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Serum maternal cytokine levels as predictors of group B Streptococcus colonization at delivery

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SERUM MATERNAL CYTOKINE LEVELS AS PREDICTORS OF GROUP B *STREPTOCOCCUS* COLONIZATION AT DELIVERY

Jelani Muhammad



A dissertation submitted to the Faculty of Health Sciences, University of Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, South Africa, 2019

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

DECLARATION

I, Jelani Muhammad, declare that the work contained herein is my own and that any assistance has been acknowledged and all sources duly referenced. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



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Jelani Muhammad

Signed on this 6th day of August 2019

I certify that the studies included in this dissertation have been approved by the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg, South Africa, as a sub-study (ethics clearance number M1611155) under a primary study (ethics clearance number M140203).



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Jelani Muhammad

Signed on this 6th day of August 2019

PRESENTATIONS

Conference presentations arising out of this work

1. **Muhammad J**, Kwatra G, Scheurmaier K, Madhi SA, Steel H, Harden LM. Maternal serum cytokines in predicting maternal Group B Streptococcus colonization at the time of delivery.

   A poster presentation at the 1st International Symposium on *Streptococcus agalactiae* Disease (ISSAD) from 20th– 23rd February 2018, Cape Town, South Africa

2. **Muhammad J**, Kwatra G, Scheurmaier K, Madhi SA, Steel H, Harden LM. Level of cytokines in relation to maternal Group B streptococcus colonization at delivery.

   Accepted for oral presentation at the 38th conference of the Physiological society of Nigeria (PSN) from 22nd – 27th September, 2019, Nigeria.

ABSTRACT

Group B *Streptococcus* (*Streptococcus agalactiae*), is a β-hemolytic Gram-positive diplococci that has been found to colonize the genito-urinary tract of pregnant women. Maternal GBS colonization during pregnancy is a leading risk factor for neonatal GBS disease with approximately half of all newborns delivered from GBS colonized mothers developing invasive GBS disease. Apart from the culture and the polymerase chain reaction (PCR) methods recommended by Centres for Disease Control and Prevention, studies have shown that cytokines produced by the immune cells in response to the presence of bacteria may also be useful at detecting bacterial infections. The aim of the study was therefore to determine the clinical usefulness of maternal serum pro-and anti-inflammatory cytokine concentrations as a clinical index of intrapartum GBS colonisation. Mothers aged ≥18 years, who were HIV-negative, had vaginal deliveries and were not receiving antibiotic treatment were included in the study. Vaginal swabs and maternal blood were collected after delivery. Vaginal swabs were used to isolate GBS using culture, and PCR methods. Maternal serum samples were processed and used for cytokine analysis using high sensitivity premixed magnetic Luminex performance assays (R&D Systems system). A total of 122 participants were recruited for the study, out of which 35 (28.7 %) were found to be colonised with GBS. The 35 colonised mothers were identified either from a positive culture result or from a positive PCR result on the swab collected at the time of delivery. The most prevalent GBS serotype was serotype Ia, followed by serotype III. All the participants were young to middle aged women with a mean (SD) age of 26.5 (6) years (range: 18 to 43). Almost all the participants 117 (96%) were of black African ancestry. Overall, there were no significant differences in maternal characteristics such as age, race, middle upper arm circumference or gravidity between the two groups. The majority of the study participants delivered at full term gestation (≥ 37 weeks gestation). The infants were healthy with median Apgar scores of 9 and 10 at 1 and 5 minutes. None of the babies developed neonatal GBS disease within 90 days of birth. There were no significant differences in serum maternal IL-6 ($P = 0.87$, unpaired t test; $t = 0.16$), IL-8 ($P = 0.19$ unpaired t test; $t = 1.48$) and IL-10 ($P = 0.95$ unpaired t test; $t = 0.06$) concentrations in women colonised with GBS and women not colonised with GBS at delivery. Thus, measuring

maternal serum IL-6, IL-8 and IL-10 may not be an appropriate clinical index of intrapartum GBS colonisation in healthy women.

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LIST OF ABBREVIATIONS

Ab:	Antibody
Ag:	Antigen
BBB:	Blood Brain Barrier
BE:	Bile Esculin
BibA:	Group B streptococcal immunogenic bacterial adhesin
BMI:	Body Mass Index
CAMP:	Christin Artesen Munsen-Petersen
CCD:	Charged Coupled Device
CD:	Cluster of Differentiation
CDC:	Centre for Disease Control and Prevention
CO_2:	Carbon dioxide
CovR:	Control of virulence Regulator
CovS:	Control of virulence Sensor
CPS:	Capsular Polysaccharide
C/S:	Caesarian Section
CRP:	C - reactive protein
DNA:	Deoxyribonucleic Acid
E. coli:	*Escherichia Coli*
ELISA:	Enzyme Linked Immunosorbent Assay
ECM:	Extra Cellular Membrane

EOD:	Early Onset Disease
EOND:	Early Onset Neonatal Disease
Fbs:	Fibrinogen binding protein
GAPDH:	Glyceraldehyde 3-phosphate Dehydrogenase
GBS:	Group B *Streptococcus*
GCP:	Good Clinical Practice
G-CSF:	Granulocyte-Colony Stimulating Factor
GM-CSF:	Granulocyte Macrophages- Colony Stimulating Factor
HylB:	Group B *Streptococcal* Hyaluronidase
i-ELISA:	Inhibition-ELISA
iNOS:	inducible Nitric Oxide Synthase
Ig:	Immunoglobulin
IL:	Interleukin
IFN:	Interferon
L. Monocytogen:	*Listeria Monocytogen*
LED:	Light Emitting Diodes
LOD:	Late Onset Disease
LOND:	Late Onset Neonatal Disease
Lmb:	Laminin binding protein
METs:	Macrophages Extracellular Traps
MIA:	Multiplex-based Immunoassay

MMP:	Matrix Metallo-Proteinase
MyD88:	Myeloid Differentiation Factor-88
NAAT:	Nucleic Acid Amplification Test
NETs:	Neutrophils Extracellular Traps
NLR:	Node-Like Receptor
NT:	Non Typable
PCT:	Procalcitonin
PCR:	Polymerase Chain Reaction
PE:	Phycoerythrin
pH:	Potential of hydrogen
PPROM:	Preterm Premature rupture of Membrane
PRR:	Pattern Recognition Receptors
RNA:	Ribonucleic Acid
RNP:	Ribonuclease P (RNase P)
RPM:	Revolution per Minute
ScpB:	Group B streptococcal C5a peptidase
SD:	Standard Deviation
SfbA:	Streptococcal Fibronectin binding protein A
Srr:	Serine-rich repeat glycoprotein
STGG:	Skim-Milk Tryptone Glucose Glycerol
TH/th:	T-helper cells

TLR:	Toll like Receptors
TNF:	Tumor Necrosis Factor
UTI:	Urinary Tract Infection
WHO:	World Health Organization

CHAPTER ONE

INTRODUCTION

Streptococcus agalactiae (Group B *Streptococcus* or GBS), is the species designation for *streptococci* belonging to Lancefield group B and is a Gram-positive β-haemolytic, encapsulated diplococci (Edwards, Nizet & Baker 2016). GBS was first identified approximately 130 years ago, in 1887, as a pathogen causing mastitis (persistent inflammation of udder tissue) in cattle (Francois*et al.*, 1972). Between 1936 and 1937, that is approximately 50 years after the discovery of GBS as a pathogenic bacterium in cattle, it was linked with causing fatal postpartum sepsis in women (Fry 1938). Around the time of its discovery as a sepsis-inducing pathogen in women, GBS was found in the cervix and vagina of some parturient women before and after labour (Hare &Colebrook 1934). Whether there was a link between GBS found in the vaginal tract and postpartum bacterial infections was much-debated though (Hare &Colebrook 1934). Throughout the 1930s and 1970s GBS was also identified as a leading cause of septicaemia and meningitis in neonates (Nyhan & Fousek, 1958, Eickhoff *et al.*, 1964; Baker and Barrett 1973; Francois *et al*, 1973). From epidemiological studies undertaken around the 1970s, it was noted that two distinct types of GBS disease were reported in neonates and infants (Barton *et al.*, 1973; Baker *et al.*, 1973; Franciosi *et al*., 1973). The early-onset disease occurred within the first ten days of birth and the late-onset disease occurred between 10 and 90 days after birth (Baker *et al*., 1973; Franciosi *et al*., 1973).

1.1 Maternal GBS colonization

Results obtained from several studies undertaken around the 1970s, indicated that the rectum and vagina are the most common sites of GBS carriage in pregnant women (Badri *et al.*, 1977; Dillon *et al.*, 1982). Ascending transmission of GBS from the genital tract is a likely manner by which early-onset neonatal GBS infection is acquired (Baker *et al*., 1973; Barton *et al*., 1973; Eickhoff *et al*., 1964; Francois *et al*., 1973; Hood *et al.*, 1961). The mode of transmission of GBS in infants that develop late-onset disease was less clear at the time (Baker and Barrett 1973). Considering the high morbidity and mortality associated with early-onset neonatal GBS disease and its association with maternal GBS colonization, studies were undertaken to determine the potential value of routine GBS identification and antimicrobial treatment of pregnant female GBS carriers, at reducing the burden of early-onset neonatal GBS disease (Boyer *et al*., 1983, Franciosi *et al*.,

1973). The positive results from these studies, showing that antenatal GBS screening and intrapartum antibiotic prophylaxis (IAP) can prevent early-onset GBS disease in neonates, lead to the implementation of guidelines in 2002. These guidelines included universal antenatal culture screening between 35 and 37 weeks' gestation and intravenous antibiotic administration during labour in women who are GBS carriers or at risk to be GBS carriers (Centres for Disease Control and Prevention (CDC) 1996; Centres for Disease Control and Prevention (CDC) 2010). GBS vaginal colonization has been associated with many risk factors, which are both biological and socio-economical in nature. Risk factors associated with GBS vaginal colonization include, a history of premature rupture of the membrane (PROM) (Alp *et al.,* 2016) and gastrointestinal and genitourinary GBS carriage (Le-Doare *et al.,* 2013). Furthermore, studies have shown that increased maternal age (≥36 years) is associated with persistent vaginal GBS colonization (Manning *et al.,* 2008; Khan *et al.,* 2015), as is being black, obese, having a low vitamin D intake, poor hygiene and literacy status (Le-Doare *et al.,* 2013; Capan-Melser *et al.,* 2015; Stapleton *et al.,* 2005).

Countries, such as the United States of America (USA), which adopted the CDC guidelines and had the capacity to ensure implementation, reported a substantial decline in the incidence of early-onset GBS neonatal infection (Schrag *et al*., 2000). However, due to limited resources most low- and middle-income countries do not have the capacity to implement IAP and rectovaginal screening. Moreover, IAP has been shown to have no effect on late-onset GBS disease (Centres for Disease Control and Prevention (CDC) 2007) and is unlikely to have an effect on stillbirth and preterm birth associated with maternal rectovaginal GBS colonization (Seale *et al.,* 2017). In South Africa, IAP has not been standardized and it has been reported that only about 25% of those who fulfil the criteria for IAP actually receive it (Dangor *et al.,* 2015). Thus, as outlined in figure 1.1 below, through maternal rectovaginal colonization, GBS continues to cause a worldwide spectrum of disease during pregnancy and post-natally (as reviewed in Lawn *et al.,* 2017). The first comprehensive worldwide estimates of the burden of GBS disease for pregnant women, stillbirths and children was published in 2015 (see Figure 1.1 below).

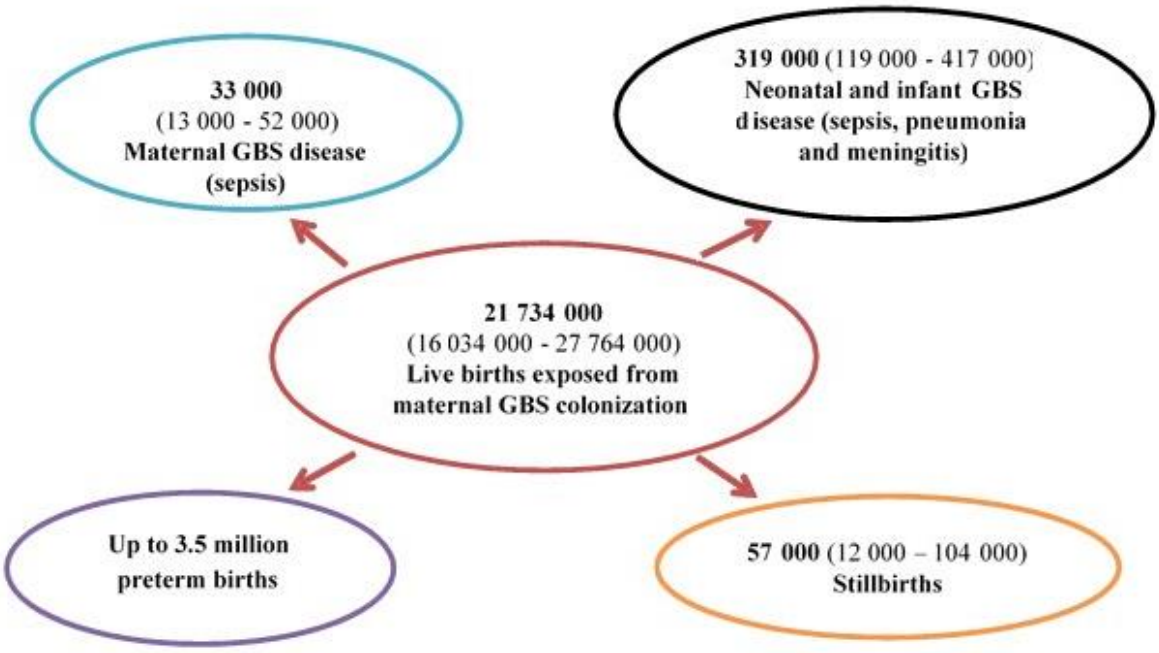


Figure 1.1: Spectrum of disease through maternal rectovaginal GBS colonization and the worldwide estimates for 2015 (adapted from Figure 2, Seale *et al.*, 2017). Values are given as calculated estimates with the uncertainty range in brackets.

