge. Therefore, to overcome the possible limitations that each bioinformatics analysis bears, we combined different approaches to analyze the RNA-seq data.

The first approach was the classical DE analysis in which we identified a distinct transcriptome profile of microglia following *S. epidermidis* infection. The distinct profile between *S. epidermidis* and saline mice became evident when visualizing the data through a PCA plot as well as through a hierarchical clustering of samples. The DEGs were then used for enrichment analysis, which revealed alterations in multiple pathways involved in the immune responses as well as alterations in developmental processes. Interestingly, alteration of complement signalling was identified. The upregulation of the complement signalling pathway might be restricted to only hippocampal microglia cells and thereby not clearly identified in **Paper I** when analyzing the overall brain. Further, no sex-linked difference was identified by the DEGs analysis.

To complement the results of the DEGs, we used another approach (WGCNA) that relies on the construction of gene networks based on their correlation. Using WGCNA, we identified four modules (i.e. clusters of genes highly

correlated) that were highly correlated with the infection. Enrichment analysis of the modules revealed alterations in several crucial biological processes involved in microglia activation, leukocyte and lymphocyte immunity, axon guidance and cell migration, proteasome, NOD-like receptor signalling as well as neurological disease-related pathways. Similar to the DEGs and the morphological analysis, no sex-linked difference was observed. Although there is a certain amount of literature that suggests sex differences in microglial activation, in our study, sex-dependent alterations were not identified, suggesting that *S. epidermidis* infection alters microglia morphology and transcriptomic program in a non-sex-dependent manner. To my knowledge, most of the studies in the literature showed sex differences in microglia activation in adult mice but not in neonates[261-265], which may explain the differences from our study.

4.6 Identification of microglia inflammasome alteration following *S. epidermidis* infection

In **Paper II**, we identified and validated the involvement of NOD-receptor signalling pathways in the pathogenesis of *S. epidermidis* infection. Although other pathways were also found to be affected, which will require future investigations, in **Paper II**, we focused on the NOD-like receptor signalling pathway, since it has been implicated in neonatal infection[156]. It was intriguing to notice that genes that were positively correlated with infection in the WGCNA were also upregulated compared to saline-injected animals. Similarly, the negatively correlated genes corresponded to the downregulated genes. NOD receptors exert crucial functions in recognizing PAMPs[77]. Activation of the NOD- signalling pathway results in activation of the caspase-1 inflammasome and production of interleukin 1β (IL-1β) and IL-18. Implications of NOD-receptor pathways have been reported in several neurological conditions, such as Alzheimer´s disease, Parkinson's disease, stroke and encephalopatitis[82]. In **Paper II**, most of the genes involved in the NOD-signalling were upregulated. To further confirm the involvement of NOD-mediated mechanisms, we measured caspase-1 protein, which is the downstream protein that reflects inflammasome activation. The identification of inflammasome activation following *S. epidermidis* infection might open new possibilities for potential treatment. Thus, blocking the inflammasome has been shown to be beneficial in HI brain injury[201,266,267]. Numerous clinical trials are now trying to understand the critical role of the inflammasome in several disorders such as Human Immunodeficiency Virus (NCT03191175), Retinal Detachment (NCT03332758), Hepatitis C (NCT04244383), Cardiovascular Disease (NCT02122575), Sepsis (NCT04635878), COVID-19 (NCT04385017) and Sepsis-Induced Acute Respiratory Distress Syndrome (NCT04870125). As sepsis can lead to multiorgan dysfunction[73], an ongoing

clinical trial is trying to understand the pathophysiology of sepsis by analyzing the importance of the activation of the NLRP3 inflammasome, correlating the finding with mortality of septic patients (NCT04635878). Interestingly, NLRP3 inflammasome activation has been proposed to be a suitable biomarker to predict the outcome of COVID-19 patients (NCT04385017)[268]. The variety of diseases in which the inflammasome is involved clearly poses this mechanism in a significant clinical perspective, which requires further investigation, especially in babies experiencing sepsis. However, to my knowledge, no ongoing clinical trials are investigating the effects of the inflammasome in infected preterm infants.

