PERIPHERAL AND LOCAL IMMUNE RESPONSES IN PREGNANCY AND PRETERM BIRTH

Dr Denise Chi Yun Chan

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Department of Metabolism, Digestion and Reproduction

Imperial College London

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DECLARATIONS

Declaration of originality

I certify that all work presented in this thesis was performed by myself unless stated otherwise.

Declaration of contribution

Optimisation experiments for the Cytek® Aurora spectral flow cytometry (Chapter 3) were performed by Maria Jaimes and the scientific team at Cytek Biosciences™. Statistical analysis was performed by Denise Chan with assistance from Lynne Sykes and David MacIntyre (Chapters 3-5). Bioinformatics for work on the vaginal microbial composition was performed by David MacIntyre (Chapter 5).

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ABSTRACT

Preterm birth (PTB) affects 11% of pregnancies worldwide and the leading cause is infection and inflammation. The maternal immune system undergoes temporal adaptions during healthy pregnancy, but how this may be altered in PTB remains largely unexplored. The vaginal microbial composition influences pregnancy outcome, although the mechanism of microbial-driven PTB is not well understood.

This thesis explores if there are peripheral and local immune signatures in women who undergo microbial-driven PTB. The key aims were to describe 1) changes in the maternal immune response in healthy pregnancy and spontaneous PTB, 2) alterations in the immune response to cervical shortening and cervical cerclage and 3) differences in the immune response to the vaginal microbial composition in term and preterm pregnancies.

Temporal adaptations in the peripheral immune response were similar in term and preterm pregnancies and were not directly impacted by the vaginal microbiota. Activation of the local innate and adaptive immune response were seen in cervical shortening, and in a proportion of women who delivered preterm. *Lactobacillus iners* and diverse microbial composition were associated with increases in local mediators of microbial recognition, complement and pro-inflammatory cytokines. This response was augmented in those who delivered preterm, suggestive of a dysregulated immune response. In contrast, cervical shortening and PTB in the presence of *Lactobacillus crispatus* was not associated with activation of the innate or adaptive immune response, therefore were likely to be driven by alternative causes. Placement of a Mersilene cerclage led to an augmented immune response and was associated with higher PTB rates compared to Nylon, and therefore should not be used.

Microbial-driven PTB involves microbial recognition and activation of the complement cascade, leading to a pro-inflammatory local immune milieu. This opens the door for the development of immune modulators, complement inhibitors and probiotics to mitigate the risk of microbial driven PTB.

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Chan D, Bura S, Lee YS, Teoh TG, Collado MC, Bennett PR, MacIntyre DA. Recognition of the vaginal and gut microbiome by the maternal immune response in pregnancy. 3rd Annual Preterm Birth Conference, Leeds, September 2017.

Chan D, Brown RG, Bennett PR. Positive predictors of pregnancy outcome following rescue cervical cerclage. RCOG World Congress, Cape Town, March 2017.

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Alqurashi M, Thurairajah S, Adan M, **Chan D**, Teoh TG, Bennett PR, Muller I, Kropf P, Sykes L. Characterisation of Normal and Low Density Granulocytes in Pregnancy, Term Labour, and Women at High Risk of Preterm Labour. 66th Annual Scientific Meeting for the Society for Reproductive Investigation, Paris, March 2019.

Chan D, Lee YS, Teoh TG, Bennett PR, MacIntyre DA, Collado MC, Sykes L. Maternal gut microbial-immune interactions during healthy pregnancy. RCOG Academic Meeting, London, January 2019.

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Chan D, Brown RG, Bennett PR. Positive predictors of pregnancy outcome following rescue cervical cerclage. 2nd European Spontaneous Preterm Birth Conference, Gothenburg, May 2016.

Chan D, Lee YS, Bura S, Arianoglou M, Teoh TG, Collado MC, Bennett PR, MacIntyre DA, Sykes L. Recognition of vaginal microbiota by the adaptive maternal immune system. 65th Annual Scientific Meeting for the Society for Reproductive Investigation, San Diego, March 2018.

Brown RG, **Chan D**, Lee YS, Smith A, Terzidou V, MacIntyre DA, Bennett PR. Rescue cervical cerclage in asymptomatic women without vaginal colonisation of Gardnerella vaginalis is associated with good neonatal outcomes. 65th Annual Scientific Meeting for the Society for Reproductive Investigation, San Diego, March 2018.

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Chan D, Lee YS, Bennett PR, MacIntyre DA, Sykes L. A comparison of toll-like receptor 1-9 induced cytokine responses in whole blood collected in the first trimester, at term and matched cord blood. RCOG Academic Meeting, London, February 2017.

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ABBREVIATIONS

17-OHP	17α-hydroxyprogesterone
ANOVA	Analysis of variance
AP-1	Activator Protein 1
BMI	Body mass index
C	Complement
CARD	Caspase-recruitment domain-containing adaptor protein
CD	Cluster of differentiation
CL	Cervical length
COX	Cyclo-oxygenase
CRH	Corticotrophin releasing hormone
csEN	Cell signaling based Elastic Net
C-STITCH	Cerclage suture type for an insufficient cervix and its effect on health
CST	Community state type
Cx Rx	Cervical treatment
CVF	Cervico-vaginal fluid
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMMPRIN	Extracellular matrix metalloproteinase inducer
FDA	Food and drug administration
fFN	Fetal fibronectin
GBS	Group B Streptococcus
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA-DR	Human Leukocyte Antigen – DR
HIV	Human immunodeficiency virus
HVS	High vaginal swab
IDO	Idoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IGFBP-1	Insulin like growth factor binding protein-1

IL	Interleukin
KIR	Killer cell immunoglobulin-like receptors
LLETZ	Large loop excision of the transformation zone
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MBL	Mannose binding lectin
MASP	Mannose binding lectin-associated serine proteases
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MTL	Mid-trimester loss
NGS	Next generation sequencing
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICE	National Institute for Clinical Excellence
NK (cells)	Natural killer (cells)
NLRP	Nucleotide-binding oligomerization domain
NMDA	N-methyl-D-aspartate receptor
NOD	Nucleotide-binding oligomerisation domain
NS	Non-significant
ORACLE	Overview of the role of antibiotics in the curtailment of labour and early delivery
OTU	Operational taxonomic units
PAMG-1	Placental alpha macroglobulin-1
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein-1
PD-L1	Programmed cell death ligand-1
PReCePT	Prevention of cerebral palsy in preterm labour
PPROM	Preterm prelabour rupture of membranes
PR	Progesterone receptor
PTL	Preterm labour
PTB	Preterm birth
RCOG	Royal College of Obstetricians and Gynaecologists
RCT	Randomised controlled trial
RDP	Ribosomal database project

rRNA	Ribosomal ribonucleic acid
SBLCBv2	Saving Babies Lives Care Bundle, version 2
SERPINH1	Serpin Family H Member 1
Sobs	Species observed
STAMP	Statistical Analysis of Metagenomic Profiles
STAT	Signal transducer and activator of transcription
TBE	Tris/Borate/EDTA
TCR	T cell receptor
Th	T helper
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
UV	Ultraviolet
VMG	Vaginal microbial group
WHO	World Health Organisation

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CHAPTER 1: INTRODUCTION

1.1 Preterm birth

Worldwide 15 million babies are born preterm before 37 weeks gestation each year. Preterm birth can be categorised based upon the gestational age at delivery; extremely preterm at less than 28 weeks, very preterm between 28 and 32 weeks and moderate to late preterm between 32 and 37 weeks [(1)]. It is the leading cause of neonatal morbidity and mortality, with 1 million babies dying each year as a result of complications of preterm birth [(2)].

Neonatal complications of preterm birth include hypoglycaemia, feeding difficulties, hypothermia, sepsis, respiratory distress syndrome, necrotising enterocolitis and intraventricular haemorrhage [(3)], with the most severe complications more commonly affecting extremely premature infants. Approximately 50% of all neonatal deaths are of preterm babies [(4)]. Those who do survive infancy can have long term neurological, respiratory, visual, hearing and developmental complications.

Preterm birth can be medically indicated (iatrogenic) due to maternal or fetal morbidity (30% cases) or spontaneous (70% cases). Preterm labour includes cases where the fetal membranes are intact at the onset of labour (40-45%) and where there is preterm prelabour rupture of membranes (PPROM) (30%) [(5)]. Spontaneous preterm birth is often considered a syndrome with multiple aetiologies and causal factors. Maternal factors influencing the risk of preterm birth include the extremes of maternal age [(6, 7)], short pregnancy intervals [(8, 9)], low BMI [(5, 10)], previous preterm birth [(11, 12)], previous excisional cervical treatment [(13)], previous mid-trimester miscarriage [(14)], black ethnicity [(15)], and uterine anomalies [(16)]. Index pregnancy risk factors include concurrent infection, multiple pregnancy causing uterine distension, male infant [(17)] and congenital abnormalities, (however the latter are often omitted from studies describing spontaneous preterm birth).

The worldwide incidence of preterm birth is approximately 11.1% of all pregnancies, however there is wide variation between different geographical regions. The lowest rates are in northern Europe and the highest rates are in Sub-Saharan Africa [(4)]. As reported by the Office for National Statistics, in the UK in 2018, the preterm birth rate was 7.9%. A breakdown by ethnic group is provided in Figure 1.1, with the lowest rates in the White British and White other groups, (7.96% and 6.56% respectively), whilst the highest rate was observed in the Black Caribbean group at 9.71%. The racial disparity is observed even in studies that correct for socioeconomic factors [(18)]. Some groups have reported on polymorphisms in the TNF-α allele (tumour necrosis factor) [(19)] and the SERPINH1 gene (Serpin Family

H Member 1), a gene encoding heat-shock protein 47 which is required for collagen synthesis [20] which are more commonly found in Black ethnicities which may partly explain the racial disparity.

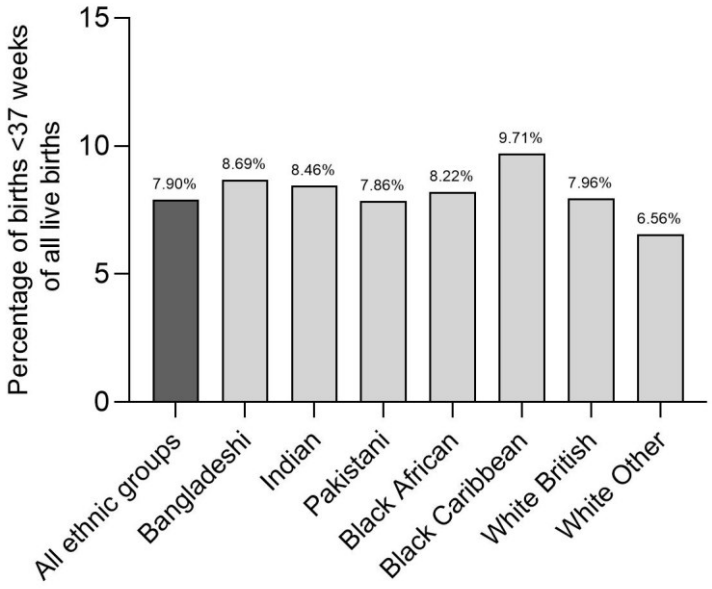

Figure 1.1 Preterm birth rate by ethnic group in the UK (2018)
Source: Office of National Statistics. Retrieved July 2020.

1.2 Prevention of preterm birth

Women at high risk of preterm labour are identified in early pregnancy when booking for antenatal care. Risk factors include previous spontaneous preterm labour, excisional cervical treatment, mid-trimester miscarriage and multiple pregnancy. The fifth element of the Saving Babies Lives Care Bundle, version 2 (SBLCBv2) from NHS England sets out recommendations to help reduce preterm birth, neonatal morbidity and mortality, with three main target areas of prediction, prevention and better preparation when preterm birth is unavoidable [21]. It is recommended that all women identified at risk of preterm birth have access to a preterm birth prevention service led by a specialist clinician. A Cochrane meta-analysis of pregnancy interventions to prevent preterm birth identified four interventions which provided clear evidence of benefit. These were cervical cerclage in singleton pregnancies in women at high risk of preterm birth, midwife-led continuity models of care, screening for lower genital tract infections before 37 weeks of gestation, and zinc supplementation in those without systemic illness [22]. Prediction and prevention of preterm birth is difficult because it has multiple underlying causes, thus interventions are unable to be applied in a 'one-size fits all' approach.

1.2.1 Screening

The SBLCBv2 recommends preterm birth prevention clinics be set up at NHS hospitals where women can have transvaginal cervical length assessments. A short cervical length (<25mm) confers a higher risk of preterm birth [23]. It is a well-established, cost-effective screening test for preterm birth and can be performed by trained clinicians (Figure 1.2). A measurement of >25mm (>10th centile) between the internal and external os of the cervix at 24 weeks gestation is reassuring and 90% of such women will deliver at term [24]. A measurement of 25mm is the threshold for clinical intervention (Figure 1.3). The NICE guidelines for Preterm Labour and Birth recommends offering prophylactic progesterone or prophylactic cervical cerclage to women with a history of spontaneous preterm birth or mid-trimester loss and cervical length ≤25mm between 16^{+0}-24^{+0} weeks gestation. Prophylactic progesterone should be considered in women with these risk factors. Cervical cerclage should be considered if the cervical length is ≤25mm with either a history of PPROM or cervical trauma [25].

Cervical length measurement has good specificity but low sensitivity and low positive predictive value [26-28]. It is a good rule out test if the cervical length is normal. There is limited use in screening low risk populations and cervical length screening is recommended for use in women with existing risk factors for preterm delivery only. In a study of over 9000 nulliparous low risk women with singleton pregnancies, transvaginal cervical length ultrasound had low predictive accuracy of 23% for spontaneous preterm birth [29].

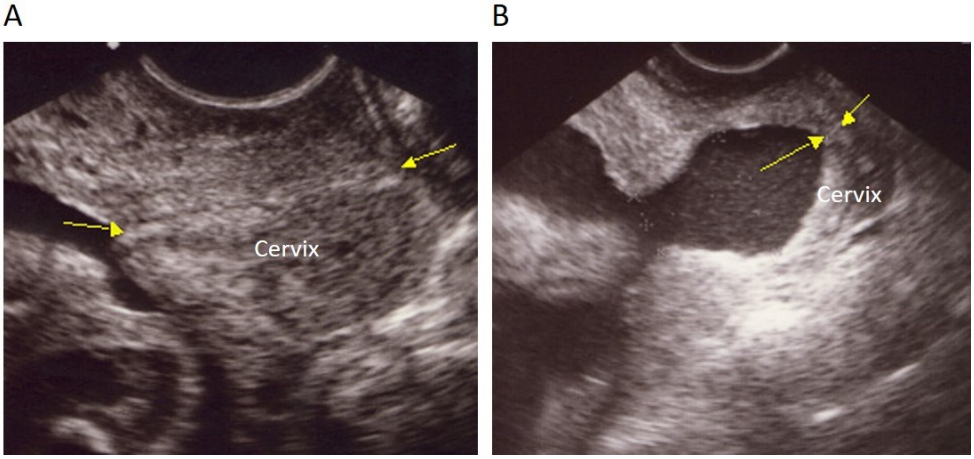

Figure 1.2 Measurement of cervical length on transvaginal ultrasound
The cervical length is measured from the internal to the external cervical os. A long, closed cervix is shown in (A), and a shortened cervix with funnelling is shown in (B). Source: St Mary's Preterm Birth Surveillance Clinic, Imperial College NHS Trust.

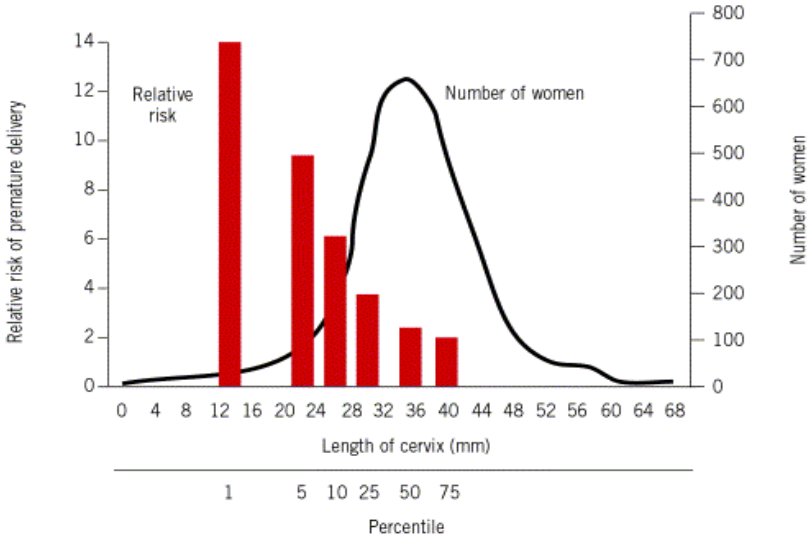

Figure 1.3 Risk of preterm delivery increases as the cervical length decreases
Cervical lengths were performed at 24 weeks gestation on a heterogenous population of 2915 women with and without risk factors for preterm birth. Cervical length measured by transvaginal ultrasound was normally distributed. The relative risk of preterm birth before 35 weeks gestation for cervical lengths below the 75th centile is displayed on bars. A cervical length less than the 10th centile, 25mm resulted in a relative risk of preterm delivery of 6.2. Reproduced from Iams et al. 1996 [23]

Cervical length measurement can be used to predict preterm birth risk in conjunction with measuring the concentration of fetal fibronectin (fFN) in cervicovaginal fluid collected by a transvaginal swab. Fetal fibronectin is an extracellular matrix glycoprotein secreted by amniocytes throughout pregnancy. Before the fusion of the decidua and fetal membranes, fFN is detectable in cervicovaginal fluid. Levels subsequently become undetectable between 18-35 weeks when the two layers fuse. Detectable concentrations reflect disruption of the extracellular matrix which could be secondary to inflammation, infection or mechanical injury. Clinically the measurement of fFN between 24-34 weeks gestation can be used to predict preterm birth, with a measurement of less than 50ng/ml having a high negative predictive value of 99% for delivery within the next 14 days [30]. The positive predictive value improves with greater concentrations. Levels between 50-200ng/ml have a positive predictive value of 20% for PTB within 14 days, whilst levels greater than 500ng/ml have a positive predictive value of 47% [31]. The QUiPP App takes into account the presence/absence of symptoms of preterm labour, risk factors for preterm birth, current gestation, shortest cervical length measurement and the fibronectin concentration to calculate the risk of preterm delivery [32]. It can help to guide when a woman at risk should be admitted to hospital and administered corticosteroids for fetal lung maturation.

Other vaginal fluid bio-marker tests which are commercially available are PartoSure® which detects placental alpha macroglobulin-1 (PAMG-1) in vaginal secretions and Actim® PROM which detects amniotic isoforms of insulin-like growth factor protein (IGFBP-1). PAMG-1 is found in high concentrations in the amniotic cavity, and low concentrations in the vaginal secretions. It has been found to correlate strongly with delivery in patients presenting with threatened preterm labour with intact membranes [33]. Insulin-like growth factor protein is secreted from placental decidual cells when there is disruption of the choriodecidual interface [34]. They are both point of care test for women already presenting with symptoms of preterm labour. Similar to fFN, their clinical value is in their negative predictive value. Their downside is their inability to predict preterm labour at an earlier time point that allows for targeted surveillance and preventative measures to be instigated.

1.2.2 Progesterone

During pregnancy, progesterone plays an important role in mediating key inflammatory and contractile pathways thus maintaining the uterus in a quiescent state. This includes receptor mediated inhibition of transcription factor NF-κB and downstream expression of pro-inflammatory genes in the cervix, uterus and fetal membranes that mediate the phenotypic changes at each of these sties required for birth, namely cervical ripening, uterine contractility and fetal membrane remodelling. Progesterone has been shown to inhibit NF-κB, COX-2 and prostaglandin production [35] and neutrophil chemoattractant IL-8 in amniocytes [36]. It also has tocolytic effects on the myometrium [37].

In early pregnancy, progesterone is produced by the corpus luteum before production is continued by the placenta. There are two isoforms of the progesterone receptor, and the balance of activity between them in gestational tissues is key to the uterus being quiescent for the majority of pregnancy and then becoming activated during labour [38]. Before labour, the anti-inflammatory actions of progesterone receptor B dominates and then at term, there is upregulation of pro-inflammatory progesterone receptor A (PR-A), which then overwhelms the anti-inflammatory activity of PR-B [39]. Murine studies demonstrate that a reduction of systemic progesterone leads to cervical remodelling and preterm labour [40], however this is not seen in humans, but rather a 'functional' withdrawal occurs due to reduced activity from PR-B, triggering labour. Further supporting the role of progesterone in maintaining pregnancy, Mifepristone is a progesterone receptor antagonist RU-486 which induces cervical ripening and is utilised in the management of medical termination of pregnancy.

Progesterone has long believed to dampen uterine contractility [41] by exerting its actions through genomic and non-genomic pathways by interacting with nuclear and plasma membrane progesterone receptors. It is the most studied pharmacological agent to prevent preterm birth. A systematic review reports equal efficacy of progesterone and cervical cerclage in cervical shortening in singleton pregnancies with a history of previous preterm birth [42]. However it does not confer any additional benefit when used in conjunction with cervical cerclage or in women presenting with threatened preterm labour [43]. There are inconsistent reports on its efficacy to prevent preterm birth which may be related to the multi-factorial causes of preterm birth, different preparations and routes of administration [44].

Many clinical trials have been performed on two different preparations, natural progesterone and the synthetic analogue of its metabolite 17α-hydroxyprogesterone (17-OHP). Natural progesterone (P4) is commonly administered as a vaginal or rectal pessary, whereas 17-OHP is administered as an intramuscular injection. The former is recommended by the National Institute for Health and Care Excellent (NICE) in the UK [25], whilst the latter is the only progestin approved by the US Food and Drug Administration for the prevention of preterm birth [45].

1.2.3 Cervical cerclage

Cervical cerclage can be performed abdominally or more commonly, transvaginally to mechanically support the cervix and protect the mucus plug. They can be ultrasound or history indicated. Recommended UK practice is to recommend insertion of a cervical cerclage if the cervical length is ≤25mm in a woman who has previously had a preterm birth, mid-trimester miscarriage or excisional cervical treatment [25]. History indicated cervical cerclages tend to be performed for women who have had a cerclage in a previous pregnancy, a history of cervical insufficiency following preterm spontaneous painless cervical dilation or trachelectomy. Evidence supports the reduction in preterm birth with the use of cerclage in women with cervical shortening ≤25mm who have risk factors for preterm birth, and in women with cervical shortening <10mm without risk factors [46]. However it is not recommended women with cervical shortening between 10-25mm without other risk factors for preterm delivery [47].

Two methods for cervical cerclage insertion have been described and are widely used in clinical practice today; Shirodkar involving bladder reflection and McDonald without bladder reflection with comparable success [48]. Non-absorbable monofilament suture (e.g Nylon) or braided sutures (e.g

Mersilene) are used for either method, which can be readily removed to allow for vaginal delivery. The braided Mersilene suture consists of woven polyester ethylene terephthalate fibres to form a 5mm wide mesh tape and has high tensile strength. The mono-filament Nylon suture is a single strand of polyamide polymer, which applies less mechanical resistance during insertion, however more surgical knots are required when securing it in place [49]. The choice of technique and suture material is driven by surgical preference. A survey of UK Obstetricians and Gynaecologists reported that braided sutures were used by the majority (175/202, 87%) but many were unsure which was the best material to use [50]. Our group have subsequently reported that the choice of suture material can impact upon the pregnancy outcome. Braided sutures were associated with vaginal microbiome dysbiosis, a local pro-inflammatory cytokine response and poorer pregnancy outcomes compared to monofilament sutures [49]. A large multi-centre UK trial (C-STITCH) to examine the effect of monofilament and braided suture material used in cervical cerclage is nearing the end of recruitment. This study aimed to recruit over 2000 women randomising study participants to receive either a monofilament or a braided cerclage, with the main outcome measures being the effect of cerclage material on pregnancy loss rate (defined as miscarriage, stillbirth and neonatal death in the first of life), and neonatal mortality up to 7 days post-delivery in women presenting with cervical insufficiency who were treated with a cervical cerclage [51].

1.2.4 Cervical pessary

The Arabin pessary is a soft silicon ring that can be inserted vaginally to surround the cervix. It alters the position of the cervical canal and redistributes the weight of the gravid uterus on the cervix. It is easily inserted, has limited side effects and is well tolerated by patients [52]. Its efficacy is likely to be influenced by the multifactorial nature of preterm birth. Some papers report that it does not reduce preterm birth in singleton pregnancies with cervical shortening [53, 54], however it may have therapeutic benefit if an individual has had previous preterm labour without a history of PPROM [55] , or as combination therapy together with cervical cerclage and progesterone [56] and in cervical shortening in women with a multiple pregnancy [57].

1.2.5 Antibiotics

Infection and inflammation are leading causes of preterm labour. Treatment of urinary tract infections, e.g pyelonephritis [58], asymptomatic bacteriuria [59], bacterial vaginosis [60], pneumonia and malaria reduces the risk of preterm birth. However there is insufficient evidence to recommend the

routine use of antibiotic prophylaxis to reduce the risk of PPROM or preterm birth [61, 62]. The ORACLE II trial (Overview of the role of antibiotics in the curtailment of labour and early delivery) found that antibiotics in spontaneous preterm labour with intact membranes without evidence of clinical infection did not reduce the study's primary outcome measures of neonatal death, chronic lung disease and major cerebral abnormalities [63]. There are reports that prophylactic antibiotics can even cause harm. The PREMET study was a randomised controlled trial of metronidazole to prevent preterm birth in women with a positive fetal fibronectin test [64]. It was stopped early due to higher rates of preterm delivery in those who received the antibiotic.

1.3 Management of preterm labour

As described, the use of cervical cerclage, cervical pessary, progesterone and antibiotics to treat concurrent infection can be utilised to delay threatened preterm labour however there are cases when delaying delivery can have deleterious effects such as prolonging the exposure of the fetus to inflammation and infection. In order to optimise care for cases of inevitable preterm birth corticosteroids, tocolytics, magnesium sulphate, prophylactic antibiotics and intra-uterine transfer to tertiary centres have all been shown to improve neonatal outcome. Approximately two thirds of preterm birth occur in women with no identifiable risk factors for preterm delivery [65], thus emphasising the importance of the following clinical interventions.

1.3.1 Corticosteroids

Corticosteroids stimulate the synthesis of pulmonary surfactant, antioxidant enzymes, and regulate pulmonary fluid metabolism to accelerate fetal lung maturation. Administration of antenatal corticosteroids to the mother reduces the risk of perinatal death, neonatal death, respiratory distress syndrome, intraventricular haemorrhage, necrotising enterocolitis, the need for mechanical ventilation and systemic infections in the first 48 hours of life [66]. The WHO recommends antenatal corticosteroid therapy for women at risk of preterm birth from 24 to 34 weeks gestation if the gestational age is accurately assessed, preterm birth is considered imminent, there are no clinical signs of maternal infection, adequate childbirth care is available and the preterm newborn can receive the appropriate care [67]. The timing of corticosteroid administration is important to confer maximal benefit which is within 7 days from administration to birth. No additional survival benefits were conferred when administering corticosteroids beyond 34 weeks gestation, and rates of neonatal

hypoglycaemia are significantly higher [68]. The usual regimen is 12mg of Betamethasone administered as two intramuscular injections 12-24 hours apart.

1.3.2 Tocolytics

Tocolytics can be used to reduce the frequency and strength of uterine contractions. In clinical practice they have a short-term role for enabling sufficient time to complete a course of antenatal corticosteroids or to facilitate intrauterine transfer to a suitable neonatal unit prior to delivery. There are various different pharmacological agents in use, including calcium channel blockers (Nifedipine), prostaglandin inhibitors (Indomethacin), oxytocin receptor antagonists (Atosiban), beta receptor agonists (Terbutaline) and Magnesium Sulphate. The first two listed have been found to be most effective at delaying delivery by 48 hours, reducing neonatal mortality, reducing neonatal respiratory distress syndrome and have less maternal side effects [69]. Caution is necessary with Indomethacin as there is increased risk of intraventricular haemorrhage [70], periventricular leukomalacia [70], necrotising enterocolitis [70] and increased risk chorioamnionitis when used following PPROM [71]. The first line agent recommended by NICE is Nifedipine, and if it is contraindicated, oxytocin receptor antagonists are the recommended second line agents [25].

1.3.3 Magnesium sulphate

As well as a recognised tocolytic agent, Magnesium sulphate has a neuroprotective role to the fetus when given antenatally to the mother, significantly reducing the rate of cerebral palsy and gross motor dysfunction [72, 73]. Multiple mechanisms may confer the neuroprotective effect of magnesium, including as a NMDA receptor antagonist, preventing calcium-induced cellular injury, reducing extracellular glutamate under ischaemic conditions, reducing calcium influx through voltage gated channels, reducing apoptosis and anti-inflammatory effects [74]. NICE recommends administering intravenous magnesium sulphate for neuroprotection of the baby to women between 24-34 weeks gestation who are in established labour or having a planned preterm delivery within 24 hours [25]. The PReCePT programme (Prevention of Cerebral Palsy in PreTerm Labour) was developed by the West of England Academic Health Science Network and is now nationwide; with the aim to reduce the incidence of cerebral palsy by offering magnesium sulphate to all eligible women in England during preterm labour [75].

1.3.4 Antibiotics

Infection and resulting inflammation are well-recognised key drivers for preterm labour. Overview of the Role of Antibiotics in the Curtailment of Labour and Early Delivery 2 (ORACLE 2) was a meta-analysis of 14 studies involving 7837 women who were randomised to antibiotic treatment, placebo and no treatment in preterm labour with intact membranes. The study concluded that in the absence of signs of infection, antibiotics should not be routinely given to women with preterm labour with intact membranes [(76)]. Despite a reduction in maternal infection, there were no neonatal benefits and of concern there was increased neonatal mortality with the use of macrolides and beta-lactam antibiotics [(76)].

In ORACLE 1, 4826 women with PPROM were randomly assigned erythromycin, co-amoxiclav, both antibiotics, or placebo. Those infants born to women in the erythromycin group only, had significantly lower neonatal death rates, chronic lung disease and major cerebral abnormality on ultrasound, compared to the placebo group. Erythromycin was found to reduce neonatal treatment with surfactant, reduce oxygen dependence at 28 days of age, and was associated with fewer major cerebral abnormalities on ultrasound and fewer positive blood cultures. Co-amoxiclav was associated with higher rates of neonatal necrotising enterocolitis [(63)]. Consequently, the Royal College of Obstetricians and Gynaecologists (RCOG) recommendation was for Erythromycin to be given for 10 days following diagnosis of PPROM, or until the women was in established labour [(77)], however ORACLE 2 did not show any long term benefits. The recommended use of Erythromycin is now debated with more recent findings that vaginal dysbiosis, characterised by *Lactobacillus* species depletion was exacerbated by Erythromycin treatment in women who were initially colonised with *Lactobacillus* species. Furthermore, an adverse vaginal microbial composition with *Lactobacillus* species depletion correlated with higher rates of early onset neonatal sepsis [(78)].

Group B streptococcus (GBS) is the most common cause of neonatal sepsis and early onset neonatal Group B Streptococcal is more prevalent in preterm infants. Therefore, intrapartum antibiotics are recommended for women in preterm labour [(79)].