Figure 1.2 shows that GBS has an ultrastructure similar to that of other Gram-positive cocci with a thick, rigid peptidoglycan layer external to the cytoplasmic membrane (Fischetti *et al*., 2006). Two polysaccharides, Group B carbohydrate and the capsular polysaccharide (CPS), have been proposed to be covalently bound to separate sites on the peptidoglycan of the GBS cell wall (Deng*et al*., 2000).

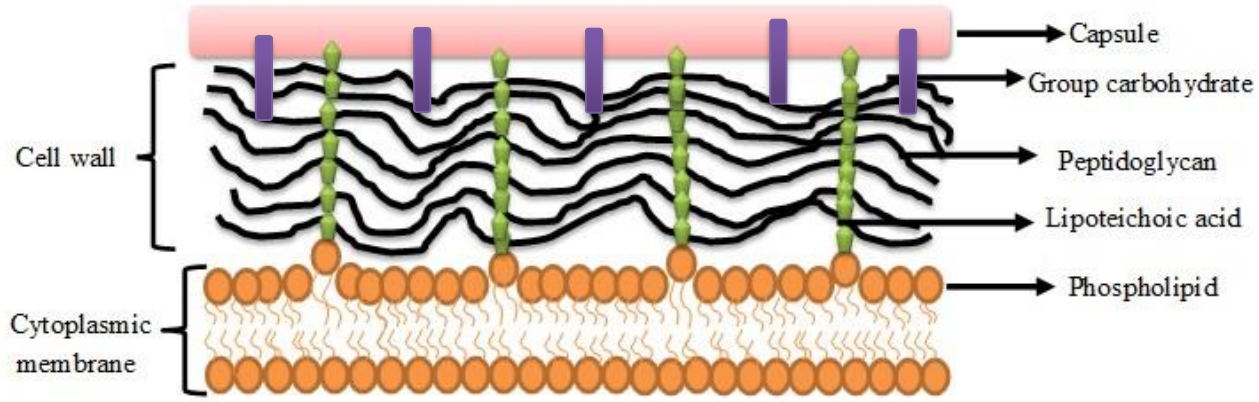


Figure 1.2: Proposed ultrastructure of GBS, adapted from Figure 1 (chapter 1, page 13) in Fischetti *et al*., 2006. GBS has a thick, rigid peptidoglycan layer external to the cytoplasmic membrane. Two polysaccharides, Group B carbohydrate and the capsular polysaccharide (CPS), have been proposed to be covalently bound to separate sites on the peptidoglycan of the GBS cell wall. Lipoteichoic acid, composed of a polymer linked to the cytoplasmic membrane through phospholipids, plays a role in bacterial growth, homeostasis and virulence (Percy & Grundling, 2014).

The current classification of GBS is founded on serology and capsule polysaccharide structure (Fischetti *et al.,* 2006). To date ten GBS serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII and IX) have been identified (Slotved *et al.,* 2007). All the serotypes are antigenically and structurally different (Cieslewicz *et al.,* 2005). "The serotype specific epitopes of each polysaccharide are created by different arrangements of four sugar components (glucose, galactose, N-acetyl glucosamine and sialic acid) into a unique repeating unit, but unfailingly these structures contain a terminal sialic acid (Neu5Ac) bound to galactosein an α2→3 linkage" (Doran and Nizet, 2004). Table 1.1 shows recent data on the global distribution pattern of GBS serotypes colonizing mothers and causing maternal and infant disease (Seale *et al.*, 2017). Overall serotypes Ia, Ib, II, III, and V account for the majority of maternal GBS colonization and GBS disease in pregnant/postpartum women and infants globally.

Table 1.1: Global distribution of GBS serotypes colonizing mothers and causing disease in pregnant/postpartum women and infants.

	Prevalence (%)								Reference
	Ia/Ib**	**Ia**	**Ib**	**II**	**III**	**IV**	**V**	**VI, VII, VIII, IX**	
Maternal GBS colonization (n = 16181)	32	21	8	11	25	1	18	2	Russel *et al.*, 2017
Maternal GBS Disease (n = 310)		31	3	14	27	0	19	0	Hall *et al.*, 2017
Infant GBS disease (n = 6500)		19	6	4	62	1	7	<1	Madrid *et al.*, 2017

** 7 studies did not differentiate between Ia and Ib

This introductory chapter focuses on maternal GBS colonization and summarizes the current understanding of the immune response to maternal GBS colonization and the screening modalities for the detection of GBS colonization during pregnancy.

1.2 Mechanism of maternal GBS colonization during pregnancy

GBS colonizes the human lower gastrointestinal tract, and in females, the lower reproductive tract, of 20 - 30% of healthy adults (Doran *e al.*, 2004). Maternal GBS colonization is clinically defined as the isolation of GBS from the vagina, rectum or perianal region at any stage of pregnancy (Seale *et al.*, 2015; Russell *et al.*, 2017). Studies have reported a difference in the prevalence of GBS colonization during pregnancy, depending on the region the study was conducted in, the population studied and the sampling and culturing methods used. Recent estimates have the worldwide prevalence of maternal GBS rectovaginal colonization at 17.9% (95% confidence interval [CI], 16.2% – 19.7%), fluctuating from 11.1% (95% CI, 6.8% – 15.3%) in Southeast Asia to 22.4% in

Africa (95% CI, 18.1% – 26.7%) (Patra & Nizet., 2018; Kwatra *et al.,* 2016). Maternal GBS colonization is associated with peripartum and postpartum infections, such as urinary tract infections, chorioamnionitis, endometriosis and sepsis; all of which may lead to preterm labour and premature delivery (as reviewed in Gibbs *et al.,* 2004 and Schuchat 1999). GBS colonization in pregnancy in most cases is asymptomatic (Schubart & Balter 2006), but identifying women who are colonized with GBS during pregnancy is essential because of the increased risk of colonized women giving birth to colonized babies (Anthony *et al.,* 1979). Studies have shown that most pregnant women colonized with GBS during prenatal screening, are more likely to also be colonized or remain colonized until delivery (Boyer et al., 1983). Thus, women who are colonized around the first trimester of pregnancy are at an increased risk of persistent GBS colonization until delivery, and the risk of being colonised at delivery increases further with a shorter period between the prenatal culture and delivery (Boyer *et al.,* 1983; Dillon *et al.,* 1982).

1.2.1 Interactions of GBS with the vaginal epithelium

The ability of GBS to survive and colonize the female genitourinary tract has been linked to its various virulence factors (Landwehr-Kenzel & Henneke, 2014; Vornhagen *et al.,* 2017; Nizet and Rubens, 2006; Shabayek & Spellerberg, 2018). Adherence to the luminal epithelial cells and/or host proteins has been shown to be an essential step for GBS colonization of the host mucosal surface (Shabayek & Spellerberg, 2018). The adherence ensures bacterial cell aggregation and biofilm formation (Shabayek & Spellerberg, 2018). As the bacteria adheres to the cell surface of the host, it is protected from the immune system by constructing colonies that are surrounded by extracellular membrane polysaccharide proteins forming a biofilm (Konto-Ghiorghi *et al.,* 2009). Biofilm formation is facilitated by specific host environmental factors, such as low pH and the presence of plasma (Costertan *et al.,* 1999; Nobb *et al*., 2009; Rosini & Margarit, 2015). Adhesins that play a significant role in mediating GBS vaginal adherence and/or invasion includes fibrinogen-binding proteins (Fbs), laminin binding proteins (Lmb), group B *streptococcal* C5a peptidase (ScpB), *streptococcal* fibronectin binding protein A (SfbA) and the GBS immunogenic bacterial adhesin (BibA) (Shabayek & Spellerberg, 2018). Furthermore, protein like pili projecting from the GBS surface, are also essential adhesins that facilitate GBS colonization, survival and

biofilm formation (Shabayek & Spellerberg, 2018). The major adhesins mediating GBS interaction with cells of the host are shown in Figure 1.3 below.

The five fibrinogen-binding proteins (Fbs) identified in GBS so far are, FbsA, FbsB, FbsC and the serine-rich repeat (Srr) glycoproteins Srr1 and Srr2 (reviewed in Shabayek & Spellerberg *et al*., 2018). Colonising GBS isolates have a much weaker fibrinogen-binding ability compared to invasive GBS isolates (Roseanau *et al.,* 2007). Results obtained from a study using an *in vivo* mouse model of GBS vaginal colonization, identified the importance of Srr1 in particular in vaginal colonisation, as mice inoculated with an Srr-1-deficient mutant showed decreased GBS vaginal persistence compared to those inoculated with the wild-type parental strain (Sheen *et al.,* 2011). *Streptococcal* fibronectin-binding protein A (SfbA) is a more recent GBS fibronectin binding protein that has been identified (Mu *et al.,* 2014) to be involved in the cellular invasion of GBS, but not the adherence of GBS (Mu *et al.,* 2014). SfbA allows GBS invasion of vaginal and cervical epithelial cells and thus could be involved in GBS colonization in the vagina (Mu *et al.,* 2014).

Apart from adherence to the extracellular matrix, adherence of the bacteria to host cells is another factor that plays a significant part in the pathogenesis of GBS colonization. In this case, GBS immunogenic bacterial adhesin (BibA) is an important adhesin found in the cell wall that facilitates GBS binding to human epithelial cells (Santi *et al.,* 2007; Santi *et al.,* 2009). Moreover, BibA has been shown to help with the survival of GBS in human blood, by affecting the classic complement pathway and phagocytic activity of neutrophils (Santi *et al.,* 2007, 2009b). Additional proteins that are important for GBS adhesion are the pili, which are present on the bacterial cell wall and extend from the bacterial surface (Dramsi *et al.,* 2006). Pili consist of three different proteins, the pilus shaft backbone protein (BP) or PilB subunits, and two ancillary proteins AP1 and AP2 located at the pilus tip (PilA subunit, the pilus associated adhesin) and pilus base (PilC subunit, the pilus anchor) (Dramsi *et al.,* 2006; Rosini *et al.,* 2006; Maisey *et al.,* 2007, 2008b; Cozzi *et al.,* 2015). PilA is involved with adherence and colonization of the host cellular epithelium, while

PilB is involved with bacterial invasion and translocation (Dramsi *et al.*, 2006; Krishnan *et al.*, 2007; Maisey *et al.*, 2007, 2008b; Pezzicoli *et al.*, 2008; Sheen *et al.*, 2011).

Not only are the surface-protruding structures, described above, involved in GBS colonisation, but so too are structures secreted by GBS, such as hyaluronidase (HyIB) (Kolar et al., 2015). Results obtained from a study using an *in vivo* mouse model of GBS vaginal colonization, identified that there was an increased mouse vaginal clearance of GBS when mice were colonised with a strain of GBS where HylB had been deleted, thus identifying the involvement of HyIB in the survival of GBS (Kolar *et al.*, 2015).

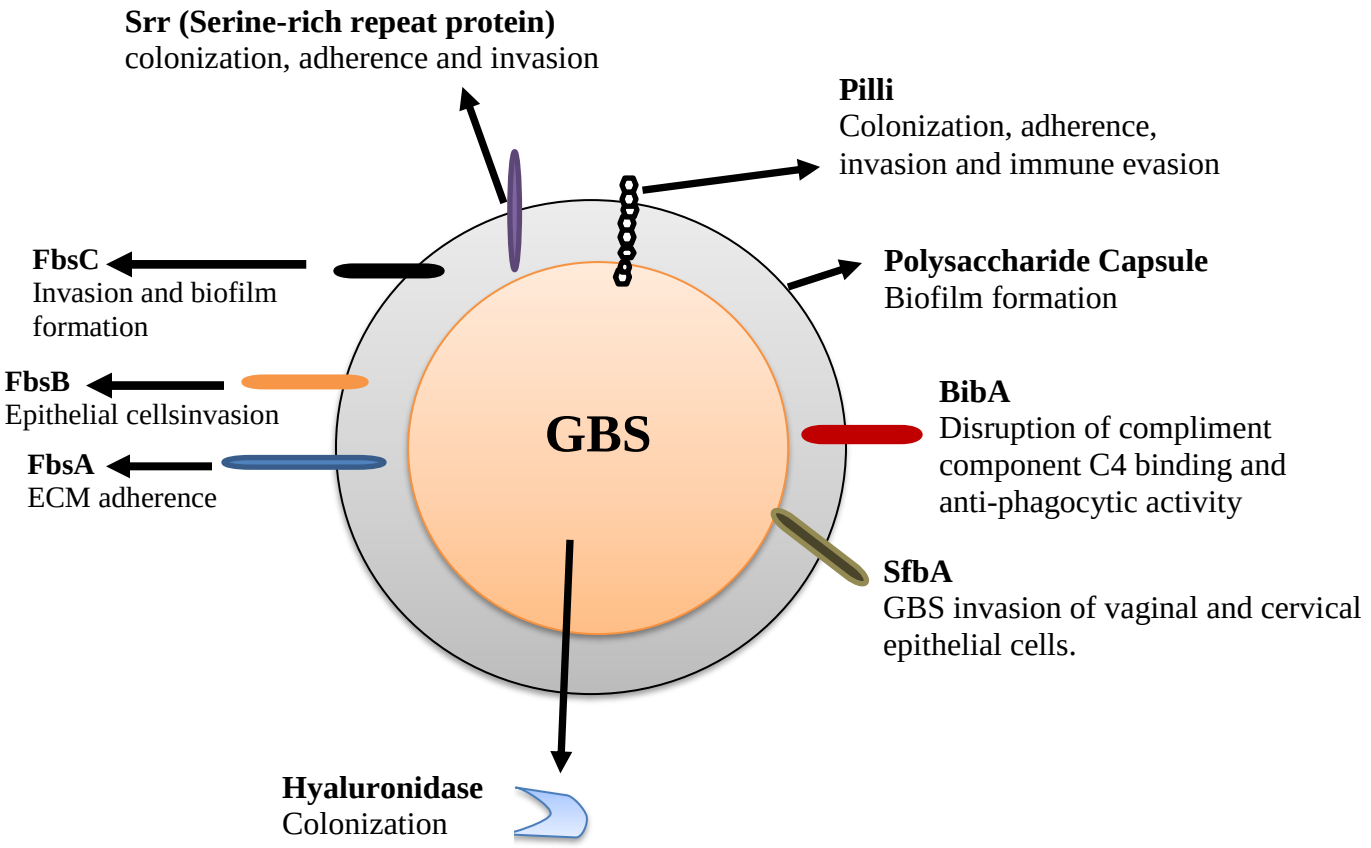


Figure 1.3: Bacterial factors that promote GBS vaginal colonization and ascending infection. Fibrinogen-binding protein (Fbs) (FbsA, FbsB, FbsC), the serine-rich repeat glycoproteins (Srr1 and Srr2), GBS immunogenic bacterial adhesin (BibA), *Streptococcal* fibronectin-binding protein A (SfbA). Protruding structures, such as the GBS pili are important adhesins in facilitating GBS colonization, persistence, invasion and biofilm formation. (Adapted from Figure 1 in Shabayek and Spellerberg 2018).