4.7 Proteomic analysis identified astrocyte activation in the hippocampus of *S. epidermidis* infected mice

Similar to the microglia findings in **Paper II**, in **Paper III** we found a distinct proteome profile of the immature hippocampus after infection. Analyzing the DEPs, we found alterations of pathways involved in multiple categories, such as Cholesterol biosynthetic processes and regulation of biological processes. However, the results became especially interesting when we complemented the enrichment analysis with IPA, finding deeper insights into how *S. epidermidis* influences the immature hippocampus. Of note, alteration of cholesterol biosynthesis is known to have detrimental effects on the brain. The concentration of brain cholesterol in newborn mice is ~3.5 mg/g[269], triplicating its amount three weeks later[269], reflecting the postnatal myelin formation. Hence, cholesterol, being an integral component of myelin, exerts a pivotal role in brain development[270]. Alteration in the cholesterol pathway might explain the increased white matter loss observed in **Paper I**. An important result in **Paper III** is the identification of LCN2 as an important regulator of the protein network. Liver[271,272] and neutrophils[273] have been shown to be the main sources of production in the periphery whereas, in the brain, reactive astrocytes are mainly responsible for its production. The proteomic results strongly suggest an involvement of astrocytes in the production of *Lcn2*, enhancing neuroinflammation and leading us to evaluate astrocyte reactivity. The upregulation of GFAP together with an increased cell soma reflects activation of astrocytes[274]. Thus, in **Paper III**, we found an increase in astrocyte soma volume in both the molecular layer of dentate gyrus as well as CA1 striatum radiatum hippocampal subregions. To better understand the production of LCN2 by reactive astrocytes, we used $GFAP^{-/-}vim^{-/-}$ mice. As these mice lack the intermediate filaments GFAP and vimentin, astrocyte reactivity is impaired. We found that preventing astrocyte reactivity also prevented the local production of *lcn2* mRNA in the hippocampus, but not the whole-brain protein levels, suggesting that LCN2 levels in the brain might be compensated for by production in the periphery. LCN2 produced in the

periphery (e.g. in the liver or neutrophils) could potentially reach the brain through the increased BBB permeability identified in **Paper II** (discussed below).

Of note, there is an interplay between microglia and astrocytes, which is crucial to influence neuronal degeneration or regeneration. [275]. Thus, activation of microglia through the production of IL-1 promotes astrocytes' activation whereas activated astrocytes might also promote or inhibit microglial activation[276,277].

4.8 Immune cell infiltration into the hippocampus following *S. epidermidis*

In **Paper II**, the microglia transcriptome analysis suggested that microglia in response to *S. epidermidis* infection change the metabolic processes which might be linked with increased communication with peripheral immune cells. Metabolic alterations are often observed in neurological conditions associated with inflammation[278]. Microglia require high energy for supporting their activation in response to several insults[279-281], which might have implications on neurological conditions[282]. It has been suggested that infiltrating leukocytes might influence microglia metabolism and vice versa[278]. In **Paper II**, to validate this process, we used LysM-eGFP k.i mice. Insertion of eGFP into the lysM locus results in labeling peripheral myeloid cells but not microglia[184]. Similar to the other experiments in this thesis, PND4 LysM-eGFP k.i mice were injected with either *S. epidermidis* or saline and immune cell infiltration was evaluated 24h after injection. In **Paper II**, we used CD31 staining to outline the vessels in combination with staining for eGFP positive cells and we could thereby distinguish peripheral immune cell localization inside or outside vessels. In support of the transcriptomic data, we found a dramatic increase of peripheral immune cells in the hippocampus parenchyma, suggesting infiltration of peripheral cells into the brain tissue and a possible exchange of communication between leukocytes and microglia. Similar events are often found in other neurological diseases such as cerebral ischemia, stroke and infection[283,284]. Consistent with results in **Paper I**, we showed that *S. epidermidis*-infected mice exhibit increased levels of CCL2 in the brain. CCL2 administration has been shown to induce leukocyte recruitment[285]. In chronic experimental autoimmune encephalomyelitis, CCL2 has been found to play a key role in the recruitment of immune cells together with the activation of glial cells[286]. Similarly, chronic cerebral LCN2 exposure has been shown to reduce hippocampal neurons, enhance microglia activation as well as increase immune cell infiltration[287]. Thus, strategies to prevent immune cells infiltration have been shown to be beneficial in neonatal HI[288,289]. Therefore, the identification of immune cell infiltration into the hippocampus of *S.*

epidermidis-infected mice and the mechanisms behind might be crucial in understanding/preventing brain injury following infection.