1.4 Physiology of labour

During healthy pregnancy, the cervix remains long and closed, the myometrium is quiescent and the fetal membranes are intact. Understanding their function during pregnancy and labour is important when considering the preventative measures and management strategies for preventing preterm birth. There is a transition at term that leads to cervical remodelling, co-ordinated uterine contractions and fetal membrane rupture, however the triggering mechanisms driving these labour events are poorly understood.

1.4.1 Cervical remodelling

The cervix provides structural support to the uterus and protects the mucous plug. Type I and type III collagen fibres in the extracellular matrix provide tensile strength [80]. The concentration of cervical collagen reduces when there is an increase in hydrophilic glycosaminoglycans, non-collagenous proteins and aquaporin water channels which disperses and solubilises the collagen fibres [81]. Coinciding with this process is a local pro-inflammatory response which sees the extracellular matrix of the cervix flooded with immune cells including T cells, mast cells, neutrophils, eosinophils and activated macrophages and up-regulation of pro-inflammatory cytokines, IL-6, IL-8, IL-1β and TNF-α [82, 83]. These cytokines promote angiogenesis, vasodilatation and increase vascular permeability further facilitating the influx of immune cells to the cervix. Also contributing to the cervical remodelling are matrix metalloproteinases (MMPs-1, -8, -9 and -13) and prostaglandin production, particularly PGE_2, all of which result in the loss of tensile strength provided by the collagen fibres. Cervical dilation is also aided mechanically as the uterus contracts and the fetal head applies pressure on to the cervix. After delivery, the mechanical stretch effect diminishes, and anti-inflammatory pathways are activated allowing repair of the extracellular matrix of the cervix [81].

1.4.2 Uterine contractions

The myometrium is the muscular layer of the uterine wall consisting of smooth muscle cells, myocytes. A sliding interaction between the actin thin filaments and the myosin thick filaments leads to muscle contraction, which is stimulated by myosin light chain kinase and inhibited by myosin light chain phosphatase [84]. Myosin light chain is activated by intracellular calcium and calmodulin [85]. Oxytocin and prostaglandin F2 alpha influence the activity of myosin light chain kinase and myosin light chain phosphatase [86, 87].

Oxytocin is a uterotonic, secreted by the posterior pituitary. Upon binding to the oxytocin receptor, phospholipase C on the plasma membrane of smooth muscle cells of the uterus produces inositol triphosphate and diacylglycerol which releases intracellular calcium stores required for muscle contraction. There is an increase in oxytocin receptors at term. Oxytocin also stimulates prostaglandin release and activation of inflammatory pathways through NF-κB [88].

Progesterone has two receptor subtypes, pro-inflammatory PR-A and anti-inflammatory PR-B. Progesterone levels remain high throughout human pregnancy, however at term there is an apparent functional withdrawal. At term PR-A expression increases and there is upregulation of uterine activator protein genes [89]. The suppressive, anti-inflammatory effect which would have been exerted by PR-B is overwhelmed by the pro-inflammatory activity of PR-A [90].

Placental derived corticotrophin-releasing hormone (CRH) increases with advancing gestation. CRH binding protein (CRHBP) limits the bioavailability of CRH, however at term, CRHBP levels fall. CRH can then bind to its receptor and its downstream effects are to increase Prostaglandin H synthase type 2 enzyme (PGH-2/COX-2) in the amnion, and to stimulate the production of MMPs in the amnion and cervix and the generation of cyclic adenosine monophosphate which stimulates myometrial contractions [91, 92]. There is also transplacental transfer of CRH and upon acting on the fetal pituitary and adrenal glands this leads to the production of corticotrophin, cortisol and dehydroepiandrosterone (DHEA). Fetal lung maturation occurs when surfactant is produced in response to cortisol.

1.4.3 Fetal membrane rupture

Together the amnion and chorion form the fetal membranes. The amnion is avascular and is the inner layer, providing most of the tensile strength. It is a major site of prostaglandin production. The chorion is highly vascular and acts as an immunological barrier protecting the fetus from the maternal immune system [93]. Prior to fetal membrane rupture, activation and thinning of the membranes occurs and this process can be thought of in the same way that the cervical remodelling pathways are activated in labour, with the release of proinflammatory cytokines, prostaglandins and MMPs [94]. Collagen turnover in the fetal membranes is controlled by the balance of MMPs and tissue inhibitor of matrix metalloproteinases (TIMPs). With advancing gestation, the activity of MMPs is greater than that of TIMPs which leads to a reduction in collagen in the extracellular matrix and thinning of the fetal

membranes particularly over the internal os of the cervix [95]. Forceful uterine contractions also contribute to membrane thinning and rupture.

1.5 Pathophysiology of preterm labour

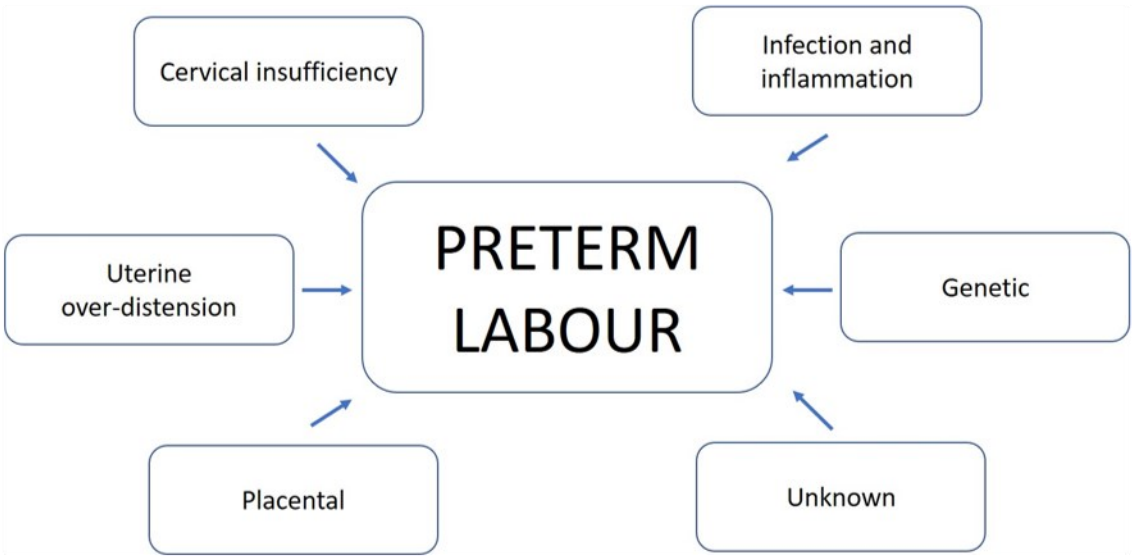

Figure 1.4 Preterm labour is multifactorial

1.5.1 Infection and inflammation

Pathological activation of the parturition pathways occurs in spontaneous preterm labour. Preterm labour is a multifactorial syndrome, (Figure 1.4) however the leading cause is infection/immune related. Bacterial infections are reported to account for 40% of cases of spontaneous preterm birth [96]. Greater than 80% of deliveries prior to 28 weeks are associated with chorioamnionitis [96]. Ascending microbes/inflammatory mediators from the vagina to the uterus is considered the most common route for infection, with bacteria commonly found in the lower genital tract being similar to those found in amniotic fluid. Another route is that of periodontal bacteria by haematogenous spread and transplacental transfer [97]. The balance between healthy commensals and pathogenic bacteria, and how the host immune response responds to this balance is likely to influence the risk of infection induced preterm birth. Addressed later in this Chapter is how the maternal immune system undergoes highly regulated, temporal adaptions to defend against pathogens whilst tolerating a semi-allogenic fetus with paternal antigens.

The first line of defence are the epithelial surfaces of the vagina and the cervix. The multi-layered squamous epithelial cells produce a mucus layer designed to prevent microbial adherence and epithelial invasion. It contains defence proteins and anti-microbials such as immunoglobulins: IgG, IgA, IgM [98], lactoferrin [99], lysozyme [100], alpha and beta defensins [100], secretory leukocyte protease inhibitor [101] and elafin [102]. These constitute the innate and adaptive immune response. The vagina is typically colonised with *Lactobacillus* species and there is a symbiotic relationship with the host immune system. High circulating oestrogen levels following menarche promotes the proliferation of vaginal epithelial cells and glycogen deposition. The breakdown products are metabolised by *Lactobacillus* species. A *Lactobacillus* species rich vaginal environment is acidic which is ill-suited for many other microbial species [103].

Disruption of this symbiotic relationship between *Lactobacillus* species and the host immune response impairs the epithelial and mucosal barriers and allows immune cells such as macrophages, neutrophils, natural killer cells and dendritic cells to migrate into the local tissues [104]. Their pattern recognition receptors (PRR) recognise pathogen associated molecular patterns (PAMPS) and inflammatory pathways are activated in order to try to eradicate the microbial pathogens. PRRs important in the female reproductive tract are transmembrane Toll-like receptors (TLR) and cytoplasmic nucleotide-binding oligomerisation domain (NOD) like receptors [105]. Ten TLRs have been described, with TLR-1, 2, 4 and 6 being the most relevant for infection induced preterm labour. TLR-2 forms heterodimers with TLR-1 or TLR-6. TLR-1:TLR-2 complexes bind to lipoproteins, peptidoglycan and lipoarabinomannan from Gram-positive and Gram-negative bacteria, and lipoteichoic acid of Gram-positive bacteria. TLR2:TLR-6 complexes recognised PAMPs of *Mycoplasma* and *Ureaplasma*. These lead to activation of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) transcription factor results setting off a cascade of inflammatory processes. Another key transcription factor is Activator Protein 1 (AP-1), and both NF-κB and AP1 can upregulate the genes encoding pro-inflammatory cytokines and chemokines: IL-6, IL-8, TNF-α and IL-1β [106, 107]. It is possible that mobilisation of phagocytic innate immune cells and the subsequent activation of the adaptive immune response may be sufficient to manage microbial invasion, however in infection mediated preterm birth, these inflammatory processes may culminate in cervical remodelling, fetal membrane rupture and uterine contractions.

Infection and immune driven preterm labour can also be due to viral pathogens [108-110]. The double-hit hypothesis refers to how viral infections may alter the host immune response to bacteria flora,

resulting in the activation of pathogenic pathways and precipitating preterm labour [111, 112]. This in effect turns an immune-tolerant environment into a pro-inflammatory state.

1.5.2 Uterine overdistension

Uterine overdistension is thought to contribute to the mechanism of preterm birth in women with multiple pregnancy, polyhydramnios, congenital uterine anomalies and fetal macrosomia. Smooth muscle cells of the myometrium undergo a degree of hypertrophy and hyperplasia as fetal growth advances; however this can be excessive if the uterus is overdistended. The increased stretch of the myometrium is thought to activate the formation of gap junction proteins, connexin-43 and connexion-26 [113], promote a local proinflammatory response (IL-6, IL-8, IL-1β, TNF-α) and upregulate prostaglandins and MMPs [114]. Uterine anomalies such as a uterine septum, unicornuate or bicornuate uterus and uterine didelphys are associated with a 7 fold increased risk of preterm birth before 34 weeks and a 3 fold increased risk of PPROM [16].

1.5.3 Cervical insufficiency

As well as providing immune protection, the cervix also serves as a barrier protecting the fetal membranes. Cervical insufficiency describes premature cervical dilation in the absence of uterine contractions. It can occur following obstetric or gynaecological trauma, e.g. cervical damage during a caesarean section performed in the second stage of labour [115, 116], or excisional cervical treatment for cervical pre-invasive disease [117, 118] or malignancy, or it can occur spontaneously without any prior complications. The mechanisms leading to cervical insufficiency are unclear in the cases without any prior complications. Genetic susceptibility for cervical insufficiency has been explored too [119], with genes involved in collagen synthesis of particular interest.

1.5.4 Placental causes

There is the theory of the 'placental clock' that influences the length of pregnancy and when labour commences [120]. Placental secretion of corticotropin-releasing hormone, with reduced concentrations of circulating corticotropin-releasing hormone binding protein may be a trigger for parturition. Increased corticotropin-releasing hormone has been found to occur earlier in some women who deliver preterm [121].

Antepartum bleeding in the first trimester of pregnancy has been associated with 2 fold increase in subsequent preterm delivery [122, 123], and the risk further increases to 7 fold if there is bleeding in the second trimester [124, 125]. The mechanism for bleeding increasing the risk of preterm birth is believed to centre around thrombin formation which is required for the conversion of fibrinogen to fibrin for clot formation. Thrombin upregulates prostaglandin production in the amnion inducing myometrial contractions [126] and triggering a local inflammatory response, increasing the expression of MMPs and local production of IL-8 [127].

There is also histological evidence that abnormal placentation, with failure of physiological transformation of spiral arteries capable of transporting blood at low resistance occurring in women who experience preterm labour [128, 129].

1.5.5 Genetic factors

Genetic predisposition according to ethnicity and collagen formation have already been described, however there are also many genetic variants in the context of inflammation-associated spontaneous preterm birth that are also being explored [130-132]. Whole exome sequencing identified mutations in genes encoding proteins involved in negative regulation of the innate immune response (CARD6, CARD8, NLRP10, NLRP12, NO2 and TLR-10) and anti-microbial proteins (β-defensin 1 and MBL2) [133]. Furthermore, the mutations in DEFB1 and MBL2 genes have been found more commonly in African populations which may help to explain ethnic differences in the rates of preterm birth. Also of interest is a polymorphism in the promoter region of the TNF-α gene which was associated with increased risk of PPROM [134]. The risk was even higher in the presence of bacterial vaginosis [19]. This is particularly intriguing as it links environmental exposures to genetic susceptibility in the pathophysiology of preterm birth.

1.6 Molecular-based profiling of vaginal microbiota

Microbiota refers to all the microorganisms in a defined environment, whilst the microbiome encompasses the entire habitat, which includes the microorganisms (bacteria, viruses and fungi), their genomes and the surrounding environment [135]. Traditional culture-based techniques to identify bacteria in a given environment have been superseded by next generation sequencing (NGS) which determines the DNA sequence of a complete bacterial genome. The main limitations of traditional culture-based techniques are the duration of time needed to culture bacteria and the inability to simultaneously culture all the bacterial communities present due to differing culturing conditions. NGS is reproducible, can readily determine the relative abundance of microorganism communities and can detect those in low abundance [136]. It is widely used to study the vaginal microbial composition.

In metataxonomics, DNA can be extracted from a given sample and regions of the bacterial 16S ribosomal RNA gene (16S rRNA) can be sequenced to identify bacteria species. The 16S rRNA gene contains nine hypervariable regions (V1-V9), which range from 30-100 base pairs in length. The hypervariable regions can vary significantly between different bacteria, whilst the highly conserved sequences between the hypervariable regions allows universal primers to be designed that can sequence the 16S gene across different taxa. Sequencing the whole gene is expensive and unnecessary as specific hypervariable regions can be selected for amplification and sequencing resulting in the same output of bacterial species identification [137]. The V1-V2 amplicon is 260 base pairs in length, with paired-end reads. Almost all of the amplified product is sequenced on both forward and reverse strands thereby improving the accuracy of the amplified product. It is the most commonly used amplicon for sequencing the vaginal microbiome.

The Human Microbiome Project was launched in 2007 by the United States National Institutes of Health to improve the understanding of microbes by NGS techniques at multiple body sites including, nasal passages, oral cavity, skin, gastrointestinal tract and urogenital tract in human health and disease (Phase 1) [138]. This developed into analysing host-microbial interactions in specific conditions; the vaginal microbiome in pregnancy and preterm birth, the gut microbiome in inflammatory bowel disease and the gut and nasal microbiomes in diabetes over time (Phase 2) [139].

The vaginal microbiota is central to reproductive health. In women of reproductive age, the vaginal bacteria consists largely of *Lactobacillus* species. The rich oestrogen environment stimulates the maturation and proliferation of vaginal epithelial cells and the accumulation of glycogen. Glycogen

metabolites such as α-dextrines, maltose and maltotriose provide substrates for the conversion of lactic acid by *Lactobacilli*. The lactic acid results in a low pH (4.0-4.5) environment that prevents the proliferation of pathogenic organisms, further promoting *Lactobacillus* species dominance [140]. Professor Albert Döderlein was the first to describe culturing bacteria from vagina secretions and discovered that lactic acid inhibited the growth of pathogens.

Ravel was the first to apply NGS to describe bacteria vaginal microbiota in 396 asymptomatic, non-pregnant women of reproductive age [141]. Using hierarchical clustering based on the relative abundance of bacterial species derived from metataxonomic profiling of vaginal samples, five distinct community state types (CST) were described. Four of these were *Lactobacillus* species dominant: CST I (*L. crispatus*), CST II (*L. gasseri*), CST III (*L. iners*), CST V (*L. jensenii*), and CST IV which is polymicrobial, consisting predominantly of *Prevotella, Sneathia, Megasphaera, Streptococcus, Atopobium* and *Gardnerella*. This last group was similar to the bacterial profile typical of bacterial vaginosis. CST IV can be divided into two subgroups, CST IV-A which included *Anaerococcus*, *Corynebacterium, Finegoldia* and *Streptococcus* species and CST IV-B which included *Atopobium* , *Prevotella* and *Gardnerella* species [142].

Other classifications have since been described as a result of sampling different populations and using different statistical methods. These are akin to CSTs but are referred to as vaginal microbial groups (VMG) or vagitypes. Classification of vaginal bacteria at genera level can also be performed whereby according to relative abundance, samples are grouped into *Lactobacillus* species dominated or depleted categories.

The vaginal microbiota is usually largely stable through the menstrual cycle despite rising and falling levels of oestrogen [143]. In pregnancy, the surge in placental production of oestrogen results in high systemic concentrations [144] driving the proliferation of *Lactobacillus* species. Healthy pregnancy is associated with a *Lactobacillus* dominant profile which is maintained during pregnancy [145, 146]. Post-partum there is a marked shift in the vaginal microbial composition due to the rapid decline in oestrogen levels, with a significant increase in microbial diversity, a decrease in *Lactobacillus* species and a shift towards the polymicrobial CST IV [145, 147].

The vaginal microbiome is also heavily influenced by ethnicity and environmental factors. Smoking and vaginal douching are associated with a reduction in *Lactobacillus* species and increase in anaerobic bacteria [148-150]. Women of black or Hispanic origin have higher prevalence of CST IV and CST III

compared to Caucasian and Asian women, whilst the dominant *Lactobacillus* species in Caucasians is *L. crispatus* [141, 145, 151].

Ascending vaginal infection to gestational tissues is the widely accepted mechanism of action in infection and inflammation related preterm birth. The bacteria detected in the fetal membranes in cases of preterm birth is similar to that found in the vagina [152] and histological evidence of chorioamnionitis is most frequently found at the site of fetal membrane rupture in the lower segment of the uterus close to the cervix [153, 154].

There are many cohort studies comparing the vaginal microbial composition in preterm and term pregnancies. There is wide variation in the ethnic groups of the study populations. This is important to note as ethnicity has strong influences on the vaginal microbial composition. In a mostly white study population, *Lactobacillus* species depletion correlated with earlier gestational age at delivery and women who delivered preterm had higher abundances of CST IV diverse species and *Gardnerella* and *Ureaplasma* bacteria [147]. The same group then studied two cohorts of women, Caucasian and Black African. They were able to replicate their findings associating *Lactobacillus* species depletion and preterm birth in the Caucasian women, but not in Black African women. *Gardnerella* was more commonly found in Black African compared to Caucasian women, and *L. crispatus* was protective against preterm birth regardless of ethnicity [155]. A more recent study comparing the vaginal microbiome profiles of 45 women with preterm deliveries, and 90 women with term deliveries mostly of African ancestry reported significantly lower abundance of *L. crispatus* in women with preterm deliveries, whilst bacterial vaginosis-associated bacterium 1 (BVAB1), *Sneathia* and *Prevotella* species were significantly higher [156]. This vaginal microbial composition correlated with higher concentrations of pro-inflammatory cytokines, eotaxin, IL-1β, IL-6 and macrophage inflammatory protein-1β (MIP-1β). Again, *L. crispatus* was more abundant in women who delivered at term.

Our group and another in Austria demonstrated that *L.* iners is more commonly associated with preterm birth, whilst *L. crispatus* is protective [157, 158]. A Canadian group narrowed down the protective effect of *L. crispatus* to be associated with decreased risk of early (before 34 weeks gestation), rather than late preterm delivery [159].

Preterm prelabour rupture of membranes (PPROM) precedes approximately a third of cases of preterm birth [160]. Microbial induced infection and inflammation can cause PPROM, or it can result because of PPROM when the protective barrier of the fetal membranes is lost. Ascending bacteria can

result in chorioamnionitis and funisitis. Our group found that in women sampled before and after PPROM, approximately half who were previously *Lactobacillus* dominant, become *Lactobacillus* deplete following membrane rupture [78]. Furthermore, in longitudinal sampling prior to PPROM, instability of bacterial community structure and a shift towards a high diversity state in the second trimester was more commonly seen in cases of PPROM compared to term delivery [161].

Women who have had previous excisional cervical treatment for cervical intraepithelial neoplasia constitute a large proportion of those referred to the preterm birth surveillance clinics. Mechanical cervical insufficiency can occur as a consequence. This is further demonstrated in a study that shows that two or more large loop excisions of the transformation zone (LLETZ) procedures increased the risk of preterm birth by 4-fold, compared to women with no prior LLETZ [117]. Additionally if the excised tissue volume exceeded $6cm^3$ or if the excised tissue thickness was greater than 12mm, there was a 3-fold increase in the risk of preterm birth [118]. Independent of this effect on the risk of preterm birth is that cervical intraepithelial neoplasia (CIN) has also been associated with increased vaginal microbial diversity and low abundance of *Lactobacillus* species in a non-pregnant population [162]. There was a negative correlation between increasing disease severity and relative abundance of *Lactobacillus* species. A recent study of women with untreated CIN 2 with *Lactobacillus* species dominance were more likely to have regressive disease when resampled at 12 months [163]. Excisional treatment also reduced the diversity of the vaginal microbiota [164]. The risk of preterm birth conferred by excisional treatment of the cervix as well as being mechanical may also originate in part by the cervicovaginal microbial environment. Although CIN may be associated with microbial changes, however once CIN is treated this association may be lost.

One possible mechanism by which *L. crispatus* can protect against preterm birth whilst *L. iners* does not are the higher concentrations of D-lactic acid in *L. crispatus*, compared to *L. iners.* A high L-lactic acid to D-lactic acid ratio influences local vaginal extracellular matrix metalloproteinase inducer (EMMPRIN) and MMP-9 concentrations [165] and therefore may lead to collagen breakdown that occurs in cervical remodelling and shortening. *L. crispatus* and *L. gasseri* produce both D-lactic acid and L-lactic acid. *L. jensenii* produces only the D-isomer and *L. iners* produces only the L-isomer [165]. Other mechanisms include the ability of *L. crispatus* to produce hydrogen peroxide which can inhibit the growth of catalase-negative anaerobic organisms by the production of hydroxyl free radicals, and its binding to the vaginal epithelium, competitively preventing other microbes from attaching to the cell surface [166].

A healthy vaginal microbial profile alone is insufficient to ensure a healthy pregnancy. Even women with high abundance of *L. crispatus* deliver preterm and most pregnant women with bacterial vaginosis do not deliver preterm. It is highly likely that an individual's host immune response to the microbial environment is central to their pregnancy outcome. Pregnancy is a unique immunological phenomenon and the complex temporal adaptions are described next.

1.7 Immunology of pregnancy

Adaptions occur to the maternal immune system in pregnancy to prevent immune rejection of the semi-allogenic fetus whilst still being able to generate a robust response to pathogens in order to protect the mother. Broadly speaking there is a degree of immune tolerance and this is notable when considering some autoimmune conditions such as rheumatoid arthritis and multiple sclerosis improve during pregnancy [167, 168]. CD4+ helper T cells subsets include Th1, Th2 and Th17 cells and they exert their cellular functions via different cytokines. Both rheumatoid arthritis and multiple sclerosis are Th1-mediated conditions. In contrast Th2-mediated conditions such as systemic lupus erythematous tends to worsen in pregnancy. To balance dampening of the Th1 response, there is a degree of enhancement of the Th2 immune response [169]. The number of circulating peripheral monocytes and neutrophils increase in pregnancy [170, 171]. At the feto-maternal interface, there is an increase in macrophages and decidual NK cells and TLR-2 and TLR-4 activity, and cervical secretions contain more defensins and lysozymes. NK cells are a type of cytotoxic lymphocyte and contribute to the first line of defence of the innate immune response. In the adaptive immune response there is a rise in lymphocytes and regulatory T cell (Treg) activity [169]. Treg cells are essential for maintaining peripheral immune tolerance, limiting the inflammatory response. The programmed cell death protein-1 (PD-1) and programmed cell death ligand 1 (PD-L1) pathway has been shown to be important in the periphery [172] and at the maternal-fetal interface [173]. On binding its ligand, PD-1 can induce T cell tolerance and regulate inflammation.

Pregnancy adaptations are highly regulated and time dependent [174]. It is broadly accepted that the first trimester is characterised by a pro-inflammatory response to aid blastocyst implantation, the second trimester is largely anti-inflammatory with a predominant Th2 response, and the third trimester is pro-inflammatory with a greater Th1 response in preparation for labour (Figure 1.5). However, pregnancy related immune modulation is much more complex and far less understood than simply the altered Th1:Th2 response.

The concept of the immune clock of pregnancy can be used to describe the timed adaptations required to achieve a successful pregnancy at term [175]. Aghaeepour et al. used mass cytometry with a cell signaling based Elastic Net (csEN) algorithm to analyse peripheral blood cells across term pregnancy in training and validation cohorts that totalled 18. Phenotypic and intracellular signaling changes of innate and adaptive immune cell types were studied following stimulation with LPS, IFN-α, IL-2 and IL-6. Key findings included a progressive increase in IL-2 production and activation of signal transducer and activator of transcription (STAT) 1 in response to IFN-α stimulation in NK cells or dendritic cells and STAT5a signaling in CD4+ T cells as pregnancy neared term. TLR-4 signaling to LPS in dendritic cells was reduced in early pregnancy compared to late gestation [175].

Apps et al. also studied the maternal peripheral immune response in healthy term pregnancy, (N=33) using flow cytometry to measure immune cell populations and serum proteomic analysis [176]. They reported reduced Th1, Th17 and CD8+ T cells across pregnancy. Changes persisting into the post-partum period were increased activity of CD4+ HLA-DR cells and CD8+ CD38+ cells. Chronological changes in normal pregnancy leads us to test if disruption of this chronology can be used to predict pregnancy complications such as preterm birth.

At the end of pregnancy, in term labour, there are sterile inflammatory processes occurring in gestational tissues. The final trigger for physiological inflammation in term labour is poorly understood, and in preterm labour, there is early pathological activation of common inflammatory pathways.

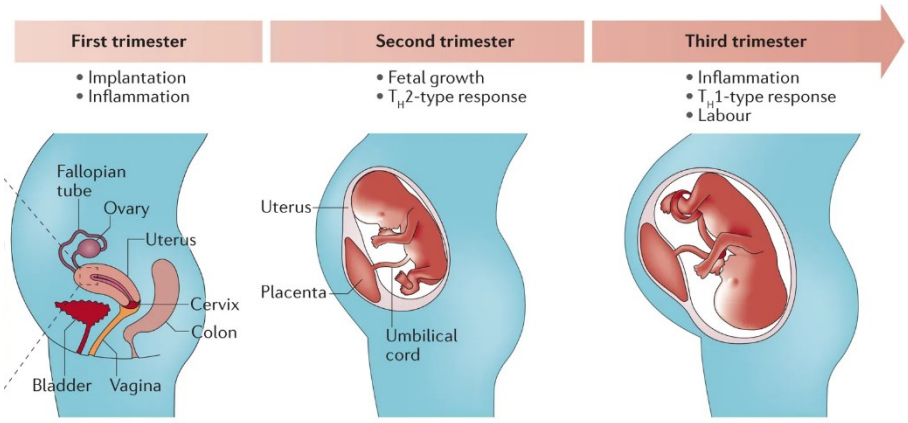

Figure 1.5 Temporal adaptions to the maternal immune system in pregnancy
The first trimester is largely pro-inflammatory to allow for blastocyst implantation. The second trimester sees a shift to an anti-inflammatory response to allow for fetal growth. The third trimester sees a return to a pro-inflammatory state to which is required for labour and delivery.
Adapted from Mor et al. 2017 [174]

The two arms to the immune system are the innate and the adaptive immune systems. The innate system is readily available and non-specifically identifies and destroys pathogens. The adaptive system recognises specific pathogen antigens and produces a specific immune response. It has memory, therefore on re-exposure to an antigen it has previously responded to, the response is more rapid and effective. There is also considerable overlap between the two arms, notably the innate immune response triggering the adaptive immune response and the adaptive response amplifying the effector mechanisms of the innate immune response.

1.7.1 Innate immune response

The innate immune response consists of physical barriers, e.g. mucosal surfaces, chemical defences, e.g. cytokines and chemokines, and cellular defences e.g. immune cells which secrete the cytokines and chemokines. The squamous epithelium of the cervix and vagina have been described in section 1.5.1. Activation of the innate immune system begins with pattern recognition receptors (PRRs) such as Toll-like receptors (TLR) recognising pathogen-associated molecular patterns (PAMPS) e.g. components of the bacterial or fungi cell walls, viral nucleic acids and flagellar proteins. This leads to recruitment of key immune cells of the innate immune response which are monocytes, macrophages, neutrophils, natural killer (NK) cells, dendritic cells, eosinophils and basophils. Immune mediators which they secrete include, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-18, IFN-γ, GM-CSF and TNF-α. Gene polymorphisms of TNF-α, IFN-γ and IL-6 have been reported to increase an individual's susceptibility to spontaneous preterm delivery [177].

NK cells are a type of lymphocyte and are usually defined as CD3- CD56+ cells. They do not have receptors for specific antigens, but instead have killer-cell immunoglobulin-like receptors (KIRs) [178]. KIRs recognise major histocompatibility complex (MHC) class I molecules expressed on nucleated cells. The downregulation of MHC class I expression on a cell is detected by NK cells which then initiates cell killing. The most prominent cytokines produced by NK cells are TNF-α and IFN-γ. However they are also a source of immune-modulatory cytokines IL-5, IL-10, GM-CSF and chemokines such as MIP-1α, MIP-1β and IL-8 [179].

Monocytes are present in the systemic circulation, comprising approximately 5-10% of circulating peripheral blood mononuclear cells (PBMCs). They have an important role in tissue inflammation and repair, and phagocytosis. Monocytes are distinguishable by the expression of the lipopolysaccharide receptor, CD14. There are 3 main subsets, classical ($CD14^{high}$, CD16-) (90%), non-classical ($CD14^{low}$,

CD16+) (5%) and intermediate ($CD14^{high}$, CD16+) (5%). These subsets represent peripheral monocytes at different maturation stages, with classical monocytes originating from the bone marrow and mature into intermediate monocytes and then into non-classical monocytes. Cytokine secreted are IL-6, IL-8, IL-10 and reactive oxygen species from classical monocytes; TNF-α, IL-1β and IL-6 from intermediate monocytes and TNF-α, IL-1β, IL-6 and IL-8 from non-classical monocytes. Upon responding to an inflammatory stimulus, monocytes invade into tissues and develop into M1 or M2 macrophages. M1 macrophages are pro-inflammatory and secrete IL-12, IL-1β, TNF-α and IL-6. There is much evidence supporting local IL-6 in cervicovaginal fluid contributing to inflammation at the maternal-fetal interface [(180)]. M2 macrophages are anti-inflammatory and are important for tissue repair, secreting IL-10 [(181)]. It has been reported that peripheral monocytes are implicated in preterm labour with increased circulating classical and intermediate monocytes in preterm labour, together with increased TLR-4 expression which in turn induces the expression of pro-inflammatory cytokines [(182, 183)]. Macrophages also express MHC class II complexes and are therefore able to present antigen to T helper cells.