1.2.2 Potential impact of GBS regulatory systems on vaginal colonization

GBS possess various regulatory systems that control several virulence factors that permit the progression from colonization to the invasive disease state (Patras and Nizet, 2018). Control systems common in bacteria have been identified and they consist of a membrane-liked histidine kinase sensor and a cytoplasmic transcriptional element (Ulrich *et al.,* 2005; Beier *et al.,* 2006). The sensor protein in the control system detects external stimuli and communicates with the regulatory protein that responds by activating specific genes that affect bacterial function (Cotter *et al*., 1998). With regards to GBS, control of the virulence sensor/regulator (CovS/CovR) system mediates the expression of pili, and BsaB, which are involved in the expression of the primary toxin of GBS, β-hemolysin/cytolysin (βH/C) and many other proteins (Jiang & Wessel., 2014; Lembo *et al.,* 2010; Jiang *et al*., 2005). CovS responds to changes in pH (Lembo et al., 2010). In response to a low pH environment, like within the vagina, CovS via the action of CovR, ensures down-regulation of some virulence factors and increases the number of genes that promote colonization (Lembo *et al.,* 2010). By reducing, the expression of virulence factors during vaginal colonization, the CovS/CovR system ensures that the host innate immune response will be suppressed and colonization will be facilitated (Patras et al., 2013). An increase in pH reduces CovS and causes an increase in the expression of the virulence factors such as BibA and beta-hemolysin (Santi *et al.,* 2009A).

1.2.3 Immune response to maternal vaginal GBS colonization

The ability of GBS to colonize and survive the vaginal epithelial environment is facilitated by its capacity to overcome many cellular and physical barriers, including the vaginal mucosal, epithelial layers and immunological response within the vagina (Patras *et al*., 2015; Carey *et al.,* 2014). Figure 1.4 shows a summary of the likely immune response to GBS within the genitourinary tract. Upon primary vaginal colonization, mast cells are activated and degranulate through the hemolytic activity of the GBS pigment (Vorhangen et al., 2017). Degranulation leads to the release of inflammatory mediators, including interleukin (IL)-6, IL-8, tumour necrosis factor (TNF)-α, and histamine, which, in turn, recruit other immune cells, such as neutrophils and macrophages, to help with bacterial clearance (Gendrin et al., 2015). Resident vaginal macrophages function in the

clearance of GBS via phagocytosis (Rogers et al., 2018) and the formation of macrophage extracellular traps (METs) (Doster *et al.*, 2018). The METs contain proteins and enzymes capable of killing GBS (Doster *et al*., 2018). Neutrophils also, facilitate GBS clearance by the formation of neutrophil extracellular traps (NETs), which also facilitate intracellular killing of GBS within the vagina (Boldenow *et al*., 2016; Gendrin *et al.*, 2015). Furthermore, GBS activates the node-like receptor protein3 (NLRP3) inflammasome in neutrophils, leading to secretion of IL-1β and other inflammatory cytokines, that all help to ensure GBS clearance (Boldenow et al., 2015). To ensure colonization, GBS will thus attempt to suppress activation of the immune system and the release of inflammatory cytokines, which facilitate GBS clearance. One such mechanism uses HyIB, which is secreted by GBS, to target and degrad host hyaluronic acid (Gochnauer et al., 1951; Baker et al., 2000). Hyaluronic acid is an anionic, nonsulfated glycosaminoglycan, distributed widely throughout connective and epithelial tissues, which plays a role in cell migration, cell signalling and regulation of inflammation (Stern et al., 2006). Host hyaluronic acid is degraded into its disaccharide components, by HyIB, which bind to toll-like receptor (TLR) 2/4 and block inflammatory signalling (Kolar et al., 2015). Activation of the immune response, by TLRs, is achieved via several intracellular signalling proteins, like the Myeloid Differentiation Factor 88 (MyD88), which leads to the production of several pro-inflammatory cytokines (Kenzel et al., 2006; Asplin et al., 2008). The importance of HyIB in controlling the immune response to GBS and thus allowing colonisation is highlighted by the observations that uterine tissue infected with HyIB-proficient GBS showed decreased levels of inflammatory cytokines such as TNF-α, IL-6, and IL-8 (Vornhagen et al., 2016).

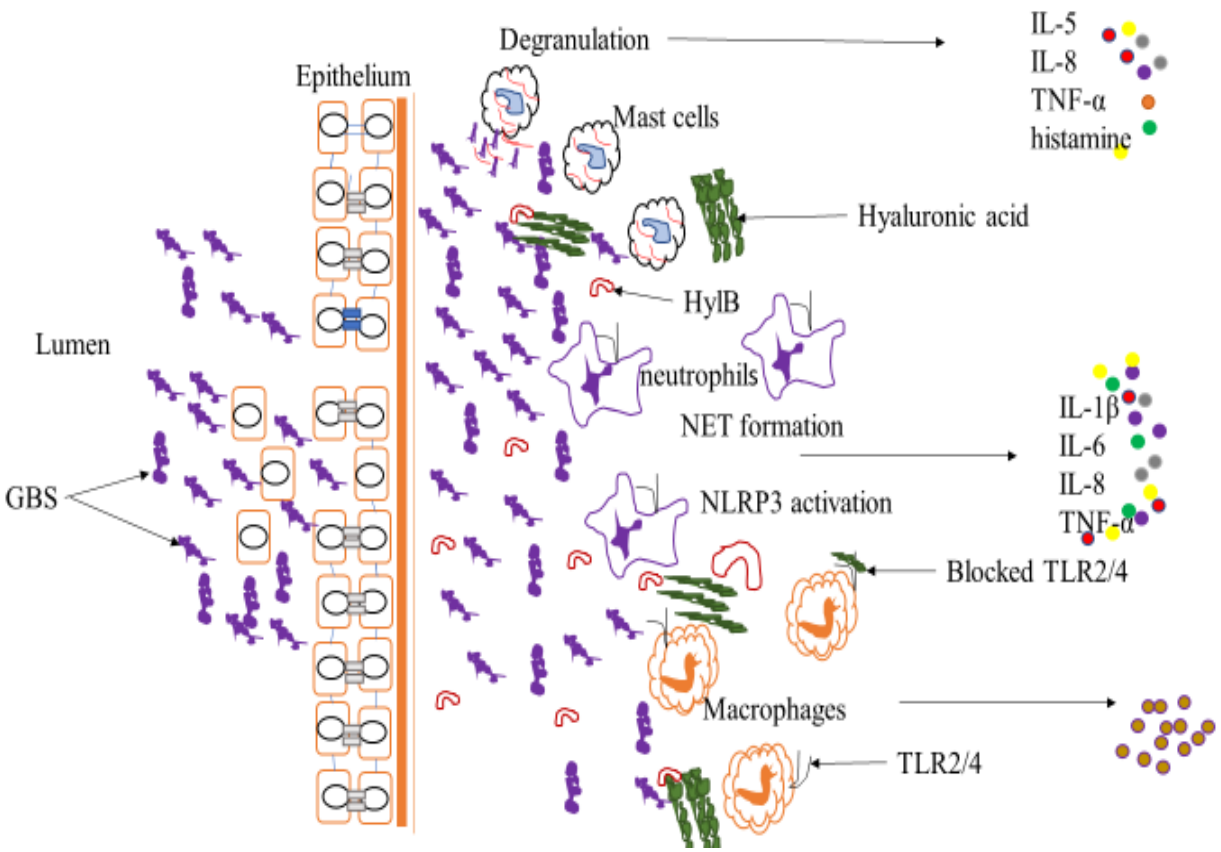


Figure 1.4: As GBS colonizes the genitourinary tract, vagina in particular, there will be activation of mast cells that will later undergo degranulation, via the action of the GBS haemolytic pigment. Degranulation of mast cells will lead to the release of inflammatory mediators, such as IL-6, IL-8, TNF-α and histamine. The release of inflammatory mediators leads to the recruitment of other immune cells, such as neutrophils and macrophages that facilitate GBS clearance (Gendrin et al., 2015). GBS also activates neutrophil node-like receptor protein3 (NLRP3) inflammasomes, which further cause the release of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α). Macrophages also function to facilitate GBS clearance through phagocytosis and the formation of extracellular traps (Rogers *et al.*, 2018). GBS HylB degrades host hyaluronic acid into its disaccharide components, which are immunosuppressive, as they bind to toll-like receptor (TLR) 2/4 on macrophages and block inflammatory signalling (Kolar et al., 2015). (Figure is adapted from Vornhagen *et al.*, 2017).

Within the cytosol, that is the semi-fluid substance filling the cytoplasm of the cell, there exists an enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), that mediates the conversion of glyceraldehyde 3-phosphate to 1-3-biphosphateglycerate in the glycolytic energy pathway (Tristan et al., 2011). GAPDH from GBS has been implicated in attenuating the immune response, by stimulating the expression of an anti-inflammatory cytokine, IL-10 (Andrade *et al.*, 2013;

Madureira *et al.*, 2011; Madureira *et al.*, 2007). Continued production of IL-10 has been shown to downregulate the recruitment of neutrophils and thus reduce bacterial clearance (Madureira *et al.*, 2011). Other studies have also reported similar findings with the observation that a reduction in IL-10 increases neutrophil recruitment and increases bacterial clearance (Babien *et al*., 2012; Andrade *et al.*, 2013; Madureira *et al.*, 2011; Madureira *et al.*, 2007). Even though it has not yet been investigated, it is therefore possible that GBS-induced increase in IL-10 could be a mechanism by which GBS colonizes the vaginal epithelial cells (Landwehr-Kenzel and Henneke, 2014).

1.3 Maternal GBS colonization screening

Pregnant women colonized with GBS have an increased risk of delivering babies with early onset GBS disease (Boyer & Gotoff, 1985). Intravenous intrapartum antibiotic prophylaxis (IAP) has been shown to have an 86 - 89% efficacy in reducing early onset GBS disease in infants born to mothers colonized with GBS (Lin *et al.*, 2001; Schrag *et al.*, 2002). In 1996, guidelines were published, which indicated that a risk based approach or a culture-based screening approach could be used in identifying women who should receive IAP (CDC, 1996). Using the risk-based approach, women are deemed to be a candidate for IAP if they deliver at a gestational age of < 37 weeks, have signs of an intrapartum fever (body temperature ≥ 38.0ºC), or have rupture of membranes for ≥ 18 hours (Hughes et al., 2017; Le Doare et *al.*, 2017). With regards to the culture-based screening approach, recto-vaginal swabs have been shown to produce more positive cases of GBS, than using only vaginal or cervical swabs (Hoogkamp-Korsanje *et al.*, 1982; Badri *et al.*, 1977; Dillon *et al.*, 1982; Philipson *et al.*, 1995; Platt et al., 1995; Quinlan *et al.*, 2000; Kovavisarah *e tal.*, 2007). The culture-based approach has been shown to be more effective than the risk-based approach in preventing early onset GBS disease in neonates, based on data obtained from a large population based study undertaken between 1998 and 1999 in the USA (Schrag *et al.*,2002). The positive results from these studies, which showed that antenatal GBS screening and IAP can prevent early-onset disease in neonates, lead to the implementation of CDC 2002 guidelines for universal antenatal culture screening between 35 and 37 weeks' gestation and intravenous antibiotic administration during labour in women who are GBS carriers (CDC, 2002, 2006).

It is important to determine the appropriate time for prenatal GBS colonization screening, as GBS status has been shown to change as the pregnancy advances (Boyer *et al.,* 1983). As GBS colonization can be temporary, women who are colonized around the first trimester of pregnancy are not predictive of colonization at delivery (Regan *et al.,* 1996). Vaginal and rectal swabs obtained from 826 women during routine prenatal visits at approximately 35 -3 6 weeks' estimated gestation, identified that the positive predictive value of antenatal cultures was 87% (95% CI 83-92) and the negative predictive value was 96% (95% CI 95-98) (Yancey *et al.,* 1996). The outcome of the antenatal culture was similar between 1 - 5 weeks before delivery, but dropped when there was 6 or more weeks between the antenatal culture and delivery (Yancey *et al.,* 1996).

It is beneficial to use an appropriate transport media to ensure that GBS viability is maintained until culturing (Teese *et al.,* 2003; Crisp *et al.,* 1998). If retained in the transport medium at room temperature, the viability of the GBS isolate can be assured for several days, but the retrieval of the isolates starts to reduce around day four, especially if the sample has been kept at high ambient temperatures (Rosa-Fraile & Spellerberg, 2017). Even with the use of transport media, the culture method is more sensitive when the specimen is kept at 4°C before culturing and when it is processed within 24 hours of collection (Hakansson *et al.,* 2008; Ostroff *et al.,* 1995; Stoner *et al.,* 2004; Rosa-Fraile *et al.,* 2005). About half of the women who are GBS carriers have a false-negative culture result when direct agar plating is done, instead of using selective enrichment broth before agar plating (Philipson *et al.,* 1995; Platt *et al.,* 1995; Baker *et al.,* 1973; Altaie *et al.,* 1994). The improved GBS identification noted when an enrichment broth is used, is likely related to the enrichment broth inhibiting the growth of other microorganisms (Philipson *et al*., 1995). Todd-Hewitt broth supplemented either with gentamicin (8 µg/ml) and nalidixic acid (15 µg/ml) [TransVag broth] or with colistin (10 µg/ml) and nalidixic acid (15 µg/ml) [Lim broth] are some of the examples of selective enrichment broths used (Fenton *et al.,* 1979).

After enrichment, isolation with subculture on blood agar plates is the suggested means to identify GBS with subsequent confirmation using the Christin Artesen Munsen-Petersen (CAMP) test (Wilkinson *et al.,* 1977) or serological identification using the latex agglutination test with group B *streptococcal* antisera (Guerrero *et al.,* 2004). Certain organisms, including GBS, produce a

diffusible extracellular haemolytic heat-stable protein (CAMP factor) that acts synergistically with the beta-lysin of *Staphylococcus* aureus to cause enhanced lysis of red blood cells. A positive reaction appears as an arrow-head zone of haemolysis adjacent to the place where the two streaked lines come into proximity (Wilkinson *et al.*, 1977). *Enterococcus faecalis* will grow on the sheep blood agar, but will show no enhanced haemolytic reaction near the line of haemolysis from *Staphylococcus* aureus. GBS produces a positive CAMP test. All *enterococcus faecalis* and other *Streptococcus* species are CAMP negative (Munch-Petersen *et al.,* 1945; Christie *et al.,* 1944). The latex agglutination test involves mixing the sample with latex beads coated with antibodies. Most of the streptococcal species bear a specific carbohydrate antigen, if the antigen is available in the sample, it will react with the antibodies, which causes the latex beads to form an agglutination reaction, and it has been shown to be a rapid and sensitive method for GBS isolation (Webb & Baker, 1980). Chromogenic agar, which undergo a colour change in the presence of beta-haemolytic colonies of GBS, are also used (Votava *et al.,* 2001; Tazi *et al.,* 2008). There are other faster methods that can be used for GBS identification directly from enrichments broths or after subculture including DNA probes (Ryan *et al.,* 1999; William-Bouyer *et al.,* 2003; Montague *et al.,* 2008; Peltroche-Llacsahuanga *et al.,*2010) and nucleic acid amplification tests (NAAT), such as polymerase chain reaction (PCR) (Goodrich *et al.,* 2007; Block *et al.,* 2008). The gene responsible for encoding the CAMP factor, is the cfb gene and it is present in almost all the GBS isolates and is primarily the main factor for the development of PCR procedures for GBS identification (Podbielski *et al.,* 1995).

The use of commercially available NAAT, using non-enriched samples, to detect GBS, has shown varying degrees of sensitivity (range: 62.5 - 98.5%) and specificity (range: 64.5 - 100%) when compared with the enrichment of samples, followed by subculture (Bergeron *et al.,* 2000; Davies *et al.,* 2004; Aziz *et al.,* 2005; Atkins *et al.,* 2006; Gavino *et al.,* 2007; Smith *et al.,* 2008; Edward et al., 2008; Money *et al.,* 2008; El Helali *et al.,* 2009; Alfa *et al.,* 2010; Scicchitano and Bourbeau, 2009). Using enriched samples increases the sensitivity of NAAT for identification of GBS to 92.5 – 100.0% (Goodrich *et al.,* 2007; Block *et al.,* 2008; Scicchitano and Bourbeau, 2009). Thus, current GBS identification options include a positive identification from culture screening or NAAT, such as commercially available PCR assays. One area, which has not been explored

substantially, is whether immune markers could be used for identifying intrapartum colonization. To date two studies have identified that assessing pro-inflammatory cytokines could potentially be an additional screening tool in identifying intrapartum or antepartum GBS colonization. One study investigating subclinical GBS infection in pregnant women, showed a five-fold increase in IL-6 in pregnant women with a chorioamnionitis with GBS subclinical infection when compared with the control group (Yi & Xinghua, 2015). The second study reported a two-fold increase in maternal plasma IL-1β in GBS positive compared with GBS negative pregnant women (Mitchell *et al.*, 2013).

As outlined above cytokine production is associated with activation of the immune system. Increased concentrations of cytokines have been shown to be a marker of various maternal and fetal infections, such as sepsis. Table 1.1 shows a summary of some studies done using cytokines as marker of diseases and immune activation. To date, enzyme-linked immunosorbent assays (ELISA), have been a widely used and accepted method for the quantification of cytokines in research studies (Leng *et al*., 2008). Determining the inflammatory profile of an individual could however require the quantification of multiple cytokines simultaneously, as shown in Table 1.1. A common method to achieve this, is the multiplex-based immunoassay (MIA) method (Leng *et al*, 2008), which can quantify many different analytes in a single sample with a small volume (Dossus *et al.*, 2009). The multiplex immunoassay based on the suspension array platform is manufactured by Bio-Plex (Bio-Rad Laboratories), FlowCytomix (Bender MedSystems), cytometric bead array (Becton, Dickinson and Company) and the partners of Luminex Corp (XMAP)] (Yiğitbaşı 2012). With the suspension array format capture antibodies are conjugated to different populations of microbeads, which can be identified by their fluorescence intensity in a flow cytometer (Yiğitbaşı 2012). Even though, ELISA is still the most recommended validated and standard method for cytokine analysis and quantification, multiplex assays have been shown to be more effective and relevant to analyze multiple panels of different cytokines with relatively small volume of sample in a cost effective manner and within a short period of time (Leng *et al*., 2008).

Table 1.2: Summary of studies measuring cytokines as markers of diseases

Disease condition	Cytokines measured	Outcome	Reference
82 patients (38 men and 44 women) with severe sepsis	Serum concentrations of the TNF-α, IL–1β, IL-6, IL-10, IL-1ra and sTNFRs	The anti-inflammatory cytokine IL-10 is the main predictor of severity and fatal outcome	Gogos *et al*, 2000
16 invasive, 20 noninvasive, and 24 pharyngeal Group A streptococcus colonization, and 21 healthy controls	Plasma concentrations of IL-1β, IL-8, IL-12, IL-18, IL-2, IL-6, IL-10, TNF-α, IFN-γ	Children with invasive GAS infections exhibited significant up-regulation of plasma levels of IFN-γ, IL-1β, IL-6, IL-8, IL-10, and IL-18, and suppression of TNF-α and IL-12 during the acute phase of their illness	Wang *et al*, 2008
A total of 506 births (105 very preterm, 237 moderately preterm, and 164 full-term) were Included. Overall, there were 63 (12.5%) births with fetal inflammatory response (FIR), 90 (17.8%) births with maternal inflammatory response (MIR), and 353 (69.8%) births with no MIR/ FIR	Cord blood concentrations of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18,sIL-6rα, IFN-γ, TNF-α, TNF-β, GM-CSF, TREM-1, TGF-β, MCP-1, MIP-1α, MIP-1β, MMP-9, sTNFRI, MIF, RANTES, BDNF, NT-3, and NT-4.	IL-1β, IL-6 and IL-8 are selectively associated with FIR. These markers may be clinically useful indicators of extensive IUI associated with poor neonatal outcome.	Mestan *et al*, 2009

Tumor necrosis factor–α (TNF-α), interleukin (IL), IL-1 receptor antagonists (IL-1ra), soluble TNF receptors (sTNFRs), soluble IL-6 receptor (sIL-6r), interferon gamma (IFN-γ), granulocyte/macrophage colony-stimulating factor (GM-CSF), triggering receptor expressed on myeloid cells (TREM), matrix metalloproteinase (MMP), monocyte chemoattractant protein (MCP), macrophage inflammatory protein (MIP), macrophage migration inhibitory factor (MIF), regulated on activation normal T cell expressed and secreted (RANTES), brain-derived neurotrophic factor (BDNF), neurotrophins (NT).

1.4 Dissertation aims, objectives and hypothesis

The findings presented in the sections above have highlighted the current understating of the host immune response to maternal GBS colonisation and the importance of detecting intrapartum GBS colonisation. Although the current recommendations for GBS identification have been shown to be effective, to date only two studies have examined whether the use of maternal serum levels of pro-inflammatory cytokines would be a valuable clinical index of intrapartum GBS colonisation in women. From these two studies, maternal IL-6 and IL-1β showed promise as clinical markers of GBS colonisation (Yi & Xinghua, 2015; Mitchell et al., 2013). Despite these two studies identifying raised maternal cytokines as being associated with GBS colonisation, they did not clearly identify when the maternal blood samples were collected during the antepartum, intrapartum or postpartum periods. In the South African clinical setting women are most likely to be tested for GBS colonization during the intrapartum period, with GBS screening not routinely being done during the antepartum period. Therefore, the aims, objective and hypothesis of my dissertation are as follows:

Aims:

1. To determine the concentration of pro-and anti-inflammatory cytokines in maternal serum obtained from GBS colonised and non-colonised women at delivery.
2. To determine the clinical usefulness of maternal serum of pro-and anti-inflammatory cytokines concentrations as a clinical index of intrapartum GBS colonisation.

Objectives:

1. To determine the GBS vaginal colonisation status of women at delivery using the standard culture technique and quantitative PCR.
2. To determine the concentration of IL-6, IL-8 and IL-10 in maternal serum obtained from GBS colonised and non-colonised women at delivery using a luminex bead-based multiplex assay.

Null hypothesis:

There will be no difference in the serum concentration of IL-6, IL-8 and IL-10 measured in maternal blood obtained from women colonised with GBS and women not colonised with GBS at delivery.

Alternative hypothesis:

Women colonised with GBS at delivery will have increased concentrations of IL-6, IL-8 and IL-10 in their blood at delivery compared to women not colonised with GBS.

Chapter Two

Methods

2.1 Study population and recruitment

The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg, South Africa, as a sub-study (ethics clearance number M1611155) under a primary study (ethics clearance number M140203). The aim of the primary study was to establish a sero-correlate of protection against invasive GBS disease in newborns aged ≤ 90 days of age. Recruitment and enrolment for the primary study took place at the antenatal clinic, antenatal ward or labour ward of the Chris Hani Baragwaneth Academic Hospital in Soweto, Johannesburg, South Africa. The sub-study undertaken for my MSc was a cross-sectional study nested within a prospective cohort of mothers enrolled for the primary study between 1 March and 31 December 2016. The rationale and procedures for the study (primary and sub-study) were explained to the participants and written informed consent was obtained from participants who agreed and volunteered to participate in the study.

For the sub-study, only mothers aged ≥18 years, who were HIV-negative and who had vaginal deliveries were included. The rationale for these inclusion criteria was based on the potential effects that these factors could have on maternal cytokine concentrations and the detection of maternal GBS colonization. In terms of maternal cytokine concentrations, studies have shown that HIV infection (Shebl *et al.*, 2012; Kedzierska & Crowe, 2001; Stacey *et al.*, 2009) and caesarean delivery can alter circulating cytokine levels (Bakheit *et al.,* 2008). Moreover, concerning caesarean delivery, it has been reported that pregnant women who have a caesarean delivery are at an increased risk of infection compared to those who have a vaginal delivery (Gibbs, 1980). As such, prophylactic antibiotics are recommended in most cases of caesarean delivery (Smail & Grivell, 2014). Thus, the use of antibiotics would mask GBS colonization in women having a caesarean delivery. Following the same rationale, mothers were excluded who had received antibiotics in the week preceding delivery. The following confounding factors were also obtained from the enrolled participants at the time of the study:

- Demographics - including participant's age and race
- Gestational age at delivery - studies have identified significant differences in the concentration of IL-6 in peripheral blood obtained from mothers who delivered at full-term

compared to those who had pre-term deliveries (Arababadi *et al.,* 2012). Gestational age was determined by fundal height, last menstrual period or ultrasound.

- Smoking and alcohol consumption during pregnancy. Smoking has been related to increased concentrations of cytokines (Tappia *et al*., 1995; Rosedalia *et al.,* 2014), as cigarette smoking causes stimulation of macrophages within the lungs, which causes inflammatory cytokine production (Tappai *et al.,* 1995). Furthermore, alcohol consumption interferes with cytokine production as it affects the functioning of both the innate and adaptive immune systems (Crew *et al.,* 1996; McClain *et al.,* 1999).
- Medical history
- Pregnancy history and outcomes
- Nutritional status - the measurement of mid-upper arm circumference (MUAC) was used as a marker of nutritional status, as MUAC has been widely shown to be a tool for nutritional assessment (Madden *et al.,* 2014; James *et al.,* 1994; Gassull *et al.,* 1984; Collins S, 1996). The circumference of the left upper arm was measured at the mid-point between the tip of the shoulder and the tip of the elbow (olecranon process and the chromium). Studies have shown that cytokine production usually increases in people who are obese/overweight compared to non-obese/overweight individuals, due to macrophages infiltrating adipose tissues (Schmidt *et al.,* 2015). Furthermore, decreased levels of cytokines have been noted in malnourished individuals (Munoz *et al.,* 1994; Doherty *et al.,* 1994).
- Specific concomitant medications of interest - antibiotics within the week of delivery. Antibiotics mediate the immune response and regulate cytokines production (Bode *et al.,* 2014).

Table 2.1 summarizes the information collected from the participants during the course of the study.

Table 2.1 Overview of the information collected during the course of the study.

Period of collection	Information collected
Antepartum/Delivery	Arm circumference, gravidity, age
Delivery	Gestational age at delivery, delivery mode and type
Post-partum	Birth weight, complications during delivery, (such as postdates, oligohydramnios, polyhydramnios, vaginal laceration/episiotomy, anaemia, post-partum haemorrhage, preterm premature rupture of membrane, premature rupture of membrane, pre-eclampsia).

2.2 Determination of GBS vaginal colonisation

A single lower vaginal swab was obtained from the participants after delivery, with the exception of two participants where it was obtained approximately one hour before delivery. The specimens were collected using a Rayon tipped swab (transwab plain medium, medical transwire, MW170) by a nurse on duty. After collection, the swab was placed in Amies transport medium without charcoal and carried in a cooler box containing ice to the Respiratory and Meningeal Pathogen Research Unit (RMPRU) laboratory where it was kept at 2 - 8°C for a maximum of 24 hours before processing. The RMPRU laboratory is based at Chris Hani Baragwaneth Academic Hospital in Soweto, Johannesburg, South Africa.

2.2.1 GBS isolation and confirmation using selective culture methodology

The isolation and confirmation of GBS was undertaken at the RMPRU Laboratory by a trained technician. The swab was removed from the Amies transport medium and rubbed onto the first quadrant of a CHROMagar StrepB agar plate. The swab was rotated whilst plating, to ensure that the entire surface came into contact with the agar. Thereafter, a sterile loop was used to streak out across the plate. After streaking the plate was incubated under aerobic conditions at 37°C in an

ambient air incubator for 18 to 24 hours. CHROMagar StrepB agar is a chromogenic selective culture medium used for the isolation and differentiation of GBS. As a chromogenic substrate, in the presence of a growing GBS colony, it generates mauve colonies that can easily be identified on inspection (Craven *et al.*, 2010) (see figure 2.1). Other bacteria appear blue, violet or even colourless on CHROMagar StrepB agar (Craven *et al.*, 2010). When no growth was observed, the CHROMagar StrepB agar plate was incubated for another 18 to 24 hours and re-examined. When GBS-like mauve colonies were observed on CHROMagar StrepB agar they were selected and subjected to further confirmatory tests, such as the catalase test, CAMP (Christie–Atkins–Munch-Petersen) test, bile esculin test (growth on bile esculin agar and bile esculin hydrolysis) and the Group B antigen agglutination test. A brief summary of the procedures and outcomes of these confirmatory tests are provided in table 2.1 below. Using culture methodology GBS was identified as follows:

- GBS-like mauve colonies identified on CHROMagar StrepB agar
- Positive CAMP test
- Growth on bile esculin agar
- No hydrolysis on esculin bile
- Negative catalase test
- Positive Group B agglutination test

Following plating on CHROMagar StrepB agar vaginal swabs were stored in skim-milk tryptone glucose glycerol (STGG) medium at -70°C. In addition to the culture methods outlined above, molecular biology-based assays have shown promise as an additional reliable tool for GBS detection (Rallu et al., 2006). In particular, a real-time polymerase chain reaction (PCR) assay has been approved for the detection of GBS DNA straight from combined vaginal and rectal swab samples (Picard & Bergeron, 2004). Thus, in addition to the tests outlined in table 2.1, PCR was used to detect GBS colonization from the vaginal swabs. All confirmed GBS isolates were stored in STGG storage medium at -70°C.

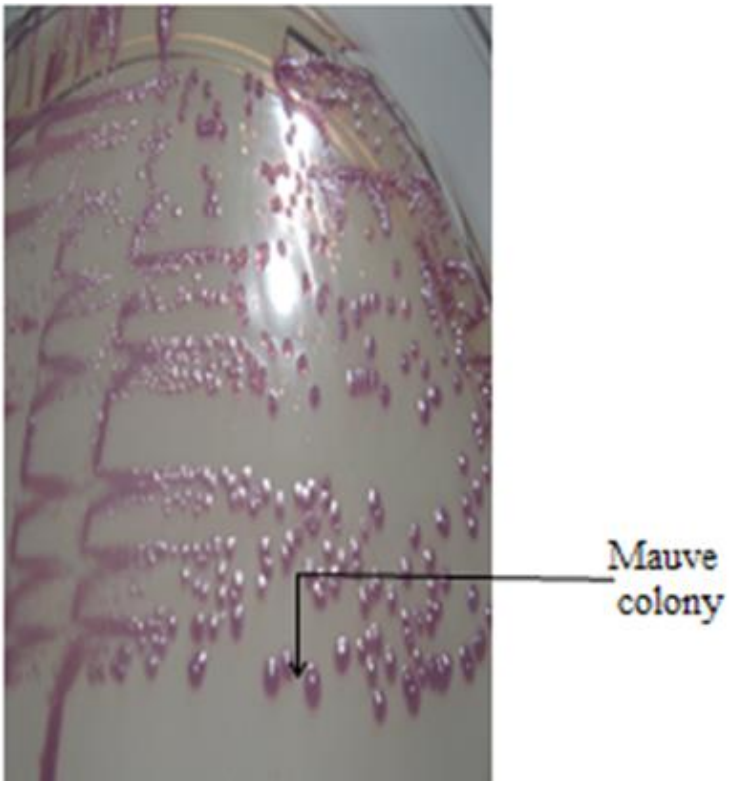
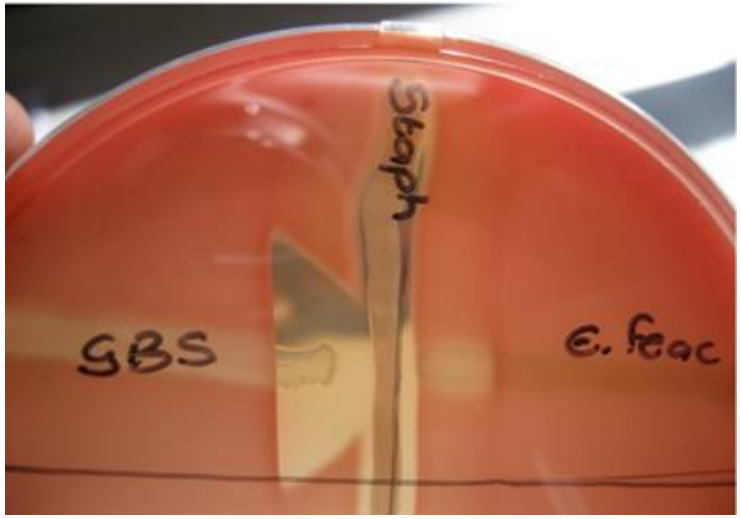


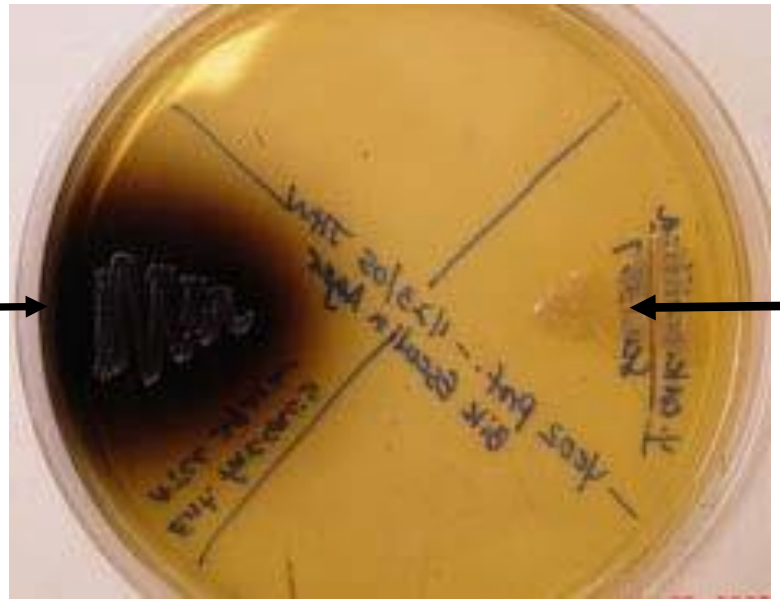
Figure 2.1. GBS primary identification on CHROMagar StrepB agar. CHROMagar StrepB agar is a chromogenic selective culture medium used for the isolation and differentiation of GBS. As a chromogenic substrate, in the presence of a growing GBS colony, it generates mauve colonies.

Table 2.2: Summary of the tests used for GBS confirmation following isolation on CHROMagar StrepB agar. Adapted from the standard operating procedure at RMPRU.

Test	Procedure	Outcome
CAMP test	A single streak of a known *Staphylococcus aureus*was made down the middle of a pre-warmed 5% sheep blood plate.A positive and negative control was streaked on both sides of, and perpendicular to the *Staphylococcus aureus* streak. The pate was incubated at 35-37˚C in 5% CO_2 for 18-24 hours.	Certain organisms (including *GBS*) produce a diffusible extracellular hemolytic heat-stable protein (CAMP factor) that acts synergistically with the beta-lysin of *Staphylococcus aureus* to cause enhanced lysis of red blood cells. A positive reaction appears as an arrowhead zone of hemolysis adjacent to the place where the two streaked lines come into proximity.*Enterococcus faecalis* will grow on the sheep blood agar but will show no enhanced hemolytic reaction near the line of haemolysis from *Staphylococcus aureus*. **GBS produces a positive CAMP test**. **All *Enterococcus faecalis* (and other *Streptococcus spp*.) are CAMP negative.**  Picture obtained from study samples.

Bile esculin test	One or two colonies of suspected GBS were touched using a sterile loop and then streaked on the surface of a bile esculin plate. The plate was incubated at 35 -37 °C at 5% CO_2 for 18 - 24 hours and the result was observed	The test is based on the hydrolysis of esculin into glucose and esculetin by a micro-organism that produces the enzyme esculinase. Esculetin reacts with ferric citrate in the medium to form a phenolic iron complex, which produces a dark brown or black colour. The test is used primarily to distinguish Group *D Streptococcus* from GBS. GBS grows as clear mucoid colonies with no hydrolysis of esculin. Group D *Streptococcus* will hydrolyze the esculin, which shows with blackening growth of the medium.

Group D *Streptococci*



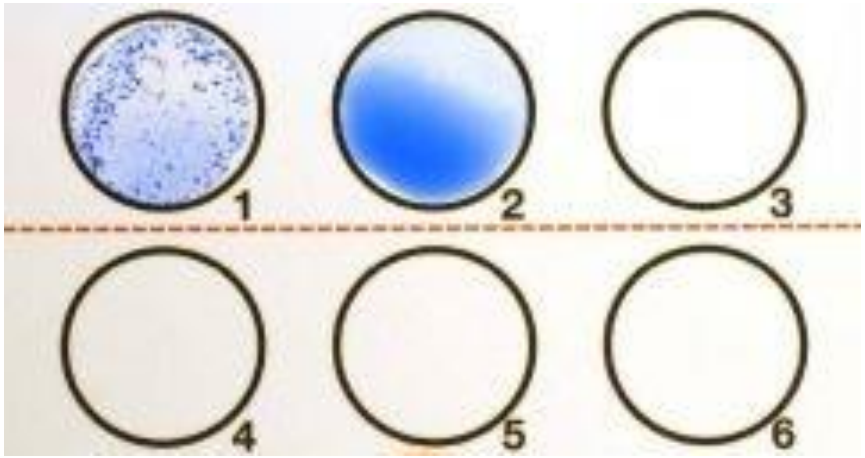
Group B *Streptococci*

Picture obtained from the study samples.

Catalase test	A drop of catalase reagent (3% hydrogen peroxide) was added onto a clean glass slide. A sterile loop was then used to transfer the GBS colonies cultured to the glass slide. The tip of the loop was thoroughly mixed with the catalase. The slide was inspected for presence of oxygen bubbles	The test is used to distinguish between *Staphylococci* and *Streptococcus. Staphylococci* are catalase positive (foams and rapidly bubbles) while *Streptococci* (GBS) are catalase negative (no bubbles seen**)**. Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize the effects of hydrogen peroxide and protect the microorganisms. Catalase mediates the breakdown of hydrogen peroxide into oxygen and water. Anaerobes, like *Streptococci* generally lack the catalase enzyme as is evident by a lack of or weak bubble production when the catalase test is performed.



Picture obtained from the study samples.

Group B agglutination test	A suspected GBS colony that is CAMP positive, bile esculin negative and catalase negative was prepared and pipetted on a test card. The blue latex Group B suspension was added to the GBS suspension and mixed with a clean pipette tip. The reading card was then gently rocked and observed for the presence of agglutination. The prolex™ streptococcal grouping latex kit was used and the test was conducted at room temperature.	The Group B agglutination test is a rapid latex test used to determine the Lancefield grouping of streptococci. This test uses the principle of antigen-antibody binding. The antigen-antibody complex forms as a visible precipitate in a positive reaction. **GBS produces a clear agglutination with the Group B reagent.** +ve agglutination -ve agglutination 

2.2.2 GBS confirmation using PCR

Swabs stored in STGG medium were removed from the freezer and thawed before the extraction process commenced. Nucleic acid from vaginal specimens were extracted using the NucliSENS® easyMAG® platform (Biomerix, Somerset, NJ, USA). The platform is intended for the automated isolation (purification and concentration) of total nucleic acid from biological specimens. The system is based on the generic and blood specific method for binding nucleic acids from complex biological samples to magnetic silica. Samples were vigorously vortexed for 60 s to ensure all biological material was suspended and then 500 µL of each sample was mixed with a lysis buffer containing a chaotropic agent to disrupt the cellular matter present in the specimen thereby releasing the nucleic acids. The lysis buffer also inactivates any nucleases present in the specimen. The isolation process was initiated by adding magnetic silica to the lysed specimen. Any nucleic acids present in the lysate will bind to the magnetic silica under high salt conditions. The magnetic silica was then washed several times using two wash buffers. The nucleic acid was released (eluted) from the magnetic silica and concentrated in a 100 µL volume of the elution buffer. Nucleic acid samples were stored at 2 - 8˚C if they were to be used within the same day for performing PCR or in the freezer at -70˚C for long-term storage.

Primers and a probe targeted to detect the cfb gene, which is responsible for encoding the CAMP factor present in almost all GBS isolates was used. The cfb gene responsible for increasing the area for hemolysis created by β-hemolysis (Phillips *et al.*, 1980; Wilkinson, 1977), is a common target used in PCR assays for GBS identification (Podbielski *et al.*,1995; Rallu et al., 2006). Human RNase P (RNP), which is a single-copy gene encoding the RNA moiety for the RNase P enzyme was used as the internal control DNA for false negative PCR reactions. The primers and probe were designed using Applied Biosystems Primer Express software based on the DNA sequence of the CAMP gene provided by the RMPRU (personal communication: Gaurav Kwatra). The primer and probe sequences used in the study were manufactured and supplied by Applied Biosystems and are given below together with the reaction mixture (Table 2.3):

CAMP

Forward Primer: 5'- GAACTCTAGTGGCTGGTGCATTG-3'

Reverse Primer: 5'- GGAGTTGTCACTTGATCAGCATGT-3'

MGB Probe: 5'-FAM-ATTTTCACCAGCTGTATTAG MGB-3'

RNP (Internal Control)

Forward Primer: 5'-AGA TTT GGA CCT GCG AGC G-3'

Reverse Primer: 5'-GAG CGG CTG TCT CCA CCA GT-3'

MGB Probe: 5'- NED- TTC TGA CCT GAA GGC TCT GCG CG MGB-3'

Two controls were included with each batch of samples: a negative control (sterile RNase free of water) and the selected GBS positive DNA control.

Table 2.3. CAMP/RNP Primer/Probe Mix

	Reagent [Stock]	**Reaction [Final]**	**1x Reaction**
CAMP/RNP	(µM)	(nM)	(µl)
CAMP Forward	10	0.2	0.5
CAMP Reverse	10	0.2	0.5
CAMP Probe	10	0.25	0.625
RNP Forward	20	0.32	0.4
RNP Reverse	20	0.32	0.4
RNP Probe	10	0.16	0.4
TaqMan Master Mix	-	-	12.5
Distilled water (sterile)	-	-	4.675
Total	-	-	25

The standard curve was set up to include 14 reference samples/standards to be tested. To ensure that the contents settle on the bottom of the each well, the 96-well plate was centrifuged for 10 seconds at 2000rpm. Thereafter the plate was placed into the real-time PCR machine (7500 fast Real Time PCR machine, Applied Biosystems, California, USA) and was run according to the stipulated cycle conditions:

- Stage 1: Activation of Taq DNA polymerase at 50°C for 10 min
- Stage 2: Denaturing process of the DNA template for 5 min at 95°C.
- Stage 3: Denaturing process of the DNA template for 5 sec at 95°C.
- Stage 4: Annealing of the complementary primers at 58°C for 10 sec.
- Stage 5: Extension period for 40 sec at 72°C.
- Repeat stage 3 to stage 5 for 50 PCR cycles

The7500 fast Real Time PCR machine software calculated the quantity of cfb target from the standard curve**.** The quantity represents the number of GBS (cfb targets) per 25 µL. To obtain the number of GBS (cfb targets)/mL sample, the number of GBS (cfb targets) was multiplied by 40. A result was considered positive if the ct value was less than 44.

2.2.3 GBS serotype testing

All GBS isolates stored in STGG medium were serotyped using the latex agglutination test. The test is based on the principles of co-agglutination. Particles of latex are coated with rabbit antisera specific for GBS serotypes Ia, Ib and II to IX. Specific antisera against Ia, Ib and II – IX (Staten serum Institute, SSI, Denmark) were used in the latex agglutination test. Two to three colonies of GBS were picked off a blood agar plate and suspended in 10 µL of sterile saline on a glass microscope slide. Equal amounts of antiserum were added and mixed with the bacterial culture. A positive reaction was recorded as any sign of agglutination for that particular serotype. GBS isolates that tested negative by latex agglutination for all serotypes were further typed by a single plex PCR method for serotypes Ia, Ib, II, III, IV and V using primer sequences described by Poyart *et al* 2007).

Specific primer sequences for the detection of group B streptococcus capsular types Ia-V

Serotype	
Ia	Forward 5’- GGTCAGACTGGATTAATGGTATGC -3’
	Reverse 5’-GTAGAAATAGCCTATATACGTTGAATGC -3’
Ib	Forward 5’- TAAACGAGAATGGAATATCACAAACC -3’
	Reverse 5’-GAATTAACTTCAATCCCTAAACAATATCG -3’
II	Forward 5’- GCTTCAGTAAGTATTGTAAGACGATAG -3’
	Reverse 5’- TTCTCTAGGAAATCAAATAATTCTATAGGG -3’
III	Forward 5’- TCCGTACTACAACAGACTCATCC -3’
	Reverse 5’-AGTAACCGTCCATACATTCTATAAGC -3’
V	Forward 5’- GGTGGTAATCCTAAGAGTGAACTGT -3’
	Reverse 5’-CCTCCCCAATTTCGTCCATAATGGT -3’
V	Forward 5’- GAGGCCAATCAGTTGCACGTAA -3’
	Reverse 5’- AACCTTCTCCTTCACACTAATCCT -3’

For this purpose, two reaction mixes were made. The primers for Ia, Ib, II, IV and V were combined in one mix and the primers for III, VI, VII and VIII were combined in another mix.

Table 2.4. GBS serotype reaction mixture

Reagents	Stock concentration	1 reaction (25 µl)	Final Concentration
EconoTaq Master Mix	2X	12.5	1X
Primer F	10µM	1	250 nM
Primer R	10µM	1	300 nM
DNA template		5	
Distilled water (Sterile)		5.5	

Amplification thermal cycles were as follows:

- Stage 1: Activation of Taq DNA polymerase at 94°C for 2 min
- Stage 2: Denaturing process of the DNA template for 30 s at 94°C.
- Stage 3: Annealing of the complementary primers at 55.1°C for 30.
- Stage 4: Extension period for 1 min and 30 sec at 72°C.
- Repeat stage 2 to stage 4 for 35 PCR cycles.
- Stage 5: Final extension for 10 min at 72°C.

The serotype of the GBS isolate was determined on the basis of matching band size of DNA fragment for capsular polysaccharide by agarose gel electrophoresis.

2.3 Determination of maternal serum cytokine concentrations

Maternal whole blood (10 – 15 ml) was collected after delivery by a nurse on duty. The blood samples were collected in an anticoagulant plastic tube (red topped Vacutainer® catalog number: 367955) and allowed to clot for 30 minutes. Thereafter it was refrigerated at 4°C, until it was transported in a cooler box that contained ice to the RMPRU laboratory where it was processed. All the blood samples collected for the study were processed within 24 hours of collection. Blood

was centrifuged at 2000 g for 10 minutes at room temperature (average room temperature was 24 °C). After the samples were centrifuged, the blood tubes were inspected to ensure that red cells were clearly separated from the serum, if there was incomplete separation, blood was centrifuged again at 2000 g for 5 minutes at room temperature. Thereafter the serum was aliquoted into clean cryovials (Thermo scientific NUNC, catalog number: 366524). Aliquoted samples were stored in a freezer at -70 °C until cytokine measurements were undertaken. Serum levels of cytokines were quantified using the commercially available R&D systems (MN, USA) human magnetic luminex performance assay, high sensitivity cytokine kit: IL-6, IL-8 and IL-10 (catalog number FCSTM09-03).

The maternal serum samples used for the sub-study were collected for the primary study and thus had to be thawed once for aliquots to be taken for the cytokine measurements. Once the samples were aliquoted they were stored at - 70 °C until analysis. On the day of cytokine measurements all reagents and samples were brought to room temperature before analysis. All samples and controls were analyzed in singles and standards in duplicate. Samples were prepared using a two-fold dilution, which was 125 µL of the serum with 125 µL of the Calibrator Diluent RD6-40. Twenty-five microliters of the microparticle cocktail was added to each well of the microplate followed by 100 µL of standard, control or sample. The assay was pipetted within 15 minutes. After adding the standards, controls and samples the microplate was covered with a foil plate sealer and incubated at room temperature for three hours on a horizontal microplate shaker at 800 rpm. After the three hour incubation, the microplate was washed three times with wash buffer. Thereafter fifty microliters of diluted biotin-antibody cocktail was added to each well and it was covered with a new foil plate sealer and incubated for one hour at room temperature on the shaker set at 800 rpm. Following the one hour incubation the microplate was washed three times with wash buffer. Fifty microliter of streptavidin-phycoerythrin was then added to each well and it was covered with a new foil plate sealer and incubated for 30 minutes at room temperature on the shaker at 800 rpm. After the 30-minute incubation, the microplate was washed three times with wash buffer. Thereafter the microparticles were re-suspended by adding 100 µL of wash buffer to each well and it was incubated for 30 minutes at room temperature on the shaker set at 800 rpm. Following the final incubation the microplate was read within 5 minutes using the Bio-Plex 200 system

(California, USA). The Bio-Plex instrument was validated monthly using a Bio-Plex validation kit and calibrated on assay days using a Bio-Plex calibration kit. The average mean fluorescence intensity of the blank was subtracted from the values for the standards, controls and samples. The standard curve for each analyte was created by reducing the data using Bio-Plex 200 system software capable of generating a five parametric logistic curve-fit. The lowest detectable concentration, termed the limit of detection (LOD), for each cytokine was (all units in pg/mL): IL-6 = 0.14; IL-8 = 0.04 and IL-10 = 0.21. Several quality parameters, including fit probability of the standard curve, coefficient of variation and inestimable concentrations were examined. All fit probabilities and coefficient of variation met the quality criteria. One sample which, had an extrapolated value for IL-10 was included; it fell between the LOD and LOQ. The limit of quantification (LOQ) is the lowest point on the standard curve (all units in pg/mL): IL-6 = 0.88; IL-8 = 0.71; IL-10 = 0.61. The extrapolated value had a reading greater than that recorded for the blank.

2.4 Data analysis

Data was analysed using SAS 9.4. Two-tailed P-values < 0.05 were considered statistically significant. The sample was divided between the GBS colonised and GBS non-colonised mothers using both culture and PCR results. A mother was classified as GBS colonised if either the GBS culture was positive or the PCR was positive. The GBS colonised and GBS non-colonised mothers were compared in terms of their demographic, clinical, gestational, delivery and infants' characteristics. To compare frequencies, the Chi-square or Fisher's exact test was used. All continuous variables were tested for normality using the Kolmogorov-Smirnoff test and checking that the mean was close to the median, with a skewness and kurtosis comprised between -1 and 1. For normally distributed (parametric) variables, means and standard deviation were reported and GBS colonised were compared to the GBS non-colonised mothers using an unpaired Student's t-test. For non-parametric variables, medians and interquartile range were reported and GBS colonised was compared to GBS non-colonised mothers using the Wilcoxon rank-sum (Mann-Whitney) test.

Serum concentrations of the different cytokines measures (IL-6, IL-8 and IL-10) did not meet the criteria for normality and were therefore log-transformed for analysis. Log-transformed serum concentration of the cytokines met the criteria for a normal distribution. Log-transformed values were used for the analyses and for figures. To test the association between GBS status and cytokine concentration, the logged values of the serum concentration of IL-6, IL-8 and IL-10 in GBS colonised and GBS non-colonised mothers was compared using a Student's unpaired t-test. To better understand a potential confounder of the relationship between GBS status and cytokine concentration, the association of the logged values of the serum concentration of IL-6, IL-8 and IL-10 with the time it took to process the whole blood sample, was tested using a Spearman's correlation analysis.

CHAPTER THREE

RESULTS

3.1 Maternal characteristics

3.1.1 Identification of the GBS colonised and GBS non-colonised mothers

A total of 122 participants were recruited for the study, out of which 35 (28.7 %) were found to be colonised with GBS. The 35 colonised mothers were identified either from a positive culture result or from a positive PCR result on the swab collected at the time of delivery. Seventeen of the 35 samples were both culture and PCR positive, nine were only culture positive and nine were only PCR positive. The PCR Ct values were significantly lower in the 17 participants who were positive for GBS with culture and PCR, compared to the nine participants who were culture negative and PCR positive (average Ct value (SD) = 43 (5) vs. 48 (2); P = 0.007 unpaired t test t=2.933).

3.1.2 GBS Serotype distribution among the GBS colonised mothers

Table 3.1 shows the distribution of GBS serotype among the GBS colonised participants. Of the samples obtained from the 35 colonised participants, eight were not typable as there was not enough genetic material left on the swab after culture. The most prevalent GBS serotype was serotype Ia, followed by serotype III.

Table 3.1. Serotype distribution among GBS colonised participants

GBS Serotype	n (%)
Ia	12 (34)
Ib	3 (8)
III	9 (26)
IV	1 (3)
V	2 (6)
N/A	8 (23)

% = Percentage n = Number of participants
N/A = Not Available (not enough material on the swab to amplify)

3.2 Demographic characteristics of GBS colonised and GBS non-colonised mothers

Table 3.2 shows the demographic characteristics of the mothers enrolled in the study. All the participants were young to middle aged women with a mean (SD) age of 26.5 (6) years (range: 18 to 43). Almost all the participants 117 (96%) were of black African ancestry. Most had been recruited from the labour ward and had already been pregnant at least once. Overall, there was no significant differences in maternal characteristics such as age, race, middle upper arm circumference (MUAC) or gravidity between the two groups. The 120 mothers tested for syphilis were negative.

Table 3.2 Maternal characteristics

		Overall (n = 122)	**GBS colonised (n = 35)**	**GBS non-colonised (n = 87)**	**P value** Statistical test used Statistical test value; Degrees of Freedom (DF)
Age, mean (SD) years		26.5 (6)	27.3 (6)	26.1 (6)	**0.36** Unpaired Student's t-test t= -0.93; DF=120
Race n (%)	Black	117 (96)	35 (100)	82 (94)	**N/A**
	Coloured	5 (4)	0 (0)	5 (6)	
Enrolment site n (%)	ANC	19 (16)	7 (20)	12 (14)	**0.77**
	ANW	4 (3)	1 (3)	3 (3)	Chi-square test X^2 =1.108; DF=3
	LW	98 (80)	27 (77)	71 (82)	
	PNW	1 (1)	0 (0)	1 (1)	
MUAC, med [IQR] cm*		28 [25-30]	28 [26-31]	28 [25-30]	**0.34** Mann Whitney U test Statistic=2224.5

Gravidity n (%)*	Primigravida	39 (32)	8 (23)	31 (36)	**0.17** Chi-square X^2 =1.873; DF=1
	Multigravida	83 (68)	27 (77)	56 (64)	

ANC = Antenatal Clinic; ANW = Antenatal Ward; LW = Labour Ward; PNW = Post Natal Ward; MUAC = Mid Upper Arm Circumference; * = information on some participants missing (Gravidity n = 4, MUAC n = 5). N/A: not available as one cell had a 0 – Fisher's Exact test could not be calculated.

3.3 Gestational and Delivery characteristics

Table 3.3 shows the main gestational and delivery characteristics. Ten participants had a history of gestational hypertension, of which 3 were GBS colonised (P = 0.65), while none of the participants had a history of gestational diabetes mellitus. Gravidity and behaviour during pregnancy (smoking and alcohol) were not significantly different between the GBS colonised mothers and the GBS non-colonised mothers. Placental weight was significantly lower in GBS colonised mothers compared to GBS non-colonised mothers (mean (SD) = 585 g (142) vs. 642 g (131), P = 0.04).

The majority of the study participants delivered at full term gestation (≥ 37 weeks gestation). There were only two twin deliveries in total. Fifty-four (46%) participants had one or other type of birth complication(s) during delivery. At delivery, 20 participants had an episiotomy, of which three were GBS colonised participants (Chi-square test; X2 =1.27; DF=1; P = 0.26) (missing information: 40 in total; 15 for the GBS colonised and 25 for the GBS non-colonised). Furthermore, 21 participants had a perineal laceration/tear during delivery, of which seven were GBS colonised (Chi-square test; X2 =1.22; DF=1; P = 0.27) (same information missing, for the same individuals as above). Ten of the participants experienced tachycardia (pulse rate above 100 bpm) during delivery, of which three were GBS colonised (Chi-square test; X2 = 0.19; DF=1; P = 0.66) (missing data for 2 GBS colonised and 3 GBS non-colonised mothers). None of the maternal participants had fever at the time of delivery (missing data for 2 GBS colonised and 3 GBS non-colonised mothers). One participant (GBS colonised) had antepartum haemorrhage and one participant (GBS non-colonised) had postpartum haemorrhage (missing information: 40 in total, 25 in GBS non-colonised and 15 in GBS colonised for both types of haemorrhage). Overall

haemoglobin levels were 11 [6 - 15] g/dL, (median [range], Unpaired Student's t-test t=0.85; DF=117; P = 0.39), with ten in total having haemoglobin less than 10 g/dL (of which three were GBS colonised participants, Chi-square test; X2 = 0.01; DF=1; P = 0.91) (data missing for 2 GBS non colonised and 1 GBS colonised mother). None of the participants had urinary tract infections, chorioamniotitis or uterine tenderness (missing information for 3 GBS non-colonised and 2 GBS colonised).

Table 3.3 Gestational and delivery characteristics

		Overall (n = 122)	**GBS non-colonised (n = 87)**	**GBS colonised (n = 35)**	**P value** Statistical test used; Statistical test value; Degrees of Freedom (DF)
Gestational hypertension	Yes	10	7	3	**0.65** Chi-square test X^2= 0.19; DF=2
	No	72	55	17	
	Unknown	40	25	15	
Smoking during pregnancy n (%)	Yes	3 (3)	0 (0)	3 (3)	**N/A**
	No	92 (75)	27 (77)	65 (75)	
	Unknown	27 (22)	8 (23)	19 (22)	
Alcohol during pregnancy n (%)	Yes	7 (6)	4 (5)	3 (9)	**0.67** Chi-square test X^2= 0.78; DF=2
	No	88 (72)	64 (73)	24 (68)	
	Unknown	27 (22)	19 (22)	8 (23)	
Gestational age at delivery n (%)	≥ 37 weeks	75 (61)	51 (59)	24 (69)	**0.31** Chi-square test X^2= 1.04; DF=1
	< 37 weeks	47 (39) [a]	36 (41)	11 (31)	
Delivery type n (%)	Single	120 (98)	86 (99)	34 (97)	**0.50** Chi-square test X^2= 0.45; DF=1
	Twin	2 (2)	1 (1)	1 (3)	

Placenta weight mean (SD)[b]	625 (136)	642 (131)	585 (141)	**0.04** unpaired Student's t-test t =2.09; DF=117
Pregnancy/ labour/ delivery complications n (%)[c]	54 (46)	14 (42)	40 (48)	**0.61** Chi-square test X^2= 0.26; DF=1

N/A: not available as one cell had a 0 – Fisher's Exact test could not be calculated.
a: 3 at 35 weeks (all GBS non-colonised), 33 at 36 weeks for the GBS non-colonised; 11 at 36 weeks for GBS colonized.
b: missing = 2 observations in GBS non-colonised and 1 in the GBS colonised.
c: Pregnancy/ labour/ delivery complications included pregnancy-induced hypertension, gestational diabetes, antepartum haemorrhage, postpartum haemorrhage, episiotomy and perineal laceration/tear.

3.4 Infant characteristics

The infants were healthy with median Apgar scores of 9 and 10 at 1 and 5 minutes (missing information for 2 GBS non-colonised mothers at 1 minute and 2 GBS non-colonised and 1 GBS colonised mother at 5 minutes) and a mean birth weight of about 3 kg (see Table 3.4). Thirteen babies experienced foetal distress at the time of delivery, five from GBS colonised mothers (Chi-square test; X2 = 0.78; DF=1;P = 0.37). Of those babies who had experience foetal distress, six had displayed an abnormal cardiotocography (3 in GBS colonised mothers, Chi-square test; X2 = 2.29; DF=1;P = 0.13). None of the babies developed neonatal GBS disease within 90 days of birth.

Table 3.4 Infants characteristics at birth

	Overall (n = 122)	**GBS colonized mothers (n = 35)**	**GBS non-colonised mothers (n = 87)**	**P value** Statistical test used; Statistical test value; Degrees of Freedom (DF)
Birth weight, mean (SD), g	3050 (486)	3020 (463)	3061 (496)	**0.67** Student's unpaired t-test; t=0.43
Apgar at 1 minute, median [range]	9 [2-10]	9 [5-10]	9 [2-10]	**0.45** MannWhitney U test; Statistic= 2219
Apgar at 5 minutes, median [range]	10 [6-10]	10 [9-10]	10 [6-10]	**0.20** MannWhitney U test; Statistic= 2162

3.5 Cytokine serum concentrations in mothers

3.5.1 Serum cytokine concentrations in GBS colonised and GBS non-colonised mothers

Figure 3.1 below shows the logged values of the concentration of IL-6, IL-8 and IL-10 in the serum of GBS colonised and GBS non-colonised mothers. There were no statistically significant differences according to GBS colonization status (IL-6 P = 0.87, unpaired t test; t = 0.16), IL-8 (P = 0.19 unpaired t test; t = 1.48) and IL-10 (P = 0.95 unpaired t test; t = 0.06).

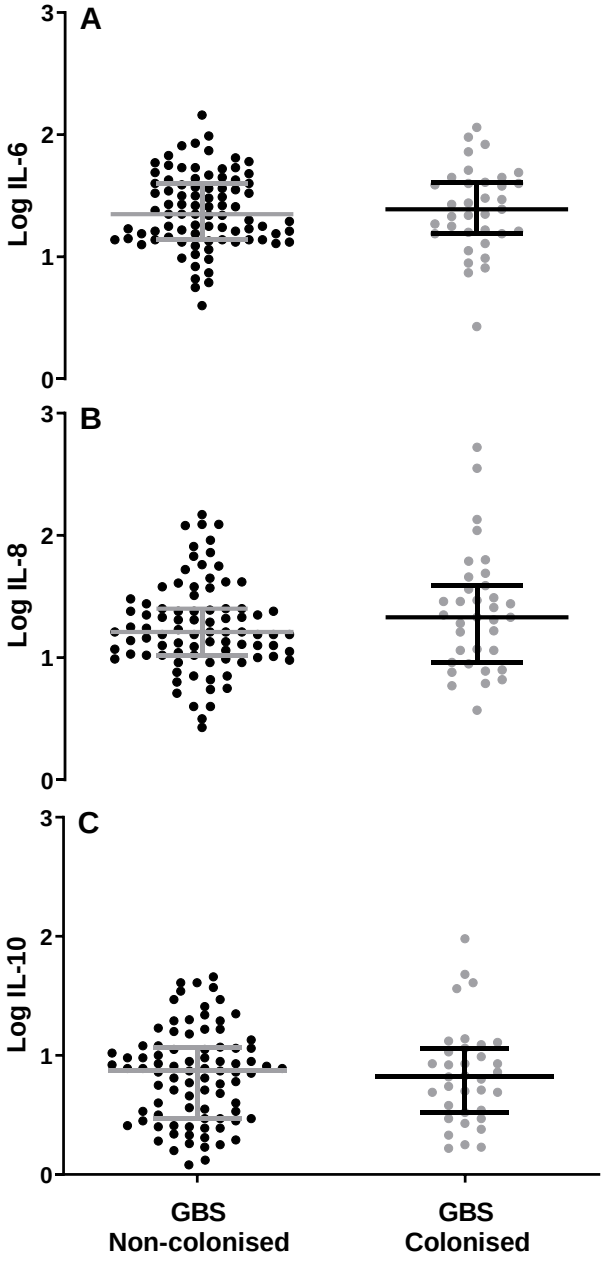


Figure 3.1: Scatter plots, median and interquartile range of log serum concentrations of (A) IL-6 (B) IL-8 and (C) IL-10 in GBS colonised and GBS non-colonised mothers. One sample, which, had an extrapolated value for IL-10 was included; it fell between the LOD and LOQ. The limit of quantification (LOQ) is the lowest point on the standard curve (all units in pg/mL): IL-6 = 0.88; IL-8 = 0.71; IL-10 = 0.61. There were no significant differences between the two groups. Unlogged cytokine data was calculated as pg/mL.

3.5.2 Maternal serum cytokine concentrations and GBS serotype

For the 27 GBS colonised participants were serotype was identified, the logged values of IL-6, IL-8 and IL-10 serum concentrations for the two most prevalent serotypes, serotype Ia (n = 12) and serotype III (n = 9) were compared. There were no significant differences in the logged serum concentration of (IL-6 P = 0.6, unpaired t test; t = 0.53), IL-8 (P = 0.14 unpaired t test; t = 1.54) and IL-10 (P = 0.38 unpaired t test; t = 0.91), see Figure 3.2) .

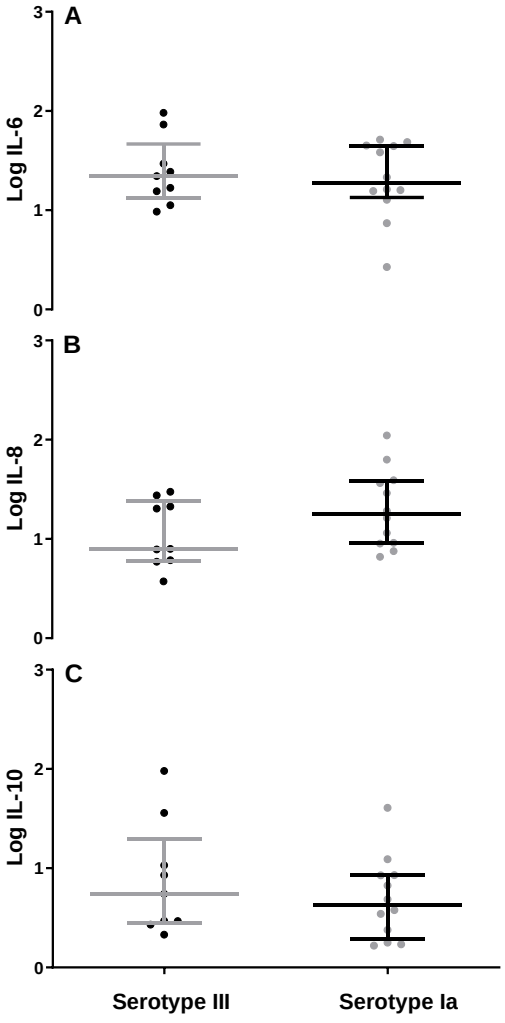


Figure 3.2: Scatter plots showing median and interquartile range of the log serum concentrations of (A) IL-6 (B) IL-8 (C) IL-10 in the two most prevalent serotypes (serotype III (n = 9) and Ia (n = 12)) from the GBS colonised participants. There were no statistically significant differences in the comparison between the serotypes and cytokine production in response to colonisation. Unlogged cytokine data was calculated as pg/mL.

3.6 Cytokine levels and time taken to process maternal blood

A significant positive relationship between the time it took to process the maternal blood and serum log IL-8 concentrations (Spearman's r = 0.23, P = 0.01) was found, whereby a longer processing time was associated with greater serum concentrations of IL-8. This relationship was not significant with serum log IL-6 concentrations (Spearman's r = -0.12; P = 0.2) or serum log IL-10 concentrations (Spearman's r = 0.02; P = 0.85). This relationship was not modified when adjusting for GBS colonization status.

CHAPTER 4

DISCUSSION

I investigated the association between maternal GBS colonisation at delivery and maternal serum levels of pro-and anti-inflammatory cytokines in healthy pregnant women in early adulthood. Within this cohort of pregnant women, 28.7% were identified to be colonised with GBS either from a positive culture result or from a positive PCR result obtained from a vaginal swab collected at the time of delivery. The most prevalent GBS serotype in colonised mothers was serotype Ia, followed by serotype III. The gestational age at delivery was > 37 weeks in 61% of the deliveries; no deliveries occurred before 35 weeks. All infants born were healthy and showed no evidence of GBS infection within 90 days of birth. A positive GBS colonisation status at delivery did not significantly increase the maternal serum concentrations of IL-6, IL-8 and IL-10 (Figure 3.1).

4.1 GBS colonisation – prevalence and methods of detection

The maternal GBS colonization rate reported in the study was 28.7%, which is higher than 20% reported in a systemic review and meta-analysis of worldwide GBS colonization rates (Kwatra et al., 2016). It is however in line with the colonization rates (28.8 - 31.6 %) reported in other studies (Mitchell *et al.,* 2013; Namugongo *et al.,* 2016; Zamzami *et al*., 2010). Rectal swabs are better for isolating GBS than are vaginal swabs and using only vaginal swabs may lead to 10 - 20% of the results being false negatives (Kwatra et al., 2016; Platt et al., 1995 Quinlan et al., 2000). Thus, in my study the GBS colonization rate obtained, may not be a true reflection of the colonization prevalence in the area, as only vaginal swabs were used. From the GBS colonised mothers, serotypes Ia, III, Ib and V, were identified, with Ia and III being the most prevalent serotypes in the cohort (Table 3.2). These findings of serotype prevalence are in line with other studies conducted in South Africa and worldwide (Dangor *et al.,* 2015; Edmond *et al.,* 2012; Hall *et al.,* 2017). The prevalence of serotype Ia and III in maternal GBS colonization is significant, as GBS serotype III and serotype Ia has been reported to be the most prevalent serotypes in invasive GBS disease (Harrison *et al.,* 1997; Zaleznik *et al.,* 1999).

4.2 Isolation and detection of GBS

In the study two methods, conventional culture and PCR were used for the isolation and detection of GBS from the vaginal swabs obtained from the study participants. The centre for disease control and prevention has recommended the use of culture based methods in the detection and isolation of GBS from pregnant women (Centres for Disease Control and Prevention (CDC) 1996; Centres for Disease Control and Prevention (CDC) 2010). Compared to the culture method, the PCR method has been reported to have a sensitivity of 97 %, a negative predictive value of 98.8 % and a positive predictive value of 100 % for GBS (Bergeron *et al.*, 2000). Together with these findings and the observed disadvantages associated with the culture method, such as limited sensitivity and requiring more time before the result can be obtained, PCR has become highly recommended in GBS detection and isolation (Carrillo-Avila *et al.*, 2018). Although my study used PCR and culture for GBS detection, the fact that only a single vaginal swab was collected could have affected the results obtained. For example, some of the samples were not serotyped because the viability of the bacteria had significantly reduced to such an extent that there was not enough genetic material left on the swabs. In addition, studies have shown that culturing rectovaginal swabs in an enriched selective broth, such as lim broth, is the most reliable method for isolation of GBS, due to its ability to prevent the growth of other microorganisms (Baker CJ, 1977). Compared to GBS detection with direct plating, culture-enhanced broth, reported the sensitivity, specificity, positive predictive and negative predictive value of lim broth at 97%, 100%, 100% and 97% respectively (Nguyen *et al.*, 1998). In addition, qPCR sensitivity has been reported to increase when combined with lim broth enriched media (El Aila *et al.*, 2011). Thus, if I had used an enriched media like lim broth in my study, more GBS colonized participants may have been identified.

4.3 GBS colonisation and cytokines

According to my study, a positive GBS colonisation status at delivery did not significantly increase the maternal serum concentrations of IL-6, IL-8 and IL-10. A previous study undertaken on subclinical GBS infections in pregnant women (n = 20 for GBS colonised and n = 60 for GBS

non-colonised), showed a five-fold increase in IL-6 in pregnant women with GBS subclinical infections when compared with the control group (Yi and Xinghua, 2015). Another study reported increased concentrations of maternal IL-β and IL-2 in GBS colonized mothers when compared to the control group, but there was no difference in the maternal concentration of IL-6, IL-8 and TNF-α (Mitchell *et al.,* 2013). Increased levels of IL-8 and IL-1α in human decidual cells has also been noted after stimulation with GBS isolated from infected neonates (Dudley *et al*., 1997). Stimulating cord blood and adult blood mononuclear cells with heat killed and active GBS showed an increased expression of IL-1β, IL-6, IL-8, TNF-α (Berner *et al.,* 2002). In an animal study, they identified that neutrophils induced IL-1β production after stimulation with GBS, and GBS hemolysin promotes IL-1β release (Mohammadi *et al.,* 2016). Results from these studies pointed to the fact that the presence of GBS can stimulate the immune system and lead to the production of cytokines by the immune cells in either colonization or disease.

My study focused mainly on maternal GBS colonization at delivery in healthy pregnant women and was not able to show any difference in cytokine concentrations between the two groups. Possible reasons for these negative findings, compared to the findings obtained by the two studies reporting increased maternal cytokines with GBS colonisation, could be related to the timing of maternal blood collection. In my study, maternal blood was collected after delivery, while in the other two studies they do not indicate when the maternal blood samples were collected. Inflammatory cytokines are reported to be involved in the initiation of labour in healthy pregnant women (Lappas *et al.,* 2006). In particular, maternal IL-6, IL-8 and IL-10 seem to increase during pregnancy in women with no history of infection during pregnancy (Opsjon *et al.,* 1993; Buka *et al.,* 2001; Curry *et al*., 2008; Kameda *et al*., 1990; Prins *et al.,* 2012). In support of the observations of increased maternal cytokines during pregnancy, increased mRNA expression of IL-6 and IL-10 has been found in the placenta and decidua in normal, term pregnancies (Saeed *et al.,* 2008). Furthermore, the increase in maternal IL-6 and IL-10 seems to be more pronounced at the time of delivery than during the first and second trimester of pregnancy (Makseed *et al.,* 2017 and Vassiliadis *et al.,* 1998). A study reported ~ 100% increase in maternal concentrations of IL-6 in women in labour compared with those that were not in labour, and the increase in IL-6 concentration did not differ across the latent or active phase of labour (Greig *et al.,* 1997). Thus, in my study the increase in maternal IL-6, IL-8 and IL-10 related to delivery may have masked

any potential increase in maternal cytokine concentration caused by GBS colonisation. Using a human epithelial cell line A549, others have investigated the cytokine response to different serotypes of invasive and vaginal GBS isolates, VIII that is predominant in Japan and GBS serotype III that is predominant in the United State (Mikamo et al., 2004). Their findings revealed that there is significant difference in cytokine production between the two serotypes, with serotype III producing more TNF-α and IL-10 than serotype VIII and serotype VIII producing more IL-8 than serotype III (Mikamo et al., 2004). In my study, I did not find a significant difference in the maternal cytokine concentrations between women colonised with serotype III and Ia; no women were colonised with serotype VIII.

In addition to labour potentially increasing the maternal cytokine concentrations in my study, the handing of the blood samples prior to storage could also have had an effect. It has been shown that the amount of time the samples are stored before separation into plasma or serum increases or decreases the measurable concentration of several endogenous inflammatory markers (Skogstrand *et al*., 2008; Flower *et al*., 2000). A significant positive relationship between the storage time prior to separation into serum and the concentration of IL-8 was found, whereby a longer storage time was associated with increased IL-8 concentrations. The increase in IL-8 concentrations after storage is most likely due to the continued production and/or release of antigens from the blood cells, or that antigens are released from binding proteins and thus can bind to the antibodies in the sample during analysis (Skogstrand *et al*., 2008). Although, a longer storage time prior to separation into serum could have affected the cytokines concentrations, it is unlikely to have affected the findings concerning cytokines and GBS colonisation status, as there was no significant overall difference in the storage time prior to separation into serum for the two different groups.

4.4 Future studies

Results from my study indicate that measuring maternal IL-6, IL-8 and IL-10 may not be an appropriate clinical index of intrapartum GBS colonisation in healthy women. To further investigate the results from my Masters study I would suggest that future studies measure additional cytokines, such as IL-1α, IL-1β, TNF-α, INF-γ and IL-17, all of which have been shown

to be increased in response to GBS, in both human and animal studies (Mitchell et al., 2013; Berner et al, 2001; Levy et al, 2003; Peat et al., 1995; Peoples et al., 2009; Wilson et al., 1985; Clarke et al., 2016; Patras et al., 2015). As with the two previous studies investigating the relationship between maternal cytokines and GBS colonisation status, my study was a cross-sectional study. A longitudinal study in which the time of colonization and colonization density could be determined, together with cytokines responses may provide a better indication of the relationship between maternal cytokines and GBS colonisation status across pregnancy.

Thus, when designing future studies I would recommend that researchers consider including more cytokines, a larger sample size, collecting vaginal and rectal swabs and using culture enriched media, like lim broth to increase sensitivity and reliability of both the culture and PCR methods in GBS isolation.

CHAPTER 5

CONCLUSION

My study aimed to determine the clinical usefulness of maternal serum pro-and anti-inflammatory cytokine concentrations as a clinical index of intrapartum GBS colonisation in healthy pregnant women in early adulthood. Almost all the participants (96%) in my study were of black African ancestry and were recruited from the labour ward at Chris Hani Baragwanath Hospital in Johannesburg, South Africa. Within the cohort of pregnant women used in my study, 28.7% were identified to be colonised with GBS either from a positive culture result or from a positive PCR result obtained from a vaginal swab collected at the time of delivery. The most prevalent GBS serotype in colonised mothers was serotype Ia, followed by serotype III. The gestational age at delivery was > 37 weeks in 61% of the deliveries; no deliveries occurred before 35 weeks. All infants born were healthy and showed no evidence of GBS infection within 90 days of birth. A positive GBS colonisation status at delivery did not significantly increase the maternal serum concentrations of IL-6, IL-8 and IL-10. Thus, based on the results from my study it does not appear that measuring maternal concentrations of IL-6, IL-8 and IL-10 will be a useful clinical index of intrapartum GBS colonisation in healthy pregnant women.

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Appendices

R14/49 Dr Jelani Muhammad et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M1611155

NAME: (Principal Investigator)	Dr Jelani Muhammad et al
DEPARTMENT:	Physiology Chris Hani Baragwanath Academic Hospital
PROJECT TITLE:	Levels of Cytokines in Maternal and Umbilical Cord Blood in Relation to Maternal Group B Streptococcus Colonization
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub-Study under Primary Study M140203
SUPERVISOR:	Dr Lois Harden and Dr Karine Scheuermaier
APPROVED BY:	Professor P Cleaton-Jones,Chairperson, HREC (Medical)
DATE OF APPROVAL:	20/01/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.** The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in November and will therefore be due in the month of November each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

25/01/2017
Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

GOOD CLINICAL PRACTICE

Basic Course for Support Staff

Dr J Muhammad

attended the course and achieved 88% for the assessment

Date: 4 April 2017

Venue: BEESA Conference Centre, 5 Sherborne Rd, Parktown

Facilitator: Mary-Anne Bopape

The Health Professions Council of South Africa approved CPD reference is as follows:

MDB08/006/01/2017 Activity No: 35657 Category: 1B Points: 6
MDB08/004/01/2017 Activity No: 35659 Category: 2L Points: 4 Ethics

This ICH E6 GCP Investigator Site Training meets the Minimum Criteria for ICH GCP Investigator Site Personnel Training identified by TransCelerate BioPharma as necessary to enable mutual recognition of GCP training among trial sponsors

COURSE CONTENT:

ICH Guideline for Good Clinical Practice - E6(R2) 9 November 2016
SA Good Clinical Practice Guidelines – 2nd Edition 2006

- Drug Development Process
- Development of GCP
- South African GCP
- Regulatory Process in South Africa
- Study Documents
- Patient Recruitment and Retention
- Informed Consent
- Investigational Product
- Safety Reporting
- Responsibilities of the Study Team
- Data Privacy
- Overview of Monitoring and Auditing

Course duration – 7hrs

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