4.9 Blood-brain barrier disruption following *S. epidermidis*

An event that often precedes leukocyte infiltration is BBB alteration[283]. Based on its dynamic structure, the BBB may vary in response to multiple factors and regulation of its integrity is indispensable for brain homeostasis[178]. Dysfunction of BBB integrity has serious consequences in several diseases, such as multiple sclerosis, epilepsy, stroke, Alzheimer's disease, seizure, autism, and psychomotor retardation syndromes[290]. BBB breakdown can alter the neural environment by allowing an increased flow of molecules passing from the blood into the brain[290]. In most neurological conditions, inflammation plays a critical role in disease development and progression. Similarly, inflammation has been implicated in the disruption of BBB integrity, which might be an underlying event of neurological conditions[291,292]. In experimental models, transient BBB breakdown was observed in several animal models such as neonatal HI[293], germinal matrix haemorrhage (GMH)[294]and infection[291]. In **Paper II**, to capture a full picture of the alterations induced by *S. epidermidis* in the immature hippocampus, we investigated BBB permeability alterations following *S. epidermidis* infection. We found a dramatic increase in the BBB permeability in the hippocampus of infected mice compared to saline animals, suggesting that BBB alteration might be a crucial event that precedes leukocyte infiltration into the hippocampus. Interestingly, disruption of BBB in neonatal rats following LPS injection was further associated with white matter development and injury[291]. Similarly, *S. epidermidis* has also been implicated in white and grey matter injury[154]. Furthermore, increased levels of CCL2, observed in **Paper I**, might contribute to BBB disruption following *S. epidermidis* infection In a mouse model of acute intracerebral hemorrhage, blockage of CCL2 resulted in neuroprotection of BBB integrity[295]. Another neuroprotective outcome might be reached by the inhibition of Caspase-1. Hence, blocking Caspase-1 results in restoring BBB insults as well as leukocytes adhesion and transmigration[296]. LCN2 has also been associated with white matter injury and BBB disruption and its inhibition provided neuroprotection[297]. Hence, BBB disruption together with leukocyte infiltration in the immature hippocampus might be driven by the microglia inflammasome and/or astrocyte reactivity. To my knowledge, the only studies that explored BBB permeability following *S. epidermidis* were performed by Lai et al 2020[247] and Brunce et al 2019[192]. The latter study demonstrated that in a piglet model of *S. epidermidis* infection, the CSF–serum ratio was increased 24h after *S. epidermidis* injection together with an increase in CSF leukocyte counts[192]. These observations are consistent with our findings at a similar time point. Similarly, Lai et al 2020 showed alteration of BBB 14h after *S. epidermidis*[196],

suggesting that BBB disruption might occur earlier than we have so far investigated.

4.10 *S. epidermidis* induces vascular changes in the hippocampus

Several cell types contribute to the formation of BBB. Among these cells, endothelial cells, pericytes as well as astrocyte end-feet are the main units of the vasculature forming the BBB[179]. It is recognized that endothelial cells have have an active role in fighting infection[298]. By expressing PRRs, endothelial cells can recognize bacteria or microbial products[298,299]. Stimulation of endothelial cells with TLR2 agonist results in an increased expression of cytokines and chemokines, increased permeability as well enhanced neutrophil adhesion[300]. Analysis of endothelial cells in post-mortem brains of infants who developed subacute sclerosing panencephalitis (a rare chronic, progressive brain inflammation disease) suggests that endothelial cells might play a pivotal role in the entry of measles virus into the brain[301]. Similarly, endothelial cells could potentially provide an entry of molecules that stimulate the activation of astrocytes and microglia following *S. epidermidis* infection. In **Paper III**, we found an alteration in hippocampal vessels following infection. These alterations included both increased diameter as well as increased vessel length. We also found increased vessel coverage of astrocyte end-feet by measuring AQP4 positive capillaries, possibly to compensate for the increased vessel length. It is known that during sepsis, regulation of the vessel diameter occurs because of the infection. However, consequences of these changes on the brain are not well understood. Mechanical dilation of vessels might be sensed by astrocyte endfeet. Many factors control cerebral blood flow such as arterial blood pressure, blood arterial CO_2, arterial O_2, and brain activity. Intriguingly, increased CO_2 indicates an increased influx of Ca^{2+} in astrocytes, determining activation[302]. On the other hand, increased astrocyte $[Ca^{2+}]_i$ influx has been shown to directly alter the diameter of cerebral vessels in both juvenile and adult mice[303-305]. Thus, reactive astrocytes might influence endothelial cells. However, microglia activation might also play a prominent role in the stability of BBB. Using two-photon microscopy, Haruwaka et al, demonstrated that microglia have a dual role in BBB permeability. Specifically, the initial migration of microglia towards endothelial cells was observed to be beneficial for the BBB. However, chronic inflammation might trigger microglia to impair BBB[306]. In the same study, they also showed that microglia processes can infiltrate the basal membrane and have contact with endothelial cells[306]. Therefore, the contact between microglia and endothelial cells might imply a mutual communication that can result in a neuroinflammatory response following *S. epidermidis*.

4.11 Lipocalin 2 in preterm infants

In **Paper III**, the proteomic analysis identified LCN2 as a key protein in the hippocampus of *S. epidermidis*-infected mice. Blood levels of LCN2 correlated with astrocyte reactivity, hippocampal vascular alteration as well as blood chemokines and cytokines, suggesting that peripheral LCN2 levels reflect hippocampal alterations. Therefore, to evaluate the clinical importance of LCN2, we studied its levels in a cohort of preterm infants born <28 weeks of gestation. Interestingly, we found that C-reactive protein (CRP), a marker of ongoing infection/inflammation, positively correlated with blood levels of LCN2, 1 and 2 weeks after birth. As in *S. epidermidis*-infected mice, LCN2 positively correlated with cytokines and chemokines, we speculated that experimental data might mirror the clinical setting. Similarly, LCN2 plasma levels in preterm infants positively correlated with several cytokines/chemokines. Overlap of LCN2-correlated cytokines/chemokines between *S. epidermidis* infected mice and preterm infants with a sign of infection showed that IL-17, IL-6, CCL3 and CCL4 were common. Identifications of the early sign of infection as well as brain alterations are important to identify babies at risk. Clinical research strongly suggests that LCN2 might represent a suitable biomarker for the diagnosis of several diseases in preterm, such as necrotizing enterocoliti[307], bronchopulmonary dysplasia[308], preterm sepsis[309,310]. Further, advances in LCN2 research indicate that LCN2 is involved in several neurological conditions and therefore, identification of its levels might open new possibilities for preterm infants with a sign of infection. It has also been suggested that LCN2 knock-down might represent a promising drug target. Indeed, attenuation of LCN2 inhibited cancer cell growth[311], reduced gliosis and production of inflammatory cytokines in diabetic mice[312], reduction of brain infarct volumes, neurologic scores, BBB permeability and glial activation[313]. Although future clinical and experimental studies have to confirm our findings, based on the results of **Paper III**, we speculated that LCN2 might have deleterious consequences on preterm infants ‘ brains and its measurement might help in the identification of high-risk babies.

5 SUMMARY AND CONCLUSION

"Everything is going to be fine in the end. If it's not fine, it's not the end"
Oscar Wilde

Perinatal infections pose preterm babies at higher risk of developing neurological impairments such as memory, attention and motor impairments[50,51] as well as an increased risk of neurodevelopmental disease[51]. Historically, *S. epidermidis* has been considered a commensal microorganism, underestimating its potential detrimental effects on preterm babies[49]. Although studies have investigated the deleterious consequences of infection/inflammation on the brain development of preterm infants[314], only three studies[154,192,193] have addressed questions of whether *S. epidermidis* might represent a real risk for the developing brain. In the present thesis, we discovered an unprecedentedly important role of *S. epidermidis* in neurodevelopment. This thesis broadly contributes to the field with the following findings, summarized in Figure 9:

1. *S. epidermidis* sensitizes hypoxic-ischemic brain injury in a sex-dependent manner
2. *S. epidermidis* alters peripheral cytokines and chemokines complement protein 5a as well as brain CCL2
3. *S. epidermidis* results in activation of hippocampal microglia
4. *S. epidermidis* alters signaling pathways in activated microglia that have analogies with neurological diseases as well as *S.aureus* infection
5. *S. epidermidis* is linked to inflammasome activation in activated microglia
6. *S. epidermidis* disrupts BBB permeability
7. *S. epidermidis* induces peripheral immune cells infiltration to the brain
8. *S. epidermidis* alters the hippocampal proteomic profile
9. *S. epidermidis* induces astrocyte reactivity
10. *S. epidermidis* induces vascular changes in the immature hippocampus
11. Proteomic analysis of the hippocampus of infected mice identified lipocalin 2 as one main regulator of *S. epidermidis* inflammatory response
12. Preventing astrocyte reactivity reduced the local production of lipocalin2 in hippocampus but not its overall protein level in the brain

13. Lipocalin 2 in blood reflects babies who experience sepsis/inflammation
14. Lipocalin 2 levels might represent a suitable marker for babies who have a higher risk of developing brain damages

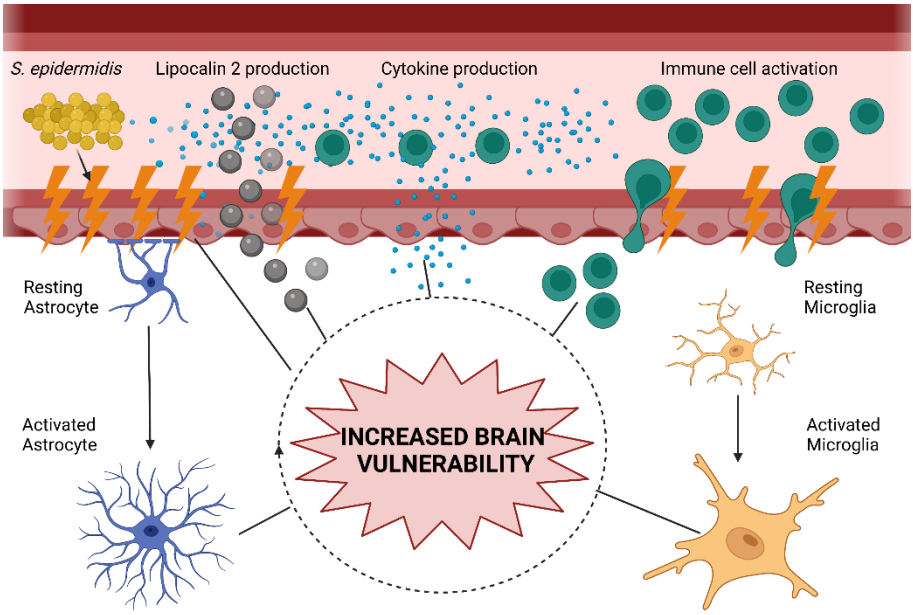

Figure 9. Summary of the findings presented in this thesis

In all the papers, we showed different aspects of the pathogenesis associated with *S. epidermidis* infection. Starting from the sensitizing effects, we demonstrated that *S. epidermidis* induces a robust neuroinflammatory response in the immature hippocampus, increasing the risk of brain damage. We strived to translate the experimental findings to preterm infants, by analyzing lipocalin 2 as a possible plasma marker of neuroinflammation. Overall, the findings in this thesis shed new light on how *S. epidermidis* affects the immature brain, representing a suitable platform for the development of novel strategies to treat/prevent potential subsequent brain injury in babies who experience *S. epidermidis* infection.

6 FUTURE PERSPECTIVES

Knowledge breeds doubts, not certainty, and the more we know the more uncertain we become. — A. J. P. Taylor

The results of this thesis clearly highlight a previously unrecognized and important contribution of *S. epidermidis* in triggering neuroinflammation in the developing brain. The findings of this thesis together with its clinical significance require further investigation to reveal new unrecognized aspects of *S. epidermidis* infection. Numerous questions remain unanswered. Some of them are listed below:

When does S. epidermidis sensitize the brain to HI injury?

In **Paper I**, we showed that *S. epidermidis*, 24h after infection, sensitizes the HI brain in a sex-dependent manner. This together with the study performed by Lai et al[247] clearly indicate that the *S. epidermidis*-induced windows of vulnerability to the HI brain injury in mice range between 14h and 24h, but not longer than 5 days when the sensitization does not occur. However, we still do not know the exact timing of the sensitization. Therefore, future studies could investigate the time window and associated mechanisms. This becomes relevant for the development of potential therapies as well as the time of administration of interventions. Similarly, as the results of **Paper II and III** point out neuroinflammation as an important contributor to the *S. epidermidis*-induced brain injury, future studies should identify the timing of the inflammation and the window of BBB disruption. Similarly, infiltration of leukocytes was observed 24h after infection. All of the experiments in **Paper II and III** were performed at 24h following *S. epidermidis*/saline injection; we did not evaluate other time points. Thus, further understanding of the timing of the events might reveal a new window of intervention for the developing brain.

In **Paper II and III** we showed that *S. epidermidis* induced robust activation of microglia and astrocytes.

What does activate the microglia and astrocytes?

From an immunological point of view, PAMPs or DAMPs are recognized by PRRs in innate immune cells, which results in their activation and thereby inflammation[69]. However, *S. epidermidis* does not cross the BBB[154], hence, other molecule(s) or cells have to be involved in the activation of microglia and astrocytes. Although we do not exclude the possibility that part of the

dying bacteria crosses the BBB, one possible candidate might be LCN2, identified in **Paper III**. In vitro, exposure of microglia and astrocytes to LCN2 resulted in glial activation[315]. However, due to the complexity of the organism, translation from *in vitro* to *in vivo* might be challenging. LCN2 can be produced by the liver, neutrophils and astrocytes and by blocking its main source of production in the brain (astrocytes), we did not show a reduction of the total protein levels, perhaps due to the disruption of BBB permeability, which allowed peripheral LCN2 to traverse across the BBB into the brain as a compensatory mechanism. Therefore, we speculate that LCN2 can trigger neuroinflammation and thereby sensitize the brain to HI. However, proving that LCN2 triggers neuroinflammation *in vivo* might represent a challenge for future projects. Although LCN2 deficiency has ameliorated neurological outcomes in neurological disorders[316], its deficiency aggravated the outcome of peripheral infection as well as higher mortality[317], perhaps due to the reduced bacteriostatic activity. Thus, other strategies have to be employed. In **Paper II**, we also found that *S. epidermidis* infection resulted in infiltration of peripheral immune cells, which might contribute to the neuroinflammation and their contribution to the activation of microglia and astrocytes still tremains unknown.

Which are the long-term consequences of S. epidermidis infection in preterms?

It is becoming clear that infection in the perinatal period has detrimental effects on brain development. Both epidemiological and experimental data pinpoint infection as a risk factor for neurodevelopmental diseases[51,55]. Recently, we showed that a single LPS injection in both male and female offspring displayed increased repetitive behavior together with a dramatic activation of microglia, suggesting that perinatal infection might be linked with the development of autistic-like behaviors[318]. Similarly, perinatal injection of *E. coli* results in behavioral abnormalities as well as white matter alteration[55]. Other studies have also found that prenatal and postnatal exposure to infection increased the risk of schizophrenia and autism in both human and animal models[319-321]. Omics analysis in both **Paper II and III** revealed potential disrupted mechanisms related to neurological disease following *S. epidermidis* infection. Many pathways were linked to neurological impairments and diseases such as Alzheimer's disease, cognitive impairments, encephalopathy, Parkinson's disease and Huntington's disease. Overall, the data in this thesis suggest that *S. epidermidis* might cause long-term abnormalities. Thus, an important extension of this thesis will be to study the long-term effects of neonatal *S. epidermidis* infection.

Is the overall brain equally affected by S. epidermidis?

Except for **Paper I**, in **Papers II** and **III** we revealed hippocampal-specific alterations. The brain displays heterogeneity in health and diseases. In schizophrenia, for example, different regions might be differentially affected based on individual variablity[322]. CNS diseases might occur in a spatial regional pattern, affecting preferentially specific brain regions[257] and therefore microglia might exhibit different phenotypes and functions in other brain regions[257]. Microglia showed different phenotypes in the different CNS compartments during aging[323] and to avoid any possible regional variations, we specifically focused on the hippocampus. Therefore, future studies should elucidate whether similar alterations can be found in other brain regions.

Is lipocalin 2 a suitable marker to detect infants who experience S. epidermidis infection?

In **Paper III**, we identified LCN2 as one of the key regulatory proteins in the hippocampus. Human and experimental studies have largely found involvement of LCN2 in neurological conditions such as ischemia/reperfusion-induced injury[313], intracerebral haemorrhage[324], traumatic brain injury[325], spinal cord injury[326] and vascular dementia[327]. We found that blood levels of LCN2 positively correlated with different blood cytokines as well as astrocyte volume in mice. Similarly, in the preterm infant cohort, blood LCN2 correlated with CRP, IL-6 and chemokines. The similar pattern of inflammatory mediators in mouse offspring with *S. epidermidis* infection and human preterm infants with increased CRP suggests a potential translation of our findings. However, our findings require further clinical investigations to confirm these observations. Thus, understanding the function and dysfunction of LCN2 in preterm infants might contribute to identifying babies with increased risk of brain injury following infection.

In conclusion, future studies should provide a more comprehensive picture of how and when *S. epidermidis* influences brain development, considering its potential important clinical translation.

ACKNOWLEDGEMENT

"La parola è una cosa profonda, in cui per l'uomo d'intelletto son nascoste inesauribili ricchezze" Gabriele d´Annunzio

When I started writing my thesis, I did not expect that the most difficult part would be writing the acknowledgment. Here it comes to the point where I´m summarizing 4 years of my PhD life in a few words (and perhaps I´m not too good at summarizing feelings). Now, in the end, I´m more and more realizing how lucky and grateful I am to have met amazing people throughout my PhD journey. If I think back to when I started, I realize how much I grew and everyone I met has contributed to my development.

Starting from the lab, I´m extremely glad to be part of the PROMISE group and each of you has contributed to this doctoral thesis. I really would like to express my gratitude to my supervisor **Carina Mallard** for allowing me to start this journey in her group. I appreciated your passion for science together with your commitment to teaching me how to develop and structure new ideas, considering all the possible problems that may arise in doing research. Thanks to you, I had the possibility to learn and develop all the knowledge I bear right now. Despite your busy schedule, you always found time to support me at any stage of my PhD. I really think it´s not easy to find a supervisor with your management skills and I´ve been learning so much from your way of working, that I embrace a part of them. I also want to thank all my co-supervisors **Maryam Ardalan, Joakim Ek and Xiaoyang Wang**. **Maryam**, it has been so fun working with you in the lab that I cannot imagine a PhD without you on my side. I really believe one day, you will be an amazing Professor. "Teamwork", that´s the sentence I heard the most from you and it was the best way to achieve goals. **Joakim**, sharing the office with you in the first 3 years has been super nice. I enjoyed every time we started talking about projects and getting lost in our ideas. Those talks inspire me so much. **Xiaoyang,** thank you very much for contributing to the success of this thesis and for the exciting discussion. It has been a pleasure to have your support throughout my PhD. I also want to thank **Henrik Hagberg** for the excellent discussion during the lab meeting. I really admire your work and I´m really glad to be part of your group.

I want to thank all the collaborators that contribute to the success of the projects presented in the thesis: **Anders Nilsson, Hanna Danielsson, Milos Pekny, Marcela Pekna, Karin Sävman, Ann Hellström, Kajsa Groustra, Emma Negoycheva**.

During my PhD, I got so much help from each of the members (present and past) that I don´t even know how to thank all of you. I really got great support from **Tetyana Chumak**, with which I spent an amazing time. Thanks to you now, I have now a fantastic thesis cover. The weekends in EBM without you would have been super boring and without your help, my thesis would have been super small. **Eridan**, we had so many nice moments in the lab and outside that I don´t even know where to start, but I think it´s enough to just come to your office and see all the dinosaurs you keep. It reminds me of all the fun times we had. Thank you **Anna-Lena Leverin** and **Pernilla Svedin** for teaching me most of the techniques in the lab and for helping in my projects; **Veronika Golubinskaya**, for the really motivating speech and nice words, **Syam Nair** and **Gisela Nilsson** for the nice discussions, **Halfdan** for all the bioinformatics tips, **Marziyeh Jabbari** for the stylish suggestions of the images, **Jacqueline Lai**, for teaching me how to grow bacteria and doing science, **Natalia Klintland**, for sharing the office, **Valentina Bucher,** for the nice talks (good luck with your PhD, you will have a nice time in the department).

Sharing is caring! I´ve been sharing so much with my two PhD friends **Aura Zelco** and **Axel Andersson** that summarizing in a few words would be limiting. Anyway, you have always been there in listening to me, making me laugh and for the MANY after work (starting from one beer and ending up forgetting what we are doing). We spent so much time together that we ended up being a lab family. Without you, my PhD would have been extra hard and I´m so grateful to have shared this experience with you.

Many thanks to all the beautiful people at the department of Physiology for creating such a great environment and cheering me up anytime I needed it, especially to **Alina Khramova** (my sunshine), for sharing the joy and frustration that sometimes can come from the PhD, **Roberto Boi**, for being there when I was bored, happy, sad, having a coffee with you was motivating me to do better, **Renee Poelman**, **Iris Stoltenborg and Maria Hammarlund**, for being such good friends, **Charlène Perian**, **Ferran Font Gironès**, **Júlia Teixidor Deulofeu**, **Lovisa Bergwall**, **Emilie Lassén**, **Marcus Ulleryd**, **Filip Mjörnstedt**, **Maria Johansson**, **Kerstin Ebefors, Bagmi Pattanaik, Alva Johansson, Jovana Zlatković Ana Domi, Saliha Musovic, Mirko Minniti, Fiona Peris, Marie Le May, Valentina Licheri, Nacho Rodriguez, Ida Carlsson, Asia Zajdel.** Good luck with everything =)

PhD life can be sometimes tough without the right people and I have been so lucky to meet so many wonderful people outside the lab (and most likely I would forget to thank some of them here). I think most of the strength and energies come from my free time and being surrounded by those friends

contributed to make me happy. Thanks especially to **Luca Cancian**, like a brother, for being my closest support and for the great friendship.

I would like to spend some words for each of my friends but if I do I know that this part would have been super long so I´m just limiting in say a big thank you to make me feel at home, **Mirko**, **Irene**, **Elsa**, **Marco**, **Liza**, **Luca M**, **Dijana**, **Marta**, **Foivos**, **Emil**, **George**, **Özgur**, **Lefteris**, **Pier, Melania.** An important person that I want to thank is **Milanka Bojchevska** (Akira-son), I´m so grateful to have you in my life.

I also want to thank my "WeLoveGBG" group (**Lydia, Tugce, Eugeni, Carlo, Marco, Katia, Martin, Katerina**) and my amazing "JSPS" friends (**Steff, Anna, Laura, Manuel**) Arigatōgozaimashita ありがとうございました

At the end of my PhD journey, I really want to thank **Franco Gravina**, the person that really believed in me, helping to move to Sweden and start this adventure. Life sometimes can be unfair but it goes on and part of my success goes to you, wherever you are! I wish you were here to celebrate this achievement with me. I´m so grateful to have had a person like you in my life

Nonostante gli anni in Svezia abbia incontrato persone fantastiche non posso non ringraziare gli amici di sempre e piú cresco e piu mi accorgo di quanto sia fortunato ad avervi nella mia vita. **Andrea Giugno, Lorena Piluso e Francesco Giuffrida** siete i migliori. Non posso poi non rigraziare il mio gruppo di amici che mi mi sono stati vicini dall´inizio della mia esperienza universitaria (**Noemi, Alessio, Denise, Valeria, Giovanni, Peppe, Maria, Daniela, Marianna, Flavia, Gloria**) e il gruppo "MIRS" (**Paola, Rachele, Sofia, Cristina, Salvo, Dario, Vincenzo**)

Quando decisi di traferirmi in Svezia non avrei mai immaginato che la mia famiglia mi sarebbe mancata cosi tanto. I primi tempi non sono stati facili ma grazie al vostro supporto sono qui a festeggiare il mio dottorato. Tutto quello che ho raggiunto lo devo a voi **Giusy** e **Filippo**. Non posso mai dimenticare tutti i sacrifici che avete fatto per farmi diventare la persona che sono ora, siete fantastici. Grazie **Papá** per incoraggiarmi ogni volta che ne ho bisogno. Non saprei come affrontare un esame senza te che mi incoraggi con i tuoi "Spacca tutto". Non saprei nemmeno come alzarmi la mattina senza te, **Mamma,** che mi dai il sorriso per affrontare la giornata. Sei la mia carica giornaliera. E poi ci viete voi, **Adriana** e **Anita**. La vita mi ha regalato tutto ció che potevo desiderare in due sorelle come voi. "Sciatu ri lu me cori, l´amuri miu si tu"

Thank You Grazie mille Tack så mycket

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