Neutrophils, eosinophils and basophils are granulocytes because of their cytoplasmic granules. They can also recognise foreign pathogens directly but can also be activated by antibody and complement components (described in the adaptive immune response). Target cells are killed by the secretion of toxic molecules inside the cytoplasmic granules and the production of reactive oxygen species. Neutrophils migrate to gestational tissues in labour and play a key role in cervical remodelling and fetal membrane activation and rupture [(83)]. Neutrophils release pro-inflammatory cytokines notably IL-8 and secrete MMPs. IL-8 is a chemoattractant which further potentiates the actions of neutrophils [(184)].

1.7.2 Adaptive immune response

The main cell types of the adaptive immune response are T and B cells. B cells are responsible for generating antibody responses. T cells regulate the immune cells via T helper and T regulatory cells and can clear virally infected or neoplastically transformed cells via cytotoxic T cells.

B cells leave the bone marrow and enter the peripheral circulation as immature transitional B cells which develop into naïve B cells. In secondary lymphoid organs, B cells encounter antigen epitopes and become activated into memory B cells and plasma cells [(185)]. The humoral activity of B cells generating protective immunoglobulins is thought to be key to avoiding an immune response to paternal antigens [(186)]. The pool of regulatory B cells expands in pregnancy and their activity is thought

to be involved in tolerance of the maternal immune response [187]. The immunoglobulin (Ig) can act as a B cell receptor, bind directly to pathogens, (blocking their ability to bind to a target cell), or recruit effector mechanisms to the target cell. B cells have a Fc region that binds to Fc receptors on macrophages and neutrophils attracting them to the target cell carrying the antigen recognised by the immunoglobulin. Antigen-Ig complexes can activate the complement system (described below). There are five different Ig classes, IgG, IgA, IgM, IgE and IgD. IgG and IgA are of particular importance in providing protection at mucosal surfaces.

Each class of Ig has a distinct heavy chain, γ, α, μ, Ɛ, β for IgG, IgA, IgM, IgE and IgD respectively. They consist of four polypeptide chains: two light chains and two heavy chains forming antigen-binding sites linked by covalent and non-covalent bonds. IgG is the most abundant peripherally circulating Ig and has four subtypes, IgG1-4. Serum concentrations of IgG1 are highest at 66%, followed by IgG2: 24%, IgG3: 7% and IgG4: 3% [188]. IgG1 and IgG3, as well as IgM can activate antibody cell-mediated cytotoxicity (ADCC) and can activate classical complement-dependent cytotoxicity (CDC). In contrast IgG2 and IgG4 act on bacterial capsular polysaccharide antigens. IgG are the only class of Ig that can transfer across the placenta.

Under the influence of T helper cells, B cells can alter the class of Ig secreted. Initially all Ig are IgM, but if required at mucosal surfaces, class switching to IgA or IgG can occur. IL-4, IL-6 and IFN-γ are important for B cell proliferation and Ig class switching [189]. IL-10 regulates B cell activity.

T cells recognise antigen through their T cell receptor. They recognise antigen in association with MHC molecules. MHC class I molecules are expressed on all nucleated cells, whilst class II molecules are expressed on B cells and antigen presenting cells. MHC class I molecules present antigen to cytotoxic T cells. MHC class II molecules present antigens to helper T cells and regulatory T cells (Treg). Cytotoxic T cells express CD8+ on the cell surface, whilst helper T cells express CD4+.

Local immune modulation occurs in syncytiotrophoblast cells at the maternal-fetal interface with expression of little to no MHC molecules. The enzyme idoleamine 2,3-dioxygenase (IDO) breaks down tryptophan. This together with tryptophan metabolites, kyneurenines inhibits T cell activation.

Helper T cells amplify the responses of cells of the innate and adaptive immune response. There are two main types of helper T cells, Th1 and Th2 cells. Th1 cytokines include IL-2, IFN-γ and TNF-α which drive inflammation and are effectors of phagocyte mediated host defence. Th2 cytokines are IL-4, IL-

5, IL-10 and IL-13 which are anti-inflammatory, whilst IL-6 has pro-inflammatory actions. The importance of a predominant Th2 response is most evident at the maternal-fetal interface. Th2 cells accumulate in the decidua basalis [190] and uterine dendritic cells can differentiate naive T cells to Th2 cells. There is little change in the peripheral Th2 response in normal pregnancy [191].

The balance between Th1 and Th2 cells for a successful pregnancy have been explored for many years [192, 193], however Th17 and Treg cells are also helper T cells and also play hugely important roles in immunotolerance. Th17 cells secrete IL-17 which has a role in the pathogenesis of autoimmune diseases and immune rejection. Treg cells have immune modulatory roles and secrete IL-10 and transforming growth factor β (TGF-β). Even before implantation, seminal plasma induces paternal antigen-specific tolerance and expands the pool of Treg cells [194]. Treg cells can supress the activation, proliferation and cytokine production of CD4+ helper cells and CD8+ cytotoxic cells and their population increases in pregnancy [195]. The expansion of Treg cells in pregnancy even has fetal specificity. After delivery, fetal specific Treg cells are maintained and can rapidly accumulate in a subsequent pregnancy [196]. Th1/Th2/Th17 and Treg cell lineages are closely associated and cell types can convert to other lineages [197].

1.7.3 Complement system

Linking the innate and adaptive immune responses is the complement system, consisting of more than 30 proteins which aid in the opsonisation and killing of bacteria. Many of these proteins circulate in an inactive state but in response to pathogens being recognised, they become sequentially activated in an enzyme cascade fashion (Figure 1.6).

There are three different pathways which converge to a common final lysis pathway. The classical pathway is triggered by antibody-antigen complexes binding to C1. This leads to C3 convertase splitting C3 into two components, C3a and C3b. C3a and C5a enhance phagocyte recruitment. C3b attaches to the surface of microbes, opsonising pathogens. The alterative pathway requires factors B, D, H and I to interact with each other and C3b to form C3 convertase. The mannose-binding lectin pathway is activated upon the binding of mannose binding lectin (MBL) to mannose particles on the pathogen. This activates MBL-associated serine proteases (MASP-1 and MASP-2) which activate C4 and C2 to form C3 convertase. The common final lytic pathway occurs when C5 is activated. C6, C7, C8 and C9 combine with C5b. The resulting membrane attack complex (MAC) induces bacterial cell lysis.

It is widely thought that there is increased peripheral complement activation in pregnancy providing increased protection from pathogens. Richani et al reported increased plasma concentrations of C3a, C4a and C5a in pregnant compared to non-pregnant women [198].

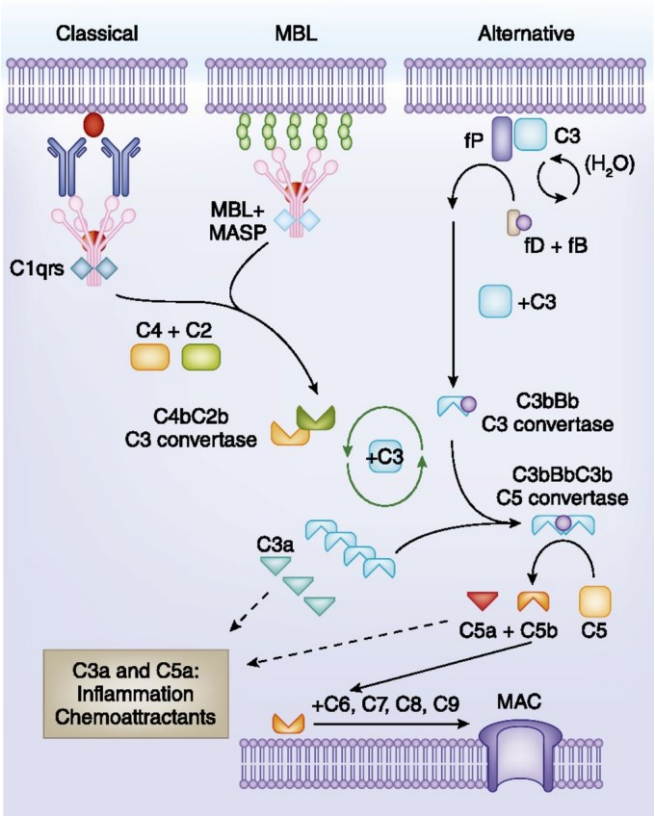

Figure 1.6 The complement system
The complement system can be activated via three pathways, the classical, the lectin and the alternative pathway. The classical pathway is initiated by antigen-antibody complexes binding to C1. The alternative pathway requires factors B, D, H and I to interact with each other and C3b to form c3 convertase. The mannose binding pathway requires MBL to bind to mannose particles on the pathogen. They all converge on the final lytic pathway. C5 is activated and C6, C7, C8 and C9 combine with C5b forming the membrane attack complex to induce cell lysis. C3a and C5a are chemoattractants that enhance phagocyte recruitment.
Adapted from Mathern et al 2015 [199].

1.7.4 Dysregulation of the immune response in preterm birth

Whilst both Th1 cytokines, IFN-γ, IL-2 and TNF-α and Th2 cytokines, IL-4 and IL-6 are all upregulated locally and peripherally in preterm labour [200, 201], differences in Treg and Th17 cells have been found in preterm and term labour. Xiong et al studied peripheral Treg cells during pregnancy and labour, reporting that the proportion of Treg cells was lower in labour at term compared to at term with no labour. Furthermore women in preterm labour had significantly reduced peripheral Treg cells compared to women with normal pregnancy, and no labour at the same gestational age [202]. Koucky et al found that reduced peripheral Treg cells and cervical shortening together were strongly predictive of preterm labour. However the study participants already had uterine contractions, cervical shortening and/or cervical dilatation and were close to delivery [203]. Th17 cells have been shown to promote inflammation at the maternal-fetal interface in preterm labour and IL-17 was significantly higher in preterm labour with evidence of chorioamnionitis [204].

In the innate immune response, mutations in TLR-2 and TLR-4, (pattern recognition molecules for bacteria), are associated with increased risk of preterm birth [205, 206]. Dysregulation of the complement system such as over activation and inadequate regulation leading to excessive inflammation in gestational tissues have been implicated in the pathogenesis of preterm birth. Lynch et al reported that significantly elevated levels of Factor B in plasma, (part of the alternative pathway) in women who deliver at preterm gestations less than 34 weeks [207]. Elevated plasma levels of C3a before 20 weeks gestation were found to correlate with adverse pregnancy outcomes including preterm birth and gestational hypertension [208]. In mouse models, vaginal administration of lipopolysaccharide (LPS) increased the deposition of cervical C3 and local macrophage infiltration. Increased collagen degradation and matrix metalloproteinase 9 (MMP-9) activity was observed in the cervix [209]. Additionally, C5a receptor deficient mice did not show these cervical remodelling changes. Looking at myometrial contraction, C5a levels were also higher in the myometrium following stimulation of LPS [210]. C3a and C5a are usually rapidly cleared by the innate immune system, therefore it is possible that elevated plasma levels of these subunits represent a degree of dysregulation. In a human study, C5a was found to upregulate pro-inflammatory labour mediators: cytokines, prostaglandins, MMPs in placenta and fetal membrane tissues via C5a receptor mediated NF-κB activation [211].

1.7.5 Justification for selected peripheral immune cell subset and activation markers for the flow cytometry panel

A literature search was performed using PubMed® to identify articles published between 2010-2020 with keyword search terms 'peripheral blood mononuclear cells', 'inflammation', 'infection' 'pregnancy' and 'labour'. This enabled the generation of a summary of key cell subsets and markers of cell activity to be explored in this study, Table 1.1.

Table 1.1 Peripheral blood mononuclear cells and markers of cell activity assessed in study

	Role
Cells	
Monocytes	Cells with key roles in tissue inflammation and repair, and phagocytosis
CD4+ T cells	Helper T cells amplify the responses of other immune cells including macrophages and neutrophils through the secretion of cytokines
CD8+ T cells	Cytotoxic T cells which induce the killing of damaged cells, cancer cells and virally infected cells
Treg cells	Suppress the activation, proliferation and cytokine production of CD4+ and CD8+ cells The population increases in pregnancy [212]
γδ T cells	Considered to have innate immune functions with the T cell receptor functioning as a pattern recognition receptor [213] and adaptive immune functions capable of rearranging T cell receptor genes to develop memory [214]
B cells	Key cells of the humoral immune response, antigen presenting cells Secretes immunoglobulins important for mucosal immunity
NK cells	Effector lymphocytes of the innate immune response Respond to microbial infections, limiting spread and tissue damage
NK T cells	Share properties with T cell and NK T cells Recognises CD1d, which binds to self and foreign lipids and glycolipids Provide support to B cells again microbial infections [215]
Markers of cell activity	
CD25	Located on the surface of T cells It is the IL-2 receptor, and has a key role in immune tolerance [216]
CD38	Located on surface of CD4+ and CD8+ T cells, B cells and NK cells Involved in cell adhesion, signal transduction and calcium signalling [217]
HLA-DR	Expressed by B cells, macrophages and dendritic cells Presents antigen to T helper cells A marker of late activation of T cells, suppressing T helper cell responses and preventing production of antibodies against self-antigens
PD-1	An inhibitory receptor which down-regulates T cell and B cell activity [218] Also known as CD279 An immune checkpoint, promoting apoptosis of antigen-specific T cells and reducing apoptosis in regulatory T cells [219]
IgD	A marker of B cell activation, expressed when B cells exit the bone marrow A key modulator of the humoral immune response [220]

1.7.6 Justification of analytes measured in cervico-vaginal fluid and plasma

A literature search was performed using PubMed® to identify articles published between 2010-2020 with keyword search terms 'plasma', 'cervico-vaginal', 'inflammation', 'infection' and 'cytokines,' 'complement', 'immunoglobulins', 'pregnancy' and 'labour'. This enabled the generation of a summary of key immune analytes to be explored in this study, Table 1.2. These analytes are involved in inflammatory processes in infection, labour, cervical ripening or angiogenesis.

Table 1.2 Immune analytes profiled in study

Analyte	Role
IL-1β	Upregulated in cervix, myometrium and amnion, increasing COX-2 and prostaglandin production in labour [221, 222]
IL-2	A pro-inflammatory cytokine produced by Th1 cells Involved in T cell proliferation and angiogenesis[223]
IL-4	An anti-inflammatory cytokine produced by Th2 cells and in the placenta and amnion[224] Stimulates IgG1 and IgE antibody production
IL-5	A Th2 cytokine, promoting growth and differentiation of eosinophils, mediates IgE production and important in defence against parasitic infection
IL-6	A pro-inflammatory cytokine produced by Th2 cells, macrophages, fibroblasts and B cells and involved in cervical remodelling[225]
IL-8	A pro-inflammatory cytokine A chemokine produced primarily by macrophages and monocytes Closely associated to IL-6, recruiting neutrophils to the cervical extracellular matrix Increased IL-8 is associated with reduced *Lactobacillus* spp. and infection induced PTB [226-228]
IL-10	An anti-inflammatory cytokine produced by Th1, Th2 cells, macrophages and B cells Down regulates pro-inflammatory cytokines including IL-8, IL-6, TNF-α, IL-1β and MMPs [224, 225, 229] in a negative feedback loop
IL-18	A macrophage derived anti-inflammatory cytokine
IFN-γ	Produced by NK and Th1 cells, increased in preterm labour [169, 230] Key effector of phagocyte-mediated host defence
GM-CSF	Induces expression of VEGF and increased in CVF prior to cervical shortening [231, 232]
TNF-α	A pro-inflammatory cytokine, and increased in systemic infection Associated with IL-6 to stimulate arachidonic acid, increasing prostaglandins, role in cervical remodelling [233] Role in vasodilation and endothelial cell activity allowing the extravasation of leukocytes [234]
C5 and C5a	C5a upregulates pro-inflammatory cytokines, prostaglandins and MMPs in gestational tissues [211]
Mannose binding lectin	Plays a key role in the primary contact of the host immune system to microorganisms [235] Polymorphisms in the MBL gene may be associated with cervical insufficiency and preterm birth [236]
C3b	Together with MBL, C3 has been shown to be important for host recognition of microorganisms in the female genital tract [237]
IgG	The most abundant immunoglobulin in cervico-vaginal fluid [238]
IgA	A key immunoglobulin at mucosal surfaces [238]
IgM	Another key mediator of mucosal immunity and a strong activator of the classical complement pathway

1.8 Mucosal immunity and the vaginal microbiota

Mucosal immunity and the vaginal microbiome are most studied in the context of human immunodeficiency virus (HIV). There is a wide gender imbalance for HIV-1, highlighted by the fact that in sub-Saharan Africa, women account for 59% of total people living with HIV-1 (Global HIV & AIDS Statistics - 2019). The mucosal immune system of the female reproductive tract is the first line of defence against HIV. The rate of sexual transmission of HIV-1 is much greater in the presence of mucosal inflammation with a reported threefold increase in the rate of transmission in women who had higher concentrations of IL-8, IL-1β, IL-1α and TNF-α [(239)]. HIV has been shown to impair the mucosal barrier facilitating microbial translocation and by interacting with TLR-2 and TLR-4 on the mucosal epithelium causing downstream pro-inflammatory cytokine release [(240)]. There are also increased CD4+ T cells in the cervix of women with local inflammation and HIV-1 preferentially infects CD4+ T cells. Viral exposure leads to recruitment of CD4+ T cells to the female reproductive tract allowing systemic infection to ensue [(241)].

It has become well recognised that bacterial diversity confers greater susceptibility to HIV-1. Bacterial vaginosis has been found to be consistently associated with increased risk of HIV infection [(242)]. The local inflammation as a result of bacterial vaginosis may account for increased susceptibility to HIV-1. Gossman et al. studied this in 236 HIV-uninfected women. There was seroconversion in 31 women (13%), despite intensive HIV prevention education. There was significantly reduced *L. crispatus* and increased *Prevotella*, *Sneathia* and other anaerobes, and locally activated CD4+ T cells in those who contracted HIV-1 [(243)]. Vaginal dysbiosis has also been associated with proteome changes related to alterations in the cervicovaginal mucosal barrier which can also contribute to increased transmission. Using mass spectrometry, protein abundances according to *Lactobacillus* dominant and dysbiotic microbial communities were determined. Increased bacterial diversity was associated with altered mucins, reduced antimicrobial histones and lysozymes and decreased IgG1 and IgG2 [(244)]. Bacterial vaginosis associated anaerobic bacteria have been shown to produce large quantities of short chain fatty acids such as acetate, propionate and butyrate [(245)]. These short chain fatty acids influence cell migration, cytokine production and phagocytosis. Probiotics to promote *Lactobacillus* colonisation, anti-inflammatories and immune modulators are all potential therapeutic strategies.

Elovitz et al. have studied the relationship between mucosal immunity conferred by β-defensin-2 and the vaginal microbial communities in preterm birth in a largely African American population [(246)]. In their population, *Lactobacillus* species depletion was associated with preterm birth. β-defensin-2, an

antimicrobial peptide and can be constitutively expressed or expression can be induced upon bacteria stimulation [247]. Cervico-vaginal fluid concentrations of β-defensin-2 were significantly lower in vaginal microbial communities dominated by *L. iners* and anaerobic species in African American women who delivered preterm. In non-African American women β-defensin-2 concentrations were also low in *L. crispatus and L. gasseri* dominated vaginal microbial communities. Nevertheless, these findings are still valuable in identifying women at risk of preterm delivery early in pregnancy.

Witkin et al found that tissue inhibitor of matrix metalloproteinases 1 (TIMP-1) in cervico-vaginal fluid was positively associated with communities dominant with anaerobic bacteria and *L. iners*, and correlated with short cervical length, whilst *L. crispatus* dominant communities had significantly lower TIMP-1 concentrations. However, they were not able to correlate TIMP-1 levels with pregnancy outcome and preterm birth [248].

Group B Streptococci (GBS), β-haemolytic gram-positive bacteria is a common vaginal commensal. Maternal vaginal colonisation with GBS is associated with preterm delivery [249], and in those cases triggers a detrimental mucosal immune response. It is the most common cause of early onset neonatal sepsis. In their nonhuman primate model, Boldenow et al were able to demonstrate that a GBS haemolytic pigment toxin could induce neutrophil cell death and suppress killing by neutrophil extracellular traps in placental membranes in vivo. Increased expression of the haemolytic pigment enabled GBS to penetrate the placental chorioamniotic membranes, infect amniotic fluid and fetal organs thus precipitating preterm labour [250]. Furthermore, Vornhagen et al have shown that certain GBS strains associated with preterm labour and neonatal sepsis have increased hyaluronidase activity compared to commensal strains detected in healthy women. They report that hyaluronidase activity confers an ability to GBS to avoid host immune detection and cause intraamniotic infection and preterm birth [251]. A GBS vaccine is being developed and it will be interesting to see its effect upon reducing the risk of preterm birth [252].

1.9 Justification, hypothesis and aims for project

The maternal immune response undergoes highly adapted, time dependent changes during pregnancy. Some of these changes have been studied in a chronological fashion in the periphery but they are not so well described in the local environment. A good understanding of the chronological immune changes during normal pregnancy will allow the subsequent study of how aberrations to these changes can culminate in abnormal pregnancy outcomes such as preterm birth. It is important in longitudinal studies in pregnancy to recruit large numbers of study participants, collect samples at defined timepoints and minimise study participant drop out. Minimally invasive testing is also a pre-requisite for study participant acceptability. In this study the peripheral immune response is studied longitudinally in healthy term pregnancy, pregnancy that is complicated by cervical shortening and pregnancy that results in preterm delivery. The peripheral immune response to interventions to prevent preterm delivery such as a cervical cerclage are also explored.

The local immune response can be studied relatively non-invasively by sampling the cervico-vaginal fluid. Whilst non-infective inflammation is the hallmark of term labour, the trigger for this shift is not well understood. Identifying when non-infective local inflammation occurs in term pregnancy will help in the understanding of infective and non-infective inflammation occurring in preterm pregnancy. The ability to identify aberrations from physiological immune adaptations in pregnancy at an earlier stage in pregnancy that allows for early surveillance and timely interventions will improve the clinical management of high-risk pregnancies. Interventions such as a cervical cerclage may induce local inflammatory changes, therefore longitudinal profiling will enable the immune changes to be tracked. If the intervention is successful, an individual's immune trajectory may be successfully modified to resemble that of an individual who has a term pregnancy.

The local immune response is strongly influenced by vaginal microbial communities. However, assessing the microbiota alone does not take into account the maternal host response to the bacteria in term and preterm pregnancies. Therapeutic strategies to eliminate pathogens known to cause vaginal infections such as bacterial vaginosis does not always prevent preterm birth. Therefore, mapping immune responses to the local vaginal microbiome can improve our understanding of the mechanistic pathways underlying infection and inflammation mediated preterm birth.

Hypotheses:

- Adaptations in the maternal immune response differ between women who deliver at term compared to preterm.
- Activation of the innate and adaptive immune response drives a proportion of spontaneous preterm births.
- Microbial driven preterm labour is dependent on dysregulation of the innate and adaptive immune response.
- Microbial driven preterm labour is dependent on activation of the innate and adaptive immune response.

Aims:

- To characterise the longitudinal profile of the peripheral and local immune response in term and preterm pregnancies.
- To examine the maternal immune response in women who undergo cervical shortening.
- To determine the immune response to vaginal microbial composition and how this differs depending on pregnancy outcome.

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

2.1.1 Chemicals, Reagents and Solvents

Table 2.1 Chemicals, reagents and solvents

Chemical/Reagent/Solvent	Company	Catalogue number
Agarose	Invitrogen	15510-019
BACTQuant Probe sequence 5' 6FAM-CAGCAGCCGCGGTA-MGBNFQ	Life Technologies	4316034, 6000pmol scale
BactQuant forward primer 100mM 0.025µmol synthesis scale, desalted Sequence 5'-CCTACGGGAGGCAGCA-3'	Sigma	Custom
BactQUANT reverse primer 0.025µmol synthesis scale, desalted Sequence 5'GGACTACCGGGTATCTAATC-3'	Sigma	Custom
BD CompBead Set Anti-mouse Ig, κ	BD	552843
Bovine Serum Albumin	Sigma Aldrich	A2153
Brilliant stain buffer RUO	BD	563794
DC™ Protein Assay Reagent A	BioRad	5000113
DC™ Protein Assay Reagent B	BioRad	5000114
DC™ Protein Assay Reagent S	BioRad	5000115
Dimethyl Sulfoxide	Sigma Aldrich	276855
E. coli DNA	Sigma	D4889
Ethanol	VWR	64-17-5
Fetal calf serum	Thermofisher	A3840001
Ficoll-Paque PLUS®	GE Healthcare	17-1440-02
Paraformaldehyde	VWR	50-00-0
Phosphate buffered saline	(In-house)	
Platinum PCR Supermix UDG Containing ROX	Life Technologies	11730-017
Nuclease free water	VWR	7732-18-5
RPMI medium (+glutamine)	Life Technologies	61870036
SBYR™ safe DNA gel stain	Invitrogen	S33102
Tris base	Sigma	77-86-1
Triton	(In-house)	
Trypan blue	Sigma	72-57-1

2.1.2 Enzymes

Lysozyme (Sigma L6876 – chicken egg white)

- Prepared to concentration; 10mg/ml in filter sterilised 10mM Tris. HCL pH 8.0

Mutanolysin (Sigma M9901/10Ku)

- Prepared to concentration; 25U/µl by dissolving 10 000 units in 400µl sterile water

Lysostaphin (Sigma L9043)

- Prepared to concentration; 4000U/ml by dissolving 23 915 units in 20mM Sodium acetate

2.1.3 Polymerase Chain Reaction

NEB OneTaq® DNA Polymerase (BioLabs)

dNTP 10mM (Sigma cat D7295)

5x Buffer (BIO-37045)

6x Loading dye (PROMEGA G190A 1006034)

Agarose (Invitrogen cat 15510-019)

SBR Safe DNA Stain (Invitrogen cat S33102)

Molecular weight marker (Bioline Hyperladder 100bp cat BIO-33056)

TBE buffer (Tris/Borate/EDTA)

Sample template DNA

16S rRNA Universal Forward and Reverse primers (PCR)

Forward 27F-5'AGAGTTTGATCCTGGCTCAG-3'

Reverse 338R-5'GCTGCCTCCCGTAGGAGT-3'

2.1.4 Antibodies

Table 2.2 Antibodies to stain PBMC for analysis on the Cytek™ Auora flow cytometer

Flow Cytometry Antibodies	Company	Cat	Control	Volume
Alexa Fluor™ 488 anti-human CD45RA	Biolegend	304114	Beads	3.5µl
BV750 mouse anti-human CD4 (clone SK3)	BD	566355	Beads	1.5µl
Brilliant violet 650™ anti-human CD279 (PD-1)	Biolegend	329950	Beads	3.5µl
PE anti-human CD25	Biolegend	356104	Beads	3.5µl
eFluor 450 anti-human CD16	eBioscience	48016842	Beads	3.5µl
APC anti-human CD127	eBioscience	17127842	Beads	3.5µl
AlexaFluor 532 anti-human CD3	eBioscience	58003842	Beads	3.5µl
Brilliant Violet 605™ anti-human CD19	Biolegend	363024	Beads	3.5µl
Brilliant Violet 480 mouse anti-human TCRγδ	BD	566076	Beads	3µl
PerCP-eFluor 710 anti-human CD14	eBioscience	46014942	Beads	3.5µl
APC/Fire™ 750 anti-human CD8	Biolegend	344746	Cells	3.5µl
PE/Dazzle™ 594 anti-human CD197 (CCR7)	Biolegend	353236	Cells	3.5µl
PE/Cy7 anti-human CD38	Biolegend	356608	Cells	3.5µl
Alexa Fluor® 700 anti-human HLA-DR	Biolegend	307626	Cells	2µl
Brilliant Violet 711™ anti-human CD56 (NCAM)	Biolegend	318336	Cells	3.5µl
Brilliant Violet 785™ anti-human IgD	Biolegend	348242	Cells	3.5µl
Brilliant Violet 421™ anti-human CD27	Biolegend	356418	Cells	3.5µl
LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit	Invitrogen	L34965	Cells	5µl

2.1.5 Kits and Equipment

Glass beads (Sartorius Stedim Cat No BBI-8541400)

Liquid Amies swab (BBL™ CultureSwab™, Becton, Dickinson and Company)

QIAamp DNA Mini Kit (Qiagen Catalogue No 51304)

TissueLyser LT (Qiagen Catalogue No 69980)

Human Premixed Multi-Analyte Kit (R&D Systems cat LXSAHM)

Human Complement Magnetic Bead panel 1 and 2 (Milliplex®) HCMP1MAG-19K and HCMP2MAG-19K

ProcartaPlex Human Antibody Isotyping Panel 7plex (ThermoFisher Scientific cat EPX070-10818-901)

Countess™ Automated Cell Counter (Life Technologies)

Cytek™ Aurora Flow Cytometer

Nikon Eclipse 90i fluorescence microscope

MoFlo XDP Cell Sorter

2.2. Methods

2.2.1 Patient recruitment

Study participants were recruited from five preterm prevention clinics, four of which were in London: Queen Charlotte's and Chelsea Hospital, St Mary's Hospital, University College Hospital London, Chelsea and Westminster Hospital and one centre in Scotland, Royal Infirmary of Edinburgh, Figure 2.1. Study participants were at high risk of preterm birth; identified by previous excisional cervical treatment, previous spontaneous preterm birth or previous spontaneous mid-trimester loss. The study was performed under ethical approval by the National Health Service (NHS) National Research Ethics Service Committee London – Stanmore, REC 12/LO/0328. Exclusion criteria were multiple pregnancies, women who had sexual activity within 72 hours of sampling, vaginal bleeding and women who were HIV or hepatitis B positive. Study participants recruited from Queen Charlotte's and Chelsea Hospital requiring a cervical cerclage were also invited to participate in the C-STITCH trial, involving randomisation to monofilament or braided suture material, with ethical approval by Cambridgeshire and Hertfordshire REC ISRCTN 15373349. Women requiring a rescue cerclage were excluded.

Peripheral blood and vaginal swabs which sampled the cervico-vaginal fluid were taken at three timepoints during pregnancy, 12^{+0} - 16^{+6}, 20^{+0} -24^{+6} and 30^{+0} - 34^{+6} weeks. A transvaginal ultrasound to measure the cervical length was performed on the same day as sampling. Detailed metadata was collected from hospital notes and the electronic patient database, Cerner Millennium Powerchart®.

When pregnancy outcomes were known, study participants were retrospectively divided into three outcome groups, a) women who had spontaneous preterm deliveries (preterm, PT), before 37 weeks gestation; b) women who delivered at term without intervention (term, T); c) women who delivered at term following an elective history or ultrasound indicated cerclage and/or progesterone (term intervention, TI). Term delivery was defined as ≥37 weeks gestation. Although all study participants who had PPROM delivered before 37 weeks, if they had delivered after 37 weeks, they would have been included in the preterm group due to the abnormal pathology. All preterm deliveries in this study cohort were spontaneous. In the event of iatrogenic preterm delivery, these would have been excluded from analysis comparing spontaneous preterm and term births.

This work was supported by the March of Dimes, the Global Alliance to Prevent Prematurity and Stillbirth and the Genesis Research Trust.

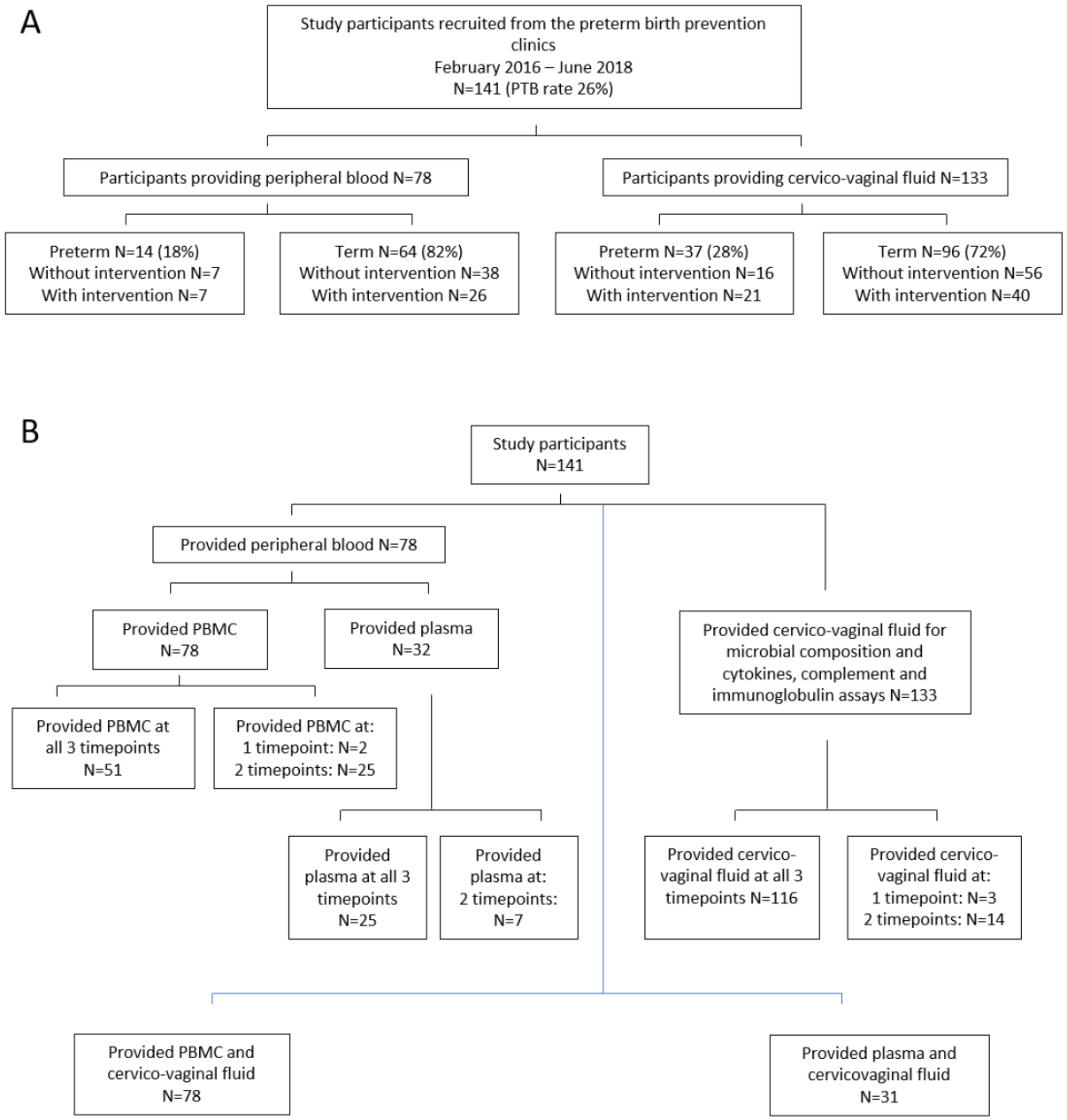

Figure 2.1 Study participants were recruited from preterm birth prevention clinics across five hospitals between February 2016 and June 2018

Of 141 women recruited to immune profiling, 78 women provided peripheral blood samples, and 133 women provided cervico-vaginal fluid swab samples (A). The preterm birth rate for the whole cohort was 26%, 18% in women who provided peripheral blood samples and 28% in women who provided cervico-vaginal fluid samples, reflecting a population at high risk of preterm birth (A). The study was designed to collect peripheral blood and cervico-vaginal fluid at three sampling timepoints, 12^{+0} - 16^{+6}, 20^{+0} -24^{+6} and 30^{+0} - 34^{+6} weeks (B). Due to some study participants not attending appointments or delivering before the third sampling timepoint, in some cases, samples were not collected at all three sampling timepoints (B). Cross-sectional analyses were performed on all samples collected according to the sampling timepoints. Longitudinal analyses were performed on matched samples collected at all three sampling timepoints for a subset of the study cohort. Where possible peripheral blood mononuclear cells (PBMC) and plasma were separated from peripheral blood. Cytokine and complement assays were performed on the plasma. Cervico-vaginal fluid was collected to determine the microbial composition and to perform cytokine, complement and immunoglobulin assays.

2.2.2 Blood sample collection and processing for peripheral blood mononuclear cells

Peripheral venous blood was collected using a vacutainer collection system in one 5ml Ethylenediaminetetraacetic acid (EDTA) bottle, BD Vacutainer® Tubes. Blood was processed within 30 minutes of collection. Blood was diluted 1:1.5 with phosphate buffered saline (PBS). This was layered on the top of 3ml of Ficoll-Paque PLUS® before centrifuging at 400x*g* for 40 minutes at room temperature (Figure 2.2). The resulting supernatant was removed and discarded. The halo containing the peripheral blood mononuclear cells (PBMC) were isolated and transferred into fresh tubes. They were washed twice with 10ml of PBS and centrifuged for a further 10 minutes each time at 300x*g*. The remaining cell pellet was resuspended in 10% dimethyl dulfoxide (DMSO) and fetal-bovine serum (FBS) in a 1:9 dilution and frozen at -80˚C, for 6 months before further use.

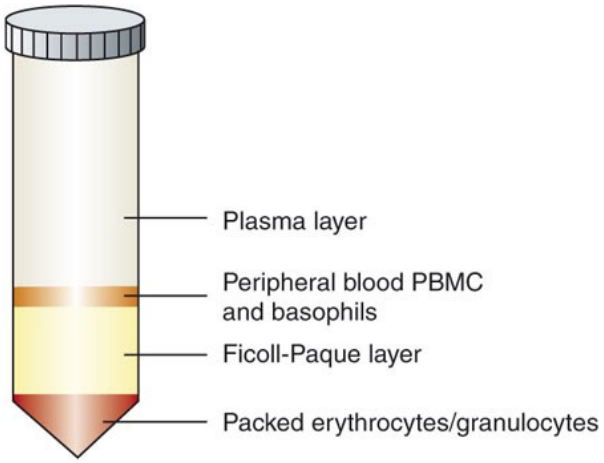

Figure 2.2 The separation of blood cell fractions following gradient centrifugation with Ficoll-Paque PLUS®
The halo formed by the peripheral blood mononuclear cell fraction (PBMC) was identified and separated from other blood cell fractions using Ficoll-Paque PLUS.

2.2.3 Flow cytometry of PBMC Aurora Cytek™

Thawing of PBMC and cell counting

Each sample of PBMC was thawed in a water bath at 37˚C, then transferred into 20ml of RPMI medium (+ glutamine). This was centrifuged at 500x*g* for 10 minutes at 21˚C. The resulting supernatant was removed, and the cell pellet was resuspended in 1ml of PBS. To 5µl of resuspended PBMC, 10µl of tryphan blue stain was added. The cell count and viability were determined on the Countess™ Automated Cell Counter. Comparison was made with matched fresh and cryopreserved/thawed samples, (Figure 2.3). The results for 3 replicates are shown in (Table 2.3). Cell viability reduced significantly following cryopreservation and thawing procedures, however the mean cell viability in cryopreserved/thawed samples was still acceptable at 88%. The cell viability of all samples used in experiments reported in this this thesis was greater than 80%.

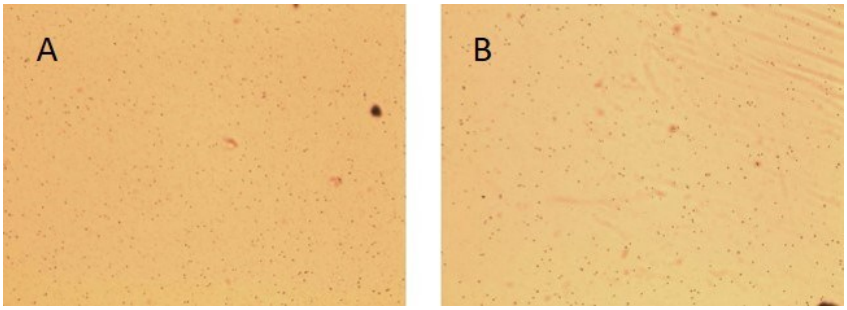

Figure 2.3 Peripheral blood mononuclear cell counting and determination of viability
In the fresh PBMC sample, the cell count was 1.2 x 10^6/ml with a cell viability of 97% (A). In the cryopreserved/thawed sample, the live cell count was comparable at 1.1 x 10^6/ml, with a cell viability of 89% (B).

Table 2.3 Cell count and viability in matched fresh and cryopreserved/thawed PBMC samples

		Fresh	Cryopreserved/thawed	p-value
Sample 1	Cell count	1.2 x 10^6/ml	1.1 x 10^6/ml	0.14
Sample 2	Cell count	1.1 x 10^6/ml	1.0 x 10^6/ml	
Sample 3	Cell count	1.0 x 10^6/ml	0.9 x 10^6/ml	
Sample 1	Cell viability	97%	89%	<0.001
Sample 2	Cell viability	99%	88%	***
Sample 3	Cell viability	96%	87%	

PBMC preparation for flow cytometry

Following gentle thawing, the PBMC cell pellet was washed in PBS and centrifuged at 400x*g* for 10 minutes. The resulting pellet was then resuspended at a concentration of 1 million cells/100ul PBS. Aqua amine, brilliant stain buffer and flow cytometry antibodies listed in Table 2.2 were added and incubated in the dark for 30 minutes at room temperature. All antibodies were added to each sample and the final staining volume per 100ul tube of cells suspended in PBS was 60.5µl. Following incubation, 1ml of staining buffer (PBS, 1% BSA, 0.1% sodium azide) was added and the sample was centrifuged at 1000rpm for 10minutes. The supernatant was discarded, and the pellet resuspended in 500µl of PBS ready to be analysed on the Cytek™ Aurora flow cytometer. Controls were an unstained PBMC sample, and single stained bead or cell controls (Table 2.2). For each single stain cell controls, the PBMC pellet was resuspended at a concentration of 1 million cells/100ul PBS. For the cell viability control, 10ul of the resuspended PBMC pellet was placed in a fresh centrifuge tube and placed on a heat block at 90˚C for 15 minutes. These cells were added back to the non-heat treated PBMC before the addition of the live/dead cell stain. This process improved the degree of separation of fluorescence from live and dead cells. Cell controls were preferentially used over bead controls; however beads were required for cell markers with lower expression. 500 000 events were collected for samples and the unstained control. A minimum of 10 000 events were collected for bead and cell single-stained controls. The event count was based on all events, prior to gating on singlets and live cells.

Spectral flow cytometry with Cytek™ Aurora

Conventional flow cytometry uses mirrors and filters to select specific wavelength ranges for detecting signals from different fluorochromes on individual photomultiplier tubes. Spectral flow cytometry differs in the optics and detectors required to obtain high resolution spectral, (Figure 2.4). Gratings or prisms are used to disperse light across a detector array allowing the full spectra from each particle to be measured [(253)].

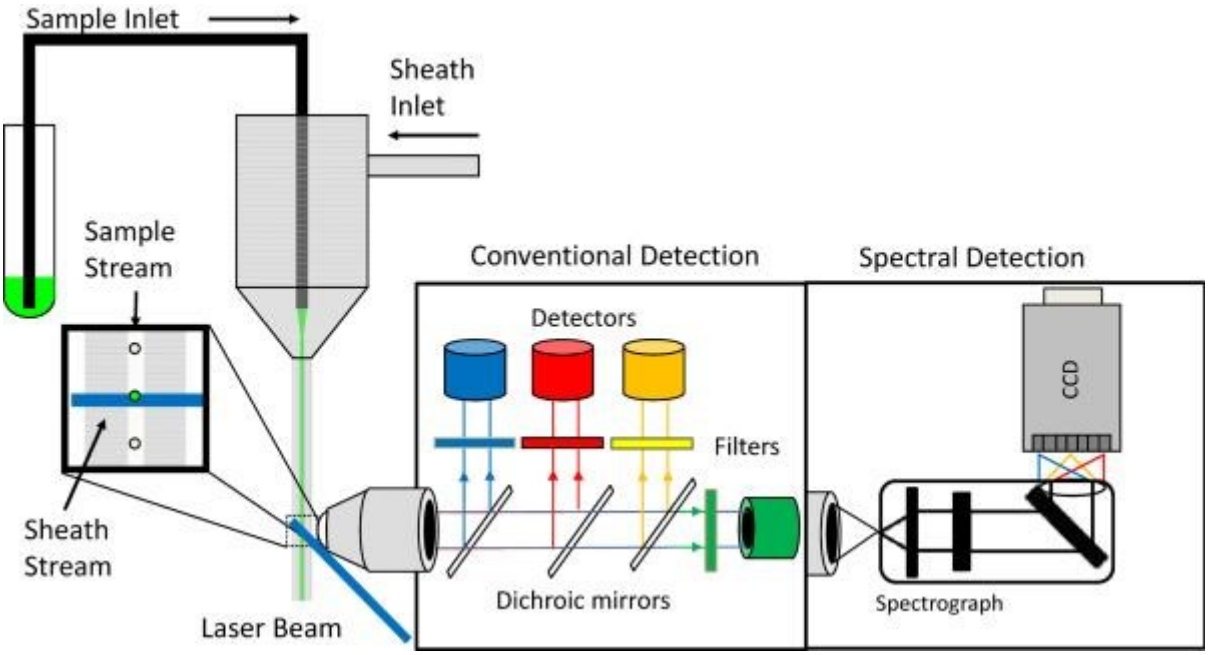

Figure 2.4 Comparison of conventional and spectral flow cytometry
Conventional flow cytometry uses dichroic mirrors and bandpass filters to select colours of light for detection on photomultiplier tubes. Spectral flow cytometry uses gratings or prisms to disperse light across a detector array. Adapted from Nolan et al. 2013 [253]

The Cytek™ Aurora has spatially separated red, blue and violet lasers. Each excitation laser has an associated solid-state multi-channel semiconductor array detector module. The entire emission spectrum is captured across the different modules and collated to create a spectral signature that combines emission information from all three excitation wavelengths (Figure 2.5). The spectral unmixing algorithms are provided on the Spectroflo® software package to calculate the contribution of each known fluorochrome's spectra to the total collected emission signal. Single stained controls were used to establish the signatures of each fluorochrome. Table 2.2 shows when bead or cell controls were used. An unstained control was also used and any autofluorescence was accounted for. Spectral unmixing is a more complex form of compensation. The laser channel with the highest fluorescence for a given fluorochrome is compared to all the other channels. Spectral unmixing is the sensitivity with which the spectrum can be reconstructed within the statistical distribution of photons across the 38+ channels. Spectral detection is the linear unmixing of a fluorescent spectrum across an array of emission detectors to statistically reassign the origin of a photon to the source fluorochrome [254].

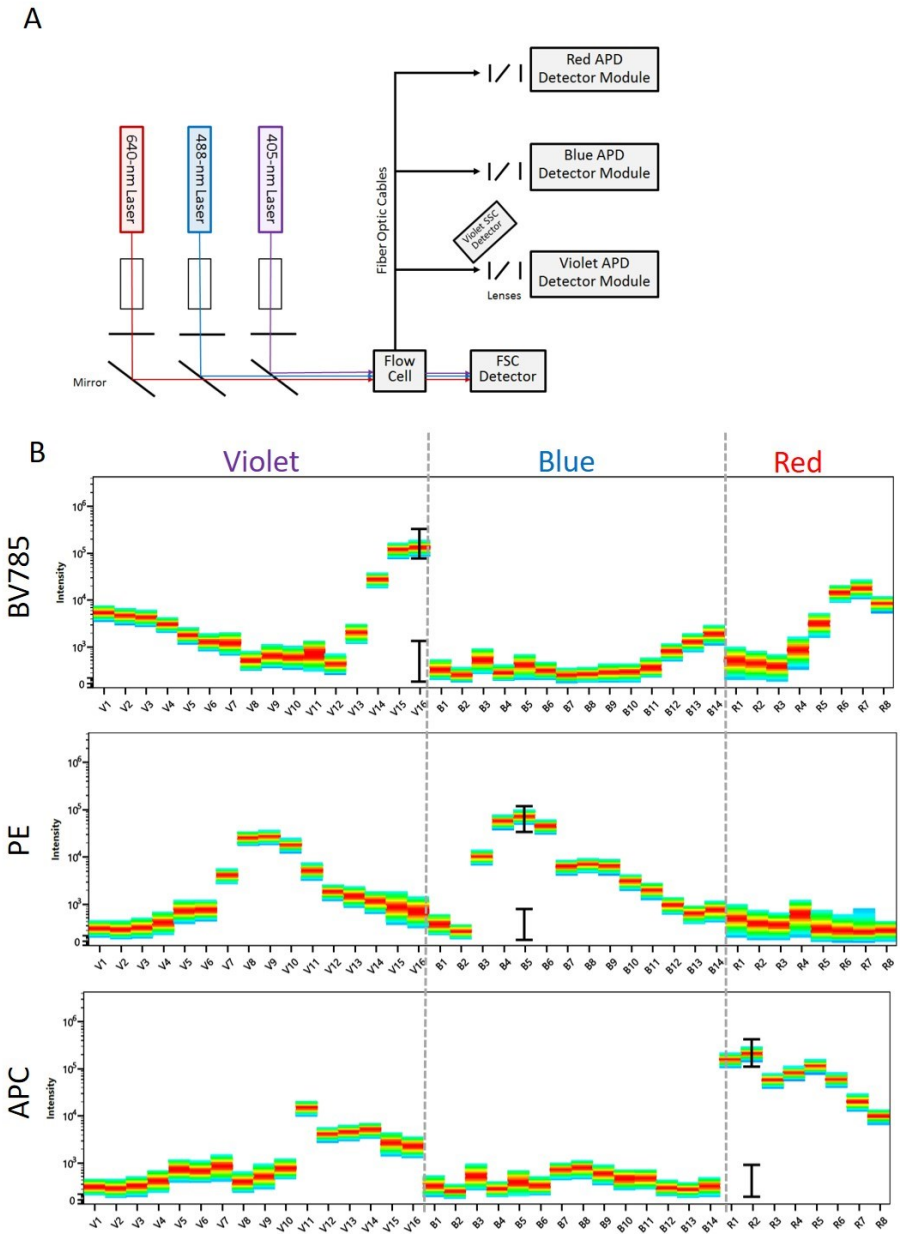

Figure 2.5 Three lasers and array detector modules, and the full emission spectral signatures
The three lasers, red 640nm, blue 488nm and violet 405nm are connected to Avalanche Photodiode detectors by fibre optic cables (A). The entire emission spectrum is captured across the different modules and a spectral signature that combines the emission signature from all three excitation wavelengths is created (B). Shown here are the spectral signatures from three fluorochromes; BV785 which has the brightest signal from the violet laser in the V16 channel, PE which has the brightest signal from the blue laser in the B5 channel and APC which has the brightest signal from the red laser in the R2 channel.

2.2.4 Plasma collection and processing

Peripheral venous blood was collected using a vacutainer collection system in one 5ml Ethylenediaminetetraacetic acid (EDTA) bottle, BD Vacutainer® Tubes and placed on ice prior to processing. The blood bottle was centrifuged at 1500x*g* for 10 minutes at 4˚C, within 30 minutes of collection. The plasma was aliquoted into 1.5ml tubes and frozen at -80˚C until the Multiplex Luminex assays were performed.

2.2.5 Cervico-vaginal fluid collection and processing

BBL™ CultureSwab™ Max V Liquid Amies (Becton, Dickinson and Company, Oxford, UK) swabs were used to collect cervico-vaginal fluid from the posterior fornix of the vagina under direct visualisation by trained midwives and obstetricians. The swab was placed directly on ice and then stored at -80˚C until the relevant laboratory experiments were performed.

The swab was thawed on ice, and the mixed liquid Amies and cervico-vaginal fluid solution was transferred into sterile DNAase/RNase free 2ml centrifuge tubes. This was centrifuged at 7000rpm for 10 minutes. The supernatant was stored in sterile DNAase/RNase free 1.5ml tubes at -20˚C with protease inhibitor and was analysed for cytokine, complement and immunoglobulin analytes. DNA extraction was performed with the remaining pellet, described in section 2.2.7.

2.2.6 Multiple Luminex® assays

Multiplex bead assays allow for simultaneous quantitative assessment of analytes. Assay plates were chosen to quantify cytokines, complement and immunoglobulin analytes in cervico-vaginal fluid, and cytokines and complement in plasma. Traditional enzyme-linked immunosorbent assays (ELISA assays) can detect and measure only a single analyte per plate. Multiplex bead assays are therefore more cost and time efficient and more sensitive than ELISA for samples with low concentrations of the target analyte [255]. The principles are described in Figure 2.6.

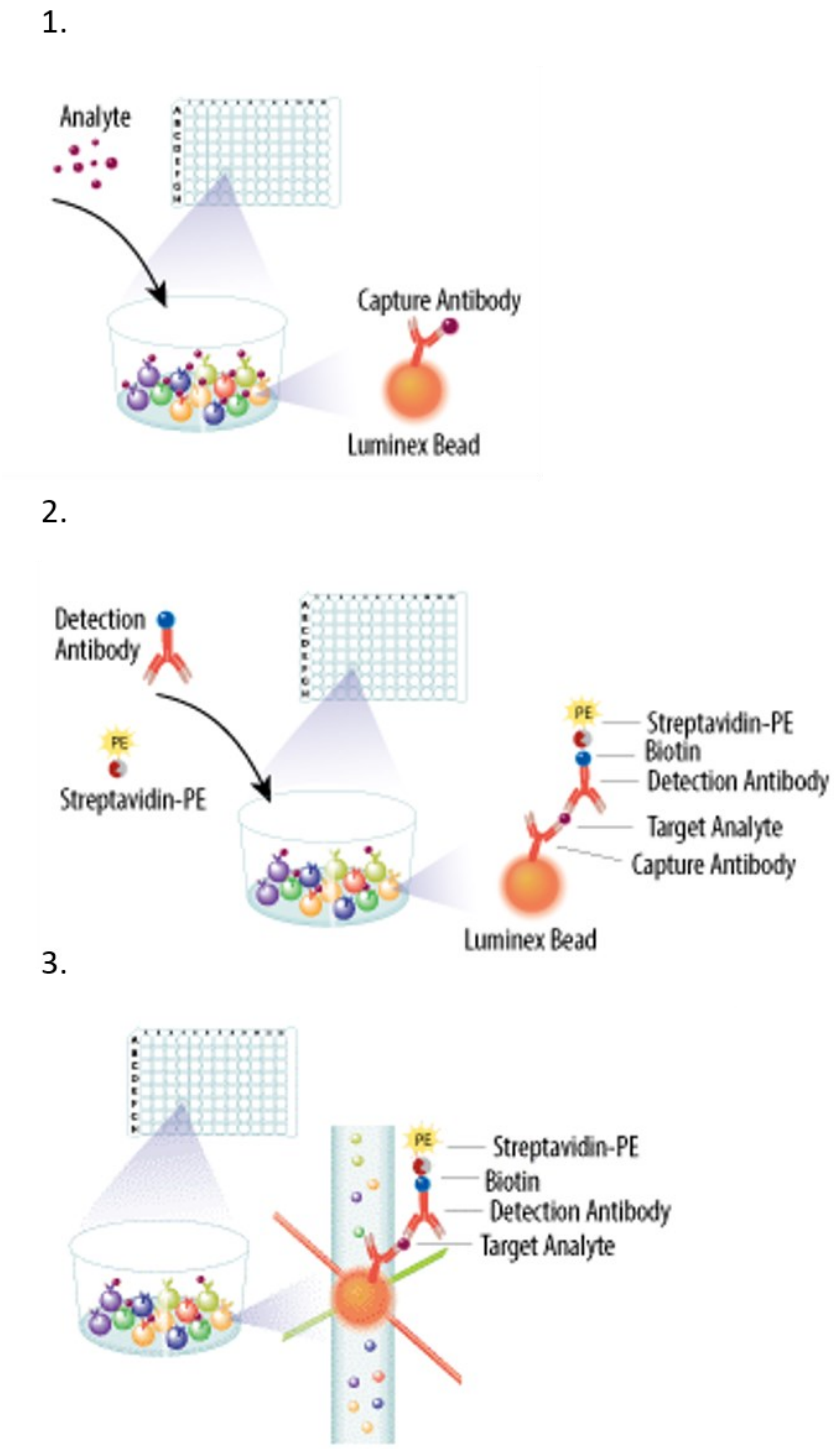

Figure 2.6 Luminex® assay principles
The sample is added to a mixture of magnetic beads which are pre-coated with analyte specific capture antibodies. The antibodies bind to the specific analytes [(1)]. Biotinylated detection antibodies specific to the analytes of interest are added forming antibody-antigen complexes. Phycoerythrin (PE)-conjugated streptavidin is added, which in turn binds to the biotinylated detection antibodies [(2)]. The magnetic beads are read on the Bio-Plex® 200. One laser classifies the bead region and determines the analyte being detected, whilst the second laser determines the magnitude of the PE-derived signal which is in direct proportion to the amount of analyte bound to the antibody (3). Taken from R&D Systems Technical Resources [(256)].

Cytokine assay procedure

The following analytes were chosen according to evidence of their involvement in the inflammatory changes related to preterm labour, cervical ripening and remodelling and fetal membrane activation or their response to the vaginal microbiota: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-18, IFN-γ, GM-CSF and TNF-α. CVF supernatant and plasma were thawed on ice and centrifuged at 2000x*g* for 10 minutes, removing cell debris. The resulting supernatant was used for the assay. For CVF samples when quantifying IL-8, a 10-fold dilution using Calibrator Diluent RD6-52. For quantifying the remaining 10 analytes in CVF samples no dilution was required and a multiplex plate was used. No dilution was performed for plasma samples and a multiplex plate was used to quantify all 11 plasma cytokine analytes. The Human Premixed Multi-Analyte Kit (R&D Systems cat LXSAHM) was used together with Bio-Plex® 200 (Bio-Rad Laboratories Ltd).

Graduated standards were prepared by reconstituting each of the standard cocktails with 100µl of Calibrator Diluent RD6-52. Six 3-fold dilutions were performed. 50µl of standard or sample was added in duplicate to assigned wells of a 96 well microplate. This was followed by the addition of 50µl of diluted microparticle cocktail. The plate was incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 800rpm. Each well was washed with wash buffer (included in the pre-mixed kit) on a magnetic plate. 50µl Biotin-Antibody Cocktail was added to each well and the plate was incubated for 1 hour at room temperature on the plate shaker. The wash step was repeated before the addition of 50µl Streptavidin-PE to each well and the plate was incubated for 30 minutes at room temperature on a plate shaker. The wash steps were repeated, and the magnetic beads resuspended in 100µl of wash Buffer. The plate was shaken for a further 2 minutes prior to reading on the analyser. All standards and samples were run in duplicate with allocated wells for blanks. The concentrations of the analytes were determined by the spectral properties of the beads and the amount of fluorescence. Standard concentrations were provided by the manufacturer and analyte detections were compared to the standard ranges and subtracting the average blank median fluorescence intensity. If analytes were detectable, but outside their specified standard range of concentrations, concentrations were calculated from extrapolations off the standard range of the immunofluorescence curve. The proportion of detectable and non-detectable concentrations for plasma samples is presented in Chapter 3, table 3.5 and cervico-vaginal fluid samples in Chapter 4, table 4.4. Non-detectable concentrations were assigned a value half of the sensitivity level of the assay. The coefficient of variation was calculated across all plates for standards and a common pooled sample on all plates to assess for plate-to-plate variation (presented in Chapter 4, table 4.3).

Table 2.4 Standard curve range and sensitivity levels for plasma cytokine multiplex Luminex® assay

Cytokine	Standard curve range (pg/ml)	Sensitivity level (pg/ml)
IL-18	10.12 - 2460	1.93
IFN-γ	58.48 - 14210	0.4
IL-2	29.63 - 7200	1.8
IL-4	14.61 - 3550	9.3
IL-8	5.19 - 1260	1.8
TNF-α	9.71 - 2360	1.2
GM-CSF	12.22 - 2970	4.1
IL-5	6.63 - 1610	0.5
IL-1β	19.51 - 4740	0.8
IL-6	4.73 - 1150	1.7
IL-10	4.77 - 1160	1.6

Table 2.5 Standard curve range and sensitivity levels for cervico-vaginal fluid cytokine multiplex Luminex® assay

Cytokine	Standard curve range (pg/ml)	Sensitivity level (pg/ml)
IL-8	2.96 - 720	1.8
IL-1β	1.81 - 3950	0.8
IL-10	0.41 - 900	1.6
IL-6	0.33 - 730	1.7
IL-4	1.55 - 3380	9.3
IL-18	1.73 - 3790	1.93
IL-5	0.66 - 1440	0.5
IL-2	6.62 - 14470	1.8
IFN-γ	4.7 - 10290	0.4
TNF-α	0.94 - 2050	1.2
GM-CSF	1.28 - 2800	4.1

The R&D assay reported a sensitivity (or mean detectable dose) which gave a statistical estimation of the level of precision between concentrations. It can be thought of how much two concentration points must differ to be able to be distinguished from each other, and the minimum value that can be distinguished from the background. This sensitivity level was below the lowest standard, therefore such low concentrations at that level are not necessarily accurate. The sensitivity level was determined by adding two standard deviations to the mean fluorescence intensity value of twenty zero standard replicates and calculating the corresponding concentration. The commercial vendor recommended that the lowest standard value on the linear portion of the standard curve as the limit of accurate sensitivity. Therefore, in some cases, a value between the limit of sensitivity value and lowest standard value detected above the background but was not quantifiable.

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Complement assay procedure

Human Complement Magnetic Bead panel 1 and 2 (Milliplex®) HCMP1MAG-19K and HCMP2MAG-19K was used together with Bio-Plex® 200 (Bio-Rad Laboratories Ltd) to detect 4 pre-specified analytes in CVF and plasma. Panel 1 detected C5, C5a and MBL, whilst panel 2 detected C3b. Cervico-vaginal fluid samples did not require dilution. Plasma samples were diluted 1:200 for panel 1 and 1:400 for panel 2.

CVF supernatant or plasma was thawed on ice and centrifuged at 2000x*g* for 10 minutes, removing cell debris. The resulting supernatant was used for the complement assay. Graduated standards were prepared by reconstituting each of the standard cocktails with 250µl of deionised water. Six 3-fold dilutions were performed. The 96 well microplate was washed with 200µl of Wash buffer prior to use. 25µl of standard or sample was added in duplicate to assigned wells of a 96 well microplate. This was followed by the addition of 25µl of Assay buffer, and then 25µl of mixed magnetic beads. The plate was incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 800rpm. Each well was wash with Wash Buffer on a magnetic plate. 50µl of detection antibody was added to each well and the plate was incubated for 1 hour at room temperature on the plate shaker. The well contents were not aspirated after this incubation step. 50µl Streptavidin-PE was added to each well and the plate was incubated for 30 minutes at room temperature on the plate shaker. The wash steps were repeated and the magnetic beads resuspended in 150µl of sheath fluid. The plate was shaken for a further 5 minutes prior to reading on the analyser. All standards and samples were run in duplicate with allocated wells for blanks. The concentrations of the analytes were determined by the spectral properties of the beads and the amount of fluorescence. Standard concentrations were provided by the manufacturer and analyte detections were compared to the standard ranges and subtracting the average blank median fluorescence intensity. If analytes were detectable, but outside their specified standard range of concentrations, concentrations were calculated from extrapolations off the standard range of the immunofluorescence curve. The coefficient of variation was calculated across all plates for standards and a common pooled sample on all plates to assess for plate-to-plate variation.

Table 2.6 Standard curve range and sensitivity levels for cervico-vaginal fluid and plasma complement multiplex Luminex® assay

Complement analyte	Standard curve range	Sensitivity
C5 ng/ml	2.74-2000	1.04
C5a pg/ml	4.12-3000	0.0051
MBL ng/ml	0.14-100	0.04
C3b ng/ml	8.23-6000	3.639

Immunoglobulin assay procedure

The ProcartaPlex Human Antibody Isotyping Panel 7plex (ThermoFisher Scientific cat EPX070-10818-901) was used together with Bio-Plex® 200 (Bio-Rad Laboratories Ltd) to detect 6 pre-specified analytes in the CVF. These were IgG1, IgG2, IgG3, IgG4, IgA and IgM (pg/ml).

CVF supernatant was thawed on ice and centrifuged at 2000x*g* for 10 minutes, removing cell debris. The resulting supernatant was used for the assay. Graduated standards were prepared by reconstituting the standard cocktails with 250µl of universal assay buffer. Seven 3-fold dilutions were performed. 50µl Antibody magnetic beads were added to each well followed by washing of the 96 well microplate with 150µl Wash buffer on a magnetic plate. 25µl of standard or sample was added in duplicate to assigned wells of a 96 well microplate. This was followed by the addition of 25µl of universal assay buffer. The plate was incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500rpm. The plate was washed with Wash buffer on a magnetic plate. 25µl of detection antibody was added to each well and the plate was incubated for 30 minutes at room temperature on the plate shaker. The plate was washed with Wash buffer on a magnetic plate. 50µl Streptavidin-PE was then added to each well and the plate was incubated for 30 minutes at room temperature on the plate shaker. The wash steps were repeated and the magnetic beads resuspended in 120µl of reading buffer. The plate was shaken for a further 5 minutes prior to reading on the analyser. All standards and samples were run in duplicate with allocated wells for blanks. The concentrations of the analytes were determined by the spectral properties of the beads and the amount of fluorescence. Standard concentrations were provided by the manufacturer and analyte detections were compared to the standard ranges and subtracting the average blank median fluorescence intensity. If analytes were detectable, but outside their specified standard range of concentrations, concentrations were calculated from extrapolations off the standard range of the immunofluorescence curve. The coefficient of variation was calculated across all plates for standards and a common pooled sample on all plates to assess for plate-to-plate variation.

Table 2.7 Standard curve range for cervico-vaginal fluid immunoglobulin multiplex Luminex® assay

Immunoglobulin analyte	Standard curve range
IgG1 pg/ml	3784.5-137945000
IgG2 pg/ml	7922.63-5775600
IgG3 pg/ml	620.16-150700
IgG4 pg/ml	1092.73-79600
IgA pg/ml	297.53-10845000
IgM pg/ml	1092.73-7324600

2.2.7 Extraction of DNA for microbiota analysis

Enzyme cocktail mix (volume per vaginal sample)

- 170µl PBS (filter sterilised)
- 50µl Lysozyme (10mg/ml)
- 6µl Mutanolysin
- 3µl Lysostaphin
- 41µl TE50
- 30µl 12% Triton

DNA extraction from the cervico-vaginal swab

As described in section 2.2.5, supernatant from the cervico-vaginal swab was used for the Luminex® assays, and the resulting pellet was used for DNA extraction. The pellet was re-suspended 300µl of enzyme cocktail mix and placed in a 37°C water bath for 1 hour. Following this enzymatic lysis step, the sample was subjected to mechanical lysis by the addition of 100µg 0.1mm acid washed glass beads and oscillation at 25Hz for 1 minute using the TissueLyser LT. The sample was centrifuged to separate the glass beads from the lysate. Further chemical lysis was performed with the QiAamp DNA Mini Kit. The lysate was added to 20µl Proteinase K and 200µl AL buffer and incubated on a heat block at 56°c for 30 minutes. 200µl Ethanol was added and the sample transferred to the QiAamp spin column and centrifuged for 8000rpm for 1 minute. The supernatant was discarded, and the column was washed with 500µl AW1 buffer at 8000rpm for 1 minute followed by 500µl AW2 buffer at 13 300rpm for 3 minutes. The DNA within the spin column was eluted in 100µl AE buffer at 8000rpm for 1 minute.

Polymerase Chain Reaction (PCR) Amplification

To confirm successful extraction of bacterial DNA from vaginal swab samples PCR amplification was carried out using 16S rRNA universal primers: forward primers 27F-5'AGAGTTTGATCCTGGCTCAG-3' and reverse primers 338R-5'GCTGCCTCCCGTAGGAGT-3'. Amplified bacteria DNA was confirmed by gel electrophoresis. Agarose gels (1.5%) were prepared using 3g agarose powder dissolved in 200ml 1x TBE and 16µl SYBR safe DNA gel stain. 16µl loading dye was added to each DNA extract and a 100bp molecular weight marker included on each gel. Each gel also contained a positive and negative control. The positive control was a previously extracted vaginal sample with confirmed 16S rRNA bacterial sequencing on an earlier experiment. The negative control was a blank sample processed in parallel with DNA extracted from vaginal samples. Samples were separated by electrophoresis at 140 volts for 15 minutes and the gel was trans-illuminated and photographed under UV light, (Figure 2.7). The presence of bacterial DNA was confirmed by PCR for all extracted samples, and there was no amplification for any of the negative controls.

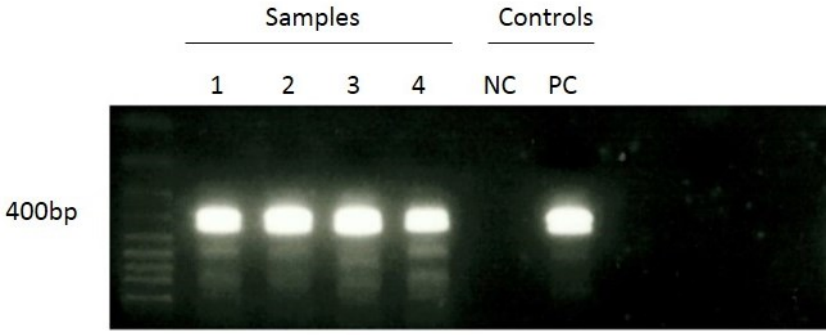


Figure 2.7 PCR confirmation of bacterial DNA.
PCR demonstrating molecular weight marker, detectable bacterial DNA amplicons of expected molecular weight 400 base pairs in samples 1-4, and a negative water control (NC) and a positive control (PC).

DNA Sequencing – vaginal samples

The V1-V2 hypervariable regions of 16S rRNA genes were amplified for sequencing using the following primers. The forward primer set (28f-YM) consisted of a mixture of the following primers mixed at a 4:1:1:1 ratio; 28F-Borrellia GAGTTTGATCCTGGCTTAG; 28F- Chlorlex GAATTTGATCTTGGTTCAG; 28F-Bifido GGGTTCGATTCTGGCTCAG; 28F- YM GAGTTTGATCNTGGCTCAG. The reverse primer consisted for 388R GCTGCCTCCCGTAGGAGT. Sequencing was performed on an Illumina MiSeq platform (Illumina Inc.) at Research and Testing Laboratory, (RTL Genomics) in Lubbock, Texas, USA.

2.1 Data analysis and statistical methods

Flow cytometry data was acquired with the Cytek® Aurora flow cytometer together with the SpectroFlo® Software Package (Cytek) and analysed using FlowJo software v10.4.2 (FlowJo, LLC).

Statistical analysis of cytokines, complement and immunoglobulins from cervico-vaginal fluid or plasma was performed using Graphpad Prism 8.4.1. The D'Agostino-Pearson omnibus normality test was applied. Differences between two groups were analysed using the t-test for parametric data, or the Mann Whitney U test for non-parametric data. Differences between three groups were analysed using the one-way ANOVA test for parametric data, or the Kruskal-Wallis test for non-parametric data. Dunnett's or Dunn's post hoc multiple comparisons test were used for parametric and non-parametric data respectively. A linear regression was used to identify temporal differences according to the gestation of pregnancy. The Fisher's exact or Chi square test was used to test for proportional differences. Spearman correlation was used to correlate concentrations of different analytes. For all tests, the level of statistical significance was taken as a p value ≤0.5.

Sequence data for the vaginal microbial composition were analysed using the MiSeq SOP Pipeline of the Mothur package [257]. Sequence alignment was performed using the Silva bacterial database (www.arb-silva.de/). Sequences were then classified using the RDP (Ribosomal Database Project) database reference sequence files and the Wang method [258]. Operational taxonomic units (OTU) taxonomies from phylum to species were determined using the RDP MultiClassifier script [259]. Rare OTUs (<10 reads per sample) were excluded. The remaining read counts were subsampled and normalised to the lowest read count. Measures of microbial diversity: inverse Simpson and Shannon indexes and richness (species observed) were calculated using Mothur and R using the Vegan package.

Examination of statistical differences between vaginal microbiota was performed at bacterial genera and species level using the Statistical Analysis of Metagenomic Profiles software package (STAMP) [260]. At genera level, samples were grouped into those that were *Lactobacillus* dominant, (>75% *Lactobacillus* species), or *Lactobacillus* deplete, (<75% *Lactobacillus* species), a commonly utilised cut-off point [78, 161] . Hierarchical clustering analysis using Ward linkage of species data was used with a clustering density threshold of 0.75 with the 20 most abundant bacterial species displayed. Thereafter, samples were classified into vaginal microbial groups (VMG) based upon Ward hierarchical clustering, VMG 1 (*Lactobacillus crispatus*), VMG 2 (*Lactobacillus gasseri*), VMG 3 (*Lactobacillus iners*), VMG 4 (Diverse species), VMG 5 (*Lactobacillus jensenii*) and VMG 6 (*Bifidobacterium spp*).

CHAPTER 3: THE PERIPHERAL IMMUNE RESPONSE IN HEALTHY PREGNANCY AND IN WOMEN WHO DELIVER PRETERM

3.1 Chapter Summary

Hypothesis

- A successful term pregnancy involves highly regulated, time-dependent adaptions to the maternal systemic immune system.
- Aberrant alterations in the maternal systemic immune response occur in a proportion of women who deliver preterm.

Aims

- To describe changes in peripheral blood mononuclear cell subsets and plasma cytokines and components of the complement system during healthy term pregnancy.
- To describe changes in peripheral blood mononuclear cell subsets, plasma cytokines and components of the complement system in pregnancies complicated by spontaneous preterm birth.
- To examine the relationship between the maternal peripheral immune response in cervical shortening.
- To describe changes in the maternal peripheral immune mediators in response to cervical cerclage as an intervention to prevent preterm birth in women with cervical shortening.

Methods

Peripheral blood mononuclear cells were collected from 78 study participants. For 51 study paricipants (65%), samples were collected at three timepoints in pregnancy, 12^{+0} - 16^{+6} weeks, 20^{+0} - 24^{+6} weeks and 30^{+0} - 34^{+6} weeks. For 51 study participants (65%), samples were collected at all three timepoints, and they formed a cohort studied longitudinally. Peripheral blood mononuclear cells were isolated from whole blood using Ficoll and cryopreserved until use. The Cytek® Aurora was used to identify and characterise populations of monocytes, T cells, Treg cells, γδ T cells, B cells, NK cells and NK T cells, using antibodies to the following cell surface markers: CD3, CD4, CD8, CD14, CD16, CD19, CD27, CD45Ra, CD56, CD127 and CD197. Cell activation status was measured by the median fluorescence intensity (MFI) of fluorochromes conjugated to antibodies directed to the following cell surface markers: CD25, CD38, PD-1, HLA-DR and IgD.

Plasma was collected from 32 study participants at the same three timepoints in pregnancy, 12^{+0} - 16^{+6} weeks, 20^{+0} - 24^{+6} weeks and 30^{+0} - 34^{+6} weeks. Plasma cytokines (IL-18, IFN-γ, IL-2, IL-4, IL-8, TNF-α,

GM-CSF, IL-5, IL-1β, IL-6 and IL-10) and plasma complement (C3b, MBL, C5 and C5a) were quantified using magnetic Luminex® immunoassays. Detailed metadata was collected from each study participant, including gestational age at delivery, treatment with progesterone and/or cervical cerclage and cervical length measurement by transvaginal ultrasound.

Statistical differences between groups were analysed using the Student's t-test or one-way ANOVA where data was normally distributed, or Mann Whitney U test or Kruskal-Wallis test for non-parametric data, with Tukey's or Dunn's multiple comparisons post-hoc test. Linear regression was used to identify time dependent changes across pregnancy.

Results

Study participants were categorised into three pregnancy outcome groups, preterm delivery before 37^{+0} weeks, term (uncomplicated) delivery and term delivery with intervention (cervical cerclage and/or progesterone). Changes in the proportion and activation of several peripheral blood mononuclear cell subsets were seen across gestational age as pregnancy advanced. There was a significant increase in CD4+ T cells and CD4+ CD25 MFI with advancing gestation in the term (uncomplicated) group, in the longitudinal cohort ($p<0.05$). Although the proportion of CD8+ T cells was stable with advancing gestation, in both the whole study population analysis and the longitudinal cohort analysis, CD8+ CD38 MFI increased with advancing gestation in the preterm and term with intervention groups ($p<0.05$). CD8+ CD25 MFI increased with advancing gestation in the term (uncomplicated) group ($p<0.05$), in the longitudinally cohort. CD8+ HLA-DR was significantly greater in the preterm group at the 12^{+0} - 16^{+6} sampling timepoint compared to both term groups ($p<0.05$). There was a reduction in NK cells with advancing gestation in both term groups between the first and third sampling timepoints ($p<0.05$), however the statistical significance was lost when analysis was corrected for multiple testing. There was a significant increase in NK CD38 MFI as pregnancy advanced in both the whole study population and longitudinal cohort analysis ($p<0.05$). The proportion and activity of monocytes, Treg, γδ T cells and B cells did not show any gestational differences or differences according to preterm and term deliveries.

Plasma IL-18 increased significantly with advancing gestation in both term outcome groups ($p<0.05$), with a trend for increase in the preterm group. There was a positive correlation between plasma IL-18 and IFN-γ, TNF-α and IL-2, consistent with a Th1 pro-inflammatory response. No aberrant cytokine production was seen in women who delivered preterm. Furthermore, no changes in plasma complement were seen as pregnancy advanced or according to pregnancy outcome. There were no

differences in the cell subset percentage or activation, or plasma analytes in women who underwent cervical shortening or had a cervical cerclage.

Conclusions

The maternal peripheral immune response is altered during pregnancy, but no marked differences were observed between women who deliver preterm compared to at term. The gestation related changes were predominantly seen in activation markers of T cells, supporting the theory of an immune clock in healthy pregnancy. These results suggest that different aetiologies of preterm birth and the associated pathophysiological mechanisms are not reflected in the maternal peripheral immune response.

3.2 Introduction

The maternal immune system undergoes time-dependent adaptations in order to concurrently maintain pregnancy with a semi-allogenic fetus and respond appropriately to pathogens [174]. This involves responding to different physiological phases of pregnancy, (implantation, placentation, fetal growth and labour), and adaptation to both the innate and adaptive immune systems. Peripheral immune signatures have been described in granulocytes with advancing pregnancy, and in granulocytes, lymphocytes and monocytes at the onset of labour [261]. Adaptions to peripheral cytokine and complement signalling pathways during pregnancy have also been explored [175, 198].

Dysregulation of the healthy immune signatures have been implicated in the pathogenesis of miscarriage and preterm birth. For example, an imbalance in the activation and regulation of the complement system has been implicated in abnormal implantation leading to first-trimester miscarriage [262]. An exaggerated cytokine response in placenta and in the peripheral circulation have been seen in first trimester miscarriages [263].

There is also a strong association between preterm birth, infection and inflammation [5, 264]. A reduction in peripheral Treg cells [202] and dysregulation of the complement system as evidenced by high peripherally circulating complement proteins [208] have been shown to be associated with preterm birth. Histological chorioamnionitis demonstrated in preterm birth is associated with significantly higher peripheral leukocyte counts [265]. Our group have previously identified several plasma microRNAs associated with inflammation which are predictive of cervical shortening and preterm delivery [266], and differences in the expression of certain peripheral exosomes in term and preterm pregnancies have been described [267].

The traditional view that pregnancy is a state of immunosuppression has been superseded by a more complex notion that the maternal immune system is dynamically responsive. When these temporal changes occur during pregnancy, or if they have an impact on the timing of labour remains unclear. There are limited studies characterising peripheral longitudinal changes as pregnancy advances. Therefore, the objectives of the work described in this Chapter were to characterise the peripheral immune cell subsets and their activations status, and quantify plasma cytokines and complement concentrations across pregnancy and in the context of both preterm and term labour outcomes.

3.3 Study design

To study the peripheral immune changes during pregnancy in women with term and preterm pregnancies, women with risk factors for preterm birth were prospectively recruited from the preterm surveillance clinics at Queen Charlotte's and Chelsea Hospital and St Mary's Hospital in London. Peripheral blood was taken from women at three timepoints during their pregnancy, 12^{+0} - 16^{+6} weeks, 20^{+0} - 24^{+6} weeks and 30^{+0} - 34^{+6} weeks gestation. Whole blood was collected in EDTA tubes. Peripheral blood mononuclear cells were separated from whole blood for flow cytometry. Plasma cytokines and complement concentrations were analysed in a subset of women. This cohort was selected at the time of an interim analysis from the first 32 women with outcome data.

The cervical length was obtained by a transvaginal ultrasound on the same day as blood sampling. Study participants were divided into 3 outcome groups retrospectively: a) women who had spontaneous preterm deliveries (preterm, PT); b) women who delivered at term without intervention (term, T); c) women who delivered at term following an elective history or ultrasound indicated cerclage and/or progesterone (term intervention, TI). Detailed metadata was collected from all study participants from the hospital notes and the electronic patient database, Cerner Millennium Powerchart®.

3.4 Statistical analysis

Flow cytometry data were analysed using SpectroFlo® Software Package (Cytek) and FlowJo software v10.4.2 (FlowJo, LLC). Hierarchical gating was performed to identify specific cell subsets. FlowJo software v10.4.2 was used to display the data graphically.

The concentrations of cytokine, complement and immunoglobulin analytes were determined by the spectral properties of the beads and the amount of emitted fluorescence using the Bio-Plex® 200 software. Standard concentrations were provided by the manufacturer and analyte detections were compared to the standard ranges and subtracting the average blank median fluorescence intensity.

Statistical analysis was performed using Graphpad Prism 8.4.1. Differences between two groups were analysed using the t-test if the data was normally distributed, or the Mann Whitney U test for data which was not normally distributed. Differences between three groups were analysed using the one-way ANOVA test if the data was normally distributed, or the Kruskal-Wallis test for data which was not normally distributed. The post hoc Dunnett's or Dunn's multiple comparisons test were used where appropriate. A linear regression was used to identify temporal differences according to the gestation of pregnancy. For all tests, the level of statistical significance was taken as a p value ≤0.5.

3.5 Results

3.5.1 Development of the flow cytometry fluorochrome panel

In collaboration with the scientific team at Cytek™, an antibody panel was developed to assess the following peripheral blood mononuclear cell types during pregnancy: lymphocytes, CD4+ T cells, CD8+ T cells, Treg cells, γδ T cells, NK cells, B cells, CD4+ NK T cells, CD8+ NK T cells, monocytes, classical monocytes, intermediate monocytes and non-classical monocytes. Fluorochrome conjugated antibodies were directed to the following cell surface activation markers CD25, CD38, HLA-DR, PD-1 and IgD. Aqua amine exclusion dye was used to exclude dead cells and analyse parameters of live cells only. As detailed earlier (see Methods 2.2.3), the Cytek® Aurora uses cutting edge technology to measure the full emission spectrum for each fluorochrome creating a unique signature which is not affected by overlapping fluorochrome emission spectrum peaks.

For this work, 18 fluorochromes were chosen based upon the Similarity Index, a measure of uniqueness between two fluorochromes on a scale of 0 to 1. Values close to 0 represented two very different fluorochromes, whilst values close to 1 indicated very similar fluorochrome emission signatures. The similarity index for each pair of fluorochromes in the panel were compared, Figure 3.1. Fluorochromes chosen were AlexaFluor 488, BV750, BV650, PE, eFluor450, APC, AlexaFluor532, BV605, BV480, PerCP-eFluor, APC-Fire 750, PE/Dazzle594, PE-Cy7, AlexaFluor 700, BV711, BV785, BV42 and LiveDead Aqua. The Complexity Index was then calculated; which was an overall measure of uniqueness of all fluorochromes in a full spectrum cytometry panel [(268)]. The Complexity Index for this 18 fluorochrome panel was 6.21. This was comparable to previously designed smaller panels of 10 fluorochromes which typically have Complexity Indices of 2-3, and larger panels of 35-40 fluorochromes where the Complexity Indices are between 40-50. The emission spectrum of the 18 fluorochromes is shown in Figure 3.2. A fluorochrome spillover spread matrix enabled the selection of combination of fluorochromes with minimal spectral overlap, Figure 3.3.

Each fluorochrome was matched to the antibody for the cell surface marker or cell activation marker which was commercially available, taking into account the fluorochrome stain index. The stain index is a parameter reflecting the ability to resolve a dim positive signal from the background fluorescence. Fluorochromes with low stain index were used to measure parameters expressed at high levels, whilst fluorochromes with high stain index were used to measure parameters expressed at lower levels, Figure 3.4.

The fluorochromes with their cell markers were as follows: Alexa Fluor™ 488 anti-human CD45RA, BV750 mouse anti-human CD4 (clone SK3), Brilliant violet 650™ anti-human CD279 (PD-1), PE anti-human CD25, eFluor 450 anti-human CD16, APC anti-human CD127, AlexaFluor 532 anti-human CD3, Brilliant Violet 605™ anti-human CD19, Brilliant Violet 480 mouse anti-human TCRγδ, PerCP-eFluor 710 anti-human CD14, APC/Fire™ 750 anti-human CD8, PE/Dazzle™ 594 anti-human CD197 (CCR7), PE/Cy7 anti-human CD38, Alexa Fluor® 700 anti-human HLA-DR, Brilliant Violet 711™ anti-human CD56 (NCAM), Brilliant Violet 785™ anti-human IgD, Brilliant Violet 421™ anti-human CD27 and LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit. Catalogue numbers can be found in the Materials and Methods section.

Optimisation experiments were performed for each cell surface and cell activation marker to determine the correct dilution and volume of antibody to use per number of cells by the Cytek™ chemical team, (see Table 2.2 in Materials and Methods). Matched histograms of single stain controls versus multi-colour samples are shown in Appendix 3. Good detection of each fluorochrome could be demonstrated if the histogram in the single stain control matched the histogram obtained in the multi-colour sample.

Similarity Indices

	Alexa Fluor 488	BV750	BV650	PE	eFluor 450	APC	Alexa Fluor 532	BV605	BV480	PerCP-eFluor 710	APC-Fire 750	PE/Dazzle594	PE-Cy7	Alexa Fluor 700	BV711	BV785	BV421	LIVE DEAD Aqua
Alexa Fluor 488	1																	
BV750	0	1																
BV650	0	0.24	1															
PE	0.14	0	0.06	1														
eFluor 450	0	0.04	0.09	0.01	1													
APC	0	0.07	0.32	0.01	0	1												
Alexa Fluor 532	0.52	0	0.01	0.6	0.02	0	1											
BV605	0	0.1	0.53	0.26	0.06	0.07	0.01	1										
BV480	0.05	0.02	0.07	0.1	0.46	0	0.01	0.17	1									
PerCP-eFluor 710	0	0.41	0.3	0.04	0	0.25	0.03	0.11	0	1								
APC-Fire 750	0	0.19	0.05	0	0	0.17	0	0.01	0	0.17	1							
PE/Dazzle594	0.1	0.02	0.16	0.69	0.01	0.03	0.47	0.39	0.07	0.18	0	1						
PE-Cy7	0	0.24	0.04	0.01	0	0.03	0.01	0.02	0	0.37	0.2	0.05	1					
Alexa Fluor 700	0	0.17	0.14	0	0	0.51	0	0.02	0.01	0.44	0.33	0	0.07	1				
BV711	0	0.69	0.45	0.01	0.08	0.23	0	0.17	0.03	0.68	0.18	0.05	0.16	0.43	1			
BV785	0	0.82	0.15	0	0.07	0.04	0	0.07	0.02	0.28	0.23	0.02	0.29	0.1	0.49	1		
BV421	0	0.05	0.1	0.01	0.86	0	0.02	0.06	0.28	0	0	0	0	0	0.09	0.08	1	
LIVE DEAD Aqua	0.01	0.02	0.1	0.19	0.28	0.01	0.04	0.32	0.87	0.01	0.01	0.14	0	0.01	0.03	0.02	0.17	1

Complexity Index: 6.21

Figure 3.1 The 18 fluorochrome panel used for analysis with the Cytek® Aurora spectral flow cytometer
The Similarity Indices between 18 fluorochromes were between 0 and 0.87. The overall Complexity Index was low at 6.21. The 18 fluorochromes were Alexa Fluor 488, BV750, BV650, PE, eFluor 450, APC, Alexa Fluor 532, BV605, BV480, PerCP-eFLUOR 710, APC-Fire 750, PE/Dazzle 594, PE-Cy7, Alexa Fluor 700, BV711, BV785, BV421 and Live Dead Aqua Amine.

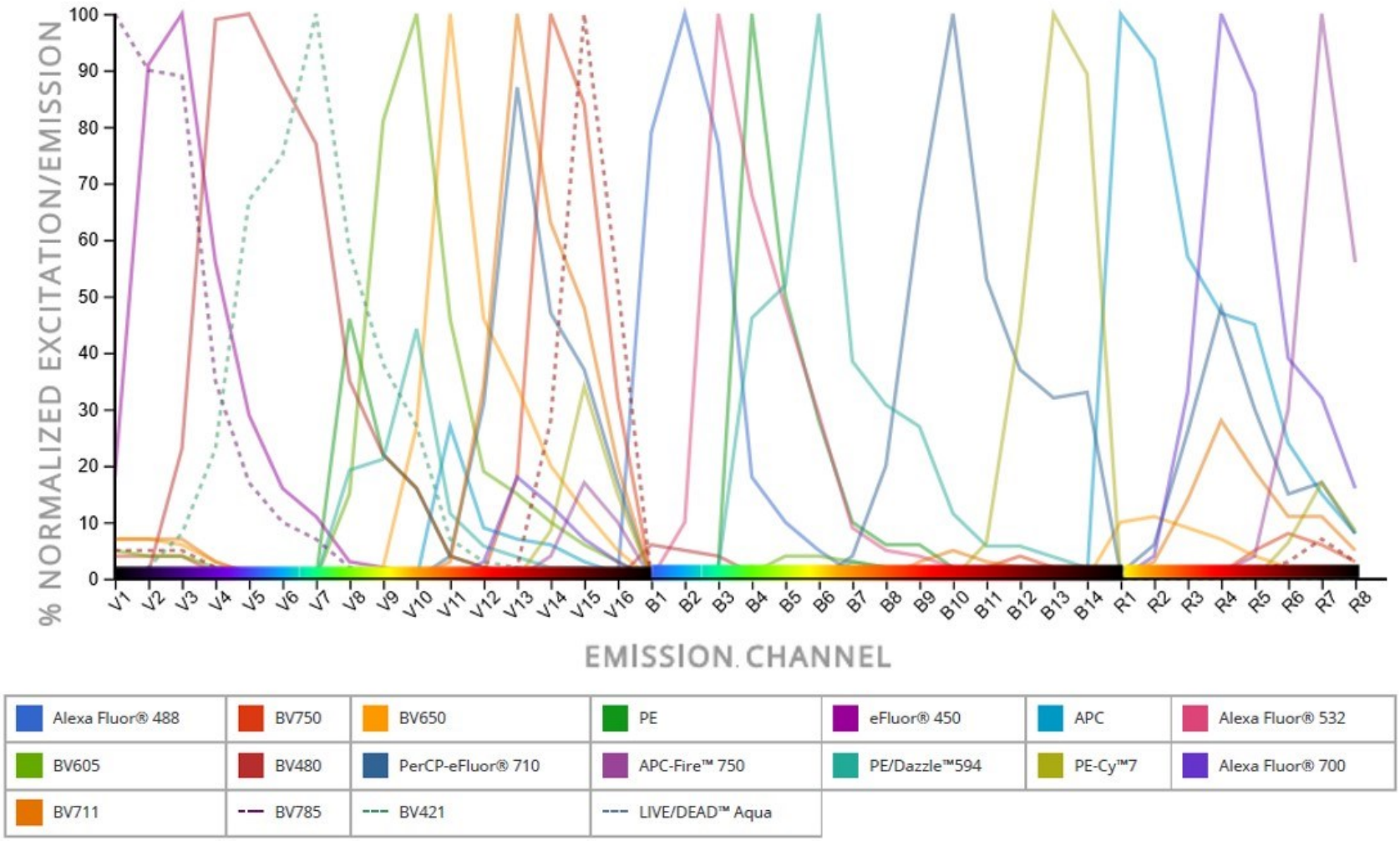


Figure 3.2 The overlapping emission spectrums of 18 fluorochromes
There are 16 channels in the violet laser (V1-V16), 14 channels in the blue laser (V1-14) and 8 channels in the red laser (R1-R8). Each fluorochrome dye is excited by all three lasers with a peak channel and secondary emission channels.

SSM	BV421	eFluor 450	BV480	BV605	BV650	BV711	BV750	BV785	Alexa Fluor 488	Alexa Fluor 532	PE	PE-Dazzle594	PerCP-eFluor 710	PE-Cy7	APC	Alexa Fluor 700	APC-Fire 750
BV421		4.08	1.26	0.45	0.35	0.07	0	0.30	1.03	0.29	0.44	0	0	0.05	0.07	0.12	0.12
eFluor 450	4.46		1.51	0.40	0.55	0.52	0.31	0.69	0	0.47	0.34	0.18	0	0.29	0	0.13	0
BV480	1.67	2.03		1.22	0.66	0.52	0.46	0.54	4.05	0.73	0.88	0.17	0.19	0.25	0	0.15	0.17
BV605	1.06	0.84	0.41		1.93	1.22	1.45	1.24	2.56	1.19	1.17	2.29	0.83	0.37	0.87	0.41	0.21
BV650	1.25	1.03	0.25	1.10		1.80	1.79	1.55	0	0.43	0	0.30	0.93	0.24	1.63	0.76	0.34
BV711	1.36	1.05	0	0.43	0.75		3.35	3.03	0.84	0.30	0.36	0.12	1.11	0.49	1.28	3.17	0.89
BV750	0.73	0.21	0.09	0.26	0.27	1.19		3.76	0.72	0.33	0	0	0.77	0.34	0.54	1.01	1.06
BV785	0.97	0.72	0	0.25	0.31	0.51	2.44		0	0.30	0	0.13	0.25	0.35	0	0.39	1.39
Alexa Fluor 488	0	0	0	0.21	0.27	0.25	0.39	0.64		2.29	0.76	0.49	0.09	0.28	0	0.30	0
Alexa Fluor 532	0.90	2.34	0	0	1.06	0.80	0	0.80	3.28		1.42	1.20	0.72	0.73	1.04	0.66	0
PE	0.35	0.19	0.57	1.11	0.54	0.32	0.27	0.24	9.48	4.70		1.02	0.61	0.18	0.46	0.15	0.12
PE-Dazzle594	0.22	0	0.35	1.44	1.06	1.09	0.55	0.38	8.50	3.84	3.03		1.37	0.47	1.50	0.32	0
PerCP-eFluor 710	0.49	0	0	0.13	1.41	4.96	2.72	2.42	1.69	0.58	1.47	0.22		1.42	2.24	4.45	1.14
PE-Cy7	0.29	0.27	0	0.24	0.43	4.38	1.13	1.31	0.89	0.39	0.93	0	4.77		0.48	1.09	2.59
APC	0	0.52	0	0.45	1.50	0.95	0.86	0.88	1.26	0.34	0	0.28	0.99	0.58		1.52	1.02
Alexa Fluor 700	1.13	0	0	0.28	0.77	1.35	1.45	1.49	0	0.39	0.66	0.25	0.97	0.54	1.75		1.55
APC-Fire 750	0	0.13	0	0.14	0.22	0.63	0.98	1.51	1.37	0.62	0.14	0	0.45	0.95	0.79	0.54	

Figure 3.3 Fluorochrome spillover spread matix

Fluorochromes were chosen to minimise spillover or spectral overlap. Spillover spread matrix values closer to zero had minimal spectral overlap.

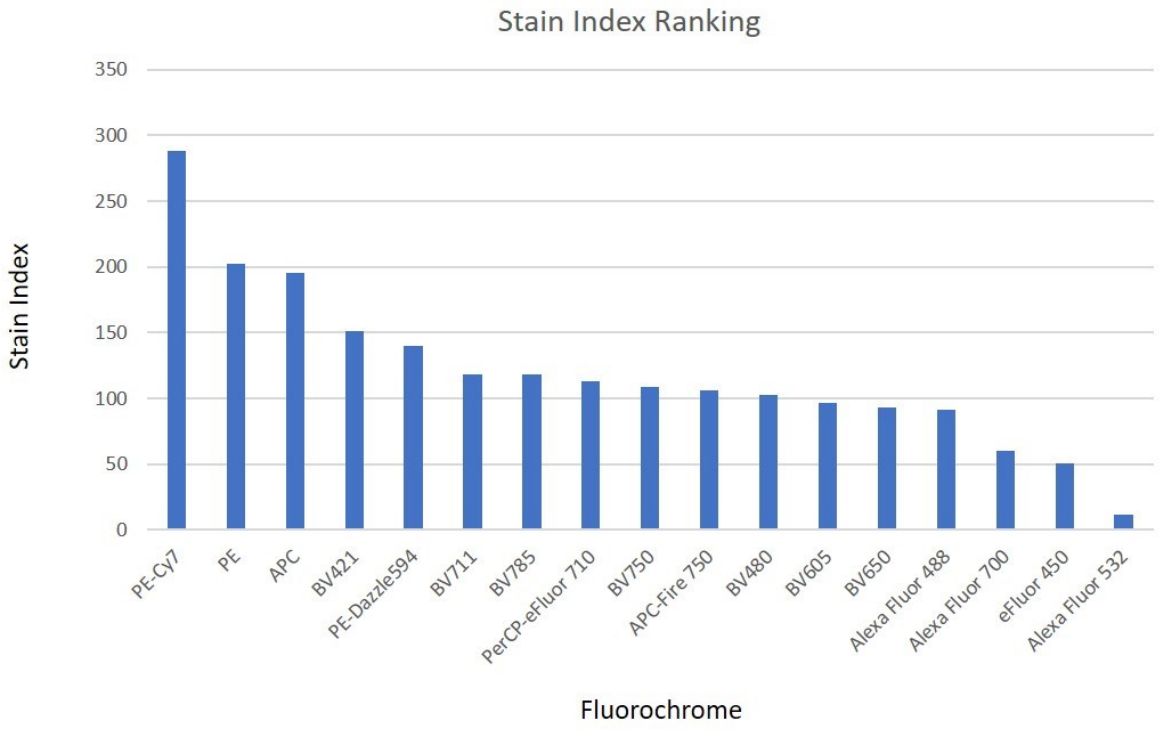


Figure 3.4 Fluorochrome stain index ranking
The stain index is a parameter reflecting the ability to resolve a dim positive signal from the background fluorescence. Fluorochromes with low stain index were used to measure parameters expressed at high levels, whilst fluorochromes with high stain index were used to measure parameters expressed at lower levels.

3.5.2 Peripheral immune cell subsets

Hierarchical gating was first performed to enable distinguishing of single cells from doublets and debris, Figure 3.5 A, with the fluorescence intensity of the aqua amine stain used to discriminate live and dead cells, Figure 3.5 B. Monocytes and lymphocytes were gated using their forward and side scatter characteristics, Figure 3.5 C. Light scatter is measured by two optical detectors; the first detector measures scatter along the path of the laser and is referred to as the forward scatter and the second detector measures scatter at ninety degrees relative to the laser and is referred to as the side scatter. Side scatter provides information about the granularity of the cell. Monocytes are larger and more granular than lymphocytes, therefore they exhibit forward and side scatter of higher intensities.

Monocyte subsets, classical, intermediate and non-classical were gated according to CD14 and CD16. CD3 was used to differentiate between T cells, NK cells and B cells, and CD19 to differentiate between NK cells and B cells. Further gating of T cell subsets, CD4+, CD8+, γδ T cells, NK T cells and Treg cells was via their cell surface markers as follows; Helper T cells were CD3+ and CD4+. Cytotoxic T cells were CD3+ and CD8+. Treg cells were CD3+, CD4+, CD25+, TCRγδ- and $CD127^{low}$. γδ T cells were CD3+ and TCRγδ+. CD4+ NK T cells were CD3+, CD56+, TCRγδ, CD4+ and CD45RA+. CD8+ NK T cells were CD3+, CD56+, TCRγδ, CD48 and CD45RA+, Figure 3.5 D.

The effector status of monocytes was measured by the median fluorescence intensity (MFI) of HLA-DR and PD-1. The effector status of B cells was measured by the MFI of IgD and PD-1. The MFI of CD25, CD38, HLA-DR and PD-1 were measured for CD4+, CD8+ and Treg cells. The MFI of CD38 was measured for NK cells.

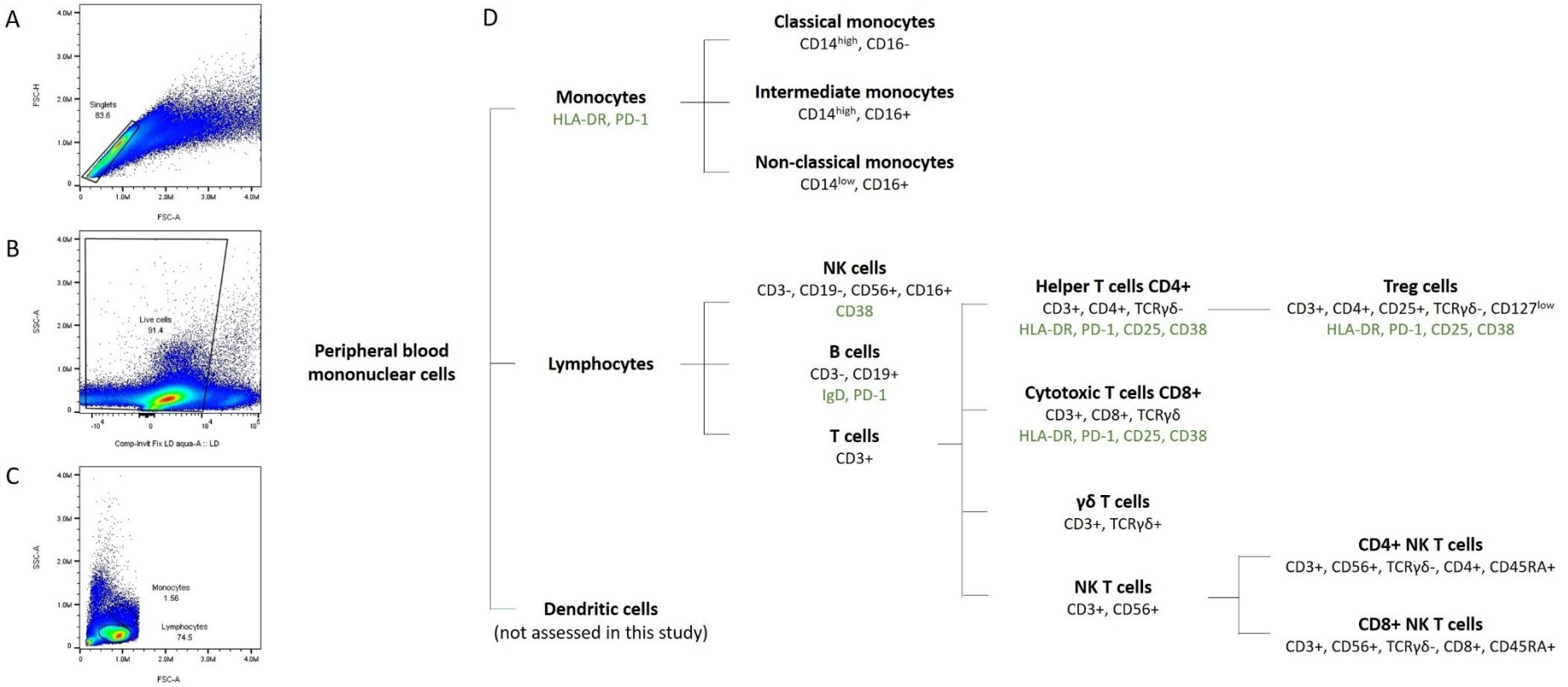


Figure 3.5 Gating strategy used for analysis of peripheral blood mononuclear cell subsets by spectral flow cytometry

Using flow cytometry, peripheral blood mononuclear cell subsets were gated from singlets and live cells followed by monocytes and lymphocytes based upon side and forward scatter characteristics. Cell subsets: classical, intermediate and non-classical monocytes, NK cells, B cells, T cells, NKT cells, γδ T cells, cytotoxic T cells, helper T cells, Treg, CD4+ NK T cells and CD8+ T cells were determined by cell surface markers. Activation markers of particular cell subsets are shown in green.

In a cohort of women with term pregnancies who did not experience cervical shortening and did not receive a cervical cerclage, the proportions of peripheral blood mononuclear cells subsets were stable with longitudinal sampling at 12^{+0} - 16^{+6} weeks, 20^{+0} - 24^{+6} weeks and 30^{+0} - 34^{+6} weeks gestation, except for CD4+ T cells which increased significantly with advancing gestation (Table 3.1). This was based upon longitudinal sampling of 22 women, who were identified as high risk of preterm delivery and were recruited from the preterm birth surveillance clinic, but delivered at term gestation not requiring progesterone or a cervical cerclage. An example image of the proportions of PBMC cell subsets is presented as a single plot based upon unsupervised clustering performed using the t-distributed stochastic neighbour embedding (t-SNE) algorithm, Figure 3.6.

Table 3.1 Major peripheral blood mononuclear subset percentages across term pregnancy.
N=22 longitudinal samples at three timepoints. Mean % (standard deviation)

	12-16 weeks	20-24 weeks	30-34 weeks	One-way ANOVA
Lymphocytes (as % of live cells)	90.0 (6.3)	89.0 (7.4)	88.3 (7.9)	p=0.45
Monocytes (as % of live cells)	10.1 (6.3)	11.0 (7.4)	11.8 (7.9)	p=0.87
CD4+ (as % of CD3+)	62.8 (6.4)	64.4 (7.9)	65.2 (7.3)	p=0.04 *
Treg (as % of CD4+)	6.9 (2.4)	6.8 (2.1)	7.7 (3.2)	p=0.54
CD8+ (as % of CD3+)	32.4 (6.1)	31.3 (7.4)	30.7 (6.8)	p=0.11
B cells (as % of lymphocytes)	9. 3 (3.5)	10.1 (3.5)	10.4 (4.2)	p=0.33
NK cells (as % of lymphocytes)	5.13 (2.3)	5.15 (2.3)	4.67 (2.6)	p=0.44

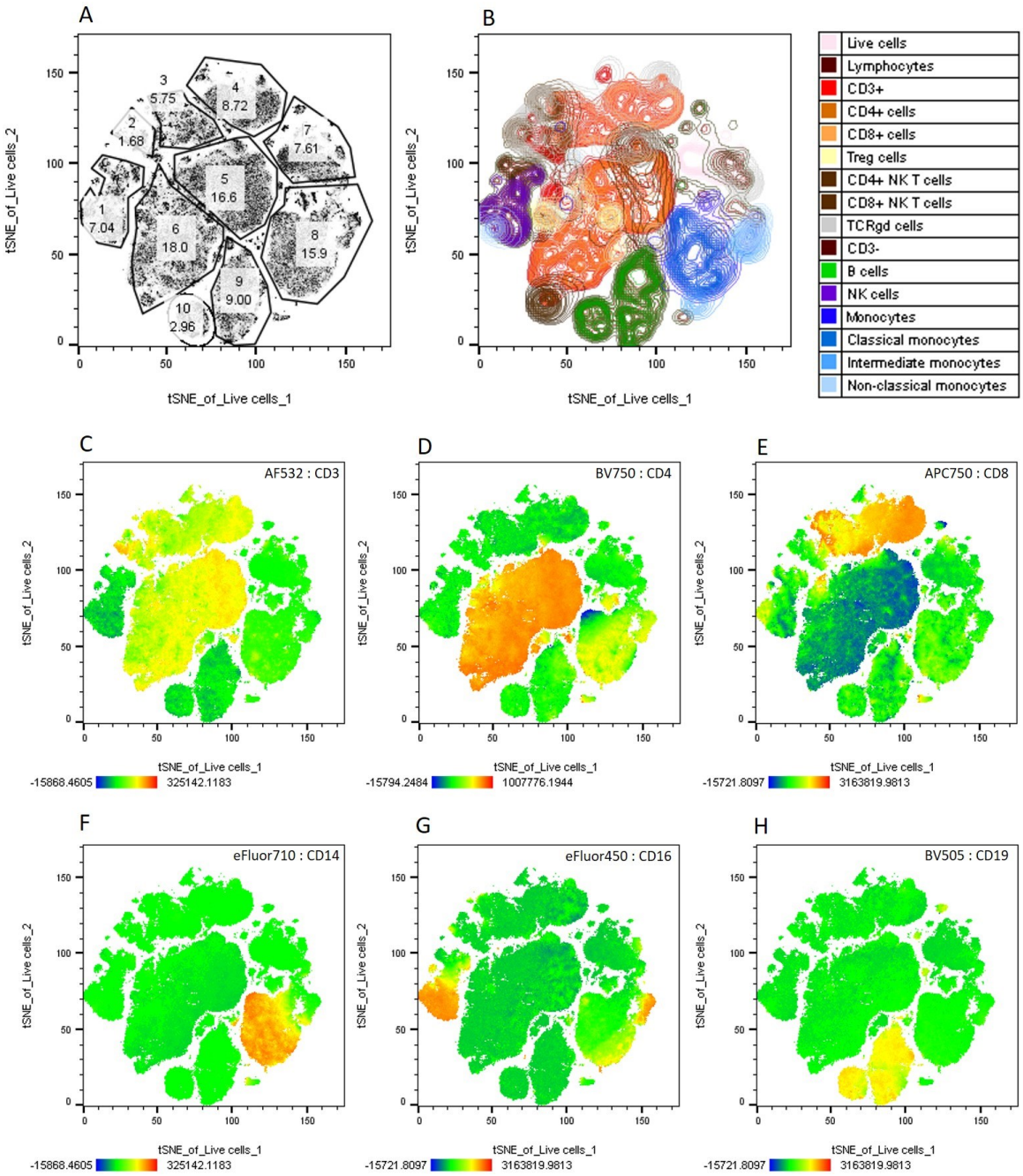


Figure 3.6 Representation of PBMC cell subsets identified by 18 fluorochrome panel

Cells were grouped into one of 10 clusters. Cluster number is indicated (top number) with the percentage of cells in each cluster (bottom number) (A). Unsupervised clustering (tSNE) visualisation of immune cells based upon phenotypic signatures with cell markers identified cell subsets, live cells, CD3+ lymphocytes, CD4+ T cells, CD8+ T cells, Treg cells, CD4+ NK T cells, CD8+ NK T cells, γδ T cells , CD3- lymphocytes, B cells, NK cells, classical monocytes, intermediate monocytes and non-classical monocytes (B). Two dimensional tSNE plots based on single immune cell flow cytometry markers are shown in C-H, CD3 (C), CD4 (D), CD8 (E), CD14 (F), CD16 (G), CD19 (H).

3.5.3 Study population characteristics for peripheral blood mononuclear cells

A total of 78 pregnant women provided blood samples whilst attending the preterm birth prevention clinics. Referral criteria for attending the clinics included previous preterm birth, previous mid-trimester miscarriage, and previous excisional cervical treatment. Most of these women delivered at term, >37^{+0} weeks gestation 64 (82%), of which N=38 (49%) were uncomplicated and N=26 (33%) received an intervention (cerclage and/or progesterone). 14 (18%) of women delivered preterm before 37^{+0} weeks gestation Table 3.2.

As there were only 14 women who delivered in the preterm, they were not further divided into two groups reflecting the differentiation conferred to the term groups which identified which women received an intervention (cerclage and/or progesterone).

Between the three pregnancy outcome groups, there were no significant differences in maternal age (p=0.89) or BMI (p=0.20), however there was a significant difference in ethnicity (p=0.001), with a larger proportion of white women in the term (uncomplicated) (76%), compared with the term with intervention (42%) and the preterm (29%). There was a smaller proportion of black women in the term (uncomplicated) (10%), compared to the term intervention (38%) and the preterm (21%).

In this cohort of 78 women, the most common reason for referral to the preterm birth prevention clinics was previous excisional cervical treatment (39%), followed by previous preterm birth (32%) and previous mid-trimester loss (25%). In total, 28 women received a cervical cerclage (36%), 7 delivered preterm before 37 weeks gestation and 21 delivered after 37 weeks. Cervical cerclages were classified as either history indicated, N=15 or ultrasound indicated, N=12. History indicated cerclages were those performed because of an obstetric history suggestive of cervical insufficiency. Ultrasound indicated cerclages were those performed due to the cervical length measuring ≤25mm on ultrasound surveillance. The type of cerclage material was also recorded. This was either braided Mersilene (36%) or monofilament Nylon (64%). Unless otherwise stated, cervical cerclages were performed trans-vaginally.

Where possible, women were sampled at three timepoints during pregnancy, timepoint 1: 12^{+0}-16^{+6} weeks, timepoint 2: 20^{+0}-24^{+6} weeks and timepoint 3: 30^{+0}-34^{+6} weeks. Data analysis was performed in two ways, firstly including all study participants (N=78), irrespective if samples were collected at each sampling timepoint (cross-sectional analysis), and secondly matched longitudinal analysis was performed for study participants who had samples collected at each of the three sampling timepoints, (N=51). As a consequence, unmatched and matched statistical analyses were performed for the individual cohorts.

Table 3.2 Clinical and demographical characteristics of the PBMC study population N=78

	Preterm (PT) (>37 weeks)	Term (T)	Term with intervention (TI)	p value (PT vs T vs TI)
N (%)	14 (18%)	38 (49%)	26 (33%)	
Gestation at delivery (weeks) median and IQ range	34+1 (32+1-36+4)	39+2 (38+3-40+1)	38+3 (38-41)	One-way ANOVA p<0.0001 *** PT vs T p<0.0001 *** PT vs TI p<0.0001 ***
Early preterm </=33+6	6	NA	NA	
Late preterm >/=34+0	8	NA	NA	
Age median and IQ range	33 (30-39)	33 (30-37)	34 (31-36)	One-way ANOVA p=0.90
BMI median and IQ range	25 (22-28)	22 (20-27)	25 (22-29)	One-way ANOVA p=0.20
Ethnicity White N (%) Black N (%) Other N (%)	 4 (29%) 3 (21%) 7 (50%)	 29 (76%) 4 (10%) 5 (13%)	 11 (42%) 10 (38%) 5 (10%)	χ^2=0.0015 **
Cervical cerclage	7 (50%)	0	21 (81%)	
History indicated cervical cerclage and material	1 (Mersilene 1)	NA	14 (Mersilene 3, Nylon 9, Abdominal 1, Unknown 1)	
USS indicated cervical cerclage and material	6 (Mersilene 3, Nylon 3)	NA	6 (Mersilene 2, Nylon 4)	
Progesterone only	0	NA	5	
Risk factor for PTB: (by patient) Cervical treatment Cervical treatment + MTL Cervical treatment + PTB MTL MTL + PTB PTB 3x1st trimester misc Short cervix at anomaly	 2 0 2 2 0 8 0 0	 21 0 1 4 2 9 1 0	 7 0 1 6 5 6 0 1	
Risk factor for PTB [(a)] Cervical treatment Previous PTB Previous MTL	 4 (29%) 10 (71%) 2 (14%)	 22 (58%) 6 (16%) 12 (31%)	 8 (31%) 12 (46%) 11 (42%)	χ^2 p<0.0039 ***

BMI=body mass index, USS=ultrasound. PT=preterm, T=term (no intervention). TI=term with intervention. Data presented as median (interquartile range (IQ)) or number (%). P values: One-way ANOVA for multiple comparisons or Chi squared for proportional data.

3.5.4 Peripheral immune cell subset signature across gestation, and in women who deliver preterm and at term

Analysis of PBMC samples obtained from all 78 study participants will be presented first. The proportion of peripheral cell subsets were assessed with advancing gestation within each the three pregnancy outcome groups, preterm (delivery $<37^{+0}$ weeks) N=14, term N=38, and term with intervention (cerclage +/- progesterone) N=26: monocytes and their subsets, (classical, intermediate and non-classical) (Figure 3.7), CD3+ lymphocytes (Figure 3.8), CD4+ T cells (Figure 3.9), CD8+ T cells (Figure 3.10), Treg (Figure 3.11), γδ T cells (Figure 3.12), B cells (Figure 3.13), NK cells (Figure 3.14), CD4+ NKT cells and CD8+ NKT cells (Figure 3.15).

Monocytes

Monocytes have important roles in tissue inflammation and repair and phagocytosis. The monocyte population was gated according to their forward and side scatter characteristics, Figure 3.7 A. The three main subsets were gated as follows: classical ($CD14^{high}$, CD16-), non-classical ($CD14^{low}$, CD16+) and intermediate ($CD14^{high}$, CD16+), Figure 3.7 B. The proportion of monocytes as a percentage of all live cells and their subsets were stable across gestation in the three pregnancy outcome groups, Figure 3.7 C-H, in the whole study population. Monocyte activation as measured by the MFI of HLA-DR and PD-1 were stable with advancing gestation in all three outcome groups, Figure 3.7 D and E, however monocyte activation was quite varied, particularly in the preterm group, possibly reflecting heterogeneity of aetiology. The most abundant monocyte subset was the classical monocytes, followed by the intermediate monocytes and then the non-classical monocytes. There was no difference in the proportion of monocytes and their cell effector status at each sampling timepoint, 12^{+0} - 16^{+6} weeks, 20^{+0} - 24^{+6} weeks and 30^{+0} - 34^{+6} weeks when comparing the three pregnancy outcome groups, ($p>0.05$) (i.e. preterm vs term vs term intervention at 12^{+0}-16^{+6} weeks, preterm vs term vs term intervention at 20^{+0} - 24^{+6} weeks, and preterm vs term vs term intervention at 30^{+0} - 34^{+6} week).

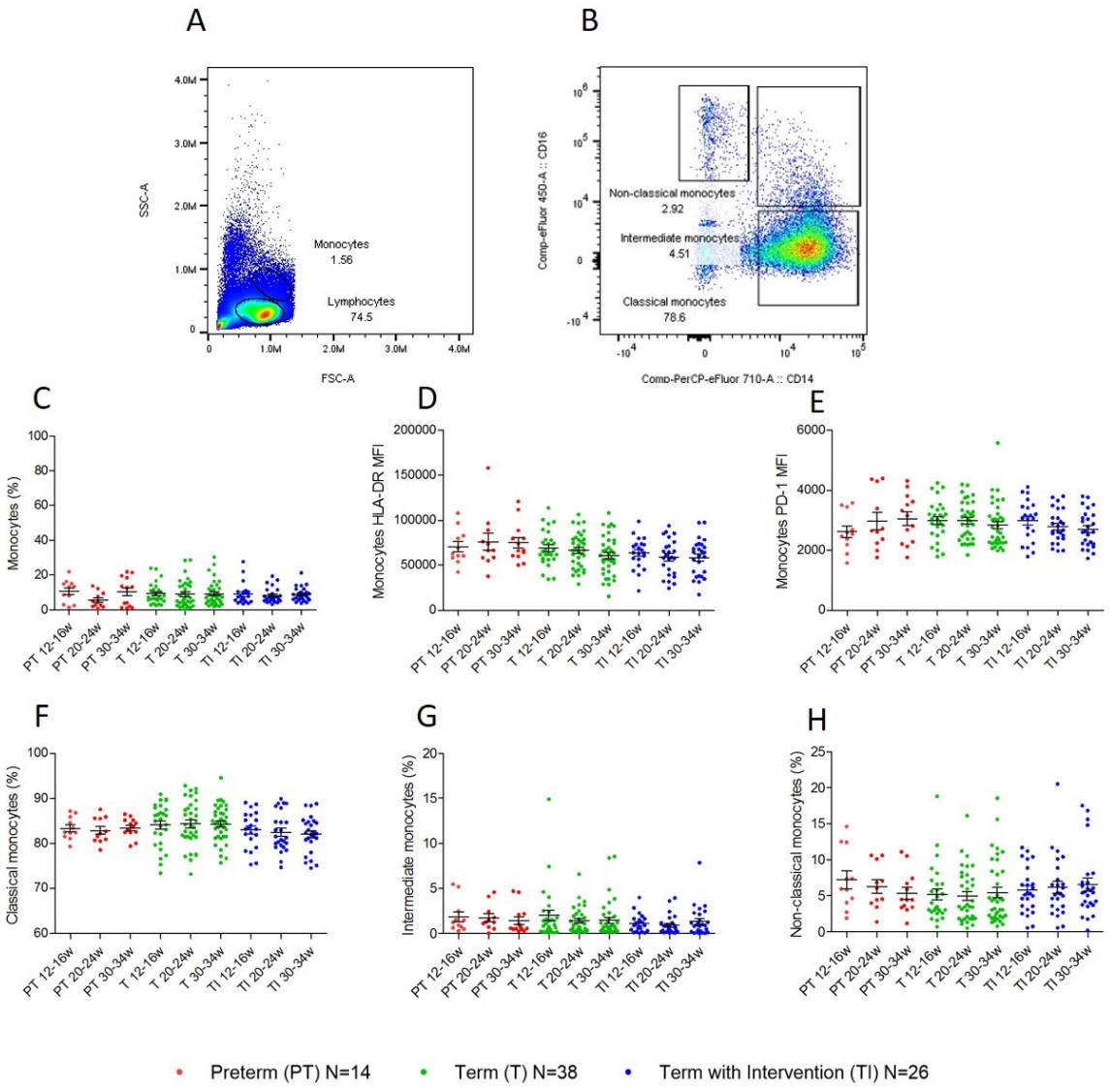


Figure 3.7 Peripheral blood monocytes, monocyte subsets and effector status across pregnancy and comparisons with preterm and term pregnancies

Monocytes were gated based upon forward and side scatter characteristics (A). Classical, intermediate and non-classical monocytes were gated upon CD14 and CD16 (B). There was no difference in the percentage of monocytes (B), or their subsets, classical monocytes (F), intermediate monocytes (G), non-classical monocytes (H), or monocyte HLA-DR median fluorescence intensity (MFI) (C) or monocyte PD-1 MFI (D) with advancing gestation or between different pregnancy outcome groups, preterm (PT), term (T), term with intervention (TI) at each of the three timepoints. (Preterm group N=14, Term (uncomplicated) group N=38, Term (intervention) group N=26). Statistical analysis was by the one-way ANOVA with Dunnett's multiple comparisons test. The results are expressed as mean ±SEM (standard error of the mean).

T cells

Lymphocytes were gated based upon their forward and side scatter characteristics, Figure 3.8 A. The proportion of CD3+ lymphocytes were stable across pregnancy and there was no difference in the proportion of CD3+ cells at each sampling timepoint, 12^{+0}-16^{+6}, 20^{+0} - 24^{+6} and 30^{+0} - 34^{+6} weeks when comparing the three pregnancy outcome groups, ($p>0.05$) Figure 3.8 C, in the whole study population.

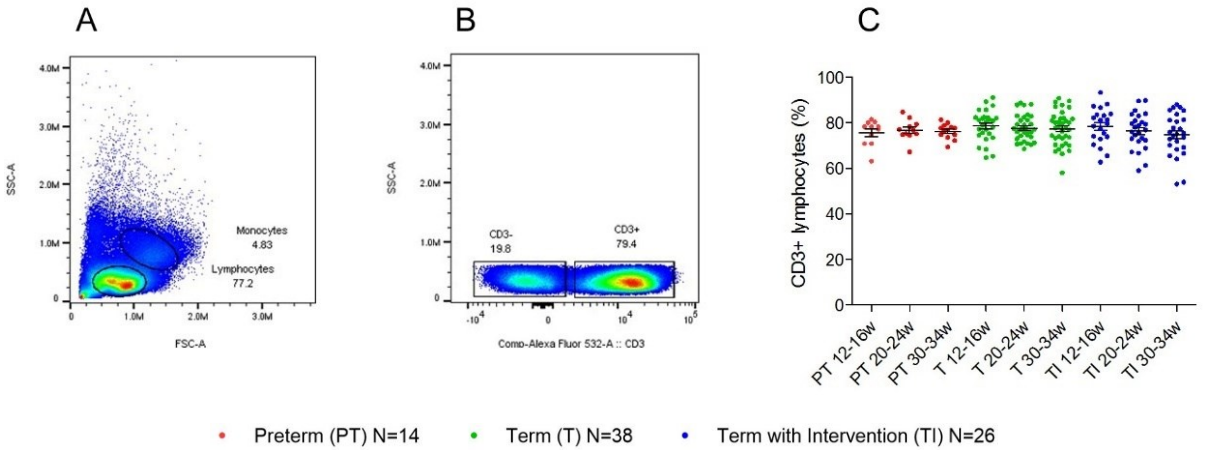


Figure 3.8 Peripheral CD3+ lymphocytes across pregnancy and comparisons with preterm and term pregnancies

There was no difference in the percentage of CD3+ lymphocytes with advancing gestation or between different pregnancy outcome groups, preterm (PT), term (T), term with intervention (TI) at each of the three timepoints (C). (Preterm group N=14, Term (uncomplicated) group N=38, Term (intervention) group N=26). Statistical analysis was by the one-way ANOVA with Dunnett's multiple comparisons test. The results are expressed as mean ± SEM.

CD4+ T cells

CD4+ T cells amplify the responses of other immune cells including macrophages and neutrophils through the secretion of cytokines. CD4+ T cells were CD3+, CD4+, Figure 3.9 A. Analysis with samples from the whole study population showed that there was no significant change in the proportion of CD4+ T cells (as a percentage of CD3+ cells) with advancing gestation in the preterm, term (no intervention) and term with intervention groups, ($p>0.05$) Figure 3.9 B. There was a trend for an increase in CD4+ T cells across gestation in the term group when data was analysed cross-sectionally, Figure 3.9 B. This relationship was later found to be statistically significant in the longitudinal cohort, Section 3.5.5, Figure 3.17 A. The effector status of CD4+ T cells as measured by the MFI of CD25, HLA-DR and PD-1 were stable with advancing gestation and there were no significant differences between the three outcome groups at the three sampling timepoints ($p>0.05$), Figure 3.9 C-F. There was a trend for a gestational increase in CD4+ CD38 in the term group, Figure 3.9 D, which was observed to be statistically significant in longitudinally sampled women, Section 3.5.5 Figure 3.17 C.

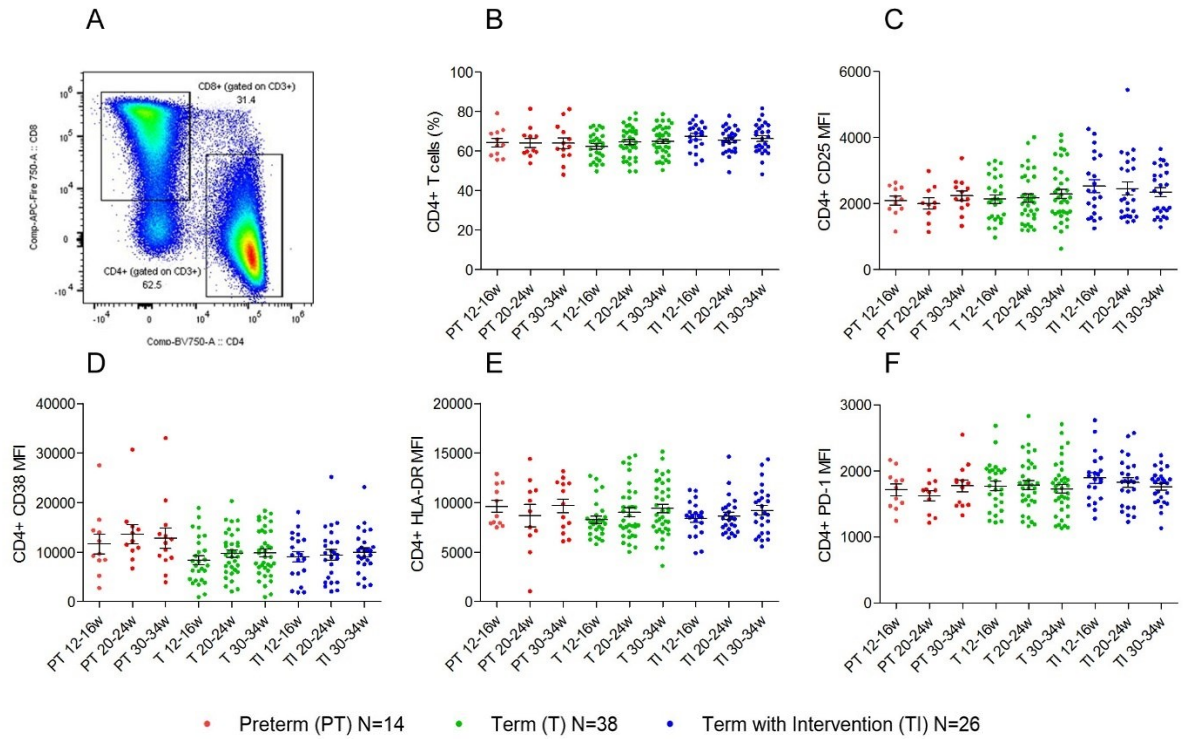


Figure 3.9 Peripheral CD4+ cells and their effector status across pregnancy and comparisons with preterm and term pregnancies

CD4+ cells were gated by CD3+/CD4+ (A). There was no significant difference in the percentage of CD4+ cells (B) or CD4+ cell CD25, CD38, HLA-DR or PD-1 median fluorescence intensity (MFI) (C-F) with advancing gestation or between different pregnancy outcome groups, preterm (PT), term (T), term with intervention (TI) at each of the three timepoints. (Preterm group N=14, Term (uncomplicated) group N=38, Term (intervention) group N=26). Statistical analysis was by the one-way ANOVA with Dunnett's multiple comparisons test. The results are expressed as mean ± SEM.

CD8+ T cells

CD8 + T cells induce the killing of damaged cells. CD8+ T cells were CD3+ and CD8+, Figure 3.10 A. The proportion of CD8+ T cells (as a percentage of CD3+ cells) was stable with advancing gestation however their activation as measured by CD38 MFI increased during pregnancy. This was statistically significant in the preterm and the term with intervention group, p=0.04 and p=0.003 respectively, with a similar trend in the term group, Figure 3.10 D. The CD8+ HLA-DR MFI was increased in the preterm group compared to both term groups at the 12^{+0} - 16^{+6} week sampling timepoint, both p=0.04, Figure 3.10 E. CD8+ CD25 MFI and CD8+ PD-1 did not show any significant changes during pregnancy or differences between preterm and term deliveries, Figure 3.10 C and F.

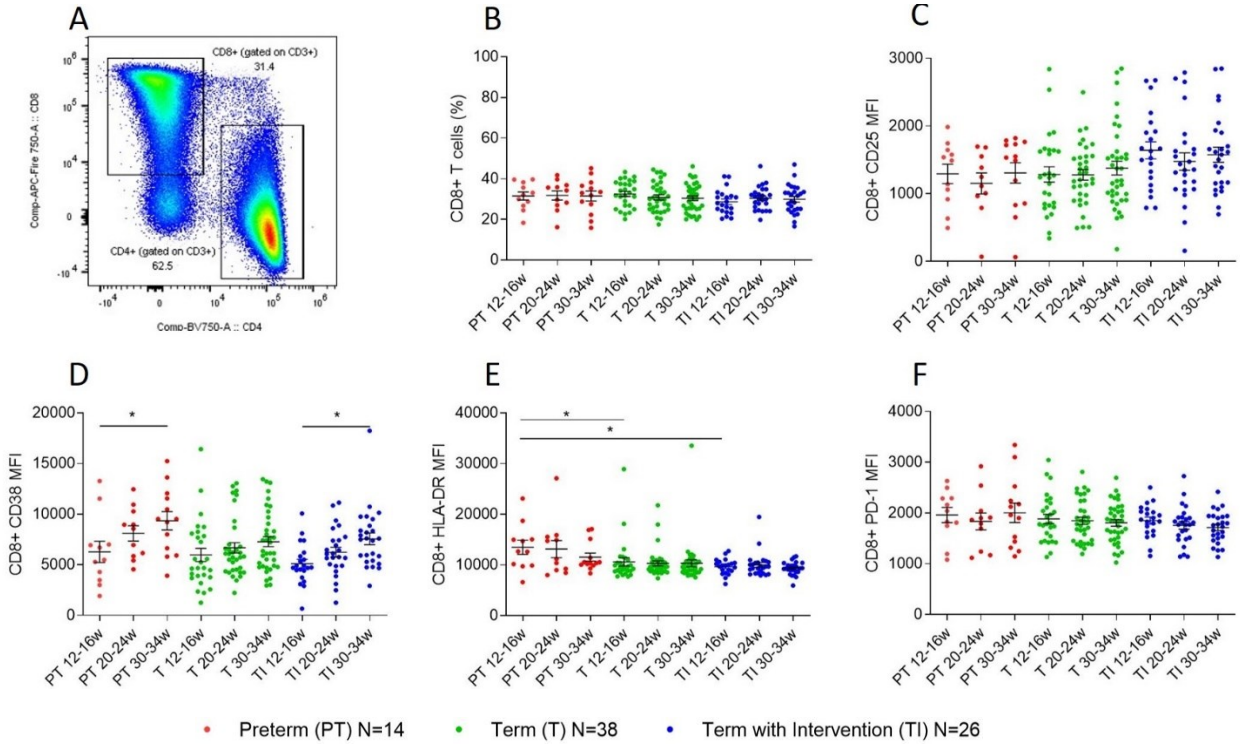


Figure 3.10 Peripheral CD8+ cells and their effector status across pregnancy and comparisons with preterm and term pregnancies

CD8+ cells were gated by CD3+/CD8+ (A). There was no difference in the percentage of CD8+ cells (B) or CD8+ cell CD25 or PD-1 median fluorescence intensity (MFI) (A,B,C and F) with advancing gestation or between different pregnancy outcome groups, preterm (PT), term (T), term with intervention (TI) at each of the three timepoints. However, CD8+ CD38 MFI increased with advancing gestation in the preterm and term intervention groups ($p<0.05$) and CD8+ HLA-DR was significantly higher at 12-16 weeks in women who delivered preterm compared to women who delivered at term ($p<0.05$) (E). (Preterm group N=14, Term (uncomplicated) group N=38, Term (intervention) group N=26). Statistical analysis was by the one-way ANOVA with Dunnett's multiple comparisons test. The results are expressed as mean ± SEM.

Treg cells

Treg cells suppress the activation, proliferation and cytokine production of CD4+ and CD8+ T cells. Ruocco reported that there is an expansion of the Treg cell pool during pregnancy [212]. Treg cells were identified as being CD3+, CD4+, CD25+, and CD127^{low}, Figure 3.11 A and B. There was no significant increase in the proportion of Treg cells (as a percentage of CD4+ cells) with advancing gestation in the preterm and term groups, Figure 3.11 C. In the term group Treg HLA-DR MFI tended to increase with advancing gestation, whilst it appeared to decrease in the preterm and the term with intervention group, Figure 3.11 D. Treg CD38 MFI however tended to increase with advancing gestation irrespective of the pregnancy outcome, Figure 3.11 F.

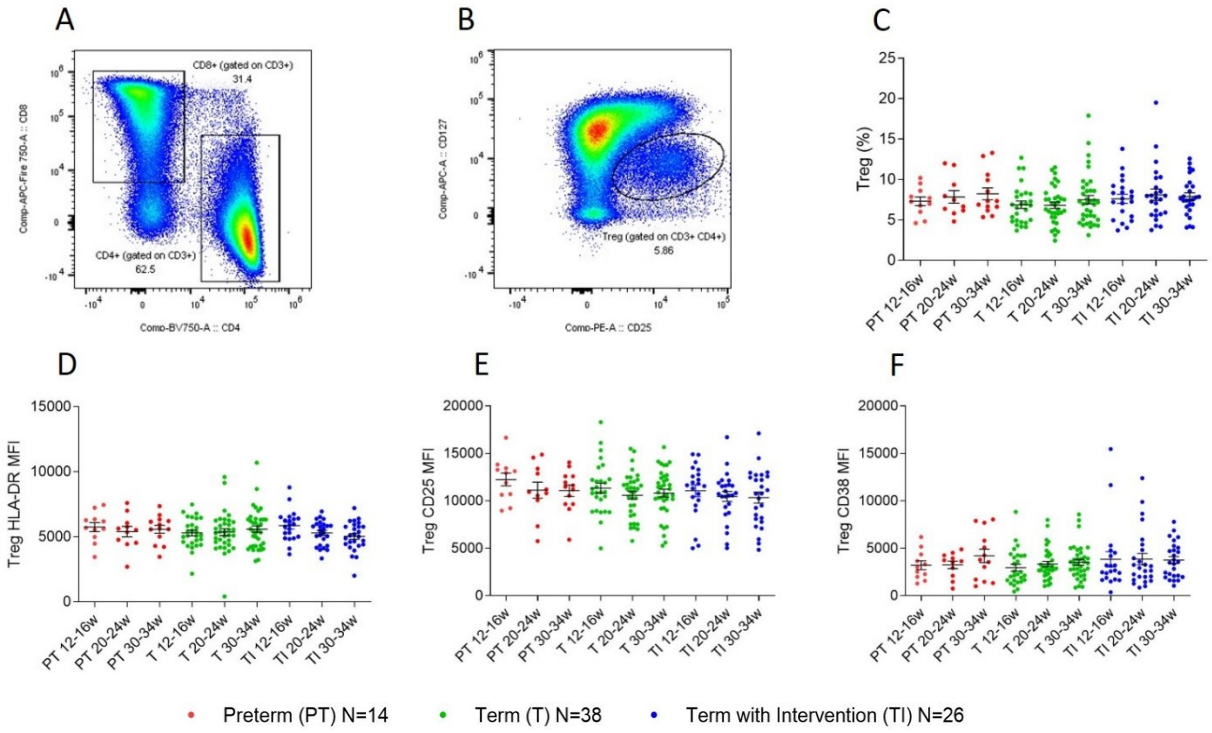


Figure 3.11 Peripheral Treg cells across pregnancy and comparisons with preterm and term pregnancies
Treg cells were gated by CD3+, CD25+ and CD127^{low} (A and B). There was no difference in the percentage of Treg, Treg HLA-DR median fluorescence intensity (MFI) (D), Treg CD25 MFI (E) or Treg CD38 MFI (F) with advancing gestation or between different pregnancy outcome groups, preterm (PT), term (T), term with intervention (TI) at each of the three timepoints (C). (Preterm group N=14, Term (uncomplicated) group N=38, Term (intervention) group N=26). Statistical analysis was by the one-way ANOVA with Dunnett's multiple comparisons test. The results are expressed as mean ± SEM.

γδ T cells

γδ T cells have roles in the innate and adaptive immune response. They were identified as CD3+ and TCRγδ+, Figure 3.12 A and B. They represented a small proportion of PBMC. The proportion (as a percentage of total lymphocytes) was no different in the three pregnancy outcome groups and there was no significant change with advancing gestation, ($p>0.05$) Figure 3.12 C.

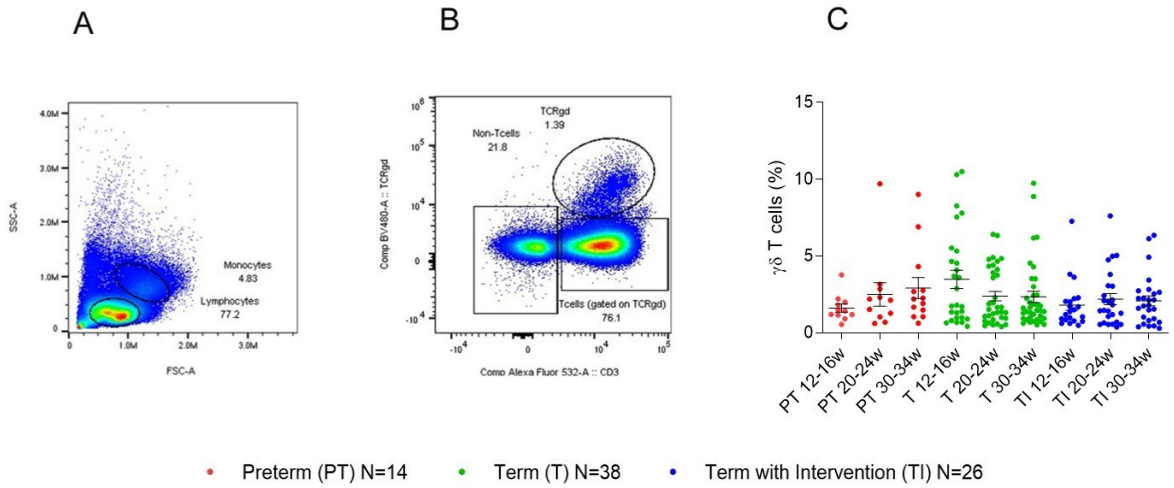


Figure 3.12 Peripheral γδ T cells across pregnancy and comparisons with preterm and term pregnancies
γδ T cells were gated upon CD3/TCRγδ (A and B). There was no difference in the percentage of γδ T cells with advancing gestation or between different pregnancy outcome groups, preterm (PT), term (T), term with intervention (TI) at each of the three timepoints (C). (Preterm group N=14, Term (uncomplicated) group N=38, Term (intervention) group N=26). Statistical analysis was by the one-way ANOVA with Dunnett's multiple comparisons test. The results are expressed as mean ± SEM.

B cells

B cells produce immunoglobulins which are key to mucosal immunity. B cells were identified with the following gating strategy, CD3-, CD19+, CD56+, Figure 3.13 B and C. The proportion of B cells (as a percentage of total lymphocytes) was stable across pregnancy within all three pregnancy outcome groups (p>0.05), Figure 3.13 D. There was no significant change in the B cell MFI of IgD and PD-1 (p>0.05), Figure 3.13 E and F. There was no difference in the proportion of B cells and their cell effector status at each sampling timepoint, 12^{+0} -16^{+6}, 20^{+0} -24^{+6} and 30^{+0} - 34^{+6} weeks when comparing the three pregnancy outcome groups (p>0.05).

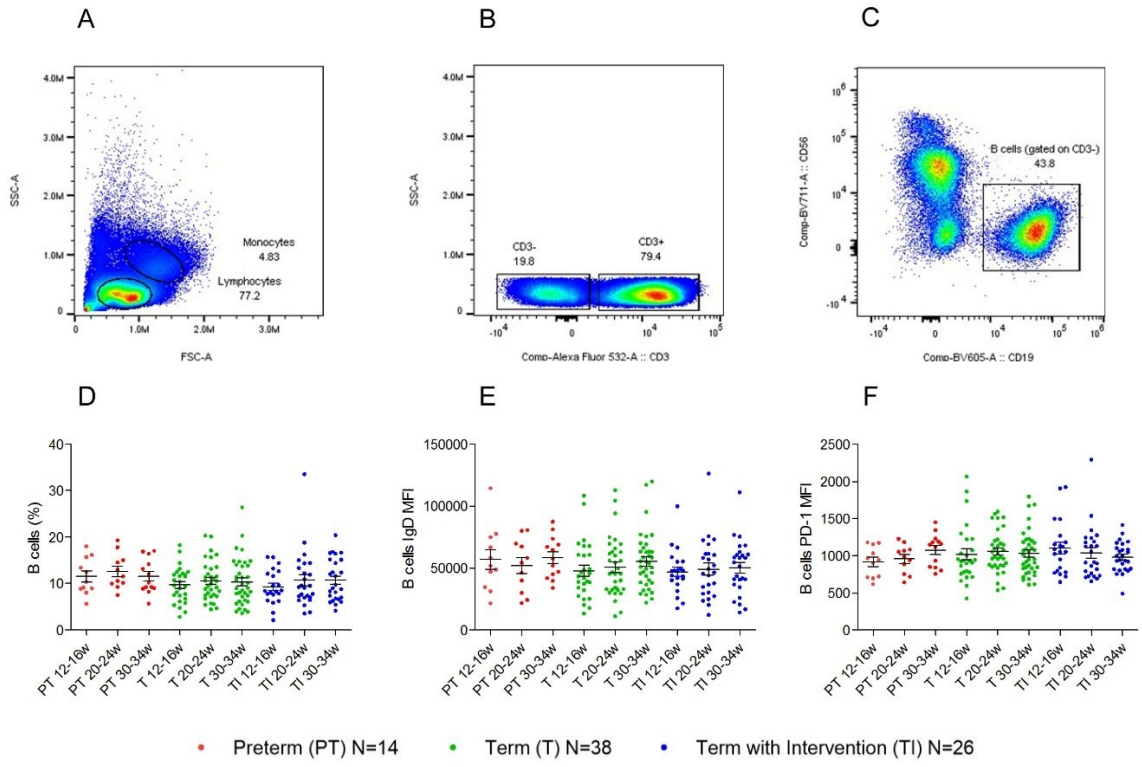


Figure 3.13 Peripheral blood B cells and their effector status across pregnancy and comparisons with preterm and term pregnancies

B cells were gated by CD3-/CD19+ (B and C). There was no difference in the percentage of B cells (D) or B cell IgD median fluorescence intensity (MFI) or B cell PD-1 MFI with advancing gestation or between different pregnancy outcome groups, preterm (PT), term (T), term with intervention (TI) at each of the three timepoints (E and F). (Preterm group N=14, Term (uncomplicated) group N=38, Term (intervention) group N=26). Statistical analysis was by the one-way ANOVA with Dunnett's multiple comparisons test. The results are expressed as mean ± SEM.

NK cells

NK cells are key effectors of the innate immune response and respond to microbial infections, limiting spread and tissue damage. NK cells were identified as being CD3-, CD19-, CD56+, and CD16+, Figure 3.14 A and B. There was a non-significant reduction in NK cells (as a percentage of the CD3- lymphocytes) in the term and term intervention groups with advancing gestation, (p=0.35 and p=0.16), Figure 3.14 C. NK CD38 MFI significantly increased with advancing gestation in the preterm and the term groups (both p=0.01), Figure 3.14 D. The NK CD38 MFI was significantly higher in the preterm compared to the term with intervention at latest sampling timepoint of 30^{+0} - 34^{+6} weeks gestation, (p=0.04), Figure 3.14 D.

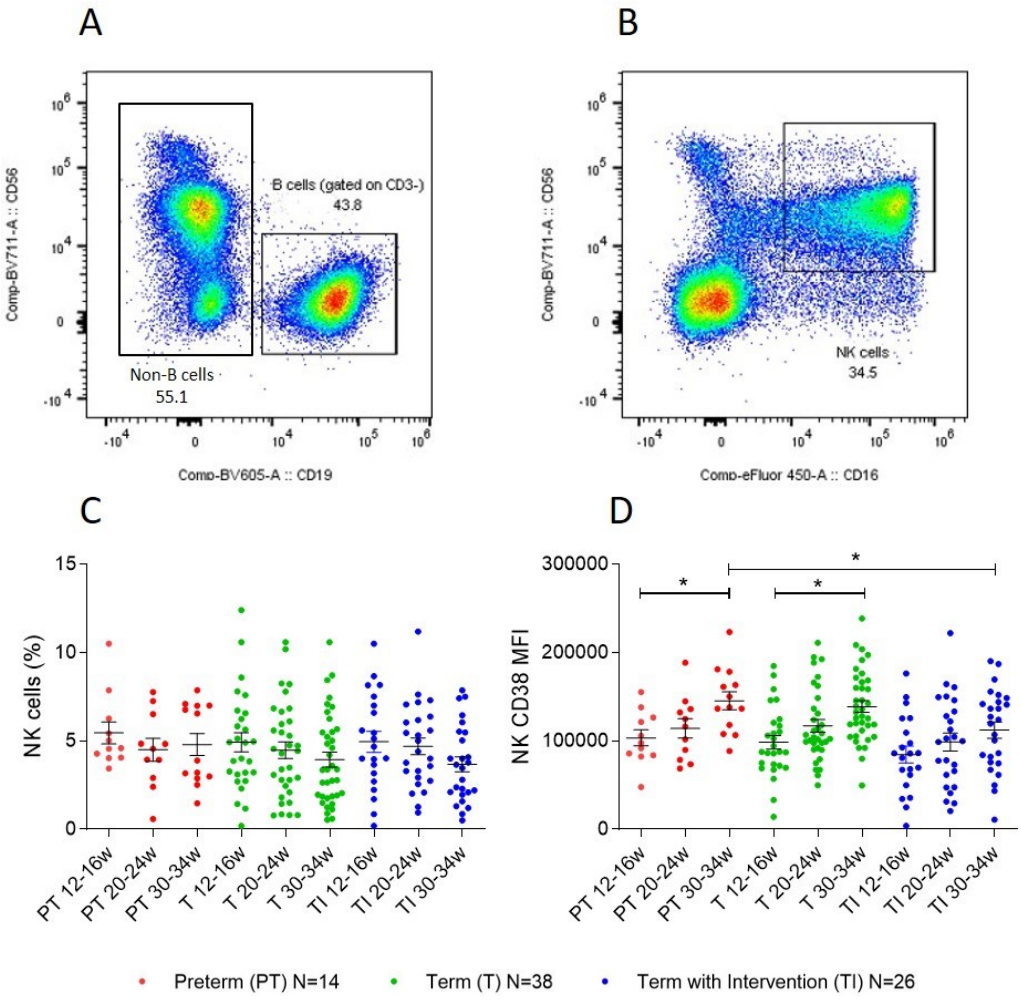


Figure 3.14 Peripheral NK cells across pregnancy and comparisons with preterm and term pregnancies
NK cells were gated on CD3-, CD16+, CD56+ (A and B). Although there was no significant difference in the percentage of NK cells with advancing gestation or between different pregnancy outcome groups, preterm (PT), term (T), term with intervention (TI) at each of the three timepoints (C), there was a significant difference with NK CD38 median fluorescence intensity (MFI) with gestation (D). It increased significantly in the PT and T group between 12-16 weeks and 30-34 weeks timepoints. In addition, at the 30-34 week timepoint, the NK CD38 MFI was significantly higher in the PT compared to the TI group. (Preterm group N=14, Term (uncomplicated) group N=38, Term (intervention) group N=26). Statistical analysis was by the one-way ANOVA with Dunnett's multiple comparisons test. The results are expressed as mean ± SEM.

NK T cells

NK T cells provide support to B cells in the defence against microbial infections. CD4+ NK T cells were CD3+, CD56+, TCRγδ-, CD4+, CD45RA+ and CD8+ NK T cells were CD3+, CD56+, TCRγδ-, CD8+, CD45RA+, Figure 3.15 A-D. CD8+ NK T cells were more abundant than CD4+ NK T cells. The proportions of both cell subsets were stable across pregnancy and they did not differ significantly between preterm and term pregnancies at the three sampling timepoints ($p>0.05$), Figure 3.15 E and F.

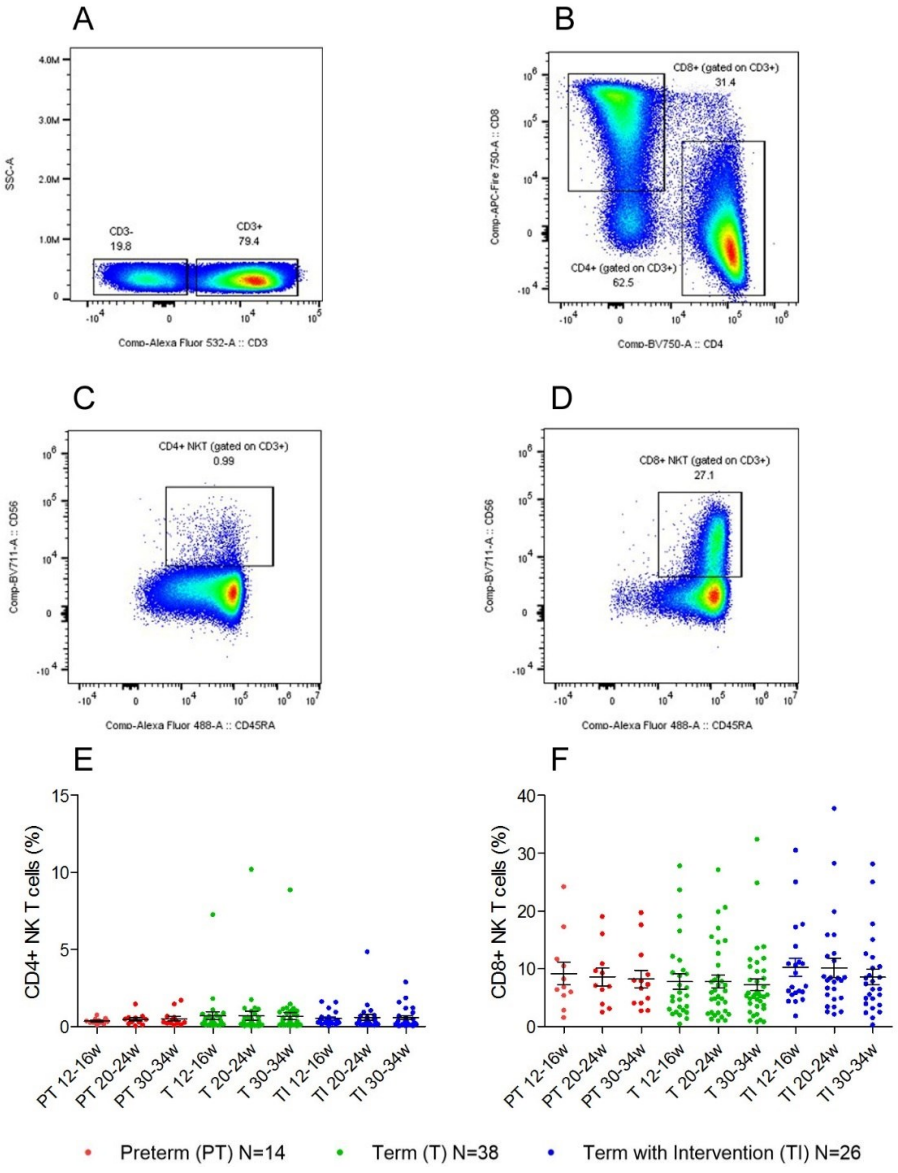


Figure 3.15 Peripheral CD4+ NK T and CD8+ NK T cells across pregnancy and comparisons with preterm and term pregnancies

CD4+ NK T cells were gated on CD3+ CD4+ CD56+ CD45RA+ (A-C). CD8+ NK T cells were gated on CD3/CD8/CD56/CD45RA (A, B, D). There was no difference in the percentage of CD4+ NK T cells (E) or CD8+ NK T cells (F) with advancing gestation or between different pregnancy outcome groups, preterm (PT), term (T), term with intervention (TI) at each of the three timepoints. (Preterm group N=14, Term (uncomplicated) group N=38, Term (intervention group) N=26). Statistical analysis was by the one-way ANOVA with Dunnett's multiple comparisons test. The results are expressed as mean ± SEM.

3.5.5 Peripheral immune cell subset signature across gestation (longitudinal cohort)

In order to assess for individual gestation effects on the peripheral immune response, the peripheral immune cell subsets were analysed in women with longitudinal samples collected at each of the three sampling timepoints, 12^{+0}-16^{+6}, 20^{+0}-24^{+6} and 30^{+0}- 34^{+6} weeks. This resulted in a study population of N=51. As described in the previous results section, women were categorised into three pregnancy outcome groups, preterm N=9, term (without intervention) N=22, and term with intervention N=20. Metadata for this longitudinal cohort is presented in Table 3.3. No significant differences in age and BMI were observed between the three pregnancy outcome groups. However, regarding ethnicity, there was a greater proportion of White women in the term (uncomplicated) group. In total, 20 women had a cervical cerclage; 13 were history indicated and 7 were ultrasound indicated.

Table 3.3 Clinical and demographical characteristics of the longitudinal PBMC study population N= 51
Longitudinal sampling was performed for each participant at all three sampling timepoints.

	Preterm (PT) (<37 weeks)	Term (T)	Term with intervention (TI)	p value (PT vs T vs TI)
N (%)	9 (18%)	22 (43%)	20 (39%)	
Gestation at delivery (weeks) median and IQ range	34+2 (32+1-36+4)	39+3 (38+3-40+1)	38+3 (38-39+1)	One-way ANOVA p<0.0001 *** PT vs T p<0.0001 PT vs TI P<0.0001
Early preterm </=33+6	3	NA	NA	
Late preterm >/=34+0	6	NA	NA	
Age median and IQ range	33 (30-40)	33 (31-38)	33 (30-36)	One-way ANOVA p=0.57
BMI median and IQ range	26 (22-28)	24 (22-27)	26 (22-30)	One-way ANOVA p=0.31
Ethnicity White N (%) Black N (%) Other N (%)	 3 (34%) 2 (22%) 4 (44%)	 17 (77%) 3 (13%) 2 (10%)	 9 (45%) 9 (45%) 2 (10%)	χ^2=0.01 **
Cervical cerclage	4	NA	16	
History indicated cervical cerclage and material	1 (Mersilene 1)	NA	12 (Mersilene 3, Nylon 6, Unknown 2, abdominal 1)	
USS indicated cervical cerclage and material	3 (Mersilene 2, Nylon 1)	NA	4 (Mersilene 2, Nylon 2)	
Progesterone only	NA	NA	4	

BMI=body mass index, USS=ultrasound. Data presented as median (interquartile range (IQ)) or number (%). PT=preterm, T=term (no intervention). TI=term with intervention. P values: One-way ANOVA for multiple comparisons or Chi squared for proportional data.

Monocytes

There was no change in the proportion of monocytes throughout pregnancy or in the monocyte activity measured by HLA-DR and PD-1 MFI with advancing gestation in this longitudinal cohort in all three pregnancy outcome groups (p>0.05), Figure 3.16 A-C. These findings are consistent with those described for the cross-sectional whole study population in section 3.5.4. The trajectory slopes were not significantly different between the three pregnancy outcome groups, Figure 3.16 D-F.

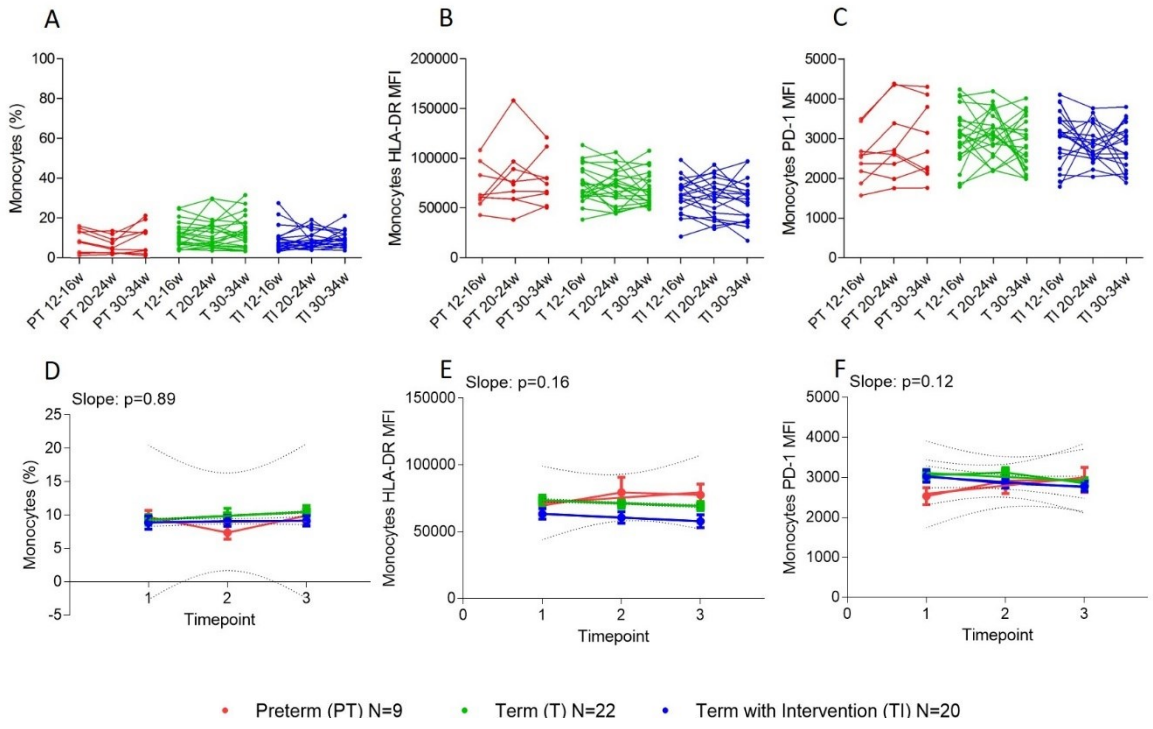


Figure 3.16 Monocytes and their effector status across pregnancy and the trajectory of change in the longitudinal cohort

The proportion of monocytes did not show any significant changes with advancing gestation (A), and the trajectories were similar across the three pregnancy outcome groups (B). The monocyte effector status as measured by HLA-DR and PD-1 median fluorescence intensity were also unchanged across pregnancy (B and C). The trajectory for monocytes, and the median fluorescence intensity (MFI) of HLA-DR and PD-1 in monocytes was similar between the three pregnancy outcome groups (D-F). (Preterm group N=9, Term group N=22, Term intervention group N=20, timepoint 1: 12-16 weeks, timepoint 2: 20-24 weeks, timepoint 3: 30-34 weeks). Statistical analysis was by the Friedman test with Dunnett's multiple comparisons test and a linear regression with the p value comparing the slopes of the three pregnancy outcome groups. The results are expressed as mean ±SEM (D-F).

CD4+ T cells

In the term (uncomplicated) group there was a significant increase in the proportion of CD4+ T cells with advancing gestation (p=0.05), Figure 3.17 A, and an increase in CD4+ CD38+ MFI (p=0.04), Figure 3.17 C.

CD4+ T cells were significantly higher in the term with intervention group at the first sampling timepoint 12^{+0}-16^{+6} weeks and remained higher for the remainder of pregnancy compared to the term and preterm group, Figure 3.17 D. The trajectory of CD4+ CD25+ MFI was significantly different between the three pregnancy outcome groups with an upwards trend in the preterm group, whilst there was more stability in the term group, Figure 3.17 E. At the first sampling timepoint, six women in the term (intervention) group had particularly high CD4+ CD25 MFI values. Further inspection of their clinical history indicated that all six had a history of preterm delivery with previous mid-trimester miscarriages and/or previous preterm births, and four of the six received history indicated cerclages, whilst the remaining two received progesterone. CD25 is the IL-2 receptor and on binding to IL-2 exerts immunoregulatory effects and is important for immune tolerance [(216)]. Therefore, it is noteworthy that the CD4+ CD25 MFI was most stable in term uncomplicated pregnancies. As already mentioned CD4+ CD38 increased significantly with advancing gestation in the term group (p=0.004), Figure 3.17 C. CD38 is involved in cell adhesion, signal transduction and calcium signalling [(217)], therefore the increase in CD38 MFI is consistent with increased cell migration and cell signalling as labour approaches.

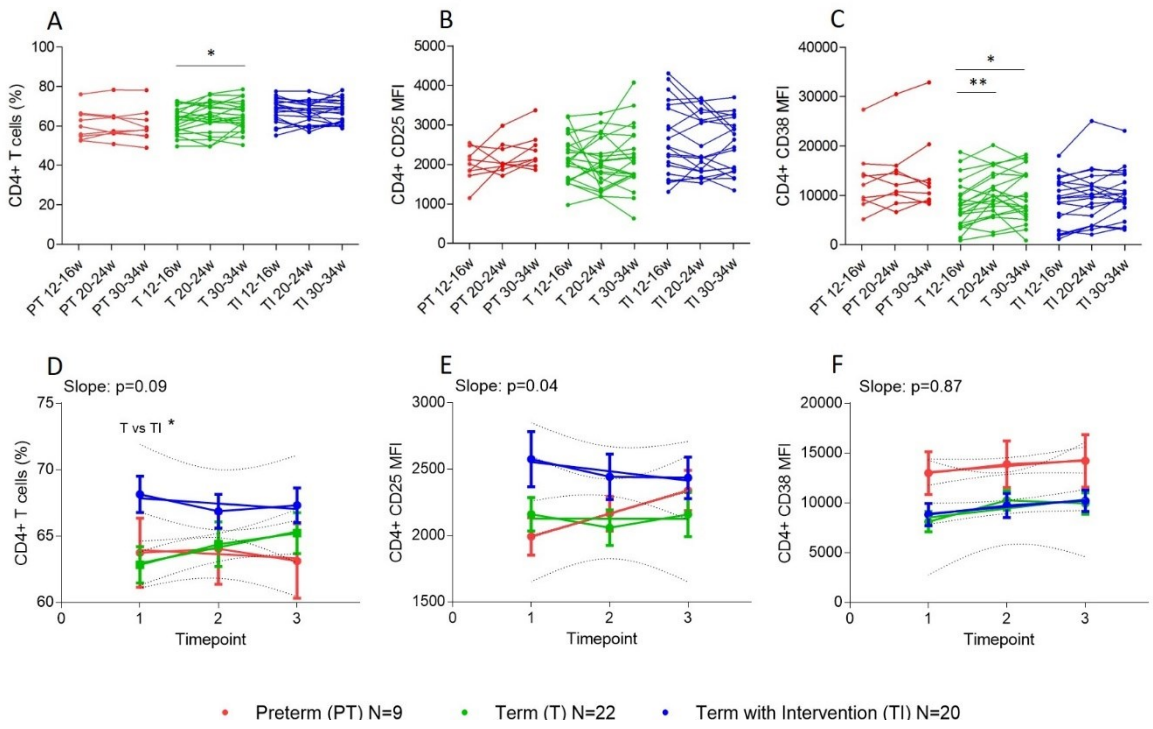


Figure 3.17 CD4+ T cells and their effector status across pregnancy and the trajectory of change in the longitudinal cohort

CD4+ T cells increased significantly with advancing gestation in the term (T) group (A), $p<0.05$ and there was a corresponding increase in CD4+ CD38 median fluorescence intensity (MFI) with advancing gestation, between 12-16 to 30-34 weeks and between 12-16 to 20-24 weeks (C). The trajectories of CD4+ CD25 MFI were significantly different across the three pregnancy outcome groups, with an increase with advancing gestation in the preterm group and a decrease in the term with intervention group (E). The trajectory slopes for CD4+ T cells and CD4+ CD38+ MFI were not significantly different across the three pregnancy outcome groups, however the CD4+ T cells were significantly lower in the term compared to the term with intervention group at the first sampling timepoint. (Preterm group N=9, Term group N=22, Term intervention group N=20, timepoint 1: 12-16 weeks, timepoint 2: 20-24 weeks, timepoint 3: 30-34 weeks). Statistical analysis was by the Friedman test with Dunnett's multiple comparisons test and a linear regression with the p value comparing the slopes of the three pregnancy outcome groups, (* $p<0.05$, ** $p<0.01$). The results are expressed as mean ± SEM (D-F).

CD8+ T cells

In the longitudinal cohort, the proportion of CD8+ T cells was stable across pregnancy in all pregnancy outcome groups, Figure 3.18 A and D, however there were differences in CD8+ cell activity as measured by the CD25 and CD38 median fluorescence intensities. CD8+ CD25 MFI increased with advancing gestation in the term group (p=0.04), Figure 3.18 B, reflecting possible increased susceptibility to IL-2 stimulation. CD8+ CD38 MFI increased with advancing gestation in both the preterm (p=0.04), and in the term intervention group (p<0.001), Figure 3.18 C, mirroring the results seen in the whole study population analysis in section 3.5.4. The rise was significantly steeper in the preterm compared to the two term groups when comparing the steepness of the slopes using a linear regression (p=0.0005), Figure 3.18 F.

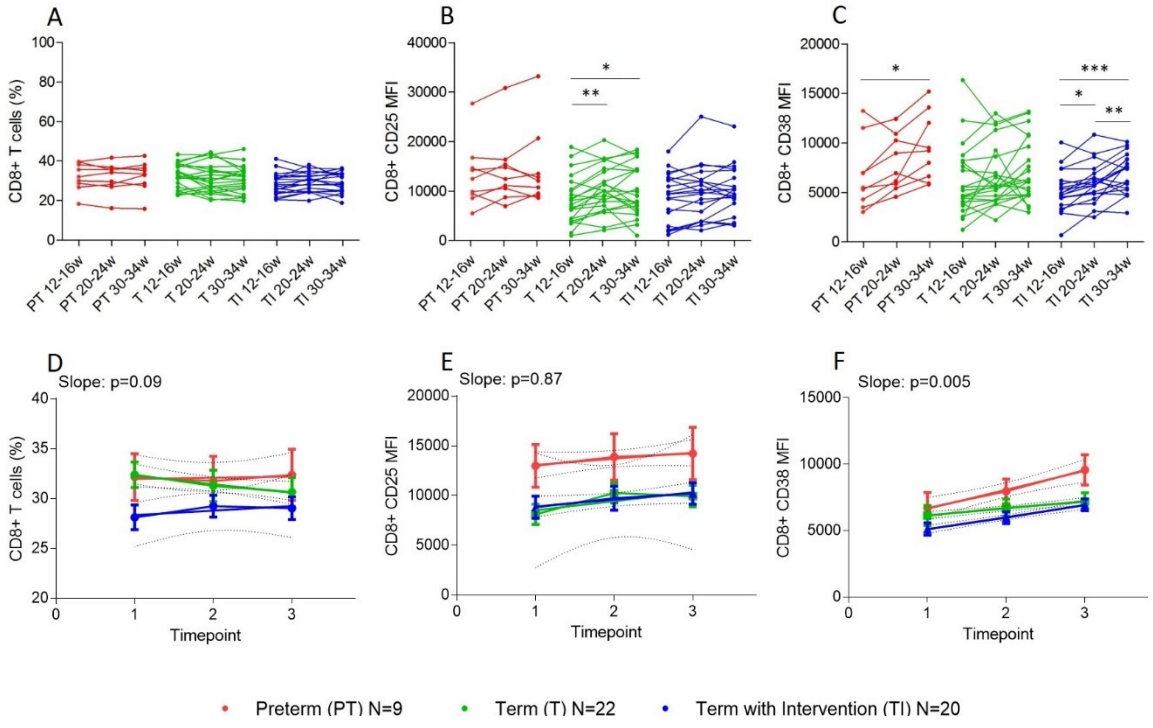


Figure 3.18 CD8+ T cells and their effector status across pregnancy and the trajectory of change in the longitudinal cohort

The proportion of CD8+ T cells was stable throughout pregnancy in all three pregnancy outcome groups, preterm (PT), term (T) and term intervention (TI), (A) with similar trajectories shown in (D). CD8+ CD25+ median fluorescence intensity (MFI) increased with advancing gestation, but only in the term group (B), and no different in trajectories between the three outcome groups (E). CD8+ CD38 MFI increased with advancing gestation in the preterm and term intervention groups and showed a significantly steeper increase in trajectory compared to the term group (F). The trajectory slopes for CD8+ T cells and CD8+ CD25 MFI were not significantly different across the three pregnancy outcome groups. (Preterm group N=9, Term group N=22, Term intervention group N=20, timepoint 1: 12-16 weeks, timepoint 2: 20-24 weeks, timepoint 3: 30-34 weeks). Statistical analysis was by the Friedman test with Dunnett's multiple comparisons test and a linear regression with the p value comparing the slopes of the three pregnancy outcome groups, (* p<0.05, ** p<0.01, *** p<0.001). The results are expressed as mean ± SEM (D-F).

Treg cells

Although there was no change in the proportion of Treg cells throughout pregnancy in the longitudinal cohort (p>0.05), the trajectory of Treg HLA-DR MFI increased in the term group and was significantly different from the preterm and term intervention groups, which showed a decreasing pattern when comparing the steepness of the slopes by a linear regression, p=0.0008, Figure 3.19. This suggests that in uncomplicated term pregnancy, the activation of Treg cells increases with advancing gestation, which fails to occur in women destined to deliver preterm.

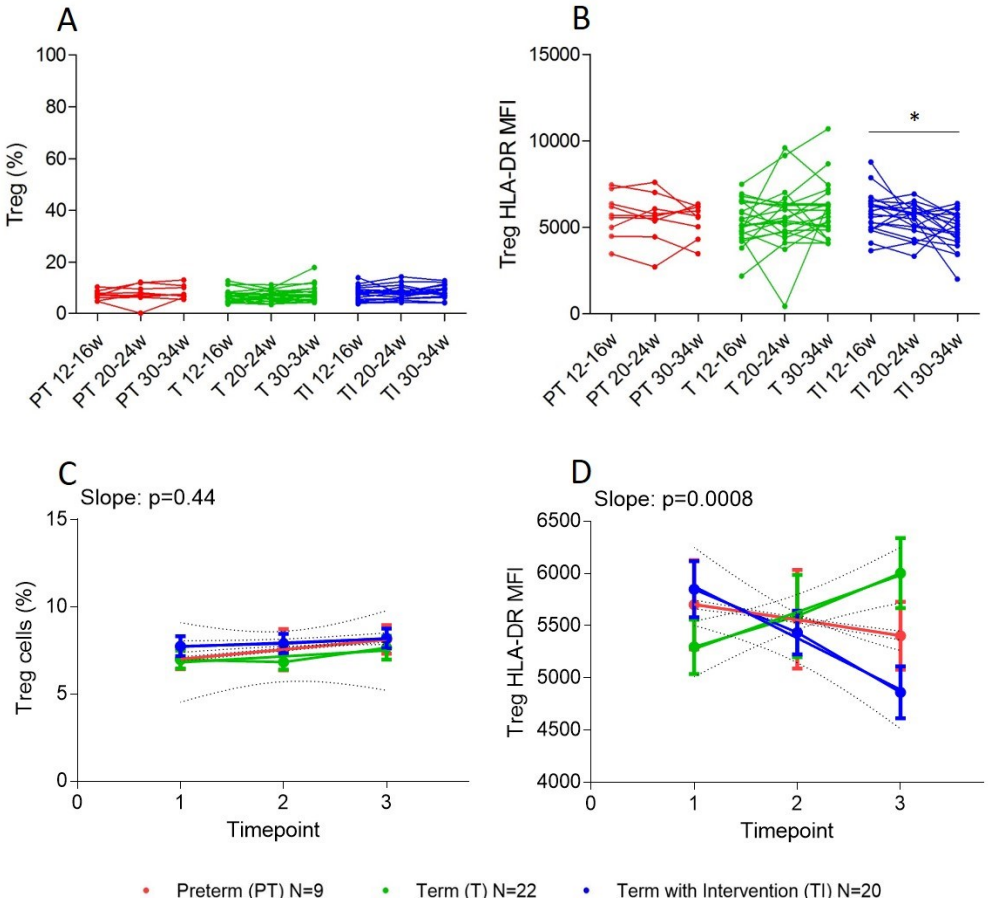


Figure 3.19 Treg cells and their effector status across pregnancy and the trajectory of change in the longitudinal cohort

The proportion of Treg cells was consistent throughout pregnancy in all three pregnancy outcome groups, preterm (PT), term (T) and term intervention (TI), (A) with similar trajectories shown in (C). The Treg HLA-DR median fluorescence intensity significantly reduced with advancing gestation in the term intervention group (B). The trajectories for Treg HLA-DR MFI were significantly different in the three pregnancy outcome groups with a marked upwards trajectory in the term group, (D). (Preterm group N=9, Term group N=22, Term intervention group N=20, timepoint 1: 12-16 weeks, timepoint 2: 20-24 weeks, timepoint 3: 30-34 weeks). Statistical analysis was by the Friedman test with Dunnett's multiple comparisons test and a linear regression with the p value comparing the slopes of the three pregnancy outcome groups, (* p<0.05). The results are expressed as mean ± SEM (C-D).

B cells

There was no change in the proportion of B cells throughout pregnancy or the B cell activity measured by IgD and PD-1 MFI with advancing gestation in this longitudinal cohort in all three pregnancy outcome groups (p>0.05), Figure 3.20. These findings are consistent with those described for the whole study population analyses in section 3.5.4. The trajectory slope for the proportion of B cells was significantly different in the preterm group compared to the two term groups (p=0.03), Figure 3.20 D, however they were similar for the B cells IgD and PD-1 MFI, Figure 3.20 E and F. IgD signalling in B cells enhances B cell humoral activity [220], whilst the PD-1 pathway inhibits B cell activation [218].

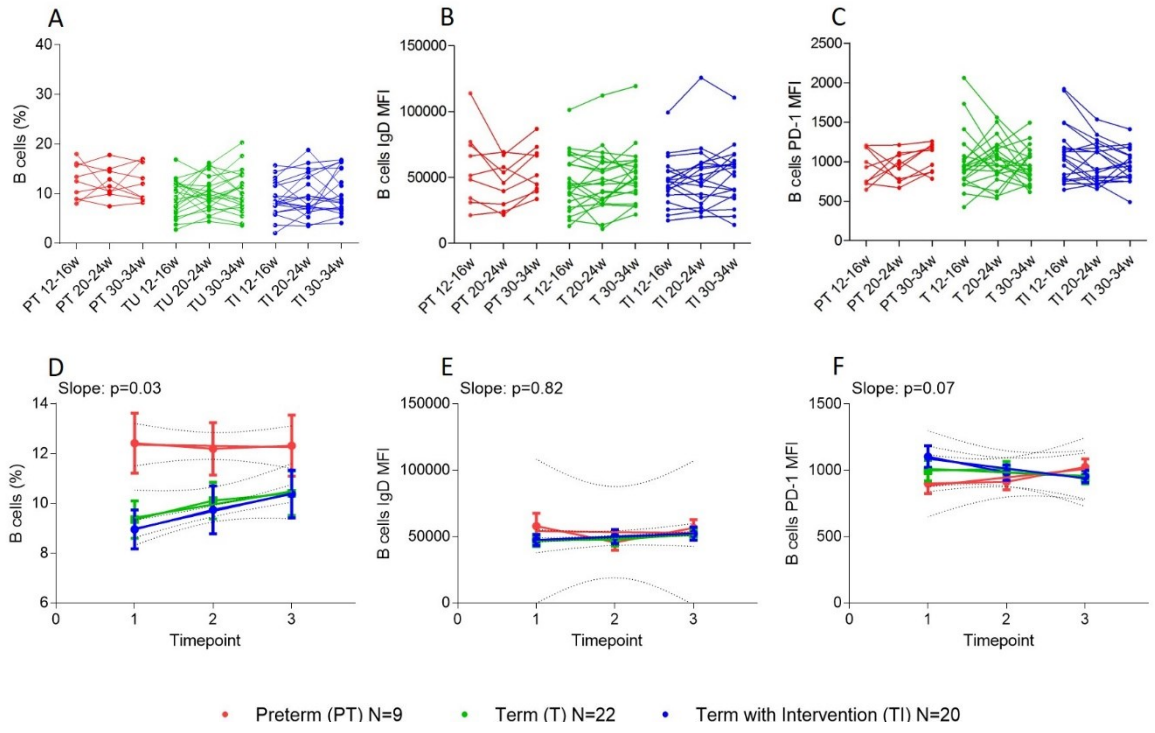


Figure 3.20 B cells and their effector status across pregnancy and the trajectory of change in the longitudinal cohort

The proportion of B cells did not show any significant changes with advancing gestation (A), however the trajectories of the preterm group was significantly different from the two term groups (D). The B cells effector status as measured by IgD and PD-1 median fluorescence intensity were also unchanged across pregnancy (B and C). The trajectory for B cell IgD median fluorescence intensity (MFI) was similar between the three pregnancy outcome groups (E). The B cell PD-1 trajectory in the preterm group showed a non-significant upwards trend compared to both term groups (F). (Preterm group N=9, Term group N=22, Term intervention group N=20, timepoint 1: 12-16 weeks, timepoint 2: 20-24 weeks, timepoint 3: 30-34 weeks). Statistical analysis was by the Friedman test with Dunnett's multiple comparisons test and a linear regression with the p value comparing the slopes of the three pregnancy outcome groups. The results are expressed as mean ± SEM (D-F).

NK cells

In the longitudinal cohort, there was a reduction in NK cells (as a percentage of the CD3- lymphocytes) in the term group between 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks, ($p<0.05$) and between 12^{+0} - 16^{+6} and 30^{+0} - 34^{+6} weeks, ($p<0.001$), however upon application of Dunnett's post hoc comparisons test, this statistical significance was lost. Additionally, there was a reduction in NK cells (as a percentage of the CD3- lymphocytes) in the term intervention group between 12^{+0} - 16^{+6} and 30^{+0} - 34^{+6} weeks ($p<0.05$), however again upon application of the Dunnett's post hoc comparisons test, this statistical significance was lost, Figure 3.21 A. NK CD38 MFI increased throughout pregnancy between the first and third timepoints within all three groups preterm ($p<0.0001$), term ($p<0.00001$) and term intervention ($p<0.001$), Figure 3.21 B, mirroring the findings in the analysis taking into account the whole study population. A steeper trajectory for this change was seen in the term group ($p=0.05$), Figure 3.21 D. CD38 signalling in NK cells is known to induce IFN-γ and GM-CSF release and NK cytotoxic activity [269].

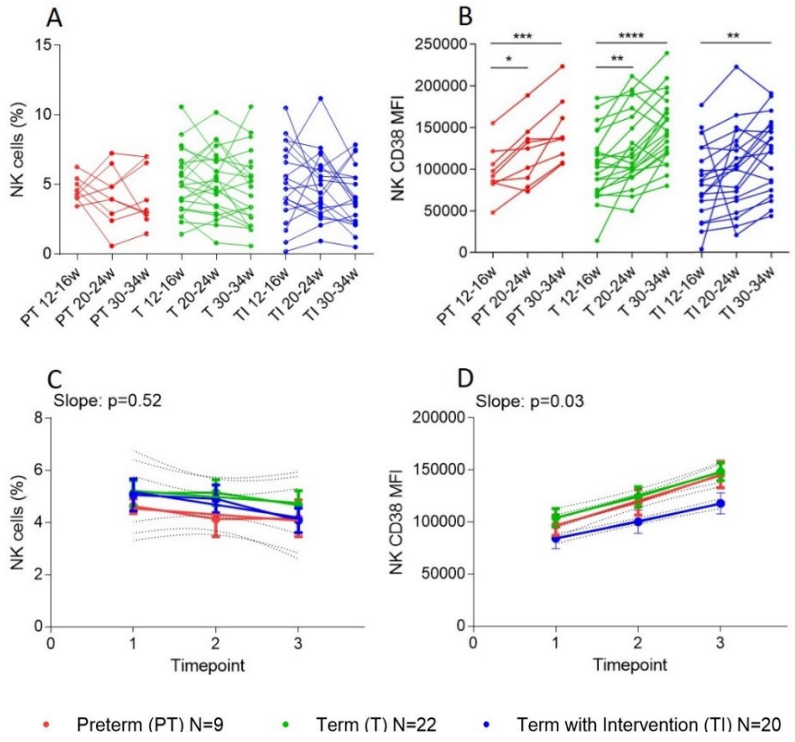


Figure 3.21 NK cells and their effector status across pregnancy and the trajectory of change in the longitudinal cohort

There was a non-significant decrease in NK cells with advancing gestation (A) and the trajectories were similar across all three pregnancy outcome groups (C). In all three outcome groups, NK CD38+ median fluorescence intensity (MFI) increased with advancing gestation (B). The steepest trajectory was seen in the term group, and the trajectory slope was significantly different for NK CD38 MFI across the three pregnancy outcome groups (D). (Preterm group N=9, Term group N=22, Term intervention group N=20, timepoint 1: 12-16 weeks, timepoint 2: 20-24 weeks, timepoint 3: 30-34 weeks). Statistical analysis was by the Friedman test with Dunnett's multiple comparisons test and a linear regression with the p value comparing the slopes of the three pregnancy outcome groups, (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ***** $p<0.00001$). The results are expressed as mean ± SEM (C-D).

3.5.6 Plasma cytokines and complement across preterm and term pregnancy

Plasma cytokines and complement concentrations were analysed in a subset of the study population with peripheral blood mononuclear cell analysis. This cohort was selected at the time of an interim analysis from the first 32 women with outcome data. Plasma was collected at 3 timepoints throughout pregnancy, 12^{+0}-16^{+6}, 20^{+0}-24^{+6} and 30^{+0} - 34^{+6} weeks on the same day as collection of PBMCs. Women were categorised into three groups based upon their pregnancy outcome. Women who delivered preterm <37 weeks gestation, N=7, women who delivered at term with no intervention N=12 and women who delivered at term with an intervention to delay preterm birth (e.g. cerclage +/- progesterone) N=13. Their clinical and demographical characteristics are shown in Table 3.4. There was no difference in age, BMI or ethnicity across the three pregnancy outcome groups. In total 16 (50%) women had a cervical cerclage.

Table 3.4 Clinical and demographical characteristics of the plasma cytokine and complement study population N= 32

	Preterm (PT) (>37 weeks)	Term (T) No intervention	Term with intervention (TI)	p value (PT vs T vs TI)
N (%)	7 (22%)	12 (38%)	13 (40%)	
Gestation at delivery (weeks) median and IQ range	34+2 (31+6-36+0)	39+1 (38+4-39+4)	38+5 (38-40)	One-way ANOVA p<0.0001 *** PT vs TU p<0.0001 PT vs TI P<0.0001
Early preterm </=33+6	4	NA	NA	
Late preterm >/=34+0	3	NA	NA	
Age median and IQ range	33 (27-39)	37 (29-38)	34 (33-37)	One-way ANOVA p=0.7157
BMI median and IQ range	26 (21-30)	25 (20-28)	23 (22-28)	One-way ANOVA p=0.8884
Ethnicity White N (%) Black N (%) Other N (%)	 3 (42%) 2 (28%) 2 (28%)	 8 (68%) 2 (16%) 2 (16%)	 5 (38%) 4 (31%) 4 (31%)	χ^2=0.7048
Cervical cerclage	5	NA	11	
History indicated cervical cerclage and material	2 (Mersilene 1, Nylon 1)	NA	8 (Mersilene 0, Nylon 5, Unknown 2, Abdominal 1)	
USS indicated cervical cerclage and material	3 (Mersilene 2, Nylon 1)	NA	3 (Mersilene 1, Nylon 2)	
Progesterone only	NA	NA	4	
Risk factor for PTB: (by pt) Cervical treatment Cervical treatment + MTL Cervical treatment + PTB MTL MTL + PTB PTB 3x1st trimester misc Short cervix at anomaly	 0 0 2 2 0 3 0 0	 5 0 1 2 0 3 1 0	 4 0 1 2 1 4 0 1	
Risk factor for PTB Cervical treatment Previous PTB Previous MTL	 2 (29%) 5 (71%) 2 (29%)	 6 (50%) 4 (33%) 2 (17%)	 5 (38%) 6 (46%) 3 (23%)	χ^2 p=0.78

BMI=body mass index, USS=ultrasound. PT=preterm, T=term (no intervention). TI=term with intervention. Data presented as median (interquartile range (IQ)) or number (%). P values: One-way ANOVA for multiple comparisons or