UiT The Arctic University of Norway

Faculty of Health Sciences

# Treatment and prevention of neonatal sepsis

Studies on inflammation model, the immunogenicity of Group B streptococcus (GBS) vaccines and natural immunity of GBS

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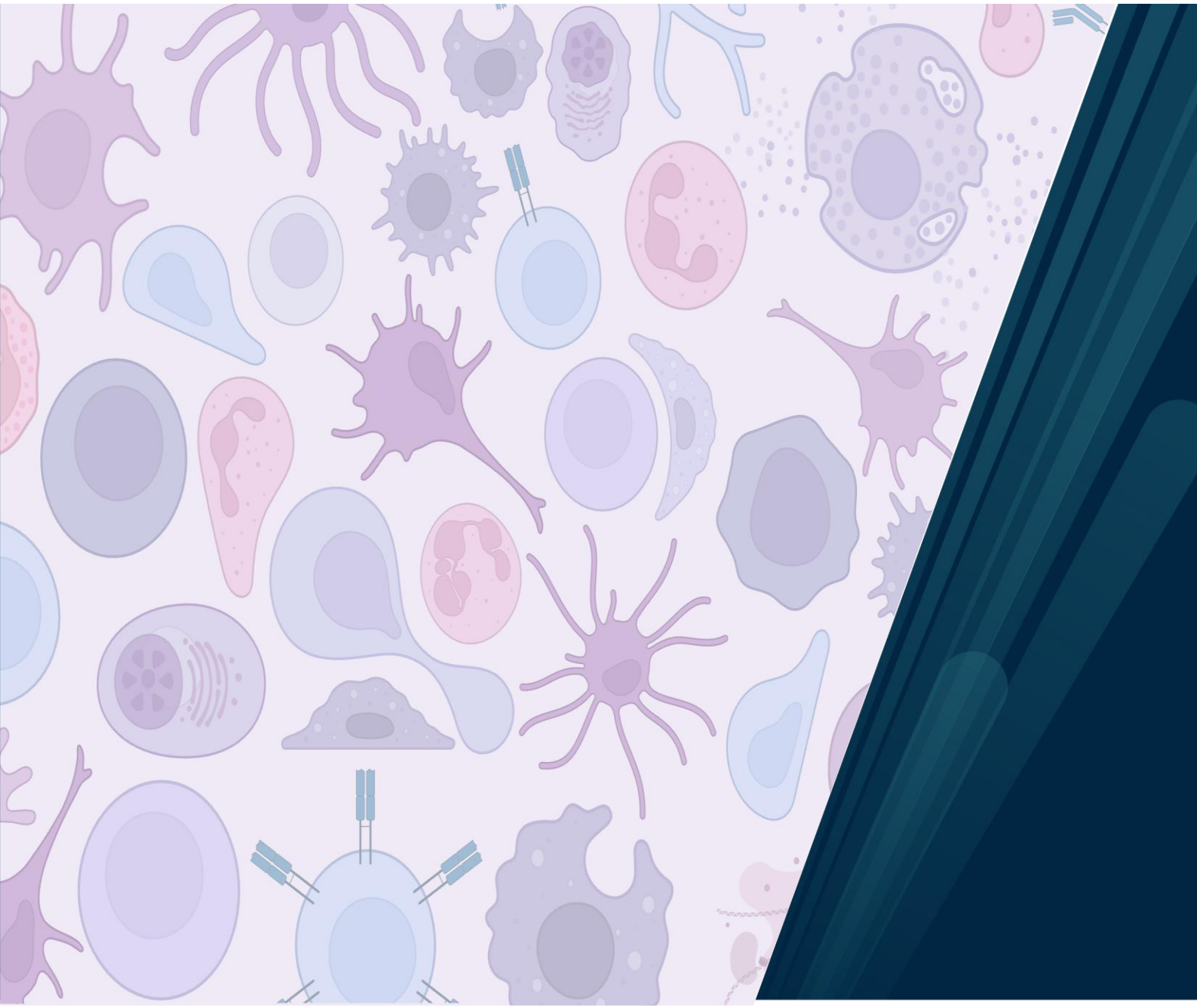

# Table of Contents

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*I dedicate this thesis to my beloved daughter, Kiara Sirianna Mwiza, whose presence on this earth lasted only one hour. Without her, this journey would have never begun.*

//Aline Uhirwa Bjerkhaug

# List of papers

**Paper I**
Bjerkhaug AU, Granslo HN, Cavanagh JP, Høiland I, Ludviksen JK, Lau C, Espevik T, Mollnes TE, Klingenberg C. Dual inhibition of complement C5 and CD14 attenuates inflammation in a cord blood model. Pediatric Research 2023; 94: 512-519.

**Paper II**
Bjerkhaug AU, Ramalingham S, Mboizi R, Le Doare K, Klingenberg C. The immunogenicity and safety of Group B Streptococcal maternal vaccines: A systematic review. Vaccine 2024; 42: 84-98.

**Paper III**
Bjerkhaug AU, Silmon de Monerri NC, Simon R, Afset JE, Cai B, Mynarek M, Anderson AS, Klingenberg C. Association between anticapsular antibodies and protection against group B streptococcus in Norwegian infants. In manuscript.

Paper not included in the PhD thesis:

Bjerkhaug AU, Granslo HN, Klingenberg C. Metabolic responses in neonatal sepsis-A systematic review of human metabolomic studies. Acta Paediatrica 2021; 110: 2316-2325.

# Abbreviations

| | |
|---|---|
| AAP | American Academy of Pediatrics (United States) |
| ACOG | American College of Obstetricians and Gynecologists |
| AE | Adverse events |
| AESIs | Adverse events of special interest |
| APC | Antigen-presenting cell |
| BW | Birth weight |
| CDC | Centers for Disease Control and Prevention |
| CI | Confidence interval |
| CoNS | Coagulase-negative Staphylococci |
| CPS | Capsular polysaccharide |
| CRP | C-reactive protein |
| EBM | Evidence-based medicine |
| ELBW | Extremely low birth weight (< 1000 g) |
| EOD | Early-onset disease |
| GA | Gestational age |
| GBS | Group B streptococci |
| HIV | Human immunodeficiency virus |
| HMOs | Human milk oligosaccharides |
| IAP | Intrapartum antibiotic prophylaxis |
| IQR | Interquartile range |
| LB | Live-born |
| LOD | Late-onset disease |
| MAC | Membrane attack complex |
| MBRN | The Medical Birth Registry of Norway |
| MedDRA | Medical Dictionary for Regulatory Activities Terminology |
| MoBa | The Norwegian Mother, Father and Child cohort study |
| MSIS | The Norwegian Surveillance System for Communicable Diseases |
| NEC | Necrotizing enterocolitis |
| NICU | Neonatal intensive care unit |
| NRL-GBS | The Norwegian reference laboratory for GBS |
| OR | Odds ratio |
| PAMPs | Pathogen-associated molecular patterns |
| PCT | Procalcitonin |
| PMA | Postmenstrual age |
| PNA | Postnatal age |

| | |
|---|---|
| PRISMA | Preferred Reporting Items for Systematic Reviews and Meta-Analysis |
| PROM | Prolonged rupture of membranes (> 18 hours) |
| PRR | Pattern-recognizing receptors |
| QoE | Quality of evidence |
| RCT | Randomized controlled trial |
| TCC | Terminal complement complex |
| TLR | Toll-like receptor |
| SAE | Serious adverse events |
| VLBW | Very low birth weight (< 1500 g) |

# Abstract in English

Worldwide, the incidence of neonatal sepsis is estimated at around 3000 cases per 100,000 live births, accompanied by a mortality rate of 17.6%. Group B streptococcus (GBS) is globally the leading cause of newborn infections. The global burden of neonatal sepsis and invasive GBS disease is skewed towards the low-income countries, but the outcome of invasive GBS disease with massive inflammation can be devastating whether born in high- or low-income countries. In this thesis I have performed three studies in relation to neonatal sepsis, in collaboration with local, national and international researchers. In **Paper I** we investigated the potential use of immune inhibitors in a neonatal inflammation blood model. We found promising results with immune-modulating treatment, reducing harmful immune components resembling the massive inflammation that can be seen in neonatal sepsis. In **Paper II** we presented insights into the immunogenicity and safety of GBS vaccines across diverse populations. Our systematic review supported the potential for a GBS vaccine to reduce the burden of GBS-related diseases, but also emphasized the need for larger studies on the effect of GBS vaccines. In **Paper III** we performed a case-control study to investigate potential protective GBS antibody levels in maternal and cord plasma from participants in The Norwegian Mother, Father and Child cohort (MoBa) study. We found that cases with invasive late-onset GBS disease had lower antibody levels, and that the placental transfer ratio also was lower in cases with GBS serotype III disease suggesting increased vulnerability when antibody levels were low. However, sample size limitations and higher antibody levels in cases than expected made interpretation of the results from this study challenging.

Overall this thesis contributes with new information on potential future adjunctive therapy for neonatal sepsis and it supports further studies on GBS vaccines to alleviate the global burden of invasive GBS disease.

# Abstract in Norwegian

Globalt er forekomsten av nyfødtsepsis estimert til rundt 3000 tilfeller per 100 000 levendefødte, med en dødelighetsrate på 17.6%. Gruppe B streptokokker (GBS) er globalt den ledende årsaken til infeksjoner hos nyfødte. Den globale byrden av neonatal sepsis og invasiv GBS-sykdom er skjevfordelt mot lavinntektsland, men følgeskadene av invasiv GBS-sykdom med massiv inflammasjon er betydelig, uavhengig om det er i høyt- eller lavinntektsland. I denne avhandlingen har jeg gjennomført tre studier relatert til nyfødtsepsis, i tett samarbeid med lokale, nasjonale og internasjonale forskere. I **Paper I** undersøkte vi bruk av immunhemmere i en inflammasjonsmodel ved hjelp av navelsnorblod. Vi fant lovende resultater når det gjelder behandling som modulerer immunforsvaret, i form av en reduksjon i skadelige immunkomponenter som man ser ved den massive inflammasjonen i nyfødtsepsis. I **Paper II** undersøkte vi systematisk immunrespons av GBS-vaksiner (immunogenisitet) i kliniske studier og sikkerhet av GBS-vaksiner på tvers av ulike populasjoner. Vår systematiske oversiktsartikkel støtter potensialet for en GBS-vaksine til å redusere byrden av GBS-relaterte sykdommer, men understreker også behovet for større studier på effekten av GBS-vaksiner. I **Paper III** gjennomførte vi en kauskontrollstudie for å undersøke beskyttende nivåer av GBS-antistoffer i blodet (plasma) fra mødre og fra navlesnoren til deltakere i Den norske mor, far og barn-undersøkelsen (MoBa). Vi fant at kasus med sen GBS-serotype III-sykdom hadde lavere nivåer av antistoffer, og at overføringsforholdet gjennom morkaken også var lavere hos kasus med sen GBS-serotype III-sykdom. Dette kan passe med økt sårbarhet for GS infeksjon når antistoffnivåene er lave. Tolkningen av resultatene fra denne studien var utfordrende, på grunn av begrensninger i prøvestørrelse og generelt høyere antistoffnivåer hos GBS kasus enn forventet. Totalt sett bidrar denne avhandlingen med ny informasjon om mulig fremtidig tilleggsbehandling for nyfødtsepsis, og den støtter videre studier av GBS-vaksiner for å redusere den globale byrden av invasiv GBS-sykdom.

# Preface

Neonatal sepsis is a medical condition that causes great concern, due to the severity of the condition, diagnostic complexity, and imperfect targeted therapy. Group B streptococci (GBS) is a significant causative pathogen in neonatal sepsis. The introduction of either a risk factor-based or GBS screening-based approach to identify “at risk infants” and administration of intrapartum antibiotic prophylaxis (IAP) has reduced the incidence of GBS early-onset disease (EOD), but it has also widely increased the use of antibiotic administration to women during delivery [1, 2]. The increased antibiotic consumption is associated with adverse effects on the gut microbiota of both the mother and the child, and may lead to an increase in antimicrobial resistance [1, 2]. In addition, there are currently no established strategies to prevent GBS late-onset disease (LOD), reduce rates of preterm deliveries, stillbirths, and maternal bacteremia [3-5].

The overall theme of this thesis is to explore potential strategies concerning the treatment and the prevention of early-onset neonatal sepsis, with a particular focus on prevention of GBS-EOD and GBS-LOD. Paper I focus on targeting the inflammatory response in neonatal sepsis caused by *E. coli* or GBS in an *ex vivo* whole blood laboratory model. In Paper II, we systematically evaluated the immunogenicity and adverse events among participant in GBS vaccine trials. In Paper III, we explored the natural immunity against different GBS strains in a Norwegian Cohort of children with invasive GBS disease versus controls. In the following introduction, I will present the hurdles in diagnosing and treating neonatal sepsis and underscore the need to pursue novel treatment options and preventative measures in the quest to reduce the mortality and morbidity associated with this condition, with a particular focus on invasive GBS disease.

# 1 Introduction

## 1.1 Neonatal Immune Defense

The immature immune system of neonates, and particularly in those born preterm, makes them vulnerable to invasive infections [6, 7]. The development of the immune system begins in early embryonic stages, yet at birth, it still shows signs of the semi-allogeneic sterile environment where it matures [8, 9]. The environment inside the womb stands in contrast to the diverse microbial environment the newborn is exposed to upon delivery [9]. This microbial colonization commences following a typical vaginal birth, originating from the maternal vaginal and gastrointestinal tracts, as well as exposure to microorganisms from the environment, skin contact and breast milk [10]. This colonization gradually evolves into a diverse and stable microbiota that largely coexists harmoniously with the host. However, certain bacteria, once they breach the neonates' protective barriers, can incite disease within the bloodstream, respiratory system, central nervous system, urinary tract, or other sterile body regions. There are also specific strains of bacteria that have been implicated in loss of feto-maternal tolerance, causing the initiation of preterm labor [11] through activation of fetal immune cells [12]. The immune system plays a pivotal role in regulating these microorganism-host interactions and sustaining a state of peaceful coexistence [13]. Neonates are equipped with passive protection against many of the microorganism they are exposed to after birth, by transfer of maternal antibodies. This protection has an estimated duration of 3 - 4 months for common infectious agents that can infect newborns [14]. However, the persistence of maternal antibodies in newborns ultimately depends on various factors, including the half-life of IgG antibodies, placental transfer ratio, IgG subclasses and vaccination status [15]. Aside from passive protection, neonates experience rapid maturation of their immune system within the first three months of life. While this protects most newborns from infections, their vulnerability to infections is significantly shaped by various genetic and environmental factors [8, 9, 14]. Preterm infants' infection susceptibility is higher due to the limited transfer of maternal antibodies, which primarily occurs in the third trimester [16]. Furthermore, they lack vernix caseosa, an antimicrobial barrier produced by fetal sebaceous glands in utero, which protects for full-term newborns in the early days of life [17].

### 1.1.1 The innate and adaptive immune system

The human immune system can be categorized into two major components: the innate and adaptive immune systems. The innate immune system acts as the initial, non-specific line of defense, providing immediate responses against a range of microbial pathogens, including viruses, bacteria, and fungi. In contrast, the adaptive immune system, while taking more time to activate, delivers highly specific and potent responses, conferring immunity against re-infection with rapid responsiveness. It is essential to recognize that these two immune components are intricately interconnected and mutually dependent for their effective responses [18, 19].

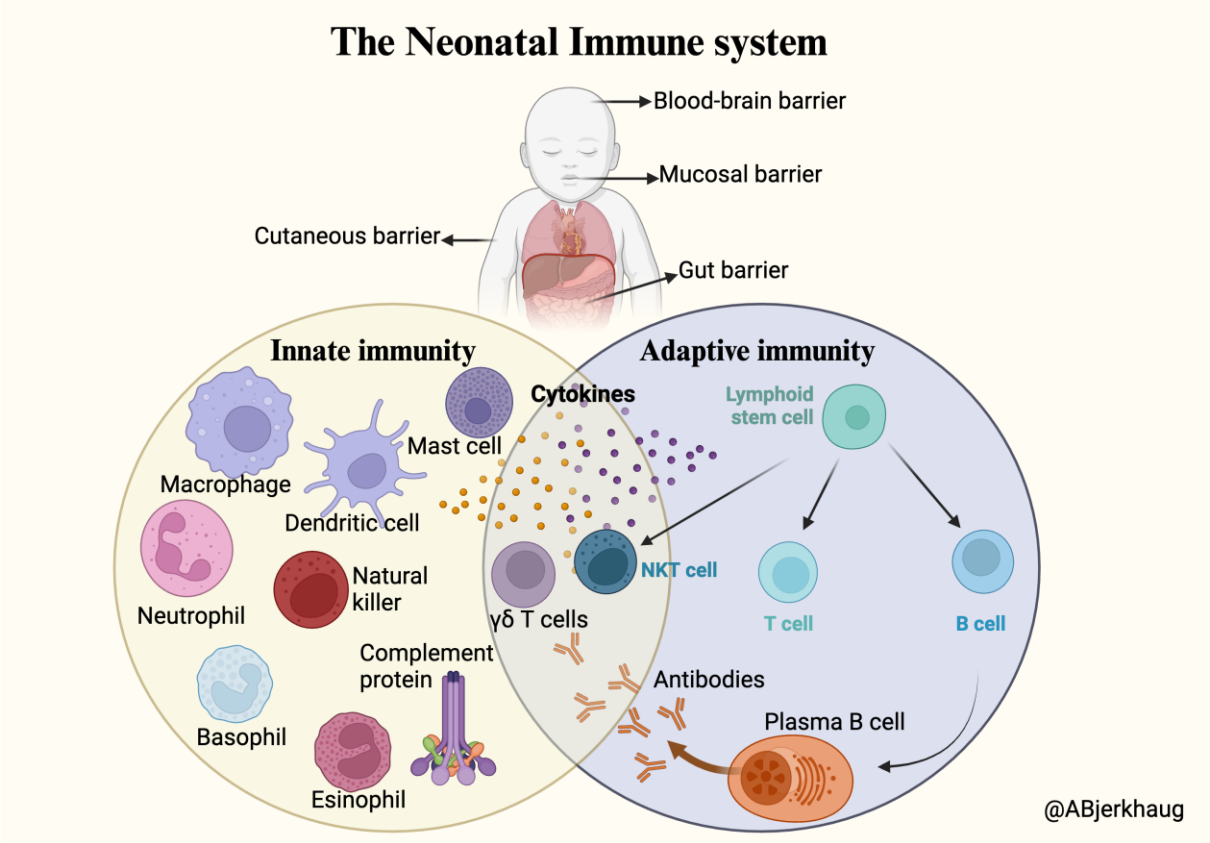


*Figure 1. Illustration of the neonatal immune system and its components. Created in BioRender.*

The innate immune system consists of i) surface barriers, primarily composed of epithelial cells that line the skin and mucosal surfaces, and ii) various cells (e.g. granulocytes, monocytes, macrophages, natural killer cells) and the complement system [18, 20], as illustrated in **Figure 1**. The epithelial cells, linked by tight junctions, and the stratum corneum layer, which is relatively thin in preterm infants, provide a protective shield against invading microbes. The epidermis contributes to immunological functions such as the recognition of microbes through pattern-recognizing receptors (PRR) and the elimination of bacteria via antimicrobial peptides (AMPs) [21]. Mucosal surfaces are also protected by epithelial cells connected with tight junctions, but have a mucus layer secreted by epithelial cells. This mucus forms a relatively impermeable gel while containing bactericidal AMPs. The granulocytes and macrophages, as phagocytes, engulf and eliminate microorganisms. Macrophages and dendritic cells, which differentiate from monocytes, serve as essential antigen-presenting cells (APCs), a crucial step in the activation of adaptive immune responses. The neonatal microbiota regulates host-microbe homeostasis and enhances resistance to sepsis. Mononuclear cells have the potential to respond robustly to commensal bacteria, producing cytokines similar to adult cells. The commensal microbiota contributes to the development of secondary lymphoid structures and helps maintain immune balance and appropriate cytokine production. Imbalances in this relationship can lead to serious long-term inflammatory and immune disorders [6, 20]. The interaction between the immune system and the microbiota plays a pivotal role in neonatal immune development [20].

The adaptive immune response is divided into two classes of lymphocytes: B cells and T cells. Plasma B cells secrete specific antibodies, immunoglobulins (Ig), which neutralize pathogens, support phagocytosis, and activate the complement system. T cells, including cytotoxic T cells (CD8+ T cells) and T helper cells (CD4+ T cells), play crucial roles. Cytotoxic T cells eliminate virus-infected and tumor cells, while T helper cells assist cytotoxic T cells, B cells, and macrophages [18, 22]. Certain B and T cells differentiate into memory cells, enabling rapid responses upon reinfection by previously encountered pathogens. Additionally, certain T cells fulfill regulatory functions (Tregs) that sustain immunological tolerance [18, 22].

PPRs, such as Toll-like receptors (TLRs), are important for both innate and adaptive immune systems to identify pathogens and differentiate them from host cells. These receptors, expressed on leukocyte membranes, particularly dendritic cells, and macrophages, recognize microbial molecules so called pathogen-associated molecular patterns (PAMPs) broadly distinct from host molecules [23]. For instance, TLR2 detects lipoteichoic acid (LTA) from Gram-positive bacteria, and TLR4 recognizes lipopolysaccharides (LPS) in the outer membranes of Gram-negative bacteria [23]. Other examples are TLR3, TLR7/8, and TLR9 that can identify double-stranded (ds) RNA, single-stranded RNA, and CpG-rich DNA, respectively, thereby aiding in the detection of viruses [24]. Upon binding to PAMPs, TLRs activate adapter proteins, ultimately regulating gene expression responsible for coordinating inflammatory responses [23, 24]. Despite infants having an equivalent number of TLRs compared to adults, they exhibit significantly different functional responses to TLR stimulation. Neonatal and cord blood mononuclear cells have reportedly reduced production of IL-1α, IL-1β, TNF (formerly known as tumor necrosis factor alpha or TNF-α), IL-18, and IL-12p70 in response to the TLR4 ligand LPS, as well as certain other TLR ligands, when compared to adult cells. However, these neonatal cells have demonstrated similar or even higher levels of IL-6 and IL-10 production in the same context [25-28]. Nonetheless, there have been varied and sometimes conflicting findings. For instance, neonatal cells have been reported to generate significantly less, comparable amounts, or even greater levels of TNF compared to adults [29-32]. Neonates also manifest reduced macrophage activation, diminished CD8+ T cells activity, and lower complement protein levels than adults [20]. These functional disparities are inversely correlated with gestational age (GA), rendering preterm infants more susceptible to infections. Furthermore, preterm infants exhibit reduced chemotaxis, impairing the recruitment of immune cells, and reduced bactericidal efficacy in neutrophil granulocytes [33]. This gap in immune profiles between preterm and term infants can persists up to three months after birth [34], indicating the importance of factors beyond maturity, like the adaptive responses to postnatal environments [35]. Despite these differences, both groups share a stereotypic trajectory post-birth, with converging immune systems at around 3 months [34]. However, we lack more studies exploring the postnatal immune system, as previous cord blood studies are somewhat unreliable due to tissue differences and ongoing changes after birth, already within the first week of life [34, 35].

Some cells and proteins act as connectors between the innate and adaptive immune system. These include γδ T cells, natural killer T (NKT) cells, antibodies, and inflammatory proteins such as cytokines [35]. In neonates, γδ T cells emerge as the initial subset within the T cell population. Diverging from their mature αβ T-cell counterparts, neonatal γδ T cells exhibit distinct functional attributes, like not having impaired IFN-γ production in neonatal αβ T cells [36]. The functional capabilities of neonatal γδ cells appear substantially influenced by prenatal conditioning, potentially linked to factors such as intra-uterine infections or distinct pathways in fetal cellular differentiation [37, 38]. Furthermore, neonatal γδ T cells display heightened functional proficiency across diverse cytokines, implying their pivotal role in early-life immune responses, particularly in the absence of fully matured αβ T-cell immunity. Notably, premature infants exhibit a lag in the expression of specific TLRs in their γδ cells, potentially impacting their responsiveness to viral nucleic acids and susceptibility to infections [37]. NKT cells express both T-cell receptors (TCRs), typical of adaptive immunity, and surface receptors found in NKT cells, which are innate immune components [39]. NKT cells play an important role in various immune responses, and one subset within NKT, known as invariant natural killer T (iNKT) cells, have demonstrated protective roles in defending the host against various microbial pathogens like bacteria, fungi, parasites, and viruses. However, in certain instances, these cells can be harmful to the host. Activation of iNKT cells can occur even without microbial antigens through multiple pathways [40]. Firstly, TCR stimulation can be facilitated by endogenous ligands presented by CD1d. Secondly, inflammatory cytokines like interleukin (IL)-12 and IL-18, generated by APCs and stimulated by TLR-agonists, are capable of triggering iNKT cell activation. Lastly, endogenous ligands have the potential to interact with inflammatory cytokines, leading to iNKT cell activation [41]. In studies using animal models of neonatal bacterial infection, there is evidence suggesting the potentially important involvement of iNKT cells in the neonatal immune response [40]. Natural IgG provides innate immune defense in cooperation with lectins. Investigating the role of natural IgG during infections, particularly through its interaction with lectins, has also revealed its ability to enhance pathogen clearance. This collaboration challenges the traditional division between innate and adaptive immunity, raising fundamental questions about their intersection and the ensuing immune pathways [42].

### 1.1.2 The complement system

The complement system is part of the innate system and consists of over 50 proteins, and it can be amplified through three activation pathways: the classical, lectin, or alternative pathway. Activation of C3 can lead to the covalent binding of the C3b fragment to foreign particles or self-tissue, resulting in C3 deposition [43]. Additionally, the activation products C3a and C5a trigger inflammation and immune cell activation by interacting with G protein-coupled receptors on various cell types. The formation of C5b-9 (membrane attack complex-MAC) creates pores in cell membrane, leading to cell lysis. Sublethal concentrations of C5b-9 can stimulate cells and enhance the expression of adhesion

molecules. Furthermore, a soluble, cytolytically inactive form of C5b-9 (sC5b-9) can initiate cytokine synthesis and vascular leakage in endothelial cells when present in plasma or serum. This soluble form can also be measured in blood and is also coined as the terminal complement complex-TCC [43].

The fetus and placenta express paternal antigens, appearing foreign to the maternal immune system, necessitating protection mechanisms. The syncytiotrophoblast, formed by the fusion of villous cytotrophoblasts, acts as a barrier between maternal blood and the fetus. It controls the complement system by keeping a low steady state level of activation to prevent harm to the fetus. Complement activation at the syncytiotrophoblast must be regulated carefully to maintain fetal and maternal health. Complement activation is crucial for normal pregnancy, aiding placental development and fetal growth. C1q, produced locally, plays a vital role in trophoblast invasion and spiral artery remodeling [44, 45]. Deficiencies in complement components, like C1q, can lead to pregnancy complications, emphasizing the importance of a functional complement system for a healthy pregnancy [46].

### 1.1.3 Transfer of antibodies across placenta and by breastfeeding

The placenta acts as the vital link between mother and fetus during pregnancy. Through thin cell layers, it facilitates the transfer of numerous substances from mother to fetus, including nutrients, oxygen, hormones, and immune mediators like cells, cytokines, and antibodies [47, 48]. Maternal antibodies transfer to the fetus occurs as maternal IgG binds with neonatal Fc receptors (FcRn) in placental syncytiotrophoblast endosomes. Using the pH difference between the endosome and fetal blood, maternal IgG is transported into the fetal circulation [48, 49]. Maternal IgG is transferred to the fetus transplacentally from the first trimester of pregnancy, although minimal amounts are transferred during this early phase (**Figure 2**) [16]. Approximately 10% of maternal IgG concentrations are estimated to be transferred by weeks 17-22 of gestation. By the 30th week of gestation, infant cord blood contains around 50% of maternal IgG levels, and in full-term, healthy pregnancies (37-40 weeks), cord blood concentrations of maternal IgG often surpass those in maternal serum at the time of delivery [16]. This suggests that the most substantial IgG transfer occurs in the final trimester, potentially due to an increase in the surface area for IgG uptake from maternal blood as gestational age advances [16].

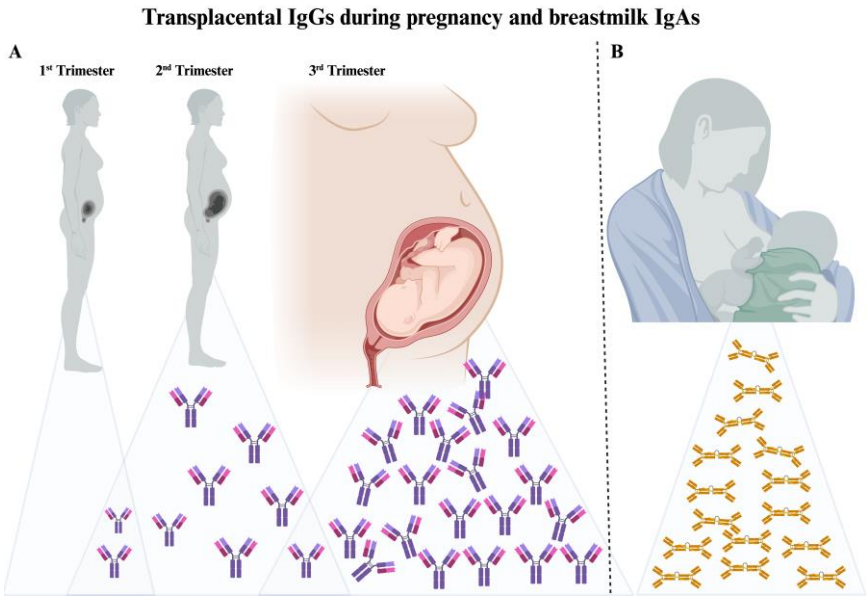


*Figure 2. Illustration of the placental transfer of IgGs during pregnancy (A) and the IgAs in human breastmilk (B). Created with BioRender.*

Birth and breastfeeding play a crucial role in shaping the infant's microbiome and overall health. At birth, the baby is exposed to various microbes, primarily from the mother, with breastfeeding contributing to the development of the gastrointestinal tract [50]. Breast milk contains essential bioactive components, including secretory IgA (sIgA), lactoferrin, lysozyme, human milk oligosaccharides (HMOs), growth factors, stem cells and cytokines, which impact immune function and gut health [51]. sIgA helps bind pathogens in the gut, while lactoferrin has antimicrobial properties and promotes the growth of beneficial bacteria [52]. HMOs serve as nutrients for beneficial bacteria, decoy receptors to prevent pathogen attachment, and promote regulatory T-cell growth [53, 54]. In very preterm infants, who lack transplacental IgG delivery, maternal milk serves as their main source of immunoglobulins [55]. The breastfed term infant’s microbiome is distinct, primarily consisting of *Bifidobacterium* and *Lactobacillus*, which are beneficial for gut health. It is a dynamic relationship with breastfeeding influencing the infant's microbiome and vice versa [55]. HMOs, complex sugars unique to breast milk, promote the growth of beneficial bacteria, such as Bifidobacteria, and help prevent pathogen attachment, reducing the risk of neonatal late-onset sepsis (LOS). Although supplemental bovine lactoferrin has shown some benefits in reducing LOS in preterm infants, larger studies did not replicate these results [56]. Preterm infants face higher risks of infection and therefore also benefit even more from breast milk which is tailored to their needs and contains higher levels of bioactive components. Preterm milk is richer in protein, fat, amino acids, sodium, and bioactive molecules, offering protection against complications such as necrotizing enterocolitis (NEC) and retinopathy of prematurity (ROP) [57]. Another component of breast milk is the presence of stem cells, identified initially through the surface marker Nestin [58]. These stem cells resemble embryonic and mesenchymal stem cells and show potential for differentiation into neural

cell lineage. These stem cells decline in count over time but hold promise for therapeutic applications, particularly in infant regeneration and repair [59].

## 1.2 Neonatal sepsis

Neonatal sepsis is characterized by hemodynamic alterations and various clinical manifestations in the first 28 days of life, or up to 44 weeks postmenstrual age in babies born preterm. Sepsis is caused by bacterial, viral, or fungal (yeast) sources [60], though a universally acknowledged definition for neonatal sepsis is still lacking [61]. Traditionally, neonatal sepsis was diagnosed by detecting a pathogen in usually sterile body fluids like blood or cerebrospinal fluid (CSF). However, sepsis in older children and adults is now defined as life-threatening organ dysfunction caused by a dysregulated host response to infection [62, 63]. In recent years, efforts have been made towards establishing a neonatal SOFA (nSOFA) to help identify organ dysfunction in, particularly, preterm infants with infections [64].

Opposing the conventional belief of sepsis progressing from an initial hyper-inflammatory stage to hypo-inflammatory reactions, recent research in adults indicates that sepsis triggers both hyper- and hypo-inflammatory responses concurrently. Moreover, these studies establish a connection between early fatalities and a sudden hyper-inflammatory phase, while delayed deaths are linked to prolonged immune suppression and recurrent infections [65, 66]. However, regarding neonatal sepsis, the specific mechanisms associated with the morbidity and mortality to either hyper-inflammation, hypo-inflammation, or a combination of both remains largely unknown [67].

Neonatal sepsis is typically divided into two categories based on the timing of symptom onset: early-onset sepsis (EOS) and late-onset sepsis (LOS). These distinctions involve differences in transmission methods, causative pathogens, as well as the guidelines and treatments recommended for each [68, 69]. Most of the neonatal literature refers to culture-proven (blood culture positive) EOS or LOS. However, a major challenge in neonatal care is the high antibiotic consumption due to so-called “culture negative sepsis”, a poorly defined and probably overused entity [70, 71].

The global incidence of neonatal sepsis is estimated at around 3000 cases per 100,000 live births, accompanied by a mortality rate of 17.6%. However, these estimates are uncertain due to insufficient data coverage across all WHO regions, often relying on information from selected countries [72].

### 1.2.1 Neonatal early-onset sepsis (EOS)

A widely accepted definition for EOS is the occurrence of bloodstream infections within the initial 72 hours after birth [73-75]. Still, according to the American Academy of Pediatrics (AAP), certain experts define EOS as the manifestation of symptoms within the initial 7 days after birth [76].

The main cause of EOS is vertically transmission of pathogens from the mother to the neonate during delivery. Neonates can be colonized by maternal bacteria in the birth canal or through aspiration of infected amniotic fluid [77]. In low- and middle-income countries, *Klebsiella* species is found in around 60% of positive blood cultures in term neonates with EOS [78], while in high-income countries, *E. coli* is the most common identified pathogen in preterm newborns with EOS and the second most common in term newborns [79]. GBS is the most common pathogen of EOS in term infants and the second most common in preterm newborns [79].

Less common bacterial pathogens associated with EOS [80] are *Enterobacter* spp., *Listeria monocytogenes*, enteric Gram-negatives, non-enteric Gram-negatives (e.g. *Hemophilus influenzae* and *Neisseria meningitidis*), Viridans group streptococci (VGS), *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) [81]. Non-bacterial agents that can be associated with EOS are *Herpes simplex virus* [82], enterovirus and parechovirus [83] and *Candida* [84].

The most recent global estimated incidence rates for EOS are approximately 3600 cases per 100,000 live births in the African region, 2000 cases per 100,000 live births in the South-East Asia Region, and 3000 cases per 100,000 live births in the Western Pacific Region [72]. In Europe, North America, and Australia it is 490 EOS-cases per 100,000 live births [85]. Premature and extremely low birthweight (ELBW) infants are notably at higher risk for EOS, with significant regional variations [72].

### 1.2.2 Neonatal late-onset sepsis (LOS)

The most common definition of LOS is a bloodstream infection occurring after the first 72 hours of life [80, 86, 87]. However, in the literature there are also experts that define LOS as the onset of symptoms occurring ≥ 7 days of life [76, 80]. LOS is associated with horizontal transmission of pathogens through the postnatal nosocomial or community environment [80, 88]. Advances in neonatology have increased survival of small premature infants, but the increased survival rate causes challenges seen as an increased incidence of LOS [88-90]. A strong focus in recent years has been on the preventative measures to reduce the burden of LOS [91, 92]. The global estimates on incidence of LOS are more uncertain [72], with one estimation indicating that LOS affects between 20% to 38% of all preterm infants within the initial 120 days of life [93]. In Norway, the blood-culture proven LOS incidence is from 9.3% to 21.6% in all preterm infants born prior to 32 or 28 weeks of gestation, respectively [94].

In high-income countries' NICU, coagulase-negative staphylococci (CoNS) and *Staphylococcus aureus* are common causes of LOS in preterm infants, in those with vascular catheters [94, 95]. Other pathogens associated with LOS [80] are *E. coli*, GBS, *Enterobacter* spp., *Pseudomonas* spp., *Acinetobacter* spp. and *Candida* spp. The distribution of these infectious agents varies depending on demographic characteristics of the patients, colonization of the nosocomial environment and the

antibiotic usage policy at the hospital [96]. In low- and middle-income countries, Gram-negative bacteria e.g. *Klebsiella* species are among the most common pathogens for LOS in preterm babies [72].

## 1.3 Group B streptococci

Rebecca Lancefield distinguished *Streptococcus agalactiae*, commonly known as group B Streptococcus (GBS) (**Figure 3**), from other streptococci in the 1930s after isolating it from milk and cows affected by bovine mastitis [97]. Until the 1960s, cases of invasive GBS disease in humans were uncommon, but there was a rise in reported instances of adult and neonatal invasive infections during that time [98-101]. Historically, *Streptococcus agalactiae* was categorized into nine serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII) based on capsular polysaccharides (CPS) [102], with a later addition of a tenth serotype (IX) in 2007 [103]. The CPSs of GBS act as virulence factors, aiding the bacterium in evading phagocytosis while triggering a serotype-specific immune response, leading to the production of protective antibodies. Although all 10 serotypes can cause disease [102], their prevalence differs globally [4, 104]. Surface proteins like Alp family proteins, serine-rich repeat proteins, C5a peptidase, and pilus islands can also contribute to classifying diverse GBS strains [105]. Moreover, certain proteins such as Rib, hvgA, and pilus island proteins have been associated with the invasiveness of GBS strains in various studies [106-109]. Whole genome sequencing has, in recent years, emerged as a reliable tool for CPS typing and antimicrobial resistance prediction [110].


*Figure 3. Group B Streptococci on Blood Agar Plate.*

### 1.3.1 The epidemiology of invasive GBS disease

GBS stands as the primary cause of sepsis and meningitis in neonates and young infants across most countries globally [111]. Invasive GBS disease can be further categorized as early-onset disease (EOD), happening within the first 7 days of life, and late-onset disease (LOD), occurring between day 7 and day 89. Almost all cases (97%) of invasive neonatal GBS disease are caused by serotypes I to V [3, 5, 112]. Among these, serotype III is responsible for almost half (43%) of EOD cases and a significant majority (73%) of LOD cases [5, 113].

In 2015, an estimated minimum of 300,000 infants under 90 days old worldwide experienced invasive GBS disease. Among them, around 200,000 infants had GBS-EOD, while approximately 100,000 had GBS-LOD [111].

In high-income countries the estimated incidence of GBS-EOD has declined from 0.7 cases per 1,000 live births in 1997 to a range of 0.21-0.25 cases per 1,000 live births in 2014 and 2015 [114, 115]. However, these figures vary considerably across countries. For example, incidence numbers (per 1000 live births) stand at 0.09 in Japan, 0.58 in Panama, 0.76 in Hong Kong, up to 1.5 in South Africa, and reaching 2.35 in the Dominican Republic. When combined, these estimates suggest an average global incidence of GBS-EOD at around 0.5 cases per 1,000 live births [5, 116-119]. Globally, the average incidence of GBS-LOD stands at 0.26 cases per 1,000 live births [5]. In the United States, the estimated incidence of GBS-LOD is slightly higher, at 0.32 cases per 1,000 live births [120].

In the Nordic countries, the estimated incidence of GBS-EOD cases per 1000 live births in 2019 was as follows: 0.28 in Denmark, 0.10 in Finland, 0.22 in Iceland, 0.25 in Sweden, and 0.22 in Norway. [121]. Figure 4 and 5 show the total incidence of GBS-EOD and GBS-LOD in Norway between 1999 and 2022 [122]. From the first epoch (1999-2009) and to the second epoch (2010-2022) the average incidence of GBS-EOD declined from 0.47 to 0.33/1000 live births whereas the incidence of GBS-LOS remained essentially unchanged at around 0.25/1000 live births between 1999 and 2022.

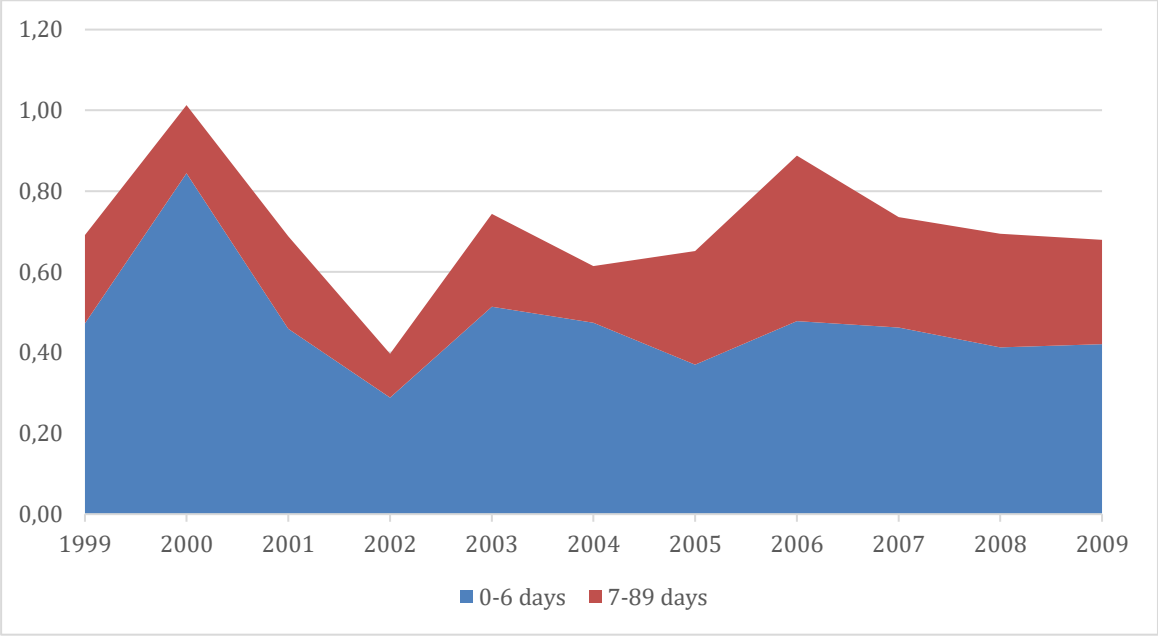


*Figure 4. The incidence of invasive GBS infections per 1000 live births between 1999 – 2009 in Norway. National data from the Norwegian Surveillance System for Communicable Diseases (MSIS).*

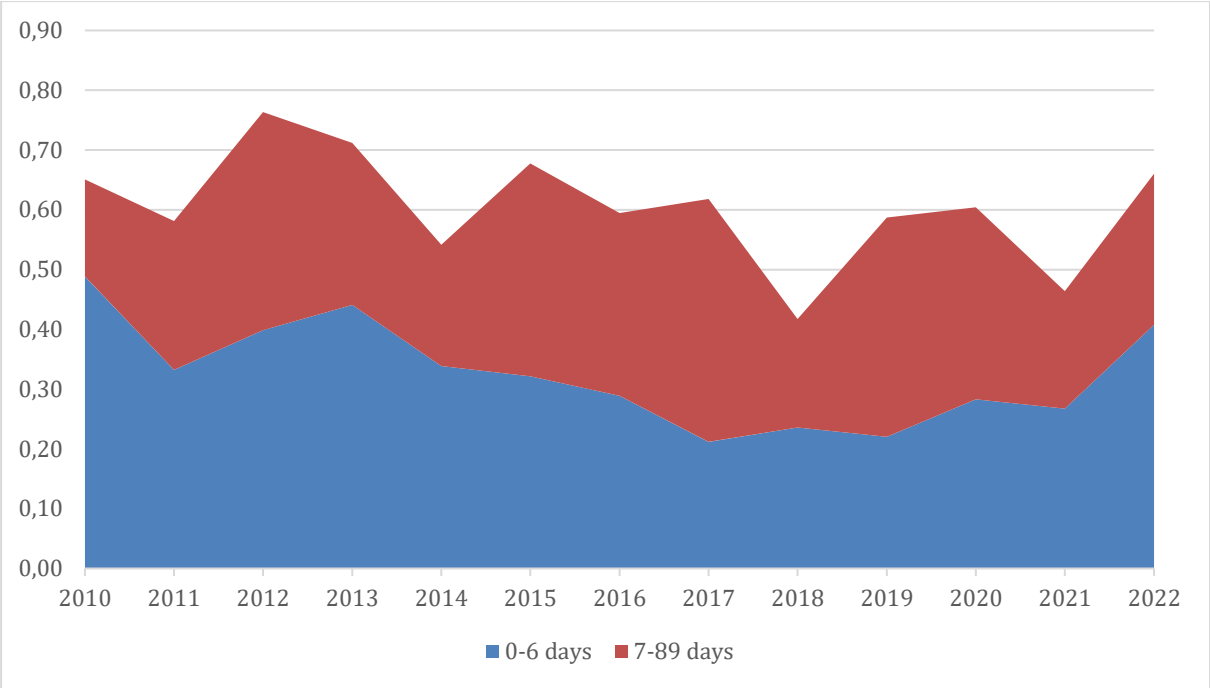


*Figure 5. The incidence of invasive GBS infections per 1000 live births between 2010 – 2022 in Norway. National data from the Norwegian Surveillance System for Communicable Diseases (MSIS).*

## 1.4 Mortality and morbidity of neonatal sepsis

Worldwide, it is estimated approximately 200,000 neonatal deaths are attributed to sepsis, with a recent study estimating a mortality of 17.6% (95% CI 10.3% to 28.6%) [72]. In Europe, North America, and Australia there is reported a mortality rate of EOS among late preterm and term neonates at 3.2% [85], and in a recent study from Sweden examining the same population it was even lower at 1.4% [123].

In very preterm infants, EOS is associated with a higher mortality and with markedly increased risk of complications such as ROP, intraventricular hemorrhage, bronchopulmonary dysplasia (BPD) and periventricular leukomalacia (PVL) [124, 125]. Studies indicate a higher mortality rate associated with Gram-negative EOS, however GA seems to be a confounding factor in these studies [1, 74].

LOS is a significant cause of mortality in preterm neonates, and treatment of sepsis is not always successful in protecting the infants from the long-term neurodevelopmental impairments [126, 127]. In a recent population-based study on LOS in very preterm infants from Norway, the overall LOS-attributable mortality was 6.3%, but markedly higher in Gram-negative (15.8%) compared with Gram-positive (4.1%) LOS-cases [94]. LOS was also associated with increased odds of development of severe BPD, PVL and ROP [94].

### 1.4.1 Mortality and morbidity of GBS disease

Invasive GBS disease leads to fatality rates, ranging from 1-8% in full-term infants and from 5-20% in preterm infants [5]. In GBS-EOD, mortality rates varies globally; high income countries estimate a 5% case fatality risk, while in African countries the case fatality can reach up to 27% [5]. On a global scale, GBS-LOD is estimated to carry a 7% case fatality rate [5]. In utero GBS disease contributes to approximately 1% of stillbirths worldwide, while this rate is estimated up to 4% in the WHO African region [111].

There is also an increased risk of neurodevelopmental impairments following invasive GBS disease in infancy. GBS meningitis is associated with markedly higher risks of attention-deficit/hyperactivity disorder, cerebral palsy, epilepsy, hearing impairment, and various developmental disorders [128, 129].

## 1.5 Diagnostics tools in suspected neonatal sepsis

### 1.5.1 Symptoms and pathogen identification

Neonates with sepsis commonly display subtle and nonspecific clinical signs, including respiratory distress, extended capillary refill time (> 2 seconds), pallor, feeding intolerance and lethargy [130, 131]. Identifying a pathogen in a blood and/or cerebrospinal fluid (CSF) culture is still considered the "gold standard" for diagnosing neonatal sepsis. Additional tests involve C-reactive protein (CRP), procalcitonin (PCT), and a complete blood count. Delayed treatment escalates mortality rates in sepsis cases, so as a precaution, empirical antibiotic treatment is typically initiated without confirmed microbiological evidence of sepsis [132-135].

Microbiological blood cultures face several challenges as the “gold standard” for sepsis diagnostics. One limitation is the susceptibility to skin microbiota contaminating the blood culture [88, 135]. In preterm infants’ small blood volumes and low bacterial colony counts may lead to false negative cultures. Previous studies have reported an average blood volume for blood cultures at around 0.5 mL [136]. This may be an inadequate volume in sepsis with low colony counts, posing a risk of false negative results. While 0.5 mL might suffice in detecting bacteria during moderate to high-grade bacteremia, it's advisable to aim for a minimum of 1 mL of blood collection [132, 137, 138]. Time to growth of bacteria in a neonatal blood culture is usually within 24 hours [139, 140], and this knowledge means that if clinical and biomarkers signs of sepsis are not very strong it is both safe and recommended to stop antibiotic treatment after 24-36 hours if the blood culture still has not shown any growth [141, 142]. Despite these notable limitations in diagnosing neonatal sepsis, blood cultures remain essential for isolating bacteria for antibiotic susceptibility testing. Unlike microbiological

independent techniques like PCR, blood cultures have the advantage of demonstrating a growth of live bacteria [143]. Several molecular nonculture techniques are also in development, but none of them have replaced the blood culture [144]. However, molecular nonculture techniques may have a specific role in attempting to identify the causative pathogen in patients that have started antibiotic treatment prior to obtaining a blood or CSF culture [145].

Early symptoms of meningitis can be subtle, often overlapping with sepsis, making lumbar puncture the primary diagnostic tool. [146, 147]. While challenging to diagnose meningitis solely through a lumbar puncture, it stands as a crucial differential diagnosis in neonatal sepsis due to its impact on antibiotic treatment, determining the type, dosage, and duration of therapy. It is reported that around 15% of neonates with meningitis may exhibit negative blood cultures, prompting discussions on whether lumbar puncture should be included as a routine investigation for neonatal sepsis [148]. In patients who have started antibiotic therapy before lumbar puncture is performed, it is still possible to identify causative pathogen by nucleic acid based PCR techniques [145].

### 1.5.2 Traditional biomarkers used in neonatal sepsis

The liver generates acute phase reactants that triggering the complement system, improve phagocytosis, regulate pro-inflammatory cytokines, and minimize tissue damage. Extensive research has been focused on sepsis biomarkers like CRP and PCT, largely because their assays are cost-effective, and the analyses are straightforward. However, CRP and PCT, being nonspecific acute phase proteins, are impacted by liver maturity and the development of organ dysfunction linked to sepsis [149, 150]. CRP serves as the frequently employed additional marker for sepsis, rising in response to IL-6 and other pro-inflammatory cytokines around 4 to 12 hours following the onset of infection or inflammation. Despite extensive examination concerning neonatal sepsis, outcomes diverge due to differing definitions of sepsis (EOS or LOS), sampling timings, study populations, sample sizes, and threshold values [150-152]. In most studies, the reported sensitivity of CRP for neonatal sepsis ranges from 50% to 77%, with specificity between 78% and 100%. CRP measurements within the initial 24 to 72 hours of suspected neonatal sepsis cases often exhibit heightened sensitivity. However, non-infectious factors such as fetal distress and maternal fever can elevate CRP levels, thereby reducing sensitivity [152, 153]. The most important benefit of CRP is probably the high negative predictive values which means that if CRP is still low 24 hours after onset of suspected symptoms and start of therapy, the likelihood of an infection is very low and this information can be used to support early discontinuation of antibiotics [152, 153].

Another commonly employed marker for neonatal sepsis is procalcitonin (PCT), the precursor of calcitonin. This protein is predominantly generated by peripheral mononuclear cells and typically rises around 2 to 6 hours following infection or inflammation. Its faster elevation compared to CRP renders PCT a potentially more efficient biomarker for the early detection of neonatal sepsis [151, 154]. The

reported sensitivity and specificity of PCT are comparable to CRP, ranging from 67% to 98% for sensitivity and from 67% to 100% for specificity [155, 156]. The inclusion of both CRP and PCT as additional tests appears to enhance sensitivity. Still, the 2018 meta-analysis conducted by Ruan *et al*. faced limitations stemming from varying definitions of sepsis and the use of different detection techniques across the studies included in the analysis [156].

Additional supplementary tests commonly used in diagnosing neonatal sepsis include the complete blood count (CBC), encompassing assessments such as white blood count (WBC), absolute neutrophil count (ANC), and the immature-to-total neutrophil ratio [157, 158]. The hematological profile indicating a rise in immature neutrophils compared to mature ones, often termed the immature-to-total (IT) neutrophil ratio, can serve as an indicator in neonatal sepsis diagnosis. Additionally, an abnormal WBC (in particular very low) or an observable left shift in the ANC, where there is an increase in immature or band neutrophils in comparison to mature ones, are noteworthy factors to consider when assessing for neonatal sepsis. These hematological alterations can signal an ongoing inflammatory response or a possible infection, contributing valuable insights to the diagnostic process [132, 159, 160]. CBC tests exhibit a broad spectrum of sensitivity, ranging from 17% to 90%, and specificity varying from 31% to 100%. These variations can be attributed to the expansive abnormal ranges within the CBC parameters, the relatively slow time required for obtaining a positive result, limitations in sampling times, and the impact of non-specific factors. The diverse ranges in these values emphasize the complexity involved in interpreting CBC results for neonatal sepsis, considering the multitude of factors that can influence these measurements, thereby affecting their accuracy in diagnosis [132, 157, 161-163].

### 1.5.3 Potential new diagnostic tools

#### 1.5.3.1 Other biomarkers

There has been substantial research on additional biomarkers (like acute phase proteins and cytokines) beyond CRP and PCT. However, meta-analyses examining cytokines such as TNF, IL-8, and IL-6 cannot conclude on benefit from using these markers, primarily because of the heterogeneity among the studies included [164-166]. IL-6 is routinely used as an early diagnostic marker for neonatal sepsis in some countries and NICUs [167, 168]. However, despite considerable research efforts, there is not yet a single test, biological marker, or a combination of markers identified as superior for diagnosing neonatal sepsis [88, 159, 169].

One new biomarker worth to mention is presepsin. Presepsin (or sCD14) is a free fragment of the membrane glycoprotein CD14 in monocytes and macrophages. After contact with infectious agents, CD14 activates an intracellular signaling pathway mediated by Toll-like receptor 4 (TLR4), initiating the inflammatory response against the microorganism [170]. A recent systematic review and meta-analysis suggests that presepsin is promising new biomarker for EOS [171].

#### 1.5.3.2 Immunometabolism and the omics

"Immunometabolism" represents an evolving area of study that acknowledges the intricate interplay between metabolism and the immune system. Within this field, various metabolic pathways—such as amino acid metabolism, fatty acid synthesis, fatty acid oxidation, glycolysis, and the tricarboxylic acid (TCA) cycle (also recognized as the Krebs Cycle)—play pivotal roles in fostering the survival, growth, functionality, and activation of innate immune cells [172, 173]. Elevated ATP levels have been associated with the potential to excessively prolong the immune response during sepsis [174]. However, the breakdown of ATP into adenosine diphosphate (ADP) and adenosine monophosphate (AMP) leads to heightened adenosine levels. Unlike ATP, adenosine serves to diminish pro-inflammatory and Th1-polarizing immune responses [172, 175]. Neonatal blood demonstrates heightened adenosine levels compared to adults, suggesting a potential for targeted manipulation of metabolizing enzymes as potential future treatment options [176, 177].

Omics technologies offer insights into genome-wide gene expression, protein translation, and metabolite production, showcasing distinct regulations seen in neonatal sepsis [178, 179]. In recent years, substantial research has emerged in genomics [180], transcriptomics [181, 182], proteomics [183], and metabolomics [184]. These methods in medicine offer distinct information. Genomics enlightens us about neonatal predispositions to sepsis [180], while transcriptomics reveals details about transcriptional changes occurring during sepsis [181, 182]. Proteomics showcases alterations in protein expression due to sepsis [183], and metabolomics elucidates the metabolites produced as a result of sepsis [184, 185].

## 1.6 Treatment of neonatal sepsis

### 1.6.1 Antibiotic treatment for EOS

Severe infections stand as one of the primary reasons for morbidity and mortality among neonates globally. However, the clinical indications/diagnostic criteria of sepsis often lack specificity, prompting the widespread empirical administration of antibiotics due to concerns regarding potentially serious outcomes, even in uninfected infants [60, 186]. In the NICU, antibiotics stand as the most prevalent medications prescribed, with a beta-lactam (benzylpenicillin or ampicillin) and gentamicin being administered more common than other common medications [142, 187, 188]. Other aminoglycosides (e.g. tobramycin, amikacin and in the future maybe plazomicin) are sometimes used due to increased levels of gentamicin-resistance among Gram-negative isolates [189, 190].

The National Institute for Health and Clinical Excellence (NICE) guidelines advocate for benzylpenicillin and gentamicin [142], whereas the AAP recommends ampicillin over benzylpenicillin as the primary treatment for EOS based on the perception that ampicillin may be more effective than

benzylpenicillin against the rare infections with *Listeria monocytogenes* [191]. Additionally, the UK NICE guidelines outline a standard antibiotic treatment duration of 7 days for infants with either a positive blood culture or those suspected of sepsis despite a negative blood culture [142].

In a cross-sectional study encompassing countries across Europe, North America, and Australia, it was reported that 2.86% (range across networks, 1.2-12.5%) of late-preterm and full-term newborns were administered antibiotics during the initial postnatal week. The study revealed a high rate of early postnatal antibiotic administration compared to the incidence of EOS [85]. The median (interquartile range) duration of treatment was 9 (7-14) days for neonates diagnosed with EOS and 4 (3-6) days for those without EOS [85]. A recent population-based study from Sweden reported that less than 2% of all late-preterm and full-term newborns were administered antibiotics during the first week of life [123]. The highest antibiotic consumption in NICUs stems from i) courses aimed at ruling out sepsis and ii) so-called culture-negative sepsis. In the SCOUT study [192], investigators evaluated antibiotic utilization among 2,500 term and preterm infants for various conditions. Only 6.9% of antibiotic usage was attributed to culture-proven infection and NEC. Conversely, prolonged antibiotic therapy (≥ 5 days) for pneumonia or culture-negative sepsis constituted 26% of the total antibiotic consumption. The median (range) duration of therapy for these conditions was 7 (5–14) days. In the SCOUT study, the highest antibiotic consumption in NICUs was from courses aimed at ruling out sepsis [192]. Increasing evidence suggests that extended antibiotic therapy in preterm infants lacking confirmed infection elevates the risk of mortality, BPD, NEC, ROP, and PVL [193-195]. A recent US-study concluded that in high-risk newborns with suspected EOS, empiric antibiotics can be safely ceased after 24 hours if the infection is not confirmed [141]. Recent literature discussing outcomes from antimicrobial stewardship initiatives in NICUs underscores the potential for enhancing antibiotic prescription protocols [196]. However, it also underscores the challenges encountered in limiting the utilization of potent antibiotics among highly vulnerable extremely premature infants [197, 198].

### 1.6.2 Antibiotic treatment for LOS

The empirical antibiotic treatment for LOS is based on factors such as exposures (including community or hospitalization status at LOS onset), local bacterial prevalence, and antimicrobial resistance (AMR) patterns [94, 199-201]. The duration, dosage, and timing of the antibiotic treatment can also variate based on factors such as GA, weight, microorganism identified, site of infection, and the antibiotic's potential to penetrate the infection site (especially in cases involving central nervous system, osteomyelitis, or endocarditis) [202].

In high-income countries, the most common cause of nosocomial LOS are Gram-positive bacteria, in particular CoNS. A beta-lactamase stable penicillin, cefazolin, or vancomycin, combined with an aminoglycoside, are the alternatives used in Norway [195, 203]. However, if there is a strong clinical suspicion of Gram-negative sepsis and/or meningitis, a third-generation cephalosporin, like

cefotaxime, can be added to the empiric regimen [199]. The routine use of broad-spectrum antibiotics like third generation cephalosporins for Gram-negative coverage is not recommended due to the higher likelihood of multi-drug resistant (MDR) bacteria developing compared to a regimen including an aminoglycoside. Furthermore, virulent late-onset pathogens such as non-*E. coli*, *Enterobacteriaceae* and *Pseudomonas* commonly exhibit resistance to third-generation cephalosporins [204]. Fungal LOS, especially in premature neonates, as well as viral infections such as herpes simplex, should also be considered [202].

In complicated hospital acquired LOS, like infections caused by ESBL and AmpC chromosomal beta-lactamase-producing Gram-negative bacteria, such as *Klebsiella spp*. and *E. coli*, necessitate treatment with carbapenems like meropenem due to their high resistance to commonly used antibiotics [205]. Meropenem offers broader antibacterial coverage, and it allows for monotherapy instead of combination therapy [206]. However, there are growing concerns regarding the emergence of carbapenem-resistant Gram-negative organisms (CROs) [202].

In Norway, penicillin remains effective against all GBS strains, while most *Staphylococcus aureus* isolates demonstrate susceptibility to oxacillin, and approximately 95% of *E. coli* strains are susceptible to gentamicin [207]. Still, there are large regional variations in consumption of vancomycin, broad-spectrum β-lactams and first-generation cephalosporins [94].

### 1.6.3 Adjunctive therapies for neonatal sepsis

Various adjunctive immunotherapeutic interventions have been examined alongside antibiotics for neonatal sepsis, but they have not conclusively improved outcomes.

Intravenous immune globulin (IVIG) is not recommended for treating neonatal sepsis based on current evidence [208]. Despite suggestions of potential benefit in preterm infants under 32 weeks' gestation with serious bacterial infection, trials have not shown clear advantages of IVIG administration in suspected or confirmed sepsis cases [208, 209].

Granulocyte transfusions or stimulating factors like granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have not demonstrated reduced mortality or morbidity in neonatal sepsis [210] or improved neurodevelopmental outcomes, general health, and educational outcomes in a five-year follow-up study [211]. They are therefore not recommended.

Lactoferrin is an important glycoprotein in human milk, that plays an important component in the innate immune defense against infections. Clinical trials on the use of lactoferrin in preterm infants were promising [126, 212, 213]. However, a Cochrane systematic review including twelve randomized controlled studies found low evidence that lactoferrin supplementation decreases the

incidence of LOS [56]. Moreover, the largest trial to date - the Enteral Lactoferrin in Neonates (ELFIN) trial - recruited 2,203 infants and failed to show any significant reduction in LOS [214].

Limited data suggests pentoxifylline, inhibiting TNF release associated with Gram-negative infection, might decrease mortality in neonatal sepsis when added to antibiotic therapy. However, small trials with methodological limitations necessitate further large-scale, unbiased studies to confirm these findings before recommending pentoxifylline routinely in neonatal sepsis treatment [215]. Currently, a large ongoing clinical trial is investigating whether pentoxifylline can improve long-term outcomes in preterm infants with LOS or NEC [216].

## 1.7 Preventive strategies for EOS and LOS

### 1.7.1 Risk factors for neonatal sepsis and initiatives to reduce infections

Maternal GBS colonization during the ongoing pregnancy, GBS bacteriuria, a history of previous infant with invasive GBS disease, prolonged rupture of membranes (PROM; lasting ≥18 hours), and maternal fever (temperature ≥38°C, that may indicate chorioamnionitis) are the most frequently linked risk factors for neonatal EOS. These factors work cumulatively, with the presence of multiple factors heightening the likelihood of developing EOS [217, 218].

A decreasing GA/BW is strongly associated with increased risk of LOS [94]. Another important risk factor is the use of central venous lines and umbilical catheters, which are frequently used in this population of small infants. These indwelling catheters provide a passageway for nosocomial bacteria, such as CoNS, and provide a surface for the development of biofilms. The longer the duration of the catheter use, the higher is the risk of a central line-associated bloodstream infection (CLABSI) [80, 219, 220]. Other risk factors are long-term use of mechanical ventilation and parenteral nutrition, hospitalization, surgery, underlying respiratory and cardiovascular diseases and late introduction of enteral feeding with breast milk [88, 96].

The understanding of risk factors for GBS-LOD is not as comprehensive as for GBS-EOD. Premature and low birth weight (LBW) infants are more vulnerable to infections due to their immature immune systems, limited placental antibody transfer, heightened gut permeability, and increased risk of hospital-acquired infections during their extended hospital stays. Additionally, prematurity disrupts the development of the microbiome, often linked to frequent antibiotic use, formula feeding, and reduced exposure to the maternal microbiome. This disruption might affect the adaptation of GBS to the neonatal environment in these infants. Recent research highlights prematurity or low birth weight and maternal rectovaginal colonization with GBS as significant risk factors for GBS-LOD [221].

Preventing LOS is the preferable strategy, rather than solely focusing on novel treatment options [126], by implementing evidence based practice like human milk feedings, proper hand hygiene and utilization of "bundle checklists" [222, 223]. In recent years there have been studies on the potential benefits of prophylactic probiotics, but the results have been inconsistent regarding nosocomial sepsis. Metanalyses have shown no significant reduction in the incidence of sepsis with the use of probiotics, though heterogeneity among trials might significantly influence the results [224, 225].

### 1.7.2 Intrapartum antibiotic prophylaxis

The introduction of intrapartum antibiotic prophylaxis (IAP) and maternal screening for vaginal carriage of GBS has reduced the GBS-EOD in the USA and many other countries [1, 121, 226]. The American Centers for Disease Control and Prevention (CDC) has been responsible for the American guidelines for prevention of neonatal GBS disease up to 2019 [227, 228]. In 2019 the American College of Obstetricians and Gynecologists (ACOG) took over the role of updating the guidelines. The guidelines from ACOG continued the focus on IAP administration in women with a positive rectal-vaginal GBS culture (culture-based approach) rather than on predefined maternal characteristics associated with GBS-EOD (risk factor-based approach). They implemented in the guidelines that all pregnant women at 36+0 to 37+6 weeks of GA should be offered a GBS rectovaginal screening culture, except pregnant women with GBS bacteriuria during the current pregnancy and women who previously gave birth to an infant with invasive GBS disease [229]. The traditional risk factor-based approach includes evaluating risk factors such as intrapartum fever ≥ 38°C, delivery before 37+0 weeks of GA, rupture of membranes ≥18 hours, previous delivery of an infant affected by GBS disease and GBS bacteriuria in the current pregnancy [229, 230].

In the Nordic countries, national guidelines for risk-based IAP were implemented at different times: in Denmark in 1997, Finland in 1998, Iceland in 1995, and Norway in 1998 [121]. Sweden lacked national guidelines for preventing GBS-EOD before 2008, although some delivery units were already utilizing risk-based IAP [121, 231].

Implementing maternal screening for rectovaginal GBS carriage and IAP has decreased the occurrence of GBS-EOD. However, it has also notably expanded the practice of administering antibiotics to women during childbirth [1, 226]. The effectiveness of IAP is constrained to GBS-EOD, and in certain countries, despite its utilization, the incidence has plateaued [1, 232]. In certain regions, a risk factor-based approach is employed rather than universal screening to identify women for IAP. While this approach aims to reduce antibiotic overuse, it potentially increases the chances of missed opportunities to identify cases for IAP, compared to a universal screening strategy. Consequently, while the risk-factor based approach may mitigate unnecessary antibiotic administration, it could result in a higher likelihood of not being able to prevent GBS-EODs [233]. Furthermore, the majority of low- and middle-income countries lack the resources needed to establish nationwide programs aimed at

identifying women for IAP to prevent GBS-EOD. Presently, there are no established strategies in place to prevent GBS-LOD or to decrease the occurrences of preterm deliveries, stillbirths, and maternal bacteremia linked to perinatal GBS infections.

### 1.7.3 Maternal immunization

Few vaccines are suitable to administer at birth or in the neonatal period [234]; mainly BCG to prevent childhood tuberculous meningitis and miliary disease [235] and a vaccine against hepatitis B [236]. However, vaccination as disease protection during early life is crucial for public health, but vaccines in the neonatal period presents significant challenges. First, the neonatal immune system, primarily Th2-skewed to prevent maternal immune system recognition of the fetus as an allograft, creates a hurdle for neonatal vaccination [237]. Second, the immaturity of neonatal leukocytes and the inhibitory effect of maternal antibodies further complicate the development of effective neonatal vaccines [237]. Finally, neonates exhibit diminished responses to T-independent polysaccharide antigens, and their antibody responses to T-dependent protein antigens are transient [238]. Therefore, strong efforts are made towards development of vaccines that can be administered during pregnancy to provide the newborn infant with protection (passive immunization) for the first months after birth [239-242].

The transmission effectiveness of maternal IgG across placenta varies notably depending on the specific antigen. Understanding these mechanisms is essential for developing effective maternal immunization strategies to enhance infant protection against neonatal pathogens during the first year of life [243]. In a regular pregnancy, IgG transfer against pertussis demonstrates remarkable efficiency, surpassing 200%. However, when it comes to IgG against GBS, the transfer efficiency is often reported at around 70%. This difference highlights the various factors that might affect how maternal IgGs are passed across during pregnancy [244, 245].

Various attributes of antigen-specific antibodies might elucidate these variations. Multiple studies have highlighted that the IgG subclass (**Figure 6**) plays a crucial role in determining the efficiency of transplacental transfer. Among these subclasses, IgG1 demonstrates the most effective transfer, whereas IgG2 exhibits the least efficiency in transfer [246, 247]. Notably, IgG subclass reactions vary distinctly across different antigens [248]. For instance, the immune response to polysaccharide antigens like the bacterial capsule of GBS or *Haemophilus influenzae* type B largely comprises the IgG2 subclass. Conversely, the response to tetanus toxoid is predominantly composed of the IgG1 subclass [248, 249].

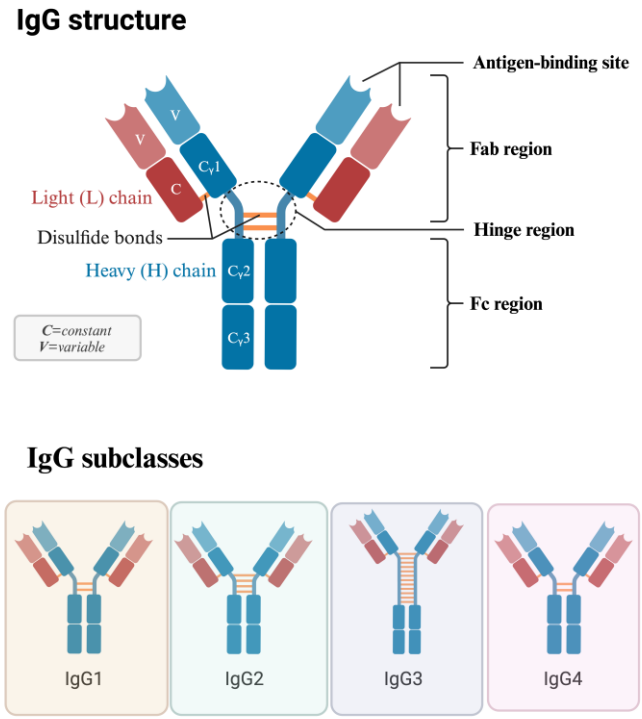


*Figure 6. IgG structure and subclasses. Created with BioRender.*

The interaction between IgG and the typical FcRn receptor might play a role in adjusting the efficiency of placental transfer of antibodies. For instance, the extensively transferred IgG1 and IgG4 subclasses exhibit notably high affinities for FcRn, while both IgG2 and IgG3 demonstrate lower affinities towards this receptor [250].

While placental IgG transfer is usually efficient in healthy pregnancies, several factors can impair it. These factors include maternal infections during pregnancy, such as human immunodeficiency virus (HIV) infection and malaria, placental pathologies, and maternal hypergammaglobulinemia [16].

Maternal HIV infection has been linked to reduced placental transfer of antibodies against certain pathogens such as *Streptococcus pneumoniae*, *H. influenzae*, GBS, pertussis, poliomyelitis, and measles [251]. Conversely, studies investigating the influence of maternal HIV on the transfer of other antibody specificities, like anti-tetanus toxoid antibodies, have yielded inconsistent findings across various studies and populations. Recent findings indicate that women undergoing prolonged antiretroviral therapy demonstrate enhanced placental transfer of IgG compared to those receiving short-term therapy [252]. Notably, some research suggests that maternal HIV might also affect the characteristics of transferred antibodies [253]. However, confirming this observation warrants extensive studies involving large cohorts of HIV-infected mothers and HIV-exposed uninfected infants [253].

# 2 Aims for the thesis

The aim of this thesis was to explore novel treatment strategies for neonatal sepsis in Paper I and to obtain new knowledge that could contribute to the development of a maternal GBS vaccine in Paper II-III. Overall, the focus was centered on pregnant women and neonates, with the overarching aim of advancing our understanding regarding strategies directed towards the prevention of neonatal sepsis and the mitigation of the devastating consequences associated with this condition.

The specific objectives were:

**Paper I: Dual inhibition of complement C5 and CD14 attenuates inflammation in a cord blood model**

The main objective of this experimental study was to compare *E. coli*- and GBS-induced inflammation and to evaluate the effects of dual C5-CD14 inhibition in an *ex vivo* human umbilical cord blood model.

**Paper II: The immunogenicity and safety of Group B Streptococcal maternal vaccines: A systematic review**

The main objective of this review was to systematically review and evaluate the immunogenicity and safety data of maternal GBS vaccines in published clinical trials until July 2023.

**Paper III: Association between anticapsular antibodies and protection against group B streptococcus in Norwegian infants**

The main objective of this case-control study was to evaluate anti-CPS antibody levels in cord blood from infants with invasive GBS disease and compare them with healthy infant controls in a 1:4 ratio, to establish IgG thresholds linked to a lower risk of invasive GBS disease. A secondary objective was to calculate the placental IgG transfer ratio in both cases and controls.

# 3 Materials and methods

This section of the thesis draws upon methods from Paper I-III. Consequently, some overlaps with the content of these articles are anticipated.

## 3.1 Study design and populations

### 3.1.1 Paper I

Paper I is an experimental study. We used an *ex vivo* whole blood inflammation model created to compare *E. coli*- and GBS-induced inflammation and to evaluate the effects of dual C5 and CD14 inhibition. Healthy mothers scheduled for an elective caesarean section at the University Hospital of North Norway in the period of October 2019 and September 2021 were invited to participate during one of their regular hospital appointments. The study was conducted in two parts: (1) we collected cord blood samples (n=16) and incubated blood with bacteria for **120 min** before analyses, and (2) we collected cord blood samples (n=14) and incubated blood with bacteria for **240 min** before analyses.

### 3.1.2 Paper II

Paper II is a systematic review of all clinical trials reporting data on the immunogenicity and adverse events following administration of a GBS vaccine in healthy pregnant and non-pregnant women, and in women with HIV. The review is reported according to the updated Preferred Reporting Items for Systematic Reviews and Meta-Analyses (24) and the study protocol was prospectively registered (PROSPERO ID: CRD42020185213). Two reviewers (Aline Uhirwa Bjerkhaug and Shouwmika Ramalingham) screened titles and abstracts independently according to predetermined inclusion and exclusion criteria, with disagreements between the reviewers being resolved through consensus with the third author (Claus Klingenberg). We extracted the following variables: paper identification (title, first author, publication year), study design, inclusion and exclusion criteria, characteristics of the population (pregnant or non-pregnant adult, adult or infant, average age/gestation week/day after delivery), study site for clinical trials, characteristics of vaccine, characteristics of analytical assays, antibody response after vaccination, placental transfer ratio of GBS antibodies and adverse effects of the vaccines. The main outcome assessed was immunogenicity defined as vaccine-elicited geometric mean antibody concentration (GMC), and secondary outcome was vaccine efficacy (if possible) and other immunological responses (e.g. opsonophagocytosis, geometric mean fold rise (GMR) of GBS antibodies), placental transfer ratio and adverse events (AEs). When appropriate, meta-analysis was conducted using the online platform recommended for Cochrane intervention reviews (RevMan Web) and we presented the effect-estimates by using the random-effect model.

We evaluated the reported AEs in all studies comparing participants that received a conjugated CPS vaccine or surface subunit protein-based vaccine versus those who received placebo. If studies reported data on AEs separately for adjuvanted or non-adjuvanted vaccines, we selected the data on AEs from adjuvanted vaccines while these commonly are more reactogenic. Many studies reported on AEs at different vaccine doses, but we collated these together when analyzing the number of AEs in the vaccine group. AEs were reported differently in studies performed more than 15-20 years ago compared to more contemporary studies, but some of the more recent trials [254-262] used the extensive MedDRA system to present AE data [263]. Three authors (Aline Uhirwa Bjerkhaug, Claus Klingenberg and Robert Mboizi) assessed AEs independently and compared the findings. To obtain similar and comparable AE data across both older and more recent vaccine trials we report rates of the following AEs; serious AEs, AEs leading to withdrawal from the vaccine study, fever/systemic illness in relation to vaccine administration and vaccine-related death. Disagreements were discussed and resolved by consensus.

### 3.1.3 Paper III

Paper III is an observational case-control study including mother-infant pairs identified retrospectively in the period 1999-2009 to investigate the GBS antibody levels in cases with invasive GBS disease (up to 89 days of age) and to compare with healthy control infants. We employed four databases: the Norwegian Mother, Father and Child cohort-study (MoBa), the Norwegian Surveillance System for Communicable Diseases (MSIS), the Norwegian reference laboratory for GBS (NRL-GBS), and the Medical Birth Registry of Norway (MBRN). Plasma from mother and baby collected in MoBa, in both cases and controls, was assessed for CPS IgG to investigate serological protective antibody levels against GBS disease. We integrated data from MoBa with information from MSIS and NRL-GBS. Clinical data from mothers of cases and controls were obtained from the MBRN and included age, smoking history, previous abortions, mode of delivery and rupture of membranes. Infant characteristics include sex, gestational age, birth weight, Apgar score, respiratory support, and mortality. Clinical data on infant cases obtained from MSIS and NRL-GBS include day of life when infection was diagnosed, source of infection and survival. The data regarding the GBS isolate serotype was collected from NRL-GBS. Four mother-child controls, where the child did not require admission to a neonatal unit in the first week of life, were selected for each case.

## 3.2 Laboratory methods

### 3.2.1 Paper I

In **Paper I** [264], all cord blood was drawn into endotoxin-free 4.5 mL NUNC tubes (Thermo Fischer Scientific, Roskilde, Denmark) and lepirudin (Refludan®, Pharmion, Windsor, UK) was added to a

concentration of 50 μg/mL blood. Several pilot experiments were performed before the main study to assess the effect of single versus dual inhibition and to compare bacterial challenge with $10^7$ GBS bacteria/mL versus $10^8$ bacteria/mL. In sub-study 1, the baseline sample (T0) was processed less than 20 min after the blood was drawn. Combined inhibitors eculizumab (final concentration 100 μg/mL blood) and anti-CD14 (final concentration 15 μg/mL blood) or isotype-matched control IgG2/4 (final concentration 15 μg/mL blood), were added to separate tubes at each of the following time points: 8 min prior to, and 15 and 30 min after adding *E. coli* strain LE392 (ATCC 33572; Manassas, VA) or a clinical GBS strain, serotype III (SO-SAG18-1, kindly provided by the Norwegian GBS reference laboratory, Trondheim, Norway) to a final concentration of $10^7$ bacteria/mL whole blood. Two positive controls were incubated with either *E. coli* or GBS. The negative control was incubated with PBS only. All samples were incubated in a Rotamix Intelli-Mixer (Norengros, Oslo, Norway) with rotation of blood at 37°C for 120 min after adding bacteria or PBS. Complement activation was stopped by placing the samples on ice and adding EDTA (Sigma-Aldrich, Steinheim, Germany) to a final concentration of 20 mM. The samples were centrifuged for 20 min at 3000 x *g* at 4°C. Plasma was collected and stored at -70°C until analyzed. Sub-study 2 followed the same protocol, but the samples were incubated for 240 min instead of 120 min.

Proinflammatory cytokines (TNF, IL-6 and IL-8) were measured using a multiplex cytokine assay (Bio-Rad Laboratories, Hercules, CA). The assay was performed according to the manufacturer's instruction. The soluble terminal C5b-9 complement complex (TCC) assay was performed according to a method developed in a research laboratory at the Nordland Hospital [265]. In short, the principle of the TCC assay is based on a monoclonal antibody aE11 reacting with a neoepitope expressed in C9 only after it is activated and incorporated into the C5b-9 complex. TCC concentrations are reported as complement activation units (CAU)/ml [265].

### 3.2.2 Paper III

The serotype determination was conducted by the Norwegian reference laboratory for GBS (NRL-GBS). During the period 1999 to 2005 CPS typing of GBS isolates at the GBS-NRL was done by immunofluorescence microscopy using rabbit antisera for CPS types (Ia, Ib, II-V) [266]. From 2006 the laboratory switched to PCR-based typing including CPS types Ia, Ib, II-VIII, and from 2010 CPS type IX [267]. However, all serotyping for GBS isolates in our case-control study was reanalyzed by both PCR and whole genome sequencing [267].

The detection of anti-GBS CPS IgG was conducted at a designated laboratory St Georges University of London (UK), with the same method as published in previous studies [268-270]. The detailed method of detection of anti-GBS CPS IgG in serum samples has previously been reported [270]. In our study, we had plasma samples and not serum. However, a bridging study (unpublished) has

compared the performance of the anti-GBS CPS IgG assay in adult serum and plasma samples and demonstrated good concordance.

## 3.3 Statistical analyses

In **Paper I** we used GraphPad Prism version 9.2.0 (GraphPad, San Diego, CA) for statistical analysis and presentation. Descriptive results are presented as means with standard deviation (SD) and medians with range or interquartile range (IQR; 25 to 75 percentiles). When comparing the effect of dual inhibition of *E. coli* or GBS induced inflammation at different time points with the positive control group, the results were analyzed by the non-parametric Wilcoxon matched-paired signed-rank test. Percentage inhibition of the positive control is presented related to the negative control as baseline. A p value $<0.05$ was considered statistically significant for all analyses.

In **Paper II**, the main outcomes assessed were immunogenicity defined as vaccine-elicited geometric mean antibody concentration (GMC), and vaccine efficacy if possible. Immunogenicity data were not possible to meta-analyze, and are therefore presented descriptively for each study. As secondary outcomes, we evaluated other immunological responses (e.g. opsonophagocytosis, geometric mean fold rise of GBS antibodies), placental transfer ratio and adverse events (AEs). We evaluated the reported AEs in all studies comparing participants that received a conjugated CPS or surface subunit protein-based vaccine versus those who received placebo. If studies reported data on AEs separately for adjuvanted or non-adjuvanted vaccines, we selected the data on AEs from adjuvanted vaccines. Many studies reported on AEs at different vaccine doses, but we collated these together when analyzing the number of AEs in the vaccine group. AEs were reported differently in studies performed more than 15-20 years ago compared to more contemporary studies. Some of the more recent trials [254-262] have used the extensive MedDRA system to present AE data [263]. AE data were meta-analyzed using the online platform recommended for Cochrane intervention reviews (RevMan Web). We calculated risk rations (RRs) with 95% confidence intervals (CI) for the AEs. We present the effect-estimates by using the random-effect model due to assumption of clinical and methodological diversity among the studies, subsequently often leading to statistical heterogeneity. Reactogenicity data were not possible to meta-analyze and therefore presented descriptively for each study.

In **Paper III**, we used the Chi Square test when comparing demographics and subject characteristics between cases and controls. For each GBS-serotype of cases and the controls, anti-GBS CPS IgG concentrations were log transformed to calculate geometric mean concentrations (GMCs) and 95% confidence intervals (CIs). Statistical comparisons of IgG concentration between cases and controls were computed using Student's *t* distribution. There was no imputation of missing serological results, and subjects with no results were excluded from the analysis; values below the lower limit of quantification (LLOQ) were assessed as half LLOQ (½ LLOQ) for calculation of GMC. Placental

transfer ratio with 95% CIs was calculated as GMCs in cord blood divided by the GMCs of plasma from mothers obtained around delivery, for each infant-mother dyad, and for each serotype.

## 3.4 Ethical considerations

The study in **Paper I** was approved by the Regional Ethical Committee (2019/834/REK nord), and all participating women gave informed written consent to participate in the study on behalf of themselves and their neonates.

For **Paper II** there was no need for seeking ethical approval.

For **Paper III**, which is a sub-study of the MoBa study, we received approval by the Regional ethics committee (REK sør-øst 2019/934). All cases from the MoBa cohort who were 18 years or older before analysis of cord blood samples, were also informed about the study and given an opt-out alternative to refuse that their samples and data could be included in this research study. The establishment of the MoBa-study in the 1990s, and initial data collection was based on a license from the Norwegian Data Protection Agency and approval from The Regional Committees for Medical and Health Research Ethics. All MoBa-participants have signed a written informed consent, and approved the use of biological specimen for health research purpose. The MoBa cohort is currently regulated by the Norwegian Health Registry Act.

## 3.5 Funding

I was funded by UiT-The Arctic University of Norway as a medical research student, and later supported by The Northern Norway Regional Health Authority (Helse Nord RHF), grant number HNF1628-22, 2022. The laboratory analysis for detection of anti-GBS capsular polysaccharide IgG was funded by Pfizer.

# 4 Summary of main results

This section of the thesis also draws upon Results from **Paper I-III**. Consequently, some overlaps with the content of these articles are anticipated.

## 4.1 Paper I

Thirty mothers participated in this study [264]. Among them, two had mild preeclampsia, one had type 1 diabetes mellitus, one had moderate anemia, one had intracranial hypertension, and the remaining 25 were deemed healthy. Scheduled caesarean deliveries were conducted at a mean (SD) GA of 38.1(0.5) weeks, resulting in the birth of 18 girls and 12 boys with a mean (SD) BW of 3316 (521) g. Notably, all infants scored 9 or 10 on the Apgar test at 5 minutes, and none required admission to the NICU. In Sub-study 2, involving 13 mothers, their mean (SD) total white blood cell counts and neutrophil counts were 8.1(1.6) and 5.8 (1.8) $\times 10^9$/L, respectively. Correspondingly, the cord blood samples from 14 neonates exhibited mean (SD) total white blood cell counts and neutrophil counts at 12.3(3.2) and 5.9 (2.1) $\times 10^9$/L, respectively.

Median (IQR) TCC cord plasma concentrations following 120 minutes of incubation with *E. coli* or GBS were 1.4 (0.7-3.6) CAU/mL and 1.5 (1.0-9.1) CAU/mL, respectively. These concentrations were significantly higher than the negative control (0.4 [0.1-0.5] CAU/mL), $p < 0.001$. Extended bacterial incubation for 240 minutes yielded similar TCC cord plasma concentrations for both *E. coli* (2.4 [1.5-4.3] CAU/mL) and GBS (1.4 [0.8-3.1] CAU/mL), with significantly elevated TCC levels compared to the negative controls.

Dual inhibition of C5 and CD14 effectively reduced cord TCC plasma concentrations when administered before bacterial challenge, as well as 15- and 30-minutes post-challenge, following both 120- and 240-minute bacterial incubations.

Incubation of cord blood with *E. coli* or GBS resulted in significant increases in plasma concentrations of TNF, IL-6, and IL-8 cytokines. Notably, *E. coli* elicited significantly higher levels of TNF, IL-6, and IL-8 compared to GBS. Dual inhibition demonstrated significant reductions in TNF, IL-6, and IL-8 concentrations after *E. coli* challenge, but no significant effects were observed following GBS challenge, except for a lower concentration of IL-6 after dual inhibition.

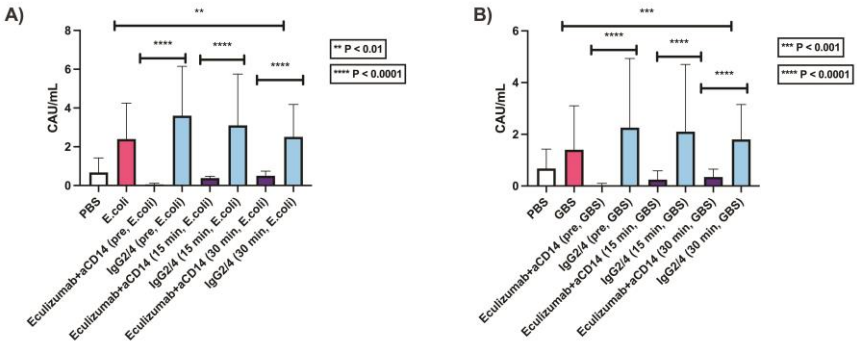


*Figure 7. Cord plasma concentration of TCC after 240 minutes incubation time with E. coli (Fig. A) and group B streptococci (Fig. B), and dual inhibition with complement C5 and co-receptor CD14 inhibitors, pre- and post-bacterial challenge (**Paper I**).*

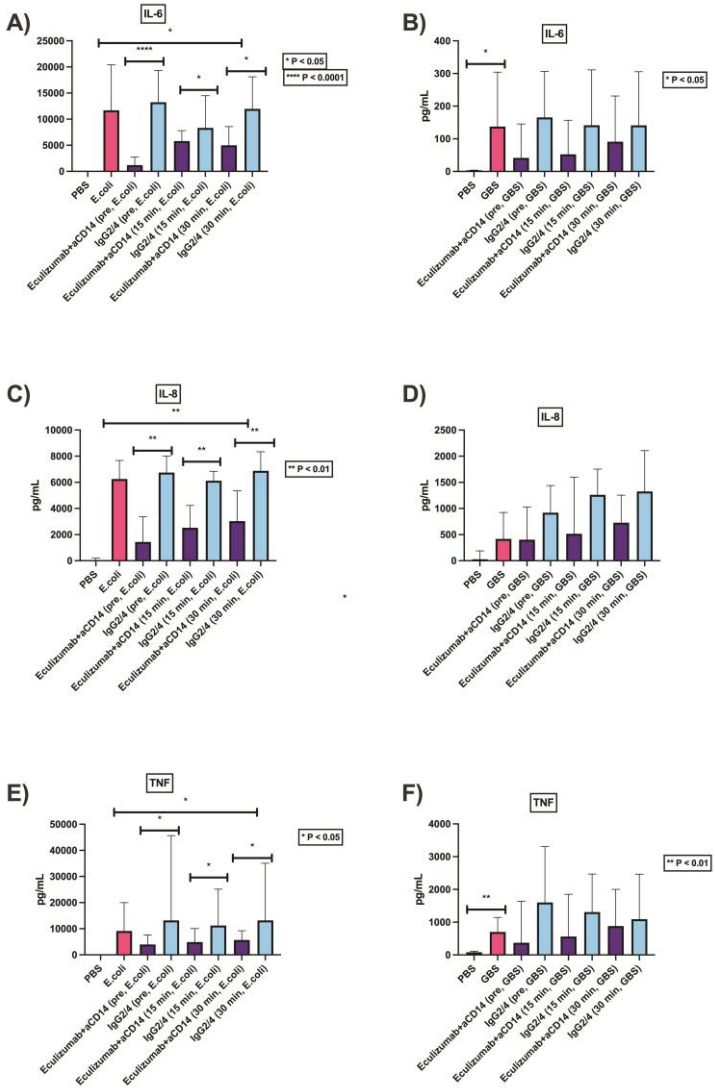


*Figure 8. Cord plasma concentration of IL-6, IL-8 and TNF after 240 minutes incubation time with E. coli (Fig. 5 A-C-E) and group B streptococci (Fig. 5 B-D-E), and dual inhibition with complement C5 and co-receptor CD14 inhibitors, pre- and post-bacterial challenge (**Paper I**).*

## 4.2 Paper II

In **Paper II** [271], a total of 1472 publications (records) were retrieved from databases, with an additional 5 publications obtained from reference list citations. Following a comprehensive review, 48 studies were deemed eligible for full-text evaluation. Exclusions primarily comprised published protocols, animal studies, and preclinical investigations. Ultimately, 26 publications were included, consisting of 20 main studies [254-262, 269, 272-281] and 6 sub-studies [282-287].

The 20 main clinical studies included 5765 participants, including 1325 pregnant women. There were predominantly Phase 1 or 2 trials. The studies varied in design, with nine being double-blind randomized controlled trials [262, 272-279], eight being observer-blind randomized trials [254-257, 259-261, 269], and three being non-randomized open label trials [258, 280, 281]. The majority reported data on GBS-IgG response, with nine also evaluating GBS type-specific opsonophagocytic killing [272-274, 277-280, 285, 286].

We found that GBS antibody response peaked within 2-8 weeks post-vaccination in healthy adults and pregnant women, with sustained elevation of GBS antibodies observed up to 6-12 months following vaccination. Dose-dependent responses were observed in several studies, particularly with surface subunit protein vaccines. Adjuvants such as aluminum salts or oil-in-water emulsions were employed, with varying impacts on immunogenicity. Placental transfer ratio varied from 0.4 to 1.4 across five studies.

A second or booster vaccine doses enhanced IgG response in individuals with initially low antibody levels.

Reactogenicity was predominantly mild, with no reported deaths attributed to vaccine administration. Serious adverse events were infrequent, with no discernible age or pregnancy-related patterns observed. Additionally, vaccination did not adversely impact pregnancy outcomes or HIV-infected individuals.

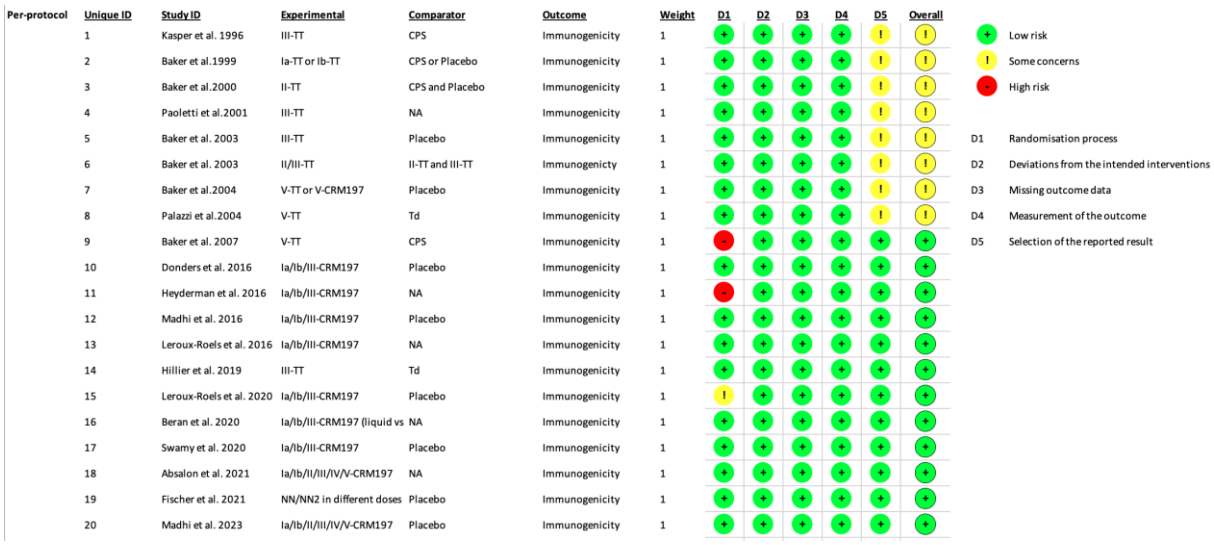


*Figure 9. Risk of bias for immunogenicity outcomes (**Paper II**).*

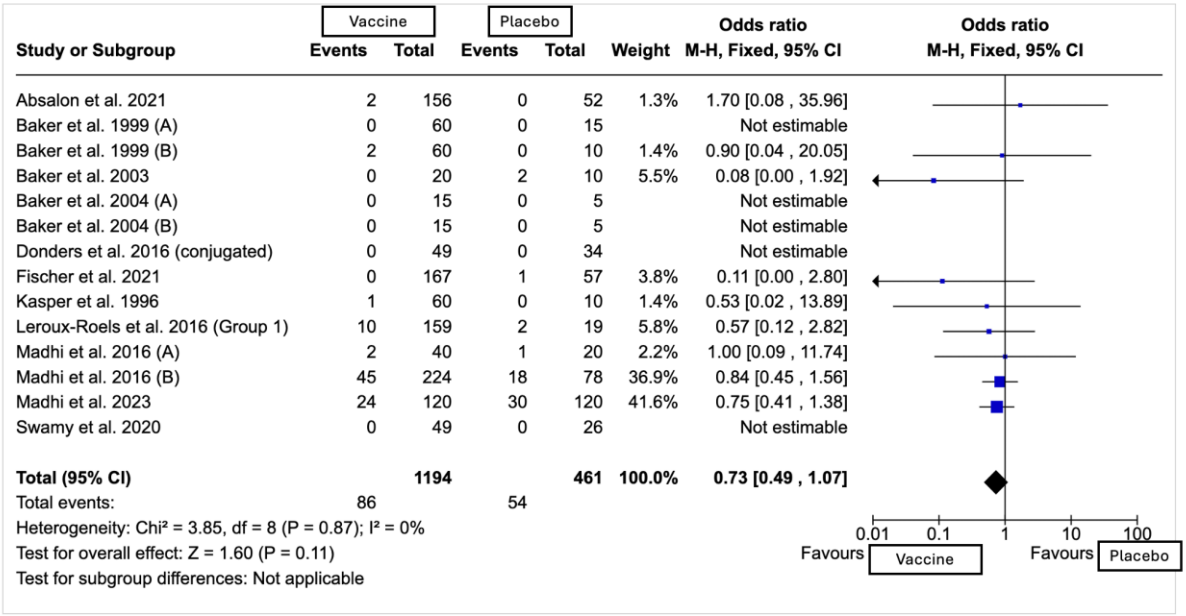


*Figure 10. Pooled results of studies comparing risk of serious adverse events between those who received a GBS vaccine versus placebo (**Paper II**). The sizes of the squares are proportional to study weights. Diamond markers indicate pooled effect sizes.*

## 4.3 Paper III

In **Paper III** we identified 57 cases of infants with invasive GBS disease within 89 days of birth in the MoBa cohort. To compare, 228 healthy term-born control infants were matched with the cases (4:1), resulting in clinical and GBS serology data from 285 mother-infant dyads from the MoBa cohort. The controls were randomly selected among the next healthy term born neonates in the MoBa-study, with cord blood available for analysis and who were not admitted to a neonatal unit in the newborn period.

In the MoBa cohort, the incidence of GBS-EOD and GBS-LOD was 0.39 and 0.11 per 1000 live births, respectively. Among the GBS cases, the serotype was known in 47 out of 57 cases; 29 (51%) were infected with serotype III group B streptococcus, 5 (8.8%) with serotype Ia, 5 (8.8%) with serotype V, 4 (7%) with serotype Ib, 3 (5.3%) with serotype IV, and 1 (1.8%) with serotype II. Analysis of serotype distribution during the study period (1999-2009) and post-study period (2009-2023) in Norway revealed that serotype III remained predominant, constituting 42-65% of all isolates in both GBS-EOD and -LOD cases.

Mothers of cases exhibited a higher frequency of spontaneous abortion before 12 weeks' gestation (17/57; 30%) compared to mothers of controls (39/228; 17%) ($P = .03$; $\chi^2$ test). Similarly, rupture of membranes for more than 12 hours was more prevalent among mothers of cases (16/57; 28%) compared to mothers of controls (30/228; 13%) ($P = .006$; $\chi^2$ test). As expected, 8.8% of the infants with GBS were born preterm, and 21% required respiratory support.

For serotype III LOD there were lower anti-CPS GMCs in cases versus the controls (P = 0.019). Moreover, when calculated as an aggregate across all serotypes, the difference between case and control GMCs for LOD was also significant (P=0.006). There were no significant differences in GMCs between cases and controls for any other serotypes.

We observed overall placental transfer ratios mainly were between 0.7 and 1.1 across all serotypes. There was a lower placental transfer ratio among cases with serotype III (0.49) than in controls (0.93), P = 0.025. For other serotypes there were no significant differences between cases and controls.

*Table 1. Geometric mean IgG concentrations (mg/mL) in cord plasma of cases and all controls (**Paper III**).*

| | Case | | Control | | All | | | |
|---|---|---|---|---|---|---|---|---|
| Serotype | n | GMC (95% CI) | n [x] | GMC (95% CI) | n | GMC (95% CI) | GMR | *P* value |
| Ia | 5 | 0.052 (0.001, 3.796) | 188 | 0.028 (0.018, 0.045) | 193 | 0.029 (0.018, 0.045) | 0.542 (0.026, 131.560) | 0.715 |
| Ib | 4 | 0.015 (0.000, 1.447) | 188 | 0.013 (0.009, 0.018) | 192 | 0.013 (0.009, 0.018) | 0.882 (0.012, 108.328) | 0.936 |
| II | 1 | 0.032 (NA, NA) | 188 | 0.202 (0.147, 0.279) | 189 | 0.202 (0.147, 0.279) | 6.325(NA, NA) | NA |
| III | 29 | 0.018 (0.009, 0.039) | 188 | 0.025 (0.018, 0.036) | 217 | 0.024 (0.018, 0.034) | 1.382 (0.317, 1.652) | 0.433 |
| III EOD | 18 | 0.027 (0.009, 0.086) | 188 | 0.025 (0.018, 0.036) | 206 | 0.026 (0.018, 0.036) | 0.930 (0.326, 3.541) | 0.901 |
| III LOD | 11 | 0.010 (0.005, 0.020) | 188 | 0.025 (0.018, 0.036) | 199 | 0.024 (0.017, 0.034) | 2.642 (0.172, 0.834) | 0.019 |
| IV | 3 | 0.010 (0.000, 8.450) | 188 | 0.012 (0.009, 0.017) | 191 | 0.012 (0.009, 0.017) | 1.283 (0.001, 615.807) | 0.889 |
| V | 5 | 0.057 (0.001, 4.539) | 188 | 0.027 (0.020, 0.038) | 193 | 0.028 (0.020, 0.039) | 0.475 (0.027, 164.666) | 0.662 |
| All serotypes | 47 | 0.022 (0.011, 0.044) | 188 | 0.027 (0.019, 0.039) | 235 | 0.026 (0.019, 0.036) | 1.240 (0.369, 1.763) | 0.585 |
| All EOD | 35 | 0.031 (0.013, 0.076) | 188 | 0.027 (0.019, 0.039) | 223 | 0.028 (0.020, 0.039) | 0.875 (0.441, 2.964) | 0.779 |
| ALL LOD | 12 | 0.008 (0.004, 0.017) | 188 | 0.027 (0.019, 0.039) | 200 | 0.025 (0.018, 0.036) | 3.434 (0.126, 0.675) | 0.006 |

Abbreviations: CI = Confidence interval; GMC = geometric mean concentration; GMR = geometric mean ratio

CIs are back transformations of a CI based on the student t distribution for the mean logarithm of the concentrations.

The GMR was calculated as the group mean difference (Control - Case) of logarithmically transformed antibody levels and back transformed to the original units.

*p* value derived from *t* test comparing GMCs in case and control cohorts

[x] Only controls from the 47 cases with serotype data were included

*Table 2. Placental Transfer Ratios in cases and controls (**Paper III**).*

| | Case | | Control | | Total | | |
|---|---|---|---|---|---|---|---|
| Serotype | n | Ratio (95% CI) | n | Ratio (95% CI) | n | Ratio (95% CI) | *P* value |
| Ia | 3 | 0.867 (0.632, 1.190) | 20 | 0.995 (0.508, 1.949) | 23 | 0.978 (0.549, 1.742) | 0.680 |
| Ib | 3 | 1.406 (0.324, 6.095) | 16 | 0.629 (0.348, 1.140) | 19 | 0.715 (0.425, 1.202) | 0.125 |
| II | 0 | NA | 3 | 1.364 (0.767, 2.429) | 3 | 1.364 (0.767, 2.429) | NA |
| III | 26 | 0.492 (0.291, 0.833) | 116 | 0.930 (0.780, 1.109) | 142 | 0.828 (0.695, 0.986) | 0.025 |
| IV | 3 | 0.489 (0.025, 9.499) | 12 | 1.044 (0.742, 1.471) | 15 | 0.897 (0.599, 1.343) | 0.386 |
| V | 4 | 0.994 (0.466, 2.121) | 20 | 1.024 (0.639, 1.642) | 24 | 1.019 (0.687, 1.511) | 0.930 |

*p* value derived from *t* test comparing GMCs in case and control cohorts

Abbreviations: CI = Confidence interval; GMR = geometric mean ratio

CIs are back transformations of a CI based on the student t distribution for the mean logarithm of the concentrations.

The GMR was calculated as the group mean difference (Control - Case) of logarithmically transformed antibody levels and back transformed to the original units.

# 5 Discussion

## 5.1 Discussion of results

In this work, we investigated the potential use of immune inhibitors in a neonatal inflammation blood model (**Paper I**). Furthermore, we present insights into the immunogenicity and safety of GBS vaccines across diverse populations, supporting their potential for reducing the burden of GBS-related diseases (**Paper II**). Finally, we investigated the potential protective anti-CPS IgG levels and placental transfer ratio in a large Norwegian mother-childbirth cohort (**Paper III**).

### 5.1.1 The neonatal immune response

The immediate immune response in neonatal sepsis depends on multifactorial components comprising the innate immune defense system [288]. However, limited data exist on the role of the complement system in neonatal inflammation and disease severity. In **Paper I**, we observed strong complement activation in cord blood after bacterial challenge with *E. coli* and GBS, as indicated by high levels of TCC. Neonatal sepsis triggers an early inflammatory response that, although less robust compared to older individuals it can be equally lethal [289]. In our experiments, extended bacterial incubation periods resulted in significantly higher levels of proinflammatory cytokines TNF, IL-6, and IL-8 after challenge with both *E. coli* and GBS, with higher cytokine release observed in response to *E. coli*. The modulation of the complement system, particularly C5 inhibition, shows promise in reducing sepsis morbidity and mortality [290]. Similar modulators are already widely present in the maternal-fetal interface of the placenta, theorized as a control mechanism for complement activation and protection against adverse pregnancy outcomes [291-293]. Our findings support previous studies indicating that dual inhibition of C5 and CD14 effectively mitigates inflammation induced by bacterial pathogens, although its efficacy may vary depending on the pathogen [290, 294-299].

Generally, unless bacteria possess specific mechanisms to evade complement killing, Gram-positive and Gram-negative bacteria are vulnerable to elimination through opsonophagocytosis [43]. In addition, the complement MAC also kills predominantly Gram-negative bacteria because they have an outer membrane containing LPS, where MAC assembles. The LPS, also known as endotoxin, in the outer plasma membrane of Gram-negative bacteria is a potent inflammation-inducing molecule that triggers the human immune system [300]. Antibody-mediated classical pathway activation is very important in defense against Gram-positive bacteria, as serospecific antibodies can weaken the serotype III capsule [301, 302]. Deficiency in maternal type III CPS-specific IgG has been shown to correlate with susceptibility of neonates to the GBS infection [303], and may contribute to explain why cases with GBS-LOD serotype III had lower anti-CPS GS antibody levels than the controls (**Paper III**). Additionally, the presence of sialic acid residues in CPS are resistance to opsonophagocytosis in GBS by complement pathway [304]. This results in reduced deposition of

opsonic C3b and iC3b fragments on the bacterial surface, which are crucial for effective phagocytosis [304]. In vitro, CPS-specific IgG effectively counteracts this resistance mechanism [305]. The level of CPS-specific IgG directly correlates with the effectiveness of opsonophagocytic killing by polymorphonuclear leukocytes [301, 302] and with the generation of the complement-derived chemoattractant C5a [305]. We therefore hypothesize that the observed significant variation in GBS induced TCC compared to *E. coli* TCC production in the participants of **Paper I** may be attributed to the lack of knowledge regarding antibody levels, in particularly for GBS serotype III.

Conjugated CPS-based GBS vaccines induced anti-GBS IgG levels that were efficiently transferred across the placenta (**Paper II**). The antibody levels detected in the infants, originating from transferred IgG, are likely a better marker for determining the risk reduction of invasive GBS disease compared to maternal antibody levels, even though the latter are easier to obtain [254, 255, 269, 276, 281]. In a study investigating serial antibody levels in vaccinated infants, functional antibodies persisted for at least two months in infants [276]. However, since maternal vaccination only provides passive immunization to the infants a decline in antibody levels is expected [242]. The placental transfer ratios ranged from 0.4 to 1.4 across the included studies in **Paper II**, and were compatible with the placental transfer ratios mainly between 0.7 and 1.1 in **Paper III**. These vaccine-induced placental transfer ratios can be influenced by vaccine-induced IgG subclasses, as studies suggest variations in transfer ratios among different subclasses [246, 306]. Additionally, factors such as naturally occurring IgG subclass distribution patterns in populations and potential influences of racial and ethnic factors on vaccination responses must be considered [307-313]. A study revealed that although sialic acid in GBS serotype III inhibits the alternative complement pathway activation, the addition of CPS-specific IgG subclass 2 significantly enhanced L-ficolin-initiated opsonophagocytic killing by increasing the activation of the alternative pathway [314].

In previous seroepidemiological investigations, most infants affected by GBS infections ("cases") have exhibited anti-CPS IgG GMC < 0.01 μg/mL, and significantly lower anti-CPS IgG GMC in cases than healthy controls [244, 303, 315-322]. Low antibody levels in infant cases with a GBS infection can be due to either poor maternal production of antibodies or limited transfer of antibodies across placenta. We lacked detailed data on the medical history of mothers to infants with GBS-EOD in **Paper III**, but some of these pregnancies may have been complicated by a more chronic placental inflammation and infection around delivery [323-328]. We speculate that a more prolonged low-grade placental inflammation in mothers of cases with serotype III may be the reason for a lower placental transfer (**Paper III**). Higher rates of placental inflammation in low- and middle-income countries are also thought to explain the low placental transfer rates found in a study from South Africa [269].

In **Paper III,** surprisingly we observed overall anti-CPS IgG GMCs ≥ 0.01 μg/mL across most serotypes in the cases with invasive GBS infection. This contrasted previous studies, but the anti-CPS

IgG GMC among controls were similar to these studies [254, 255, 269, 276, 281]. However, in LOD-cases with serotype III we found lower anti-CPS IgG levels in LOD-cases versus the controls (**Paper III**), which is in line with the hypothesis that low antibody levels are associated with increased risk of infection. For EOD cases our study could not demonstrate similar association, of unclear reasons. **Paper III** had some limitations that made interpretation of data challenging. We observed a trend towards declining rates of GBS-EOD in Norway from 0.47 to 0.33/1000 livebirths between 1999-2009 and 2010-2022, but we did not have national data on IAP use during this period. An increased use of IAP since its introduction in Norway in 1998 [121], may be one reason for declining GBS-EOD incidence. We speculate therefore that the use of IAP may have masked GBS-EOD cases in the healthy control group, as the antibiotic use would prevent the neonates with potential low antibodies levels from acquiring GBS-EOD. Continuing in this line of reasoning, the antibody levels in controls were similar to previous studies [269, 322], but the maternal rectovaginal GBS colonization rates around delivery was 34% in a Norwegian study from 2005 [329] and 26% in a Norwegian study from 2009-2011 [330]. These colonization rates are higher than reported in Northern Europe (20.6%), North America (22%) and overall in developed regions (18.4%) [331]. Colonization with GBS not only contributes to the risk of GBS disease in infants but also influences the levels of anti-GBS-CPS IgG in pregnant women and neonates [244, 303, 315-321, 332]. Theoretically, one could have expected the Norwegian healthy controls to have higher levels of antibody levels than previous studies [269, 322]. Understanding the colonization status in combination with the IgG subclass levels in the included MoBa participants might have provided valuable insights into the dynamics of GBS transmission and its implications for maternal and neonatal immunity.

### 5.1.2 Maternal Immune Response

**Paper I** focus on complement system activation by analyzing TCC concentration, a key indicator of complement activation. Strong complement activation was observed in cord blood after bacterial challenge with *E. coli* and GBS. Low levels of ficolin-3 and mannose binding lectin (MBL), which activate the lectin complement pathway, have been linked to increased susceptibility to infections [333, 334]. In a case-control study, neonates with Gram-positive sepsis had lower ficolin-3 concentrations, while those with Gram-negative sepsis had lower MBL concentrations [333]. Although serotype distribution was not known in **Paper I**, we know maternally transferred immunoglobulins targeting GBS serotype III can weaken the bacterial capsule, enhancing complement activation [335]. Extended bacterial incubation periods led to increased cytokine release, particularly TNF, IL-6, and IL-8, with differences observed between *E. coli* and GBS challenges. Our findings in **Paper I** contrast to Mohammed *et al* who reported no significant difference in cytokine release *E. coli* and GBS, but instead a more pronounced cytokine release in cord blood versus adult blood [336]. In our study, we focused on neonatal immune response and selected pregnant women with unknown colonization status and antibody levels for GBS CPS.

In **Paper II** and **III**, the systematic review and the seroepidemiological study revealed insight into the maternal immune response after GBS vaccination and the association between antibody levels and risk of GBS infection. The review of GBS-IgG levels across studies in **Paper II** revealed challenges in data interpretation due to variations in vaccine serotypes, assays, and assessment protocols. Protective maternal thresholds that translate to adequate infant protective levels remained undefined, with suggestions of "protective" maternal levels of around 1 μg/mL for anti-GBS-CPS IgG concentrations [318, 321]. The surface subunit protein vaccines' "protective" thresholds for anti-protein IgG concentration remained elusive due to assay variations and study design differences [320, 337, 338]. In **Paper II**, most vaccine studies showed antibody concentrations above the arbitrary threshold of 1 μg/mL in non-pregnant adults and pregnant women post-vaccination. In **Paper III**, due to limited number of cases we were not able to identify a specific protective blood anti-CPS IgG threshold for both EOD and LOD. Furthermore, serotype Ia elicited a significantly higher IgG response compared to other serotypes (**Paper II**). Still, serotype III is more common worldwide [331] as it also clearly was in our cohort (**Paper III**).

Conjugation of GBS-CPS with toxoid proteins proved essential for achieving adequate immune responses, and this principle is widely recognized in other CPS-based vaccines, such as the pneumococcal glycoconjugate vaccine [339]. Adjuvants, notably aluminum salts, demonstrated a notable enhancement in immunogenicity when incorporated into surface subunit protein vaccines [262]. This effect was not observed in conjugated CPS-based vaccines [256, 275]. An increased immunogenicity after adding adjuvants may cause a placental inflammation and consequently theoretically less antibodies crossing the placenta. Thus, adjuvants may have the opposite effect of what you would expect if being used in a maternal vaccine. In contrast, commercial polyvalent pneumococcal CPS vaccines include adjuvants (aluminum salts) to stimulate immune responses in infants aged two months and older [340].

In the Norwegian adult population there has been an average annual increase of 6.4% from 1999 to 2019, with a shift in the distribution of CPS serotypes from the three dominant types (V, Ia, and III) to the six most common serotypes (Ia, Ib, II, III, IV and V) [341]. Hence, a hexavalent GBS vaccine may be preventative for invasive GBS infections in a larger demographic than just GBS disease in neonates. Although long-term antibody decline was observed in vaccinated adults in **Paper II**, functional antibodies persisted for up to two years post-vaccination in adults [272-274, 277-280, 285, 286]. **Paper II-III** enhance the significance of understanding the natural immunity and mechanisms behind the variations observed in vaccine studies [242].

### 5.1.3 GBS vaccine safety

**Paper II** assessed the safety profile of GBS vaccines, and the results were reassuring, though caution is warranted in interpreting the data. The review included a relatively small number of participants, with only 1325 pregnant women among 5765 participants. Distinguishing between pregnancy-related complications and vaccine-related adverse events (AEs) poses challenges in maternal vaccine studies due to overlapping symptoms. Factors like maternal age, obstetrical history, and health conditions influence pregnancy outcomes and must be considered in interpreting AEs [342].

Identifying rare and severe adverse effects necessitates a larger cohort, exemplified by occurrences like vaccine-induced immune thrombotic thrombocytopenia following specific COVID-19 vaccines [343], or as observed in recent respiratory syncytial virus vaccine trials linked to preterm births [344]. Although no increased rates of premature births were observed in the GBS vaccine trials (**Paper II**), the small sample size limits definitive conclusions. Therefore, establishing a robust Vaccine Adverse Event Reporting System (VAERS) and maintaining vigilant safety monitoring post-licensure of a maternal GBS vaccine is imperative.

### 5.1.4 Dual inhibition of C5 and CD14 - a novel therapy in sepsis?

Earlier research on complement system-modulation during sepsis focused on targeting complement C3 due to its pivotal role in amplifying the immune response [294, 296]. However, inhibiting C3, while effective in reducing inflammation, may increase the risk of infection by impeding the primary complement defense mechanism through C3-opsonization [294]. In **Paper I**, we opted to utilize a C5 inhibitor. Blocking C5 prevents the formation of C5b, which initiates the assembly of C5b-9, and importantly, it inhibits the production of the potent proinflammatory complement protein C5a. Furthermore, this approach does not interfere with the opsonization of microbes by C3b [43]. Dual inhibition of C5 and CD14 showed promising results in reducing TCC plasma concentration and reduced key cytokines (IL-6, IL-8 and TNF), indicating a potential therapeutic approach for neonatal sepsis (**Paper I**). However, there are challenges when comparing our results with previous studies, since many studies report data from *in vitro models* or studies with isolated blood cells that show a much higher cytokine production [345-348].

There are already some promising results in animal models for the potential use of C5 inhibition for reduction of sepsis mortality and morbidity [290]. Additionally, C5 inhibition drugs, such as eculizumab, are already in widespread clinical use, and with relatively good safety data in other pediatric conditions [349, 350]. Animal studies of polymicrobial sepsis have also shown clear beneficial effects of the dual C5 and CD14 inhibition with improved hemodynamic parameters, and morbidity and survival [297, 351]. Still, there is some concern about the persistent C5 inhibition due

eculizumabs long elimination half-life. Furthermore, we did not evaluate possible interactions with most used antibiotics in our model (**Paper I**).

## 5.2 Discussion of methodology

### 5.2.1 Methodological considerations

**Paper I** primarily delves into investigating the immune response associated with neonatal sepsis and how this can be modified with potential new treatment strategies.

In neonatal care, initial clinical symptoms of the two most common EOS pathogens (*E. coli* and GBS) cannot be differentiated [121, 123, 202]. In severe cases of sepsis, empiric therapy must be started before we know the bacterial etiology [94, 123, 202]. Thus, after careful methodological considerations the two most common bacteria that cause sepsis in neonates [85, 94, 125] were included in the study protocol. While various designs are available in the basic research sphere, we opted for a whole blood inflammation model that encompasses all components of blood and where the study could be executed under physiological conditions. This model allows for crosstalk between inflammatory mediators and is an advantage in comparison to in vitro models often investigating single components or response pathways [345-348].

Both **Paper II** and **Paper III** concentrate on invasive GBS disease but adopt distinct approaches to address similar inquiries. The fundamental question explored in both papers revolves around the advancement of a maternal GBS vaccine. The objective in **Paper II** was to systematically gather global knowledge pertaining immunogenicity and adverse effects of GBS vaccines. **Paper III** builds upon the knowledge gap identified in **Paper II**. In contrast to **Paper II**'s global perspective, **Paper III** utilizes data and biological samples from a unique Norwegian mother-child cohort study, supplemented with information extracted from various Norwegian databases, to comprehensively analyze GBS seroepidemiology in infants with GBS-EOD and GBS-LOD.

### 5.2.2 Internal validity

Internal validity refers to the degree to which our work can accurately reflect the true relationship between variables, without being influenced by confounding factors or biases. It is essential for drawing valid conclusions from the study findings [352].

The collection, handling, and laboratory analysis of maternal venous blood samples and cord blood samples in the inflammation study (**Paper I**) and in the MoBa cohort (**Paper III**) were conducted according to standardized procedures by skilled and experienced personnel. While the probability of

random error was reduced as much as possible, it can never be eliminated. In the inflammation study (**Paper I**), we implemented a comprehensive multistep laboratory method involving sample collection and experimentation, which could have introduced variability. We believe that utilization of a detailed protocol and consistent execution during experimentation, along with analysis conducted by other skilled researchers, mitigated potential errors and reduced the chances of detecting false associations (type I statistical error). The sampling in the MoBa cohort (**Paper III** was done by numerous midwives during the 10-year study period, but after a strict protocol. High quality biobanking of plasma samples and avoidance of frequent freeze-thaw cycles by banking in small aliquots reduced potential measurement bias [353]. The sample sizes in **Paper I-III** could unfortunately not minimize random errors, and with small samples there is an increased the risk of not detecting weak associations (type II statistical error).

Interpreting the study results also requires consideration of bias, also known as systematic error. Systematic error can be categorized in three main categories: selection bias, confounding, and information bias [352].

**Paper I-III** exhibit a degree of **selection bias** concerning the study population, with a higher risk of bias observed in the inflammation study (**Paper I**) and seroepidemiological study (**Paper III**). These studies primarily enrolled Caucasian participants. Regarding the function of the complement system (**Paper I**), concentrations of ficolin-3 and MBL can be affected both by intrauterine infections and genetic factors. MBL-deficiency affects e.g. about 30% of the white population [333]. Furthermore, the comprehensive informed consent form being in Norwegian (although also available in English), may have biased inclusion towards well-educated Norwegian women (**Paper I** and **Paper III**). In **Paper III**, the incidence of GBS-EOD in the MoBa study cohort was in line with national data [354]. We observed that the GBS-LOD incidence was lower at 0.11/1000 livebirths. We speculate that one of the reasons might be that the educational level of mothers and rates of breastfeeding are higher in MoBa-study participants than in the general Norwegian population [355]. Human milk with bioactive substances and its microbiota is important for the neonatal gut microbiome and enhances the infant's immunity against infections [50, 51, 356, 357].

To minimize confounding in **Paper I**, we restricted the analysis to healthy pregnant women expecting term infants and further limited to elective cesarean section. The later thought to standardize the blood collection and minimize chances of inflammation due to vaginal delivery affecting our results, although a recent study reported that antimicrobial peptides known to be involved in anti-inflammatory are upregulated in vaginal delivery compared to cesarean sections [358]. In the systematic review (**Paper II**), most included studies were rated as having an overall low risk of bias for immunogenicity data. However, there were concerns regarding 8 out of 20 studies due to insufficient information about whether the data was analyzed according to a predetermined analysis

plan before unblinded outcome data became available for analysis. The latter increases the risk of information bias. In **Paper III**, we identified several potential confounders which could influence the association between anti-CPS IgG levels (exposure) and GBS-EOD/GBS-LOD (outcome). First, the number of cases with complete serotype data was limited, and mainly sufficient for statistical analyses only for serotype III or all serotypes combined. Second, we did not have corresponding data on maternal GBS-colonization. Consequently, we were unable to pair cases with healthy controls with similar colonization status. Third, we lacked detailed medical history from the mothers of the healthy control cases, like gestational diabetes or perinatal infections that could have impacted on the placental transfer ratios [49]. Finally, the IAP usage is not known for mothers in the MoBa cohort, and potential variations in use among control mothers could theoretically influence the risk of infections among the controls.

### 5.2.3 External validity

External validity concerns the extent to which research findings can be generalized or applied to real-world settings. It relies on internal validity, as the validity of generalizing study results to other contexts depends on the soundness of the study design and the absence of biases or confounding variables that could affect the internal validity of the research [352].

**Paper I-III** encompass a predominantly homogeneous population (selection bias), primarily Caucasian, except for the systematic review, which incorporated participants from African regions. As previously discussed, the demographic uniformity may influence the generalizability of the findings across diverse populations, warranting further investigation into potential ethnic and demographic variations in outcomes. However, the outcomes from the inflammation and seroepidemiological studies are anticipated to be relevant to women of Nordic heritage, whereas the systematic review encompasses data from participants of African origin as well.

## 5.3 Strengths and limitations

The *ex vivo* model in **Paper I** assesses the innate immune response in a system with fresh cord blood containing both cellular and humoral immune response components. In **Paper II**, the strengths of our systematic review include our rigorous and sensitive search strategy following an *a priori* registered protocol. This is followed up in **Paper III**, where the study integrates samples from both cases and controls with comprehensive information from multiple databases, involving both mothers and children.

In **Paper II-III**, we targeted an area of global concern and importance. GBS vaccines have been a focus of clinical trials since the 1990s, still only around 5600 participants were identified in the 20

studies in the systematic review (**Paper II**). One of the key constraints was the inability to conduct a meta-analysis for the primary outcome of immunogenicity (IgG GMCs) due to the heterogeneous use of seroassays across studies. However, the international consortium known as GASTON (Group B Streptococcus: Standardization of Laboratory Assays) has reached a consensus on a unified protocol for GBS antibody assays [270]. This standardized procedure marks a significant milestone in their collaborative efforts to ensure consistency and reliability in GBS-related research [359, 360]. This standardized assay has also been used in **Paper III** to analyze the plasma samples of the mother-child dyads, which makes this one of the few studies that are both comparable and reproducible. The latter also applies to the *ex vivo* model in **Paper I**, due to the meticulous protocol published for further exploration of the inflammatory response in neonates.

There are several limitations with the chosen methods in all included papers. All studies featured a relatively small sample size and generally homogeneous grouping. Our inflammation model (**Paper I)** had limitations in exploring certain well-known intracellular and extracellular bacterial antigen-specific mechanisms, which regulate cytokine secretion, by not conducting *in vitro* experiments [288]. The primary challenge in conducting a meta-analysis for the main outcome of immunogenicity (IgG GMCs) was due to differences in (non-comparable) seroassays used in the included studies (**Paper II)**. Although we employed a standardized method for serocorrelates in **Paper III**, our primary limitation was the lack of complete data on serotypes for all GBS isolates, and no data on the use of IAP within the study cohort.

In both **Paper I** and **Paper III** there were concerns regarding the healthy controls. In our inflammation model, although we included five mothers with well-managed medical conditions, their cord blood response could potentially have been misleading, albeit their results did not significantly differ from those of the other participants. In our seroepidemiological study, we selected a healthy control group from the MoBa cohort, which may have been a particularly selected group as MoBa participants in general were somewhat higher educated than the general population.

Finally, some limitations arise from necessary choices made in the laboratory protocol (**Paper I**). In our inflammation model, we opted for lower GBS loads and shorter observation periods compared to previous studies [361]. Nonetheless, our findings demonstrated that a GBS concentration of $10^7$ CFU/mL was adequate, as evidenced by elevated IL-6 levels indicative of neonatal sepsis [362]. Due to blood volume constraints and experimental complexity, we could not include additional experiments with eculizumab and anti-CD14 separately. We used heat-inactivated bacteria due to experimental protocol intricacies, corroborating prior findings of robust immune response [363]. Heat-inactivated GBS induces TLR2-dependent antimicrobial gene activation, with no destruction of LPS or LTA [364].

# 6 Conclusions

Our investigation into the potential application of immune-modulating treatment (**Paper I)**, such as the dual inhibition of C5 and CD14, demonstrated significant reductions in detrimental immune components, including TCC, TNF, IL-6, and IL-8. Furthermore, we conducted experiments using an inflammation model, where the immune inhibitors were added up to 30 minutes after the bacterial challenge, yielding consistent results. These findings highlight the efficacy of immune modulation in mitigating inflammatory responses and suggest its potential future therapeutic usage in clinical settings.

Our systematic review (**Paper II)** highlights the extensive data pertaining to the immunogenicity, reactogenicity, and adverse events associated with maternal GBS vaccines tested in clinical trials, and with data accumulated over several decades of research. Furthermore, it sheds light on the knowledge regarding the placental transfer ratio of these vaccines. This comprehensive analysis underscores the current evidence base surrounding maternal GBS vaccination, offering valuable insights for future vaccine development. The restricted number of pregnant women included in GBS vaccine studies is also an important finding, and clearly shows that new and larger studies are needed. This systematic review also emphasizes the existence of significant uncertainties regarding the determinants of the antibody response, particularly among individuals with low baseline GBS antibodies. Moreover, our findings align with the recent initiative aimed at standardizing measurement methods to enhance direct comparison and extrapolation of results.

Our main finding in **Paper III** was that antibody levels in serotype III LOD-cases were lower than in the healthy control infants. Additionally, the placental transfer ratio in all GBS serotype III cases were lower than in the healthy control infants. These data are in line with previous hypotheses that low antibody levels are associated with increased risk of GBS infections. However, the lack of similar findings for the EOD cases is more challenging to interpret. It may be partly explained by small sample size, lack of data on IAP usage and high maternal GBS carriage rates in Norway during the study period. These limitations underscore the importance of comprehensive data collection, the need for larger sample size and standardized methodologies in research endeavors aimed at understanding the dynamics of GBS epidemiology and vaccine efficacy.

# 7 Future perspectives

## 7.1 Immunological studies of adjunctive therapy for neonatal sepsis

The immediate immune response in neonatal sepsis relies on various components of the innate immune defense system. However, our understanding of the complement system's role in neonatal inflammation and disease severity is limited [288]. Further research incorporating mechanisms behind observed immune responses could provide a more comprehensive understanding of cytokine responses to bacterial antigens [288]. The assessment of antibacterial efficacy incorporation with the inclusion of live bacteria may enrich future findings, and further support the transition of the “dual inhibition concept” from our work into experimental animal models of neonatal sepsis.

## 7.2 GBS vaccine

Several key factors need to be addressed before a vaccine for a particular disease like Group B Streptococcus (GBS) can be introduced [271, 365].

First, a universally accepted definition of GBS-related disease should be established. This would ensure consistency in diagnosis and reporting across different regions and healthcare systems. This standardization is crucial for accurate surveillance and assessment of disease burden [5].

Secondly, one should implement consistent and comprehensive monitoring systems that enable ongoing surveillance of GBS-related infections globally. This includes standardized protocols for data collection, analysis, and reporting to facilitate comparison and tracking of disease trends over time [242, 366, 367].

Thirdly, maternal anti-GBS antibodies are linked to protection against EOD (**Paper II**) and potentially serotype III LOD (**Paper III)**. However, without a defined efficacy correlate, a phase III trial might be necessary for vaccine licensure. Selecting appropriate trial sites requires high GBS incidence rates in large birth cohorts, along with strong clinical and microbiological diagnostic capabilities [368]. Exploring alternate licensure pathways, like identifying serological protection correlates, followed by phase IV studies on vaccine effectiveness against invasive GBS disease, is also important [368]. However, a randomized, placebo-controlled efficacy trial offers the added benefit of assessing GBS's role in various neonatal complications, including culture-negative sepsis, stillbirths, prematurity, and low birth weight [242, 368, 369].

Fourthly, the access to routine prenatal care is essential for the successful implementation of a future GBS vaccine program [370]. By integrating vaccination into existing prenatal care services, healthcare providers can ensure that pregnant individuals receive timely and appropriate immunization to protect themselves and their newborns [366].

Lastly, an effective communication and education initiatives would aid to inform healthcare professionals, parents, and expectant parents about GBS and the potential benefits of vaccination [371, 372]. This includes raising awareness about the risks associated with GBS infection, the importance of prevention strategies such as vaccination, and addressing any concerns or misconceptions related to vaccination safety and efficacy [371-373].

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# Paper I

Bjerkhaug, A.U., Granslo, H.N., Cavanagh, J.P., Høiland, I., Ludviksen, J.K., Lau, C., … Klingenberg, C. (2023).

## Dual inhibition of complement C5 and CD14 attenuates inflammation in a cord blood model

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BASIC SCIENCE ARTICLE

# Dual inhibition of complement C5 and CD14 attenuates inflammation in a cord blood model

Aline U. Bjerkhaug[1]✉, Hildegunn N. Granslo[1,2], Jorunn Pauline Cavanagh[1], Ina Høiland[1], Judith Krey Ludviksen[3], Corinna Lau[3], Terje Espevik[4], Tom Eirik Mollnes[3,4,5] and Claus Klingenberg[1,2]



**BACKGROUND:** *Escherichia coli* and Group B streptococci (GBS) are the main causes of neonatal early-onset sepsis (EOS). Despite antibiotic therapy, EOS is associated with high morbidity and mortality. Dual inhibition of complement C5 and the Toll-like receptor co-factor CD14 has in animal studies been a promising novel therapy for sepsis.

**METHODS:** Whole blood was collected from the umbilical cord after caesarean section ($n = 30$). Blood was anti-coagulated with lepirudin. C5 inhibitor (eculizumab) and anti-CD14 was added 8 min prior to, or 15 and 30 min after adding *E. coli* or GBS. Total bacterial incubation time was 120 min ($n = 16$) and 240 min ($n = 14$). Cytokines and the terminal complement complex (TCC) were measured using multiplex technology and ELISA.

**RESULTS:** Dual inhibition significantly attenuated TCC formation by 25-79% when adding inhibitors with up to 30 min delay in both *E. coli*- and GBS-induced inflammation. TNF, IL-6 and IL-8 plasma concentration were significantly reduced by 28–87% in *E. coli*-induced inflammation when adding inhibitors with up to 30 min delay. The dual inhibition did not significantly reduce TNF, IL-6 and IL-8 plasma concentration in GBS-induced inflammation.

**CONCLUSION:** Dual inhibition of C5 and CD14 holds promise as a potential future treatment for severe neonatal EOS.

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**IMPACT:**

- Neonatal sepsis can cause severe host inflammation with high morbidity and mortality, but there are still no effective adjunctive immunologic interventions available.
- Adding CD14 and complement C5 inhibitors up to 30 min after incubation of *E. coli* or Group B streptococci in a human umbilical cord blood model significantly reduced complement activation and cytokine release.
- Dual inhibition of C5 and CD14 is a potential future therapy to modulate systemic inflammation in severe cases of neonatal sepsis.

## INTRODUCTION

Despite advances in neonatal medicine, early-onset sepsis (EOS) still remains a significant cause of morbidity and mortality due to cases with severe host inflammation and limited protective responses.[1,2] EOS is caused by vertical transmission of bacteria colonising the gut and the birth canal. Together, *E. coli* and Group B streptococci (GBS) cause approximately 2/3 of all EOS cases. Both bacteria may cause severe disease with high risk of later sequela or death, in both term and in particular preterm infants.[3,4]

The complement system and the cytokine network are key players of the innate immune system, centrally involved in the host inflammatory response.[5] The complement system is activated by three routes; the classical pathway, the lectin pathway and the alternative pathway (Fig. 1).[6,7] These pathways merge at C3, which when activated, binds to bacteria. This opsonisation leads to enhanced phagocytosis and is the main mechanism of the complement system in bacterial defence. Other complement mediated mechanisms are lysis of some Gram-negative bacteria by the membrane form of the terminal C5b-9 complex and through C5a-enhanced synthesis of inflammatory mediators and degranulation of granulocytes.[8] Both *E. coli* and GBS activate the complement system,[7–11] but in severe cases this activation can be excessive and lead to harmful inflammation.[12] During sepsis, cytokine concentrations increase exponentially through a multitude of different pathways, including activation of pattern recognition receptors, e.g. Toll-like receptors (TLRs).[9–11] Under normal conditions, TLR-activations induce a local and self-limited response. However, in sepsis TLR-activation can be improper and uncontrolled, leading to a fatal systemic imbalance.[12]

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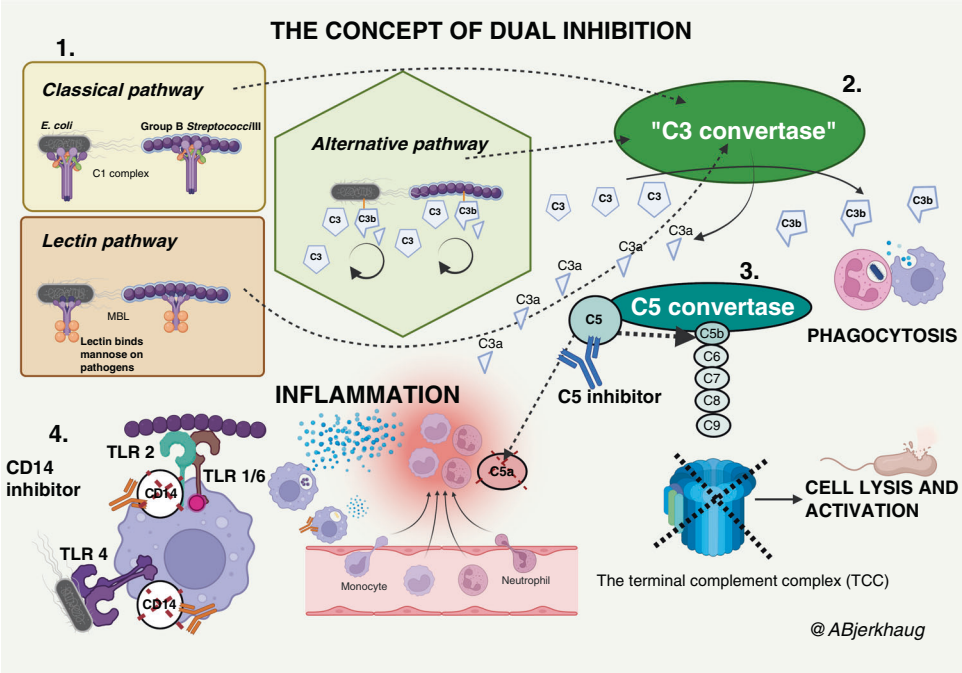


**Fig. 1 Graphical abstract: cartoon of the dual inhibition of complement C5 and the TLR CD14 co-receptor.** (**1**) *E. coli* and GBS entering the bloodstream are rapidly opsonized by complement components and immunoglobulins. (**2**) C3 convertases cleave the central and the most abundant component of the complement system, complement C3, that gives rise to the inflammatory fragment C3a and opsonisation fragment C3b. (**3**) The membrane form of the terminal C5b-9 complex induces lysis of bacteria and in sub-lytic amounts activates cells, while the role of C5a includes enhanced synthesis of inflammatory mediators and degranulation of granulocytes. **(4)** Dual inhibition targeting complement C5 and CD14 reduces inflammatory response without interfering with the C3 opsonisation.

A range of adjunctive inflammatory interventions have failed to show convincing beneficial effects in treatment of neonatal sepsis.[13] Complement factor C5 and the TLR co-receptor CD14 are new potential targets for sepsis therapy.[12] C5 inhibition reduces the proinflammatory effects caused by C5a and the terminal C5b-9 complex (Fig. 1). CD14 is a co-receptor for several TLRs,[14] including TLR2 and TLR4, expressed on macrophages and neutrophils (Fig. 1). CD14 plays an important role in the detection of lipopolysaccharides (LPS) from Gram-negative bacteria, but also other pathogen-associated molecular patterns such as lipoteichoic acids (LTA) from Gram-positive bacteria.[12,14–16] Studies in Gram-negative ex vivo and animal models have indicated that the dual inhibition of C5 and CD14 may be beneficial in attenuating the detrimental effects of complement activation, and to modulate the cytokine storm in fulminant sepsis.[12] Similar beneficial effects have been observed in experimental models with *Staphylococcus aureus*.[17] However, in most of these studies the dual inhibition of C5 and CD14 has been "prophylactically" administered, as proof of concept, before induction of sepsis.[12]

In neonates with sepsis, empiric antibiotic therapy must cover the most commonly seen pathogens, and therapy is often started after the baby has become symptomatic.[18,19] A similar approach would be necessary for empiric immunomodulatory treatment. The main objective of this study was to compare *E. coli*- and GBS-induced inflammation and to evaluate the effects of dual C5-CD14 inhibition in an ex vivo human umbilical cord blood model.

## METHODS

### Study groups and blood collection

Mothers scheduled for an elective caesarean section at the University Hospital of North Norway in the period of October 2019 and September 2021 were invited to participate. In sub-study 1, we collected cord blood samples ($n = 16$) and incubated blood with bacteria for 120 min before analyses (Fig. 2). After an interim analysis of data, we found that the cytokine release after 120 min, especially after GBS incubation, was lower than expected from previous studies with other bacteria (*E. coli* and *S. aureus*).[17,20–23] We then performed new pilot studies in both adult and cord blood (Supplementary Fig. 1a–h). Subsequently we decided to perform sub-study 2 where we collected cord blood samples ($n = 14$) and now incubated blood with bacteria for 240 min before analyses.

### Bacterial strains and culture conditions

*E. coli* strain LE392 (ATCC 33572; Manassas, VA) and a clinical GBS strain, serotype III (SO-SAG18-1, kindly provided by the Norwegian GBS reference laboratory, Trondheim, Norway) were used in all experiments. We slightly adapted a previously described heat inactivation protocol[20] for both bacteria. *E. coli* was grown in Luria-Bertani (LB)-medium and GBS in Todd Hewitt medium overnight, then washed once ($3200 \times g$, 10 min at 4 °C) with 50 mL phosphate-buffered saline (PBS; Sigma-Aldrich, Steinheim, Germany). After resuspension in PBS, *E. coli* and GBS were separately heat-inactivated for 1 h at 60 °C. Growth controls after heat-inactivation confirmed that all bacteria were killed. *E. coli* and GBS were batched and frozen at −70 °C.

Upon use, the heat-inactivated strains (*E. coli* and GBS) were thawed and washed six times in PBS, as described above. Absolute bacterial counts were obtained by diluting the bacteria 1:500 with PBS, followed by transfer of 2450 µL to a tube designed for compatibility with the flow cytometer. We added 50 µL CountBright® (Life Technologies Corporation, OR). The samples were run on a flow cytometer (BD Biosciences, NJ) and the concentration of *E. coli* and GBS were calculated using the formula provided in the CountBright® instruction manual. A batch suspension of $7.17 \times 10^7$ *E. coli*/mL PBS and $6.17 \times 10^7$ GBS/mL PBS was made and kept at 4 °C for up to maximum four months.

### Inhibitors

The complement C5 inhibitor, eculizumab (Soliris®) was obtained from Alexion Pharmaceuticals (Boston, Ma). The recombinant anti-human CD14 IgG2/4 antibody (r18D11) and an isotype-matched control were produced in our laboratory, as previously described.[24]

### Ex vivo human cord blood model

Both the original ex vivo whole-blood model[25] and the post-challenge model[22] have been described in detail previously. The major advantage of

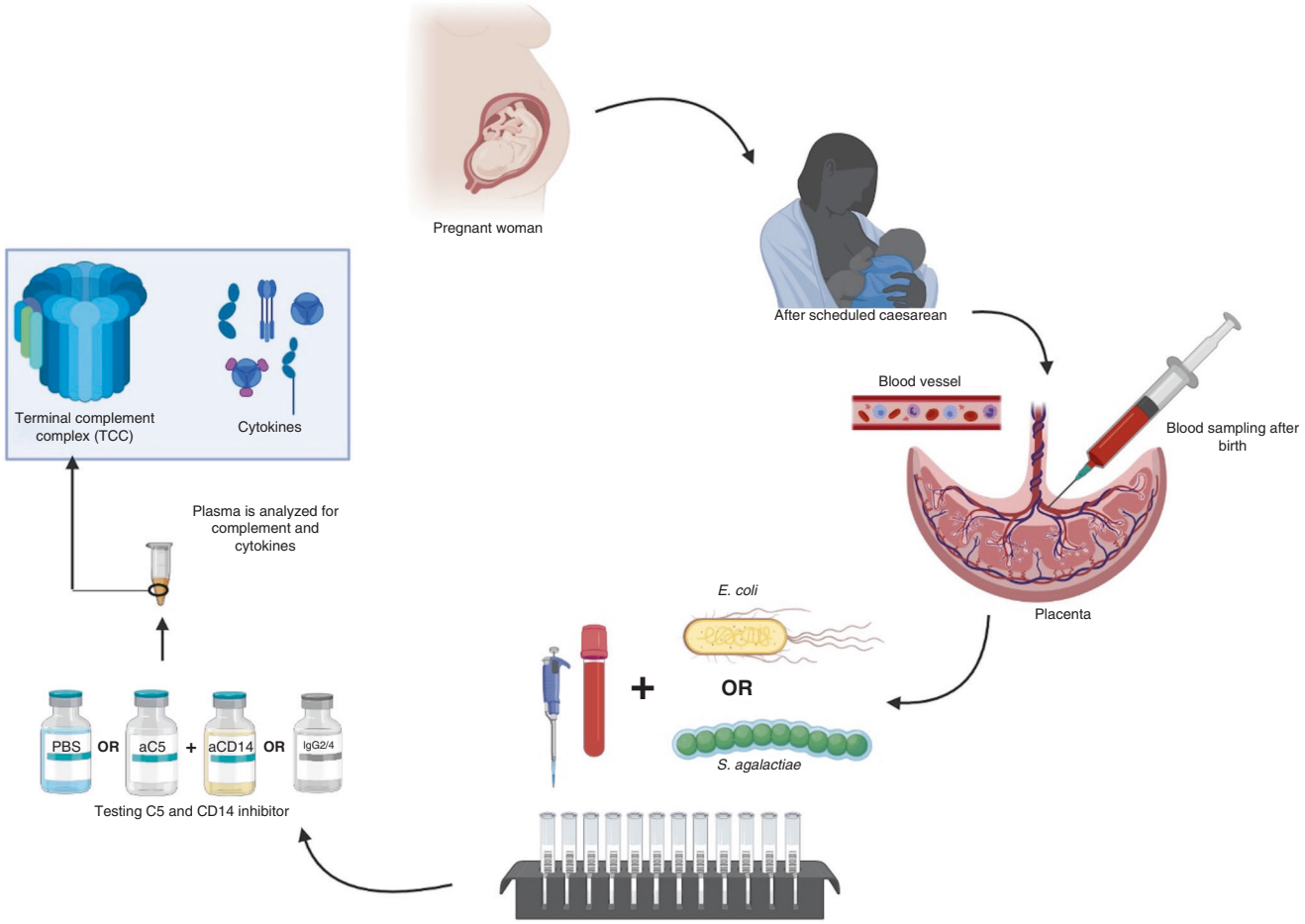


**Fig. 2** Graphical abstract: cartoon of the experimental set-up of the study.

the ex vivo human whole-blood model is the use of the thrombin-specific inhibitor lepirudin, which does not interfere with the complement system or the inflammatory network, in contrast to other frequently used anticoagulants such as EDTA, citrate and heparin.[24]

A time course for our study is shown in Supplementary Fig. 2. In the current study, we aimed to optimise cord blood sampling volumes, but volumes obtained were usually just enough for all the planned analyses including controls and inhibition experiments. All cord blood was drawn into endotoxin-free 4.5 mL NUNC tubes (Thermo Fischer Scientific, Roskilde, Denmark) and lepirudin (Refludan®, Pharmion, Windsor, UK) was added to a concentration of 50 µg/mL blood. Several pilot experiments in adult blood were performed before the main study in order to assess the effect of single versus dual inhibition and to compare bacterial challenge with $10^7$ GBS bacteria/mL versus $10^8$ bacteria/mL (Supplementary Fig. 3a–h).

In sub-study 1, the baseline sample (T0) was processed less than 20 min after the blood was drawn. Combined inhibitors eculizumab (final concentration 100 µg/mL blood) and anti-CD14 (final concentration 15 µg/mL blood) or isotype-matched control IgG2/4 (final concentration 15 µg/mL blood), were added to separate tubes at each of the following time points: 8 min prior to, and 15 and 30 min after adding *E. coli* or GBS to a final concentration of $10^7$ bacteria/mL whole blood.[22,25] Two positive controls were incubated with either *E. coli* or GBS. The negative control was incubated with PBS only. All samples were incubated in a Rotamix Intelli-Mixer (Norengros, Oslo, Norway) with rotation of blood at 37 °C for 120 min after adding bacteria or PBS. Complement activation was stopped by placing the samples on ice and adding EDTA (Sigma-Aldrich, Steinheim, Germany) to a final concentration of 20 mM. The samples were centrifuged for 20 min at 3000 × *g* at 4 °C. Plasma was collected and stored at −70 °C until analysed. Sub-study 2 followed the same protocol, but the samples were incubated for 240 min instead of 120 min.

### Cytokine multiplex assay

Pro-inflammatory cytokines (TNF, IL-6 and IL-8) were measured using a multiplex cytokine assay (Bio-Rad Laboratories, Hercules, CA). The assay was performed according to the manufacturer's instruction.

### Enzyme immunoassays for complement activation products

The soluble terminal C5b-9 complement complex (TCC) assay was performed according to a method developed in our laboratory and described in detail previously.[26] In short, the principle of the TCC assay is based on a monoclonal antibody aE11 reacting with a neoepitope expressed in C9 only after it is activated and incorporated into the C5b-9 complex. TCC concentrations are reported as complement activation units (CAU)/mL.[26]

### Data presentation and statistics

GraphPad Prism version 9.2.0 (GraphPad, San Diego, CA) was used for statistical analysis and presentation. Descriptive results are presented as means with standard deviation (SD) and medians with range or interquartile range (IQR; 25–75 percentiles). When comparing the effect of dual inhibition of *E. coli* or GBS induced inflammation at different time points with the positive control group, the results were analysed by the non-parametric Wilcoxon matched-paired signed-rank test. Percentage inhibition of the positive control is presented related to the negative control as baseline. A $p$ value <0.05 was considered statistically significant for all analyses.

## RESULTS

Thirty mothers were included in this study. Two had mild preeclampsia, 1 had a well-controlled diabetes mellitus type 1, 1

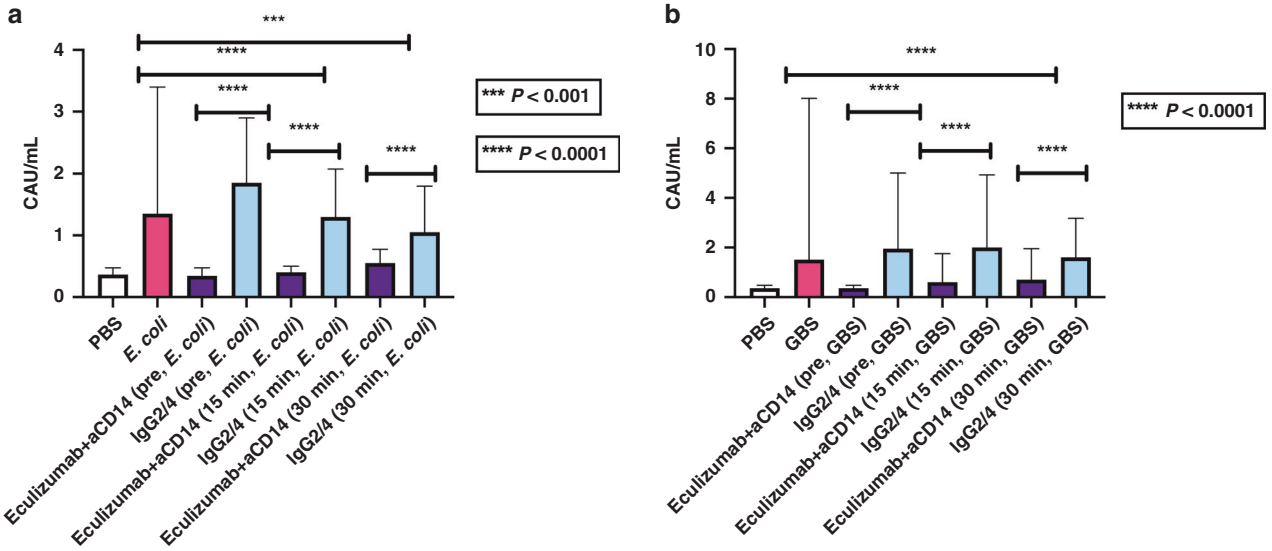


**Fig. 3 TCC cord plasma concentrations after 120 min incubation time.** Cord plasma concentration of TCC after 120 min incubation time with *E. coli* (**a**) and group B streptococci (**b**), and dual inhibition with complement C5 and co-receptor CD14 inhibitors, pre- and post-bacterial challenge (Sub-study 1; cord blood $n = 16$). Graphic presentations of bar charts with median and IQR values. PBS phosphate-buffered saline, Pre 8 min prior to adding bacteria, 15 and 30 min after adding bacteria, TCC terminal complement complex.

had mild anaemia, 1 had a benign intracranial hypertension, and the other 25 were healthy. Scheduled caesarean delivery was performed at mean (SD) 38.1 ± 0.5 weeks gestation; 18 girls and 12 boys. The mean (SD) birth weight for the 30 babies was 3316 ± 521 g. All Apgar-5 min scores were 9 or 10. No infants were admitted to the neonatal intensive care unit. Mothers in sub-study 2 ($n = 13$) had mean (SD) total white blood count $8.1 \pm 1.6 \times 10^9$/L and mean (SD) neutrophils $5.8 \pm 1.8 \times 10^9$/L. Corresponding values in cord blood ($n = 14$) were mean (SD) total white blood count $12.3 \pm 3.2 \times 10^9$/L and mean (SD) neutrophils $5.9 \pm 2.1 \times 10^9$/L.

Median (IQR) TCC cord plasma concentration after 120 min incubation with *E. coli* was 1.4 (0.7–3.6) CAU/mL, which was similar to concentrations of 1.5 (1.0–9.1) CAU/mL after 120 min incubation with GBS (Fig. 3a, b). The stimulated TCC concentrations were significantly increased compared to negative control at 0.4 (0.1–0.5) CAU/mL ($p < 0.001$). Incubation of bacteria for 240 min in sub-study 2 (Fig. 4a, b) also showed similar TCC cord plasma concentrations with *E. coli* (2.4 [1.5–4.3] CAU/mL) and GBS (1.4 [0.8–3.1] CAU/mL), and significantly higher TCC after bacterial incubation than in the negative controls. Dual inhibition of C5 and CD14 effectively reduced cord TCC plasma concentrations when administered before bacterial challenge, but also when added 15- and 30-min post-challenge after both 120- and 240-min bacterial incubation (Figs. 3 and 4).

Plasma concentrations of TNF, IL-6 and IL-8 after 240 min incubation with *E. coli* or GBS are shown in Fig. 5. There was a significant increase in the plasma concentrations of all cytokines after incubation of cord blood with *E. coli* or GBS (Fig. 5). Incubation with *E. coli* elicited significantly higher TNF, IL-6 and IL-8 levels compared to GBS. Significant reductions in TNF, IL-6 and IL-8 concentrations after the dual inhibition established 8 min prior to, and 15 and 30 min after *E. coli* challenge are summarised in Fig. 5a, c, e. The dual inhibition also resulted in lower crude concentrations of IL-6 after GBS challenge, but differences were not significant (Fig. 5b). For IL-8 and TNF there were no clear effects of dual inhibition after the GBS challenge.

## DISCUSSION

This study shows that *E. coli*- and GBS-induced complement activation in cord blood is significantly reduced after dual inhibition of complement C5 and CD14 up to 30 min after bacterial challenge. Moreover, dual C5 and CD14 inhibition in the post-challenge experiments also significantly reduced TNF, IL-6 and IL-8 plasma concentration in cord blood after *E. coli*-induced inflammation. The uniqueness of the ex vivo cord blood model with both complement and cytokine biomarkers makes this study a novel contribution to the understanding of acute innate inflammatory response in EOS, and lay the grounds for further investigations of a potential new adjunctive therapy.

The immediate immune response in neonatal sepsis depends on the multifactorial components, which together make up the innate immune defence system. However, there is still limited data on the impact of the complement system and its role in neonatal inflammation and disease severity.[14] Low concentration of ficolin-3 and mannose binding lectin (MBL), factors activating the lectin complement pathway, have been associated with increased susceptibility to infections.[27,28] In a case-control study, neonates with Gram-positive sepsis had significantly lower ficolin-3 cord blood concentrations than controls, whereas infants with Gram-negative sepsis had lower MBL cord blood concentrations.[27] In contrast, maternally transferred immunoglobulins targeting GBS serotype III can weaken the bacterial capsule, facilitate C3b deposition and thereby enhance complement activation.[7] In our study, we focused on the general activation of the complement system by analysing the TCC concentration, which is regarded to be the best single indicator of complement activation.[29] We found a strong complement activation in cord blood after bacterial challenge with *E. coli* and GBS. High levels of TCC have been observed in adult patients with sepsis complicated by disseminated intravascular coagulation.[30] A factor to consider in our study is that we included mothers and neonates with predominant Caucasian ethnicity. Concentrations of ficolin-3 and MBL can be affected both by intrauterine infections and genetic factors. MBL-deficiency affects e.g. about 30% of the white population.[27]

Neonatal sepsis is associated with an early inflammatory response that is less robust compared to children and adults, but not necessarily less lethal.[31] In our initial experiments (sub-study 1) the bacterial incubation period was 120 min, and we found only a modest cytokine release, in particular after GBS stimulation. A previous cord blood in vitro study reported a

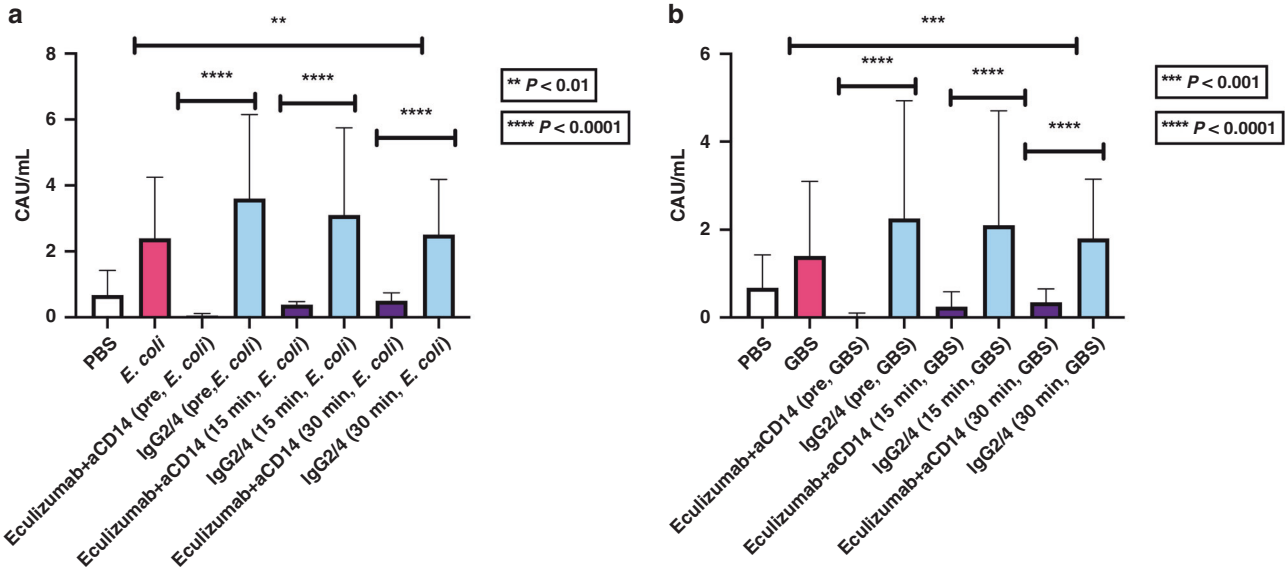


**Fig. 4 TCC cord plasma concentrations after 240 min incubation time.** Cord plasma concentration of TCC after 240 min incubation time with *E. coli* (**a**) and group B streptococci (**b**), and dual inhibition with complement C5 and co-receptor CD14 inhibitors, pre- and post-bacterial challenge (Sub-study 2; cord blood, $n = 14$). Graphic presentations of bar charts with median and IQR values. PBS phosphate-buffered saline, Pre 8 min prior to adding bacteria, 15 and 30 min after adding bacteria, TCC terminal complement complex.

time-dependent release of proinflammatory cytokines, where TNF was detected first (after 60–120 min), followed by increased concentration of IL-8, IL-6 and IL-1β.[32] We therefore extended the bacterial incubation period to 240 min (sub-study 2) and then detected markedly higher TNF, IL-6 and IL-8 concentrations after bacterial challenge compared to at 120 min. We also found a higher cytokine release after challenge with *E. coli* versus GBS. This stands in contrast to Mohammed et al. who reported no significant difference in cytokine release between the same two bacteria.[32] Other studies also report a high neonatal cytokine release in response to pathogenic *E. coli*, in particular in the preterm infant.[33,34]

There are challenges when comparing our results with previous studies. Many studies report data from in vitro models or studies with isolated blood cells. In our ex vivo model we evaluate the results of the inflammatory crosstalk between a range of cell lines, which is an approach closer to the biology of the innate immune system, and thus theoretically closer to the pathophysiology of neonatal sepsis. Specific factors that may explain variations from other studies are preparation of blood samples, types of stimulators, duration of incubation of blood samples and different assays used for cytokine detection. Experimental studies claiming to mimic EOS vary from in vitro to ex vivo models. In our ex vivo whole blood model we do not claim to have a sepsis model, but a model that reflects the complement activation and release of central pro-inflammatory cytokines induced by bacteria commonly causing EOS in neonates. Using lepirudin as anticoagulant improves the translational value of our results. We found lower cytokine release in our study compared to studies using in vitro models[35–38] suggesting a limitation of the reductionistic in vitro models.[32] This is a further argument to pursue the use of ex vivo models before moving to animal models.[25] We chose the post-challenge approach since it would be more clinically relevant to start empiric therapy after the patient has experienced a septic insult. However, due to limited cord blood we did not include all the timepoints used in our previous post-challenge model.[22] We found that adding inhibitors after 15 and 30 min would be more relevant than already after 5 min. In order to compare our model to previous whole blood models, we also include a pre-challenge timepoint as a reference point. It is common to allow between 5 and 10 min of stabilisation for an active drug before a sepsis challenge,[24,25] so we selected 8 min as the appropriate pre-incubation period in this study.

Activation of the complement system plays an important role in sepsis pathophysiology.[39] Complement acts as a first-line sensor of danger and may accentuate the inflammatory explosion, in an orchestrated effort with other first-line sensors like TLRs.[39] We therefore decided to use an "empiric" approach with dual inhibition of both the C5 molecule of the complement system and CD14, an important co-receptor for TLRs. It is well known that anti-CD14 has no effect on the complement system.[22] However, the dual inhibition of C5 and CD14 was used for all experiments to simplify the protocol, and also because it was not feasible to include separate inhibition studies in the complex experimental set up with limited blood volume. Previous studies have targeted complement C3 because of its central role in the response amplification. However, even though C3 inhibition strongly reduce inflammation, it may also lead to an increased risk of infection by inhibiting the main complement protection through C3-opsonization.[12] Therefore, we chose to use a C5 inhibitor. Inhibition of C5 prevents formation of C5b that induce the assembly of C5b-9 and most importantly, it prevents formation of the potent proinflammatory complement protein C5a. Moreover, this approach does not affect the opsonization of microbes by C3b.[12] Our results are in line with previous studies showing that C5 inhibition significantly reduces TCC plasma concentration in *E. coli*-induced inflammation.[12] Similar to a previous post-challenge whole blood study,[22] we observed a significant inhibition of TCC even when the dual inhibition was added up to 30 minutes after the bacterial challenge, and the effect was similar in *E. coli*- and GBS-induced inflammation. These are promising results for the potential use of C5 inhibition for modulation of the neonatal immune system to reduce sepsis mortality and morbidity. Keshari et al. have already shown that inhibition of C5 protects against organ failure and reduces mortality in a baboon model of *E. coli* sepsis.[40] Another advantage of C5 inhibition is that drugs, such as eculizumab, are already in widespread clinical use, and with relatively good safety data in other paediatric conditions.[41,42] Still, there is some concern that persistent C5 inhibition, due eculizumabs long elimination half-life, potentially may lead to an

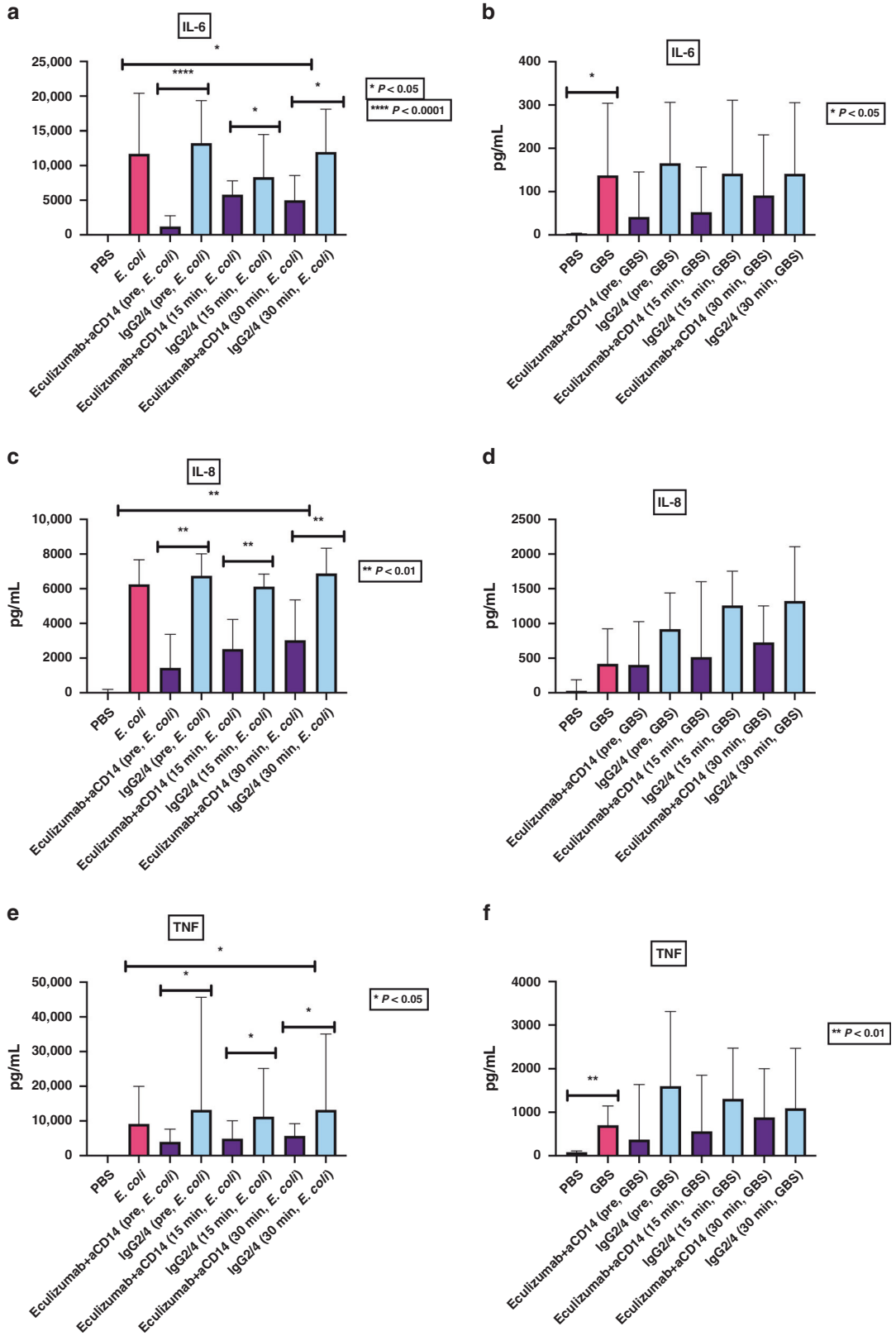


**Fig. 5 Cytokine cord plasma concentrations after 120 min incubation time.** Cord plasma concentration of IL-6, IL-8 and TNF after 240 min incubation time with *E. coli* (**a**, **c**, **e**) and group B streptococci (**b**, **d**, **f**), and dual inhibition with complement C5 and co-receptor CD14 inhibitors, pre- and post-bacterial challenge. (Sub-study 2; $n = 14$). Graphic presentations of bar charts with median and IQR values. PBS phosphate-buffered saline, Pre 8 min prior to adding bacteria, 15 and 30 min after adding bacteria.

increased risk of subsequent infections. Thus, C5 inhibition in acute sepsis should optimally be treated with C5 inhibitors having shorter elimination half-life.

A previous study has shown that IL-8 release in an LPS-induced inflammation model was significantly reduced by anti-CD14.[43] In our model we showed a significant, but modest reduction of the TNF, IL-6 and IL-8 release after dual inhibition to the *E. coli* challenge. We did not observe any significant reduction in the already quite low cytokine release in response to the GBS challenge. Skjeflo and co-workers showed that the simultaneous inhibition of CD14 and complement efficiently reduced the inflammatory response induced by various strains of *Staphylococcus aureus* in a similar human whole blood model, as used in our experiments.[17] However, in contrary to Gram-negative-induced inflammation, the responses were primarily dependent on complement, whereas CD14 inhibition played a less important role in the Gram-positive *S. aureus* model.[17] Our results point to similar findings for GBS-induced inflammation, and we found no obvious inhibition of cytokine release using the dual inhibition in the GBS-arm of our study. However, animal studies of polymicrobial sepsis have shown clear beneficial effects of the dual C5 and CD14 inhibition with improved hemodynamic parameters, and morbidity and survival, and the dual inhibition may thus be relevant for a broader range of sepsis pathologies.[44,45]

Our study has strengths and limitations. The most obvious strength is the ex vivo model assessing the innate immune response in a system with fresh cord blood containing both cellular and humoral immune response components. We assessed a novel immunological approach with promising results in adult and animal pre-challenge models and found similar potential beneficial effects in the neonatal inflammatory ex vivo model. However, the study also has limitations. First, our model did not allow us to address some of the known intracellular and extracellular bacterial antigen-specific mechanisms which can be done in in vitro experiments.[14] Second, five mothers with well-managed underlying medical conditions were included, but baseline values and post challenge values (complement and cytokines) of these five did not deviate from the remaining 25 included. Third, other studies have used a higher GBS load of $10^8$ CFU/mL or extended observation time up to 24 h, and these authors suggested that this is necessary to mimic the first stages of neonatal sepsis.[46] However, a GBS concentration of $10^7$ GBS/mL should be adequate, and indeed the median IL-6 value observed after GBS challenge was markedly above a suggested cut-off levels for neonatal sepsis.[47] Fourth, due to limited blood volume and the complexity of the experimental set-up it was not possible to add an experimental part using eculizumab and anti-CD14 as separate and single inhibition agents. Finally, due to the complexity of the experimental protocol and the need to activate the blood within 20 min of sampling we decided to use heat-inactivated bacteria and limit the outcomes analysed, like in other studies.[17,20–23] Previous vaccine studies have shown that heat inactivated GBS elicit a clear immune response, and our findings confirm this.[48] Moreover, heat-inactivated GBS induce TLR2-dependent antimicrobial gene activation,[49] and neither LPS nor LTA are destroyed by the heat inactivation process. However, the heat-inactivated GBS and *E. coli* strains/serotypes used in the current study may not be representative for all GBS and *E. coli* strains that infect neonates.

In conclusion, promising results in this umbilical cord blood inflammation model, using bacteria with clinical relevance for EOS, along with similar findings in previous adult blood models and animal studies indicate that the dual inhibition of C5 and CD14 might be a future approach for treating severe cases of neonatal sepsis. Further experiments in animal models may be the first step to assess this novel strategy.

## DATA AVAILABILITY

The data sets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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## AUTHOR CONTRIBUTIONS

Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data: A.U.B., H.N.G., J.P.C., I.H., J.K.L., C.L., T.E., T.E.M., C.K. Drafting the article: A.U.B. and C.K. Critically revised the manuscript and approved the final version to be published: all authors.

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## COMPETING INTERESTS

The authors declare no competing interests.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Regional Ethical Committee (2019/834/REK nord). All participating women gave informed written consent to participate in the study. All participants signed a written consent.

## ADDITIONAL INFORMATION

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# Paper II

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## The immunogenicity and safety of Group B Streptococcal maternal vaccines: A systematic review

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# The immunogenicity and safety of Group B Streptococcal maternal vaccines: A systematic review

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ABSTRACT

*Purpose:* To systematically review immunogenicity and safety data of maternal group B streptococcal (GBS) vaccines in published clinical trials until July 2023.
*Methods:* EMBASE, MEDLINE, Cochrane Library and clinicaltrial.gov. databases were searched for clinical studies that reported immunogenicity and/or safety of GBS vaccine in non-pregnant adults, pregnant women and infants between 1st of January 1996 to 31st of July 2023. Pairs of reviewers independently selected, data extracted, and assessed the risk of bias of the studies. Discrepancies were resolved by consensus. (PROSPERO CRD42020185213).
*Results:* We retrieved 1472 records from the literature search; 20 studies and 6 sub-studies were included, involving 4440 non-pregnant participants and 1325 pregnant women with their newborns. There was a significantly higher IgG Geometric Mean Concentration (GMC) and IgG placental transfer ratios in vaccinated compared to placebo groups, with peak response 4–8 weeks after vaccination. Placental transfer ratio varied from 0.4 to 1.4 across five studies. The different clinical trials used different assays that limited direct comparison. There were no significant differences in the risk of serious adverse events (adjusted OR 0.73; 95 % CI 0.49–1.07), serious adverse events leading to withdrawal (adjusted OR 0.44; 95 % CI 0.13–1.51), and systemic illness or fever (adjusted OR 1.05; 95 % CI 0.26–4.19) between the vaccine and placebo groups.
*Conclusions:* The published clinical trials show significant IgG GMC response in subjects receiving the conjugated capsular polysaccharide and surface subunit protein vaccines compared to placebo. In current clinical trials of experimental GBS maternal vaccines, there have been no observed serious adverse events of special interest directly linked to vaccination.

## 1. Introduction

Group B streptococcus (GBS) or *Streptococcus agalactiae* is widely recognized as the primary cause of severe bacterial infections in newborns during the initial weeks following birth [1–3]. Every year, it is estimated that around 200,000 newborns worldwide are affected by early-onset GBS disease and approximately 160,000 newborns affected by late-onset GBS disease. Maternal and infant GBS disease is also associated with approximately 2 million stillbirths, nearly 0.5 million preterm births, at least 91,900 deaths in children, and over 37,000 cases of moderate to severe neurodevelopmental impairment in children who survive invasive GBS infections [4].

Research on GBS vaccines started almost five decades ago by demonstrating a correlation between level of GBS antibodies and risk of neonatal infection [5–8]. Several GBS virulence factors have been identified as potential vaccine candidates, including the GBS capsular polysaccharides (CPS) and key surface subunit proteins. All 10 CPS-serotypes of GBS can cause disease [9], but the prevalence of the different CPS-serotypes varies worldwide [10,11]. The six CPS-serotypes Ia, Ib, II, III, IV and V are responsible for the majority of invasive infections and are included in the current vaccines in development [1,3,12]. GBS surface subunit proteins, such as Alp family proteins,

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serine-rich repeat proteins, C5a peptidase, and pilus islands, are also associated with invasiveness of GBS strains and are included in vaccines in various stages of clinical development [13–16].

Maternal vaccination leads to increased placental transfer of maternal antibodies [17]. This approach is employed to safeguard infants against many infections e.g. pertussis [18,19], tetanus [20], coronavirus 2 (SARS-CoV-2) [21], and influenza [22]. The development of a successful maternal GBS vaccine has great potential to alleviate the global burden of invasive GBS infections and to reduce antibiotic use in labour [1,3,10]. The purpose of this review is to systematically review and evaluate immunogenicity and safety data of maternal GBS vaccines in published clinical trials until July 2023.

## 2. Methods

This review follows the updated Preferred Reporting Items for Systematic Reviews and Meta-Analyses [23] and is registered in the international prospective register of systematic reviews; PROSPERO ID: CRD42020185213.

### 2.1. Search strategy and selection criteria

We identified articles by searching electronic databases EMBASE, MEDLINE, Cochrane Library and clinicaltrial.gov. from 1st of January 1996 up to the 31st of July 2023, with the search terms in the following combinations: “Streptococcus agalactiae” OR “Streptococcus Group B” OR “GBS6″ OR ”GBS“ AND ”Vaccine“ OR ”Streptococcal vaccine“ OR ”Maternal vaccine“ OR ” Maternal immunization“ OR ”Maternal immunization“ OR ”Active immunization“ OR ”Active immunization“ OR ”conjugate“ OR ”trivalent“ OR ”second dose“ OR ”immunogenicity“. Identified studies were collated and duplicates/triplicates were manually removed. All English-language published clinical trials (randomised and non-randomised) were eligible if they included an experimental GBS vaccine and reported on immunogenicity of the vaccine in human participants. The exclusion criteria were animal studies, studies dealing with screening and epidemiology, cost-effectiveness and attitudes towards a potential GBS vaccine. We also excluded studies reporting data solely on non-conjugated CPS vaccines, as non-conjugated CPS vaccines have been shown to be clearly inferior to conjugated CPS vaccines [24]. Full-text was read for studies eligible for inclusion to verify its suitability for inclusion. Reference lists of included studies and recent reviews were examined to identify additional studies. We did not conduct searches in the “grey literature”, i.e. unpublished studies, non-peer reviewed studies, conference abstracts and studies not indexed in high-quality databases.

### 2.2. Data extraction

Two reviewers (A.U.B. and S.R.) screened titles and abstracts independently according to predetermined inclusion and exclusion criteria, with disagreements between the reviewers being resolved through consensus with the third author (C.K.). We extracted the following variables: paper identification (title, first author and publication year), study design, inclusion and exclusion criteria, characteristics of the population (pregnant or non-pregnant adult, adult or infant, average age/gestation and week/day after delivery), study site for clinical trials, characteristics of the vaccines, characteristics of analytical assays, antibody response after vaccination, placental transfer ratio of GBS antibodies and adverse events after vaccination.

### 2.3. Data synthesis and analysis

The main outcomes assessed were immunogenicity defined as vaccine-elicited geometric mean antibody concentration (GMC), and vaccine efficacy if possible. Immunogenicity data were not possible to *meta*-analyse, and are therefore presented descriptively for each study. As secondary outcomes, we evaluated other immunological responses (e.g. opsonophagocytosis, geometric mean fold rise of GBS antibodies), placental transfer ratio and adverse events (AEs). We evaluated the reported AEs in all studies comparing participants that received a conjugated CPS or surface subunit protein-based vaccine versus those who received placebo. If studies reported data on AEs separately for adjuvanted or non-adjuvanted vaccines, we selected the data on AEs from adjuvanted vaccines. Many studies reported on AEs at different vaccine doses, but we collated these together when analysing the number of AEs in the vaccine group. AEs were reported differently in studies performed more than 15–20 years ago compared to more contemporary studies. Some of the more recent trials [25–33] have used the extensive MedDRA system to present AE data [34]. Three authors (A.U.B, C.K and R.M) assessed AEs independently and compared the findings. In order to obtain similar and comparable AE data across both older and more recent vaccine trials we report rates of the following AEs; serious AEs, AEs leading to withdrawal from the vaccine study, fever/systemic illness in relation to vaccine administration and vaccine-related death. Disagreements were discussed and resolved by consensus. AE data were *meta*-analysed using the online platform recommended for Cochrane intervention reviews (RevMan Web). We calculated risk rations (RRs) with 95 % confidence intervals (CI) for the AEs. We present the effect-estimates by using the random-effect model due to assumption of clinical and methodological diversity among the studies, subsequently often leading to statistical heterogeneity. Reactogenicity data were not possible to *meta*-analyse and therefore presented descriptively for each study.

### 2.4. Risk of bias of included studies

We used version 2 of the Cochrane risk-of-bias tool for randomized trials (RoB 2), with five domains of bias, to assess study quality [35]. The clinical studies were assessed by the adherence to the intervention (the “per-protocol” effect) and we evaluated the failures in implementing the intervention that could have affected the outcomes.

## 3. Results

### 3.1. Study selection

We retrieved 1472 records from databases and an additional 5 records from citations of reference lists. From these 1477, 48 studies were eligible for full-text review. The majority of excluded studies were published protocols, animal studies and preclinical studies. After full-text review we ended up including 26 publications of which 20 reported data from a main study [25–33,36–46] and six reported data from a sub-study of the main study [47–52]. Fig. 1 demonstrates the selection process of the included main studies and sub-studies.

### 3.2. Characteristics of the included studies

The 20 main clinical studies included a total of 5765 participants, of which 1325 were pregnant women. The characteristics of included studies and the main findings are summarized in Table 1. All studies were either Phase 1 or 2 trials. Nine of the included studies were double-blind randomized controlled trials (RCT) [33,36–43], eight were observer-blind randomized trials [25–28,30–32,46] and three were non-randomized open label trials [29,44,45]. All studies reported data on the elicited GBS-IgG response, except for one study that focus on vaginal GBS colonization [28]. Nine studies evaluated the GBS type-specific opsonophagocytic killing in adult study participants [36–38,41–44,50,51] and one study evaluated this only in sera from infants [40].

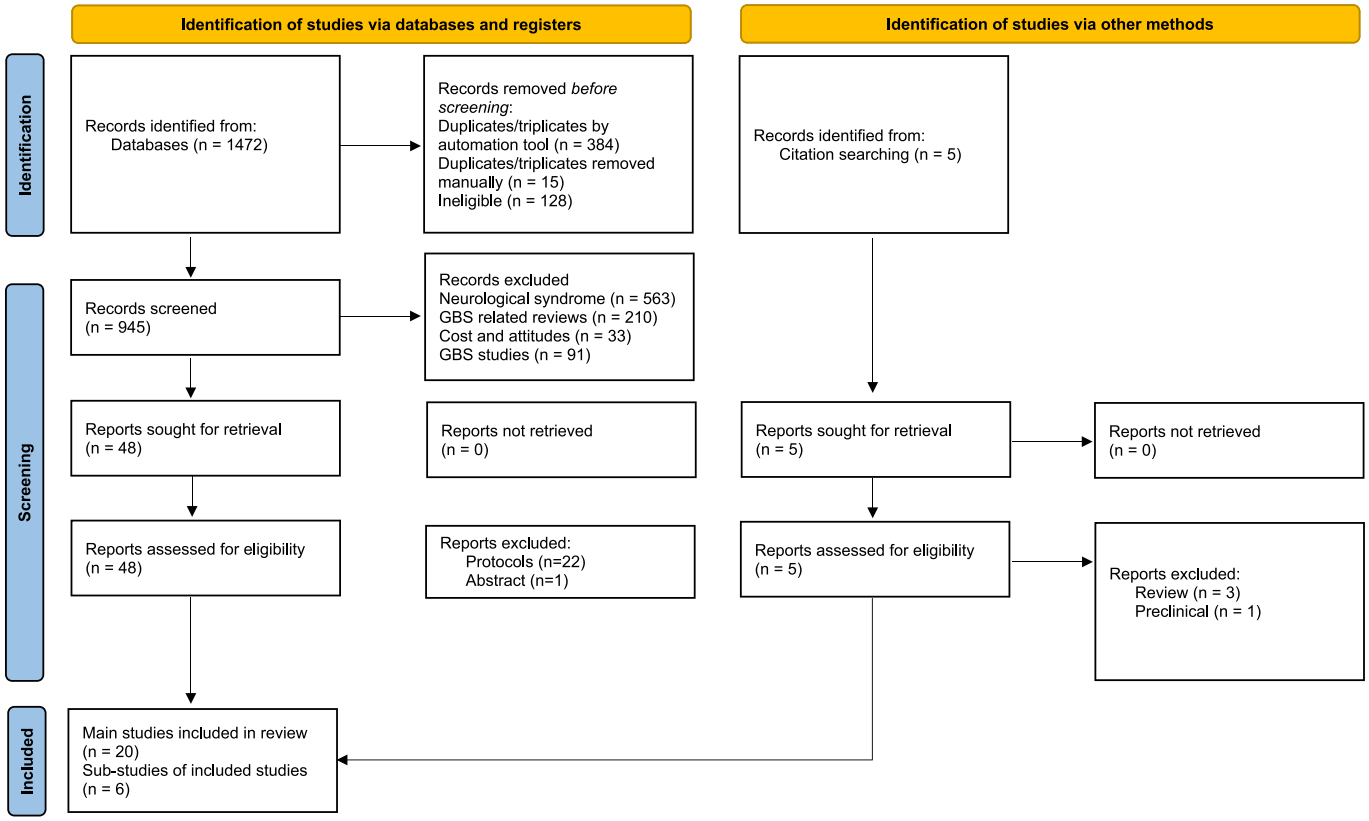


**Fig. 1.** PRISMA overview of systematic search results.

### 3.3. Risk of bias

Fig. 2 shows a summary of findings from the risk of bias assessment of the primary outcome immunogenicity. The overall risk of bias was rated as low for immunogenicity data in 12 of the 20 main studies [25–33,44–46]. Eight studies were downgraded to "some concerns" because they had insufficient information about whether the data was analyzed according to a predetermined analysis plan before unblinded outcome data became available for analysis [36–43]. Despite two studies [44,45] being open label and having a high risk of bias, and one study [29] being partially non-randomized and scoring "some concerns" in "Domain 1″, it was unanimously agreed that the open-label nature of these studies would not impact the immunogenicity data based on the judgement of the other domains.

### 3.4. Immunogenicity

Most studies showed that the GBS antibody GMC response peaked around 4–8 weeks after vaccine administration in healthy adults and pregnant women [31,32,36–43,45]. However, among pregnant women who received vaccinations, three studies reported that the levels of antibodies continued to increase for a minimum of 3 months after childbirth [25,26,31]. The GMC response remained markedly elevated compared to placebo up to 6–12 months after vaccination in both healthy adults and pregnant women [26–28,32,36–39,41–44,46]. Three studies evaluated antibody levels in infant serum during the first 3–6 months after birth [25,31,45]. One of these studies showed a GBS antibody half-life of 42 days in infants without HIV infection [45]. The other two studies found that infant antibody levels were 22–25 % of birth levels three months after birth [25], and while IgG GMCs in vaccinated infants declined with age, they remained 3–9 times higher than in the placebo group at day 90 [31]. One study found that breast milk sIgA GMCs were significantly higher in the Ia/Ib/III-vaccine group compared to the placebo group [31]. In five studies, including the surface subunit protein vaccine, the GMC response was dose-dependent [33,37,38,50] and correlated with *in vitro* opsonophagocytic activity [37,38,43,50]. In four other CPS vaccine dose–response studies there were no significant differences when increasing the dosage [26,27,32,44]. However, while the hexavalent CPS vaccine did not demonstrate a significant difference in testing various doses in non-pregnant adults [32], the interim descriptive analysis of the recent vaccine study in pregnant women suggests that the immune response in pregnant women was dose dependent [46].

The conjugated vaccines included in this review utilized diphtheria (D) toxoid, tetanus (T) toxoid or CRM197 (a non-toxic variant of diphtheria toxin) as conjugates. In the trials comparing a non-conjugated versus a conjugated GBS type-specific CPS vaccine there was a significant higher increase in the IgG GMC response in recipients of the conjugated vaccines versus the unconjugated vaccines [36–38,44]. The response for the conjugated CPS vaccine showed lower levels of IgG GMC in the HIV-infected pregnant women and their infants, compared to the HIV-uninfected pregnant women and their infants (44). A clinical trial investigating the surface subunit protein vaccine in immunocompromised women (NCT04596878) has been completed, but the results are not yet published.

A variety of adjuvants were used in many of the trials including aluminium salts [27,39] or oil-in-water emulsion adjuvant (e.g. MF59 ®) [27]. For the CPS vaccine studies, these adjuvants did not clearly increase immunogenicity [27,39]. In contrast, the surface subunit protein vaccine adjuvanted with aluminium hydroxide elicited a significantly higher GMC response compared to the same vaccine without adjuvant [33]. One study compared the effect of a fully liquid versus a lyophilized formulation of a trivalent (serotypes Ia, Ib and III) GBS vaccine, and found no differences in IgG GMCs 30 days after receiving the single-dose administration of each vaccine formulation in healthy non-pregnant women [30]. A detailed summary of the immunogenicity

**Table 1**
Included clinical studies om maternal GBS vaccines, immunogenicity data and placental transfer ratio.

| Ref. nr. | Main study First author, year, country | Sub-study | Vaccine antigens and dose | Population | N | Intervention | **1.** Geometric mean concentrations (GMC) of GBS-IgG in μg/mL (95 % CI)<br>**2.** Geometric mean fold rise (GMFR) of GBS-IgG<br><br>Placental transfer ratio |
|---|---|---|---|---|---|---|---|
| [36] | Kasper 1996 USA | Guttormsen 2002 | CPS III (monovalent) Dose: 3.6, 14.5 or 58 μg | Healthy non-pregnant adults | 100 | III-TT vs III-non-conjugated | **(1)** Four weeks after first dose: GMC against serotype III was 1.0 (0.3–3.6), 2.5 (1.9–7.3) and 4.2 (1.8–9.9), for three different doses, respectively.<br>**(2)** Promoted GBS type-specific OPK up to 4 weeks post vaccination. |
| [37] | Baker 1999 USA | Brigtsen 2002<br><br>Edwards 2012 | CPS Ia and Ib (monovalent) Dose Ia-TT: 3.75, 15 or 60 μg Dose Ib-TT: 3.94, 15.75 or 63 μg | Healthy non-pregnant adults | 190 | Ia-TT vs Ia-non-conjugated vs Placebo Ib-TT vs Ib-non-conjugated vs Placebo | **(1)** Four weeks after first dose: GMC against serotype Ia was 1.5 (0.6–4.3), 13.1 (4.3–39.8) and 25.5 (12.6–51.4), for three different doses, respectively.<br>**(2)** Four weeks after first dose: GMC against serotype Ib was 2.9 (1.1–7.1), 10.7 (3.2–35.7) and 14.2 (5.8–35.0), for three different doses, respectively.<br>**(3)** No cross-immunization.<br>**(4)** Promoted GBS type-specific OPK up to 24 months. |
| [38] | Baker 2000 USA | – | CPS II (monovalent) Dose: 3.6 or 14.3 or 57 μg | Healthy non-pregnant adults | 75 | II-TT vs II-non conjugated vs Placebo | **(1)** Four weeks after first dose: GMC against serotype II was 12.7 (6.9–23.2), 39.4 (17.9–86.4) and 39.2 (21.5–71.2), for three different doses, respectively.<br>**(2)** Promoted GBS type-specific OPK up to 4 weeks post vaccination. |
| [39] | Paoletti 2001 USA | – | CPS III (monovalent)<br><br>Dose: 12.5 μg | Healthy non-pregnant adults | 96 | III-TT vs III-TT with $AlPO_4$<br>2nd dose of III-TT (without adjuvant) | **(1)** Four weeks after first dose: GMC against serotype III was 3.6 (1.1–12.3).<br>**(2)** Four weeks after 2nd dose: Only a booster effect, with a GMFR of 4, was observed after initial immunization in the eight participants who had undetectable III CPS-specific IgG before the first dose. |
| [40] | Baker 2003 USA | – | CPS III (monovalent)<br><br>Dose: 12.5 μg | Healthy pregnant adults, 30–32 w GA | 30 | III-TT vs Placebo | **(1)** Four weeks after vaccination 95 % of recipients had a GMC $>$ 1.0<br>**(2)** Four weeks after vaccination the GMFR was $>$ 50-fold increased, and it persisted at delivery and 2 months postpartum.<br>**(3)** Placental transfer ratio 1.4.<br>**(4)** Promoted GBS type-specific OPK in infant sera 2 months after birth. |
| [41] | Baker 2003 USA | – | CPS II and/or III (mono- or bivalent) Dose: 3.6 μg or 12.5 μg or combined 3.6/12.5 μg | Healthy non-pregnant adults | 75 | II-TT and III-TT vs. bivalent II/III-TT | **(1)** Four weeks after first dose of 3.6 μg: GMC against serotype II was 6.7 (3.3–13.5).<br>**(2)** Four weeks after first dose of 12.5 μg: GMC against serotype III was 2.0 (0.7–5.8).<br>**(3)** Four weeks after first dose of 3.6/12.5 μg: GMC against serotype II/III was 13.8 (5.8–32.8).<br>**(4)** Promoted GBS type-specific OPK up to 4 weeks post vaccination. |
| [42] | Baker 2004 USA | Edwards 2012 | CPS V (monovalent) Dose: 50 μg | Healthy non-pregnant adults | 35 | V-TT vs V-$CRM_{197}$ | **(1)** Four weeks after first dose V-TT: GMC against serotype V was 8.9 (3.5–22.4).<br>**(2)** Four weeks after first dose V-$CRM_{197}$: GMC against serotype V was 6.5 (2.7–16.0).<br>**(3)** Promoted GBS type-specific OPK up to 24 months. |
| [43] | Palazzi 2004 USA | – | CPS V (monovalent) Dose: 38.5 μg | Healthy non-pregnant adults | 32 | V-TT vs V-Td | **(1)** Four weeks after first dose V-TT: GMC against serotype V was 2.2 (0.7–6.8).<br>**(2)** Promoted GBS type-specific OPK up to 4 weeks post vaccination. |
| [44] | Baker 2007 USA | – | CPS V (monovalent) Dose: 38.5 μg | Healthy non-pregnant adults | 60 | V-TT vs V-non-conjugated | **(1)** Four weeks after first dose V-TT: GMC against serotype V was 11.8 (3.7–37.2).<br>**(2)** Promoted GBS type-specific OPK up to 4 weeks post vaccination. |

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**Table 1** (*continued*)

| Ref. nr. | Main study First author, year, country | Sub-study | Vaccine antigens and dose | Population | N | Intervention | **1.** Geometric mean concentrations (GMC) of GBS-IgG in μg/mL (95 % CI)<br>**2.** Geometric mean fold rise (GMFR) of GBS-IgG<br><br>Placental transfer ratio |
|---|---|---|---|---|---|---|---|
| [25] | Donders 2016 Belgium and Canada | Fabbrini 2018 | CPS Ia/Ib/III (trivalent) Dose: 5 μg | Healthy pregnant adults, 24–35 w GA | 172 | Ia/Ib/III-$CRM_{197}$ vs placebo | **(1)** Maternal GMCs at delivery, were against serotypes Ia 5.2 (3.4–8.1), serotype Ib 2.4 (1.5–3.9) and serotype III 1.9 (1.2–3.1).<br>**(2)** Infant GMC at birth, were against serotypes Ia 0.3 (0.2–0.3), serotype Ib 0.2 (0.2–0.3) and III 0.3 (0.2–0.3).<br>**(3)** No interference with diphtheria vaccine.<br>**(4)** Promoted GBS type-specific OPK at delivery.<br>**(5)** Placental transfer ratio 0.7–0.8. |
| [45] | Heyderman 2016 Malawi and South-Africa | – | CPS Ia/Ib/III (trivalent) | Pregnant women with/ without HIV and newborns | 536 | Ia/Ib/III -$CRM_{197}$ | **(1)** Four weeks after vaccination of HIV-uninfected participants GMCs against serotypes were for Ia 6.6 (4.4–10), Ib 5.4 (3.6–7.9) and III 5.4 (3.7–7.8).<br>**(2)** Four weeks after vaccination of HIV-infected (low CD4 count) participants GMCs against serotypes were for Ia 2.7 (1.7–4.1), serotype Ib 2.6 (1.6–4.2) and serotype III 1.5 (1.0–2.4). |
| [26] | Madhi 2016 South Africa | Madhi 2017 | CPS Ia/Ib/III (trivalent)<br><br>Dose: 2.5 or 5 μg | Healthy non-pregnant and pregnant women and newborns | 697 | Ia/Ib/III -$CRM_{197}$ vs placebo | **(1)** Four weeks after vaccination of pregnant women (merged data for dose 2.5 and 5 μg) GMC against serotypes were for Ia ~ 20 (10–40), Ib ~ 5.5 (2–9) and III ~ 3.5 (2–6).<br>**(2)** GMCs were lowest in those whose baseline concentration was lower than lower limit of detection, particularly for serotype Ib and III.<br>**(3)** Placental transfer ratio 0.5–0.8.<br>**(4)** Vaginal colonization unchanged at delivery. |
| [27] | Leroux-Roels 2016 Belgium | – | CPS Ia/Ib/III (trivalent)<br><br>Dose: 5 or 20 μg | Healthy non-pregnant women | 678 | Ia/Ib/III -$CRM_{197}$ With/without adjuvant and vs placebo | **(1)** Two months after vaccination with trivalent 20 μg vaccine without adjuvants GMCs against serotypes were: Ia 16 (6.9–38), Ib 3.9 (1.6–9.6) and III 2.8 (1.2–6.7).<br>**(2)** GMCs were lowest in those whose baseline concentration was lower than lower limit of detection.<br>**(3)** Two months after vaccination against the three serotypes the GMFRs were 14–89 in the vaccine groups, and remained at 4–5-fold above baseline two years after vaccination. |
| [28] | Hillier 2019 USA | – | CPS III (monovalent) Dose: 12.55 μg | Healthy non-pregnant adults | 667 | III-TT vs tetanus/ diphtheria toxin | **(1)** One month after vaccination with the III-TT vaccine GMCs against serotype III was ~ 12 (10–16).<br>**(2)** The GMFR was 40 one month after vaccination (III-TT) compared to baseline values.<br>**(3)** III-TT resulted in significant delay in rectovaginal GBS colonization. |
| [29] | Leroux-Roels 2020 Belgium | – | CPS Ia/Ib/III (trivalent) Dose: 5 μg | Healthy non-pregnant adults | 80 | Ia/Ib/III -$CRM_{197}$, no adjuvant. Second dose 4–6 years after first dose vs a first dose | **(1)** One month after second dose vaccination (both doses without. adjuvant) GMCs against serotypes were: Ia 142.4 (54–379), Ib 56.3 (22–145) and III 111.3 (42–294).<br>**(2)** Two months after second dose, 90–98 % of women with undetectable baseline concentrations before first dose reached the 8 μg/mL threshold across all three serotypes.<br>**(3)** Two months after first dose, 36–56 % of women reached the 8 μg/mL threshold across all three serotypes. |
| [30] | Beran 2020 Czech Republic, Belgium, USA | – | CPS Ia/Ib/III (trivalent) | Healthy non-pregnant adults | 1050 | Ia/Ib/III -$CRM_{197}$ Fully liquid vs lyophilized | **(1)** One month after vaccination with a liquid trivalent vaccine (5 μg) the GMCs against serotypes were: Ia 6.8 (5.5–8.4), Ib 2.9 (2.4–3.6) and III 2.4 (2.0–3.0).<br>**(2)** One month after vaccination the GMFR was 8–16 higher than at baseline. |

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**Table 1** (*continued*)

| Ref. nr. | Main study First author, year, country | Sub-study | Vaccine antigens and dose | Population | N | Intervention | **1.** Geometric mean concentrations (GMC) of GBS-IgG in μg/mL (95 % CI)<br>**2.** Geometric mean fold rise (GMFR) of GBS-IgG<br><br>Placental transfer ratio |
|---|---|---|---|---|---|---|---|
| | | | | | | | **(3)** GMCs were lowest in those whose baseline concentration was lower than lower limit of detection. |
| [31] | Swamy 2020 USA | – | CPS Ia/Ib/III (trivalent) | Healthy pregnant women, 24–34 w GA, and newborns | 75 | Ia/Ib/III -$CRM_{197}$ vs placebo | **(1)** One month after vaccination GMCs against serotypes were: Ia 9.0 (4.7–17.0), Ib 7.3 (3.5–15) and III 3.6 (1.5–8.6).<br>**(2)** The GMFR was 13–23 fold higher in vaccine vs placebo recipients on day 31 and persisted until postpartum day 90.<br>**(3)** At birth, antibody GMCs in cord blood of infants born to GBS vaccinated. women were 8–39-fold higher than in infants born to placebo recipients.<br>**(4)** Placental transfer ratio 0.6–0.8. |
| [32] | Absalon 2021 USA | – | CPS Ia/Ib/II/ III/ IV/V (hexavalent)<br><br>Dose: 5 or 10 or 20 μg | Healthy non-pregnant adults | 365 | Ia/Ib/II/III/ IV/V -$CRM_{197}$ in different doses vs Placebo. With/without adjuvant. | **(1)** One month after vaccination with GBS6 10 μg (no AlPO4) GMCs against serotypes were: Ia 41.8 (17.7–98.6), serotype Ib 3.6 (1.4–9.3), serotype II 57.0 (31.9–101.8), serotype III 12.8 (6.2–26.4), serotype IV 4.9 (2.9–8.3) and serotype V 5.1 (2.4–11.0).<br>**(2)** One month after vaccination. GBS serotype-specific IgG GMFR. ranged from 25 to more than 200 for each serotype.<br>**(3)** The GMFR remained 10–56 for all doses and formulations of GBS6 at 6 months after vaccination compared with placebo. |
| [33] | Fischer 2021 UK | Pawlowski 2022 | Protein subunit NN/NN2<br>Dose: 10 or 50 or 100 μg | Healthy non-pregnant adults, (non-vaccinated pregnant women and newborns, n = 304) | 240 | NN/NN2 in different doses vs placebo. With/without adjuvant. | **(1)** Four weeks after vaccination, two doses of 50 μg, the GBS-NN IgG GMC was 6.0 (3.9–9.3). Maximal response was 16.9 (11.3—25.4) 85 days after vaccination.<br>**(2)** For the 2-dose (50 μg) regimen 100 % and 89 % of the subjects achieved antibody levels above the arbitrary thresholds of 1 and 4 μg/ml, respectively.<br>**(3)** Added effect of a second dose most pronounced for subjects with pre-existing IgG levels below the median of the entire cohort.<br>**(4)** The natural occurring placental transfer ratio 1.1–1.2. |
| [46] | Madhi 2023 South Africa | | CPS Ia/Ib/II/ III/ IV/V (hexavalent)<br>Dose: 5 or 10 or 20 μg | Healthy pregnant women, 27–36 w GA, and newborns | 360 | Ia/Ib/II/III/ IV/V -$CRM_{197}$ in different doses vs placebo. With/without adjuvant. | **(1)** Maternal GMCs at delivery, after vaccination with GBS6 20 μg (no AlPO4), were against serotypes: Ia 40.3 (23.9–68.2), serotype Ib 1.3 (0.6–2.9), serotype II 27.6 (15.6–48.9), serotype III 6.4 (2.8–14.4), serotype IV 2.5 (1.5–4.2) and serotype V 0.9 (0.4–2.0).<br>**(2)** Infant GMC at birth after maternal vaccination with GBS6 20 μg (no AlPO4), were against serotypes: Ia 29.6 (17.0–51.5), serotype Ib 0.7 (0.3–1.8), serotype II 20.8 (10.7–40.5), serotype III 3.2 (1.3–7.7), serotype IV 2.1 (1.2–3.7) and serotype V 0.6 (0.2–1.4).<br>**(3)** Placental transfer ratio 0.4–1.3 across the different serotypes. |

Abbreviations: Ref. nr., reference number; CI, confidence interval; CPS, capsular polysaccharide; OPK, Opsonophagocytic killing; GMC, Geometric mean antibody concentration; TT, Tetanus toxoid conjugated vaccine; $CRM_{197}$, non-toxic mutant form of the 58-kd diphtheria toxin; Td, Tetanus-diphtheria toxoid vaccine; NN/NN2, N-terminal domains of the Rib and AlphaC proteins vaccine; $AlPO_4$, aluminium phosphate; GA, gestational age.

outcomes is presented in Table 1.

A second dose or a booster dose improved IgG GMC in study participants with low initial CPS-specific IgG GMC after the first dose in both the conjugated CPS-vaccine and the surface subunit protein vaccine [29,33,39]. There was no additional benefit of a booster for participants with an adequate initial CPS-specific IgG GMC concentration [27,39]. In HIV-infected pregnant women, the serotype-specific antibody concentrations were lower compared to the HIV-uninfected pregnant women [45].

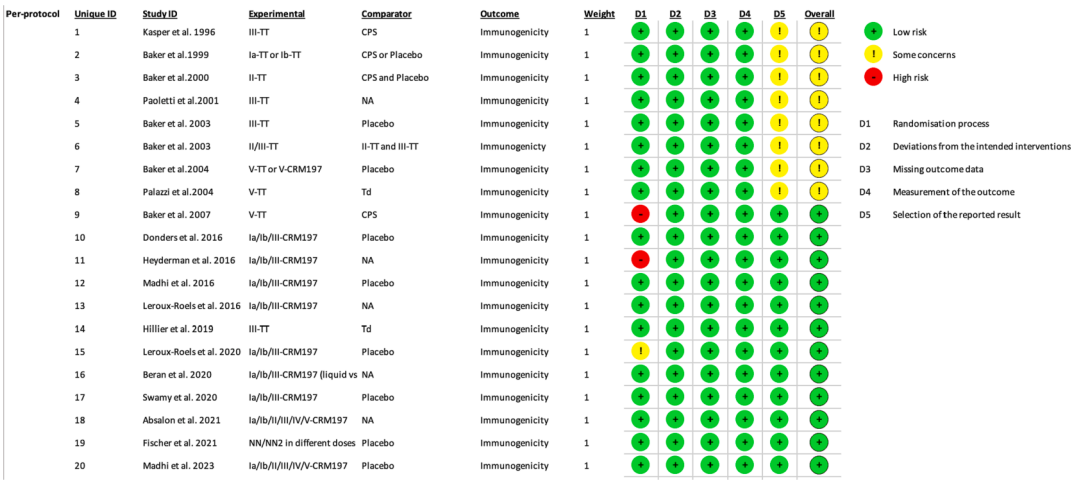



**Fig. 2.** Risk of bias for immunogenicity outcomes.

### 3.5. Placental transfer ratio

The placental transfer ratio, defined as the ratio between the level of GBS-specific antibodies in maternal serum during pregnancy and corresponding level in cord blood or infant serum shortly after birth, was investigated in five vaccine studies [25,26,40,45,46]. In three studies the IgG placental transfer ratios were 1.42 for III-TT [40], 0.66–0.79 for Ia/Ib/III-CRM$_{197}$ [25] and 0.49–0.79 for Ia/Ib/III-CRM$_{197}$ [26]. In one study the placental transfer ratio was 0.49–0.72 both in the HIV-uninfected group and the HIV-infected groups [45]. In a recent study reporting data from the hexavalent vaccine, the placental transfer ratio ranged from approximately 0.4 to 1.3. In this study the highest antigen dose provided IgG GMCs in infant sera associated with an estimated 75 % risk reduction of perinatal GBS disease in 57–97 % participants, depending on the serotype [46]. For the surface subunit protein vaccine the natural placental transfer ratio was 1.22 for αC-N-specific IgG and 1.12 for Rib-N-specific IgG, but not assessed after vaccination [51].

### 3.6. Reactogenicity and adverse events

All 20 original articles reported data on reactogenicity and more severe AEs/safety in non-pregnant adults [26–30,32,33,36–39,42–44], pregnant women [25,26,31,40,41,45,46] and infants [31]. Mild vaccine reactogenicity symptoms such as pain at the injection site, tenderness or local swelling were described in all studies. These were more frequently reported in studies comparing adjuvanted versus not adjuvanted vaccines [26,27,32,37,38,42,44,46]. The most frequent solicited systemic AEs were fatigue and headache [25,26,37,42]. Most solicited AEs were mild or moderate.

There were no reported deaths relating to the trial vaccines across the 20 clinical trials [25–33,36–46]. Fig. 3a-c shows an overview of serious AEs, AEs leading to withdrawal from the vaccine study and fever/systemic illness in 11 of 20 of the clinical trials included in this review [25–27,31–33,36,37,40,42,46], while Table 2 shows an overview of reactogenicity and AEs across the 20 studies. One study presented a significantly lower rate of serious AEs in the CPS vaccine conjugated with tetanus-toxoid versus tetanus-diphtheria toxoid group [28]. We did not identify any age pattern for AEs [26–30,32,33,36–39,42–44], and no higher incidence of pregnancy-related AEs reported after vaccination [25,26,31,40,41,45,46]. There were no increased systemic AEs reported after the second dose when comparing it to the first dose administration [29,33,39].

In two studies [30,33] reporting on pregnancies after receiving the GBS-vaccine, none of the adverse pregnancy outcomes were assessed as related to vaccination (Table 2). The capsular conjugate vaccine studied in HIV infected pregnant mothers showed no effect on CD4 count and viral loads [45].

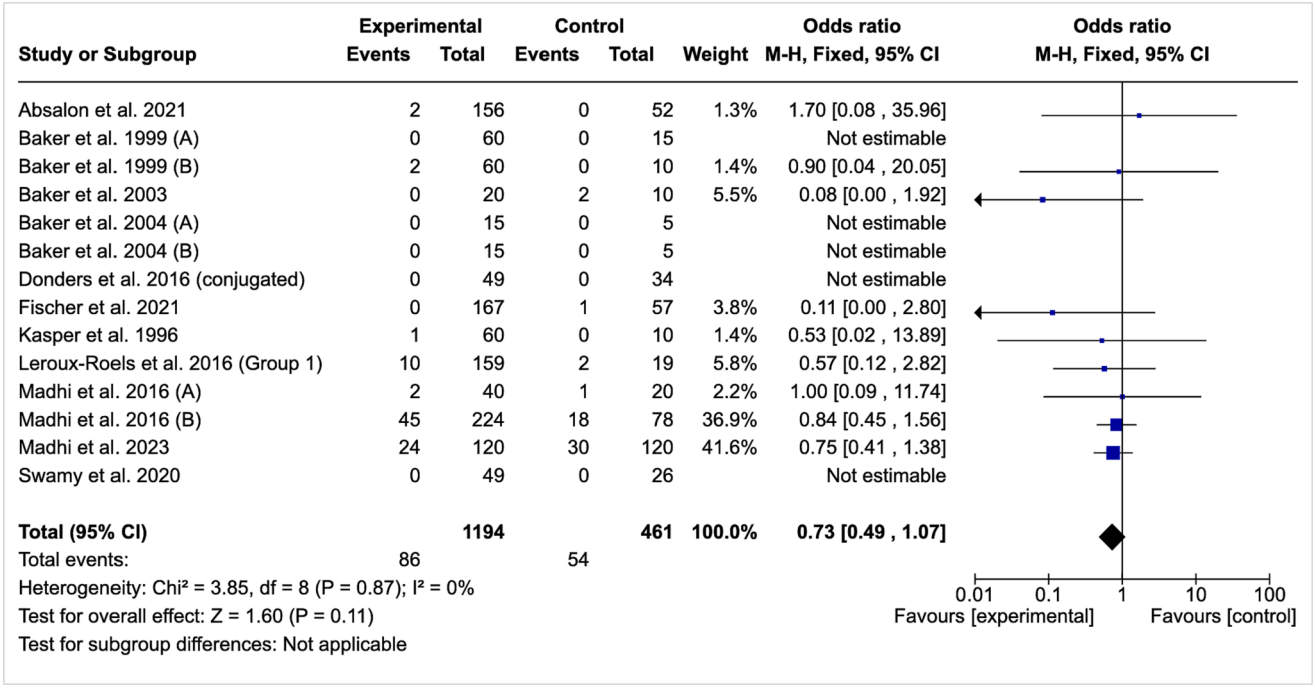
## 4. Discussion

The global public health impact of perinatal GBS disease is a matter of great concern and the development of GBS vaccines for maternal immunization is therefore top priority [53].

In this systematic review we identified and included a total of 20 primary studies published between 1996 and 2023. There were 5765 participants, of which only 1325 were pregnant women. Our review revealed large disparities in the methods used to measure immunogenicity and how AEs were reported. Still, there are three key findings. First, the vast majority of participants, exposed to conjugated CPS vaccines or the surface subunit protein vaccine, exhibited markedly increased IgG GMC concentrations compared to placebo. There were also an increase in the antibody GMC following a second dose in those who had low baseline antibody GMC, and antibody levels remained clearly above baseline values for at least 6–12 months [25,26,29–33,36–45]. Second, placental transfer ratios ranged from 0.4 to 1.4 indicating that antibody crosses the placenta and can protect infants from invasive GBS disease [25,26,40,45,51]. Third, we found low levels of reactogenic events and serious AEs regarding the experimental vaccines, in non-pregnant adults [26–30,32,33,36–39,42–44], pregnant women [25,31,40,41,45,46] and infants [31].

### 4.1. Immunogenicity

Evaluating the reported GBS-IgG levels in the studies included in this review was challenging as they varied by different serotypes covered in the vaccines, the immunogenicity assays and reagents used, and the different time schedules for assessment across studies. Thus, data were not possible to *meta*-analyze and were summarized for each study separately. Naturally acquired anti-GBS IgG concentrations associated with a reduced risk of disease among infants are reported from seroepidemiological studies [46,54–57]. However, it is important to note that suggested protective thresholds are based on a limited number of cases versus controls in seroepidemiological study, which poses a limitation to the findings. There is also no uniform agreement on how to establish "protective" GBS-IgG levels, and there is limited data in particular for the low-prevalent CPS-serotypes vaccines and the surface subunit protein vaccines [58]. Some data suggest that anti-GBS-CPS IgG concentrations at around 1 μg/mL or higher are "protective" [55,59]. In all studies evaluating CPS-IgG levels in our review the majority of elicited anti CPS-IgG concentrations were above 1 μg/mL in non-pregnant adults



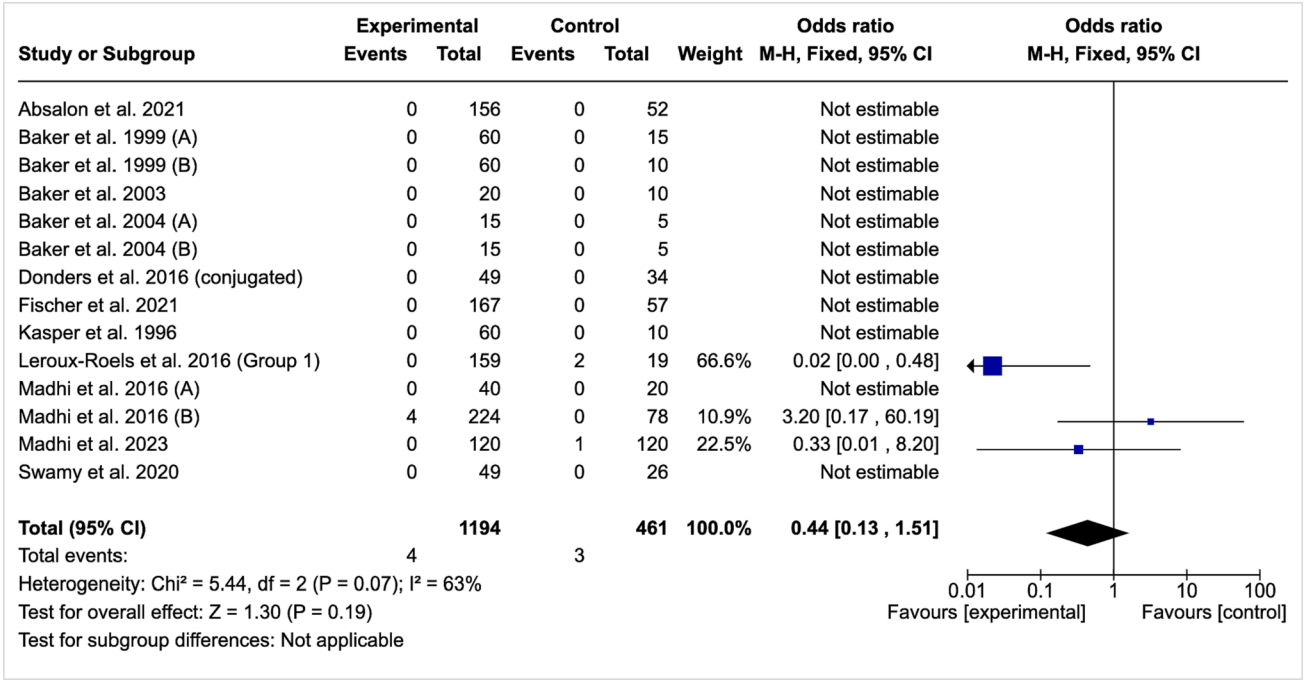




**Fig. 3.** Forest Plots of adverse effects in studies comparing a GBS vaccine versus placebo. **a.** Pooled results of studies comparing risk of serious adverse events between those who received a GBS vaccine versus placebo. The sizes of the squares are proportional to study weights. Diamond markers indicate pooled effect sizes. **b**. Pooled results of studies comparing risk of serious adverse events leading to withdrawal from the study between those who received a GBS vaccine versus placebo. The sizes of the squares are proportional to study weights. Diamond markers indicate pooled effect sizes. **c.** Pooled results of studies comparing risk of fever/systemic illness between those who received a GBS vaccine versus placebo. The sizes of the squares are proportional to study weights. Diamond markers indicate pooled effect sizes.

and pregnant women 4–8 weeks after vaccination. For the multivalent conjugated CPS vaccines, we observed different immunogenicity among the different serotypes. The main pattern was a markedly higher IgG GMC response to serotype Ia versus the other serotypes Ib, II, III, IV, V [25–27,30–32,37,41,46], though the potential clinical importance of this observation in unclear. Regarding the surface subunit protein vaccine, there is no specified threshold for protective anti-protein IgG concentration [60–62], and comparing the studies estimating this has been challenging due to variations in assay methods, protein sources, absence of a common reference serum, and differences in study designs [58].

Polysaccharides are weak vaccine antigens and therefore often conjugated to an immunogenic protein, eliciting a strong T-cell dependent response with establishment of B-cell memory and long-term

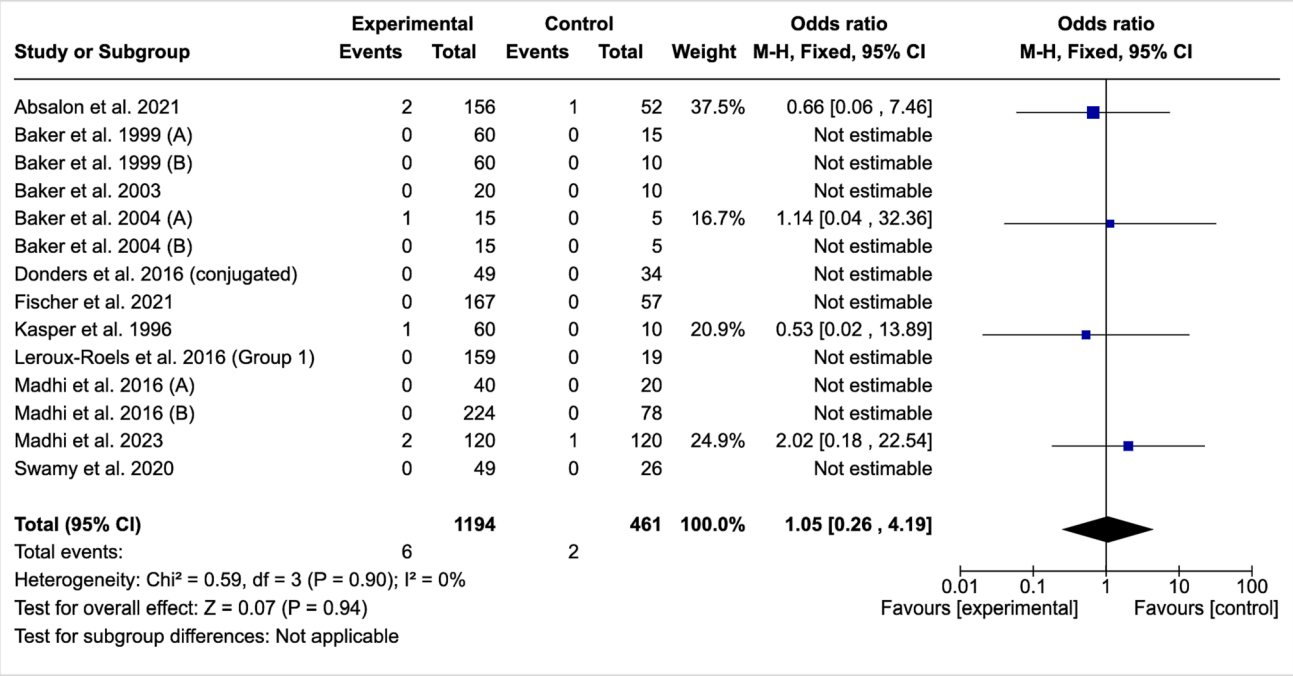



**Fig. 3.** (*continued*).

immunization [63]. Toxoids are often selected as the carrier proteins due to their inherent immunogenicity and the potential for a booster effect in previously immunized recipients [64]. Conjugation of the GBS-CPS with a toxoid protein carrier was essential to achieve an adequate immune response in the studies in this review comparing conjugated and non-conjugated vaccines [36–38,44]. This principle is well known from other CPS-based vaccines like the pneumococcal glycoconjugate vaccine [63]. For the surface subunit protein vaccine conjugation was not needed as proteins are more antigenic than polysaccharides. Including GBS surface subunit proteins in future vaccines offers advantages over unrelated proteins like tetanus toxoid or CRM197. It could simplify coverage for additional strains beyond the CPS serotypes included and enhance protection against some strains. However, using a range of carrier proteins in some conjugate vaccines may increase reactogenicity and potentially suppress the immune response to CPS [65].

Vaccine adjuvants are also often added to enhance the ability of a vaccine to elicit strong and durable immune responses, especially in immunologically compromised individuals like immature neonates and immunosuppressed individuals [66]. Adjuvants, e.g. aluminum salts, may also reduce the antigen dose needed and subsequently the number of immunizations [67]. For the surface subunit protein vaccine, adding an adjuvant enhanced immunogenicity [32]. When examining the conjugated CPS-based GBS vaccines in our review, we did not observe any indications that adjuvants enhanced immunogenicity [27,39]. However, it is important to acknowledge that these vaccines were predominantly evaluated in immunocompetent adults. In contrast, the commercial polyvalent pneumococcal CPS-vaccines contain aluminum salts, in order to elicit immune response in young infants from the age of 2 months and upwards [68]. We belive it is less likely that future commercial CPS-based GBS vaccines for pregnant women will be manufactured with adjuvants.

The majority of the trials had a follow-up period of 6 months [25,31–33,36,38,40,41,45,46] and some even longer [26,28,29,37,39,42–44]. These studies report a decline of antibody levels in both the mother and child, in line with the expected gradual decrease of antibodies levels over a period of 6–12 months. While the majority of studies did not assess the functionality of maternal antibodies, earlier research has demonstrated that functional GBS antibodies can endure for as long as two years after vaccination [36–38,41–44,50,51] and at least 2 months in infants [40].

### 4.2. Placental transfer

Our review found that conjugated CPS-based vaccines resulted in induction of anti GBS-IgG which were effectively transmitted across the placenta. The infant antibody levels, derived from transferred IgG, is also most likely more relevant for defining a level for risk reduction of acquiring invasive GBS disease compared to maternal antibody levels. Only five studies provided data from infant sera after maternal vaccination [25,26,40,45,46]. Overall, the placental transfer ratios varied between 0.4 and 1.4 across these five studies. Evaluating placental transfer ratios from different vaccines should ideally also include presentation of vaccine induced IgG subclasses. Studies indicate the IgG1 has the highest transfer ratio and IgG2 the lowest [69,70]. However, the placental transfer ratio could also be affected by the IgG subclass distribution pattern in a population [69,70]. Similarly, earlier vaccine studies have indicated that vaccination response can be influenced by racial and ethnic factors [71–77].

### 4.3. Safety and adverse events

Overall, the safety profile of GBS vaccines evaluated in this systematic review were reassuring. However, our data must be interpreted with caution. First and foremost, the number of participants included in our review were only 5765 participants, of which only 1325 were pregnant women. Secondly, distinguishing between pregnancy related complications and symptoms, and vaccine-related AEs is challenging in maternal vaccine studies. This difficulty arises because both pregnancy and vaccines can lead to similar symptoms, such as nausea, making it a complex task to determine whether these symptoms are solely attributable to normal pregnancy experiences or are indicative of AEs. Factors like maternal age, obstetrical history, and health conditions influence pregnancy outcomes. Understanding these factors is vital for interpreting AEs in clinical vaccine trials [78]. Additionally, a much higher number of participants will be needed to detect rare and severe side effects, like the vaccine-induced immune thrombotic thrombocytopenia

**Table 2**
Adverse events reported in 20 GBS vaccine studies.

| Ref. nr. | First author, year, country | Population | N | Intervention | Reactogenicity | Adverse events (AEs) and serious adverse events (SAEs) | Adverse events of special interest (AESI) |
|---|---|---|---|---|---|---|---|
| [36] | Kasper 1996 USA | Healthy non-pregnant adults | 100 | III-TT vs III-non-conjugated vs placebo | **(1)** 7 % experienced serious redness/swelling at the injection site in the 14.5 μg III-TT group.<br>(2) No severe systemic reactions reported. | None | 1.7 % in III-TT group had a temperature of 100.38°F coupled with RTI that resolved within 24 h. |
| [37] | Baker 1999 USA | Healthy non-pregnant adults | 190 | Ia-TT vs Ia-non-conjugated vs Placebo<br>Ib-TT vs Ib-non-conjugated vs Placebo | **(1)** Ia-TT vs Ia-non-conjugated vs Placebo: None experienced serious pain or redness/swelling at the injection site.<br>**(2)** Ib-TT vs Ib-non-conjugated vs Placebo: 3.3 % experienced serious pain or redness/swelling at the injection site in the 63 μg Ib-TT group.<br>**(3)** No severe systemic reactions reported. | None | No significant changes in CBC or blood chemistry values noted 2 days after vaccination in all groups. |
| [38] | Baker 2000 USA | Healthy non-pregnant adults | 75 | II-TT vs II-non conjugated vs Placebo | **(1)** 10 % experienced serious redness/swelling at the injection site in the 57 μg II-TT group and none in the II CPS group.<br>**(2)** 6.7 % experienced chills, malaise, headache, and temperature to 37.8 °C up to 36 h after immunization in the 14.3 μg II-TT group. | None | Not retrievable |
| [39] | Paoletti 2001 USA | Healthy non-pregnant adults | 96 | III-TT vs III-TT with $AlPO_4$<br>2nd dose of III-TT (without adjuvant) | **(1)** III-TT vs III-TT with $AlPO_4$: 6.7 % experienced serious pain at the injection site both with and without adjuvant.<br>**(2)** 2nd dose of III-TT (without adjuvant): 2.8 % experienced serious redness/swelling at the injection site.<br>**(3)** 2.8 % in the 12.5 μg, first dose, III-TT experienced severe systemic reactions. | **(1)** 1 experienced fever of 100.4°F associated with chills, malaise, and headache 18 h after receiving the first dose of GBS III-TT conjugate (accidently) combined with GBS II-TT.<br>**(2)** No SAEs reported. | Not retrievable |
| [40] | Baker 2003 USA | Healthy pregnant adults, 30–32 w GA | 30 | III-TT vs Placebo | **(1)** No serious pain or redness/swelling reported at the injection site in either group.<br>**(2)** No severe systemic reactions reported. | None | Not retrievable |
| [41] | Baker 2003 USA | Healthy non-pregnant adults | 75 | II-TT and III-TT vs. bivalent II/III-TT | **(1)** No serious pain or redness/swelling at the injection site reported in the groups.<br>**(2)** 2 with reported severe systemic reactions. | **(1)** AEs: 2 experienced fever of 100.6°F and 100.4°F at 11 h and 17 h after immunization with monovalent GBS III–TT and bivalent II/III-TT, respectively. Fever combined with chills, mild headache, malaise, and myalgia.<br>**(2)** No SAEs reported. | Not retrievable |
| [42] | Baker 2004 USA | Healthy non-pregnant adults | 35 | V-TT vs V-$CRM_{197}$<br>Placebo | **(1)** No serious pain or redness/swelling reported at the injection site in the groups.<br>**(2)** 6.7 % in the V-TT group experienced systemic reactions. | **(1)** AEs: 3.3 % in V-TT experienced headache, malaise, myalgia, and nausea a few hours after immunization.<br>**(2)** No SAEs reported. | Not retrievable |
| [43] | Palazzi 2004 USA | Healthy non-pregnant adults | 32 | V-TT vs Td | **(1)** No serious pain or redness/swelling at the injection site reported in the groups.<br>**(2)** No severe systemic reactions reported. | **(1)** AEs: 1 reported fatigue and myalgia within a few hours of immunization in the V-TT group, while in the Td group 1 reported moderate fatigue on day 2 after | Not retrievable |

(*continued on next page*)

**Table 2** (*continued*)

| Ref. nr. | First author, year, country | Population | N | Intervention | Reactogenicity | Adverse events (AEs) and serious adverse events (SAEs) | Adverse events of special interest (AESI) |
|---|---|---|---|---|---|---|---|
| | | | | | | immunization.<br>**(2)** No SAEs reported. | |
| [44] | Baker 2007 USA | Healthy non-pregnant adults | 60 | V-TT vs V-non-conjugated | **(1)** 6.7 % in the 38.5 μg V-TT group experienced serious pain at the injection site.<br>**(2)** No severe systemic reactions reported. | None | 1 reported fever, sore throat, malaise and myalgias 6 h after vaccination, coupled with RTI that resolved within 24 h. |
| [25] | Donders 2016 Belgium and Canada | Healthy pregnant adults, 24–35 w GA | 172 | Ia/Ib/III-CRM$_{197}$ vs placebo | **(1)** No serious pain or redness/swelling reported at the injection site in the vaccine group, while 0–6 % in the placebo group reported severe local reactions.<br>**(2)** 0–6 % in placebo group reported systemic reactions. | **(1)** AEs: 63 % [95 % CI 48.1–75.9 %] and 74 % [95 % CI 56.7–87.5 %] reported in vaccine and placebo, respectively.<br>**(2)** SAEs: reported in 24 % and 31 % of infants in the vaccine and placebo groups, respectively. No SAEs in maternal groups. | Obstetric outcomes were similar between the vaccine and placebo groups.<br><br>1 neonatal asphyxia occurring 28 days after maternal vaccination. |
| [45] | Heyderman 2016 Malawi and South-Africa | Pregnant women with/without HIV and newborns | 536 | Ia/Ib/III-CRM$_{197}$ | **(1)** 2 %, 0 % and 4 % reported severe pain at injection site in HIV-infected low CD4 cell count, HIV-infected high CD4 cell count and HIV-uninfected, respectively.<br>**(2)** Fever only reported in HIV-infected low CD4 cell count group (n = 3). | **(1)** AEs: 7 %, 13 % and 23 % reported AE possibly related to vaccine in HIV-infected low CD4 cell count, HIV-infected high CD4 cell count and HIV-uninfected, respectively. In infants the rates were 0 %, 2 % and 1 %, respectively.<br>**(2)** SAEs: None at least possibly related to vaccination.<br>**(3)** Similar rates of maternal and infant SAEs reported across all groups.<br>**(4)** No differences in obstetric outcomes and pregnancy events were recorded across the three groups.<br>**(5)** No association between vaccine administration and change in viral load was seen in the HIV-infected groups. | 1 maternal death in the HIV-infected high CD4 cell count group.<br><br>4, 2 and 2 neonatal deaths in HIV-infected low CD4 cell count, HIV-infected high CD4 cell count and HIV-uninfected, respectively. |
| [26] | Madhi 2016 South Africa | Healthy non-pregnant and pregnant women and newborns | 697 | Ia/Ib/III-CRM$_{197}$ vs placebo | **(1)** No serious pain or redness/swelling at the injection site reported.<br>**(2)** Systemic reactions were reported by 95 % and 90 % of the women in the vaccine and placebo groups, respectively, with the most reported reactions being myalgia, headache, and fatigue. Similar rates for pregnant and non-pregnant women. | **(1)** AEs: Unsolicited were reported by 30 (75 %) participants in the vaccine group and 16 (80 %) participants in the placebo group, with 40 % per group (23 % for pregnant) considered possibly related to study vaccination. Similar rates for pregnant and non-pregnant.<br>**(2)** SAEs: None at least possibly related to vaccination. | Obstetric outcomes were similar between the vaccine and placebo groups.<br><br>3 stillbirths were recorded in placebo group (4 %) and 4 (2 %) in vaccine groups. |
| [27] | Leroux-Roels 2016 Belgium | Healthy non-pregnant women | 678 | Ia/Ib/III-CRM$_{197}$ With/without adjuvant (AlOH or MF59) and vs placebo | **(1)** No serious pain or redness/swelling at the injection site reported.<br>**(2)** 50 %–85 % across vaccine groups, and 58 %–65 % in the placebo groups reported systemic reactions. | **(1)** AEs: On average 26 % (no adjuvant), 14 % (AlOH) and 0 % (placebo) in enrolment group 1. On average 11 % (MF59 half), 18 % (MF59 full) and 5 % (placebo) in enrolment group 2. Similar rates in possibly related to vaccination.<br>**(2)** SAEs: None at least possibly related to vaccination. | None mentioned |
| [28] | Hillier 2019 USA | Healthy non-pregnant adults | 667 | III-TT vs tetanus/diphtheria toxin | **(1)** No serious pain or redness/swelling at the injection site reported.<br>**(2)** 41 % in the III-TT and none in the Td groups reported systemic reactions (headache, malaise, muscle aches). | **1)** AEs: Around 9.8 % considered to be vaccine associated in all groups.<br>**(2)** SAEs: None at least possibly related to vaccination. | None mentioned |

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**Table 2** (*continued*)

| Ref. nr. | First author, year, country | Population | N | Intervention | Reactogenicity | Adverse events (AEs) and serious adverse events (SAEs) | Adverse events of special interest (AESI) |
|---|---|---|---|---|---|---|---|
| | | | | | **(3)** Women who received Td vaccine reported local symptoms of greater severity compared to women who received GBS III-TT vaccine | | |
| [29] | Leroux-Roels 2020 Belgium | Healthy non-pregnant adults | 80 | Ia/Ib/III-CRM$_{197}$ vs non-vaccinated Second dose 4–6 years after first dose vs a first dose | **(1)** 7 % in the GBS without adjuvant group experienced serious pain at the injection site. **(2)** No severe systemic reactions reported. | **(1)** AEs: Across groups, 29 %–67 % of women reported unsolicited AEs within 31 days postvaccination. **(2)** SAEs: None at least possibly related to vaccination. | 2 in the prior GBS group reported RTI and hot flush after immunization, while 2 in the no prior GBS group reported injection site erythema and nasal congestion after immunization. |
| [30] | Beran 2020 Czech Republic, Belgium, USA | Healthy non-pregnant adults | 1050 | Fully liquid vs lyophilized trivalent GBS vaccine | **(1)** 0.2 % experienced serious pain and redness/swelling at the injection site in the fully liquid vaccine group, while 0.2 % experienced serious pain in the lyophilized vaccine group. **(2)** No more than 2.1 % experienced severe systemic reactions in either group. | **(1)** AEs: 11 % and 10 % of women in Liq and Lyo, respectively. **(2)** SAEs: None at least possibly related to vaccination. | 10 women became pregnant during the study; 5 singleton liveborn babies, 1 stillbirth, 2 abortions (one spontaneous and one therapeutic) and 2 pregnancies lost to follow-up. |
| [31] | Swamy 2020 USA | Healthy pregnant women, 24–34 w GA, and newborns | 75 | Ia/Ib/III-CRM$_{197}$ vs placebo | **1)** No serious pain or redness/swelling at the injection site reported in the groups. **(2)** 1 % (vaccine) and 2 % (placebo) experienced severe systemic reactions (fatigue). | **(1)** AEs: None related to maternal vaccination. **(2)** SAEs: 15 % and 12 % of infants in the vaccine and placebo groups, respectively. None related to maternal vaccination. | 16 % in the vaccine group experienced ten AESI in total (amniotic cavity infection, arrested labor [five cases], gestational hyper- tension, pre-eclampsia, premature separation of placenta, prolonged labor) and 15 % in the placebo group experienced six AESI (anemia, cholelithiasis, breech presentation, pre-eclampsia, umbilical cord prolapse, nephrolithiasis). None related to vaccine. |
| [32] | Absalon 2021 USA | Healthy non-pregnant adults | 365 | Ia/Ib/II/III/ IV/V-CRM$_{197}$ in different doses vs Placebo | **(1)** No serious pain or redness/swelling reported at the injection site in the groups. **(2)** No severe systemic reactions reported. | **(1)** AEs: Rates ranging from 12 % in the 10 μg without AlPO4 group to 29 % in the 20 μg with AlPO4 group and placebo group. Most common upper respiratory tract infection and sinusitis. **(2)** SAEs: Reported on 3 GBS6 with AlPO4 recipients (diabetic ketoacidosis, suicide, metrorrhagia) and none in the GBS6 without AlPO4 and placebo groups. | None of the changes in laboratory values after vaccination were associated with clinical findings. |
| [33] | Fischer 2021 UK | Healthy non-pregnant adults, (non-vaccinated pregnant women and newborns, n = 304) | 240 | NN/NN2 in different doses vs placebo (Part A) and comparing effects of single dose versus booster (Part B). With/without adjuvant. | **(1)** No serious pain or redness/swelling reported at the injection site in either group. **(2)** No severe systemic reactions reported. | **(1)** AEs: Similar across vaccine and placebo (gastrointestinal, nervous system and infections and infestations system organ classes). **(2)** SAEs: None at least possibly related to vaccination. | 12 pregnancies reported (6 in placebo and 6 in GBS-NN); 7 liveborn, 4 spontaneous abortions (2 in each group), and 1 lost to follow-up. |
| [46] | Madhi 2023 South Africa | Healthy pregnant women, 27–36 w GA, and newborns | 360 | Ia/Ib/II/III/ IV/V -CRM$_{197}$ in different doses vs placebo. With/without adjuvant. | **(1)** No serious pain or redness/swelling reported at the injection site in either group. **(2)** Severe systemic events were reported in 4 GBS6 recipients and 4 placebo recipients (fever). | **(1)** AEs: 45 to 70 % in the GBS6 groups and 61 % in placebo group reported (fetal distress syndrome most common). Only headache and vomiting related to vaccine. **(2)** SAEs: None at least possibly related to vaccination. | 1 stillbirth in GBS6. 1 fatal motor vehicle accident. None related to the vaccine. |

(*continued on next page*)

**Table 2** (*continued*)

| Ref. nr. | First author, year, country | Population | N | Intervention | Reactogenicity | Adverse events (AEs) and serious adverse events (SAEs) | Adverse events of special interest (AESI) |
|---|---|---|---|---|---|---|---|
| | | | | | | **(3)** 24 women in the GBS6 with AIPO4 and 43 in the GBS6 without AlPO4 groups reported SAEs. **(4)** Similar number on infants reported an SAE in both groups. | |

Abbreviations: Ref. nr., reference number; CPS, capsular polysaccharide; OPK, Opsonophagocytic killing; GMC, Geometric mean antibody concentration; TT, Tetanus toxoid conjugated vaccine; $CRM_{197}$, non-toxic mutant form of the 58-kd diphtheria toxin; Td, Tetanus-diphtheria toxoid vaccine; NN/NN2, N-terminal domains of the Rib and AlphaC proteins vaccine; $AlPO_4$, aluminium phosphate; GA, gestational age.

observed after the adenoviral vector covid19-vaccine [79]. The current GBS vaccine candidates are based on bacterial surface subunit protein products and by definition inactivated or killed vaccines, and considered more safe than live vaccines. This safety extends to pregnancy, where purified macromolecule vaccine types such as subunit vaccines, conjugate vaccines, and inactivated toxoids are considered suitable. Nevertheless, continuous safety monitoring remains crucial to assess their appropriateness for this vulnerable population [80]. A recent maternal vaccination trial against respiratory syncytial virus indicated that the vaccine might increase the rate of premature births [81]. Our data did not show any signal towards increased rates of premature births, but with only 1325 pregnant participants in GBS vaccine trials this potential side effect could not be ruled out in our dataset. Hence, it is crucial to establish a robust Vaccine Adverse Event Reporting System (VAERS) and maintain vigilant safety monitoring post-licensure of a maternal GBS vaccine.

### 4.4. Strengths and limitations

The strengths of our systematic review include our rigorous and sensitive search strategy following an *a priori* registered protocol. Additionally, we targeted an area of global concern and importance. GBS vaccines have been focus for clinical trials since the 1990s, still only around 5800 participants were identified in the 20 studies in this systematic review. A greater volume of data is necessary, even in cases where a vaccine's licensure relies on sero-correlation information rather than clinical efficacy. Another key constraint was the inability to conduct a *meta*-analysis for the primary outcome of immunogenicity (IgG GMCs) due to the heterogeneous use of seroassays across studies. The international consortium known as GASTON (Group B Streptococcus: Standardization of Laboratory Assays) has reached a consensus on a unified protocol for GBS antibody assays. This standardized procedure marks a significant milestone in their collaborative efforts to ensure consistency and reliability in GBS-related research [82,83]. Our evaluation of adverse events data revealed no significant issues concerning the various GBS vaccine candidates. Comparable levels of reactogenicity and adverse effects were noted in both the intervention and control groups. However, limited sample sizes prevent us from drawing a definitive conclusion regarding adverse effects.

### 4.5. Implication and conclusion

All candidate maternal GBS vaccines presented good immunogenicity and safety data. A multivalent CPS-based vaccine or a broad-spectrum surface subunit protein vaccine are the most promising vaccine candidates. This systematic review also highlights that there are still significant uncertainties in the determinants of the antibody response, particularly in people who have low baseline GBS antibodies. Our findings also support the recent initiative to standardize measurement methods in order to facilitate direct comparison and extrapolation of results.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors AUB, SR and RM declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. CK is involved in a seroepidemiologocal study, partly funded by Pfizer, but does no receive personal honoraria in this study. KLD's University has received funds from Pfizer, Minervax and GSK for unrelated vaccine work. KLD has received no personal honoraria.

## Data availability

Data will be made available on request.

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# Paper III

Bjerkhaug, A.U., Silmon de Monerri, N.C., Simon, R., Afset, J.E., Cai, B., Mynarek, M., Anderson, A.S., Klingenberg, C.

## Association between anticapsular antibodies and pro &ection against group B streptococcus in Norwegian infants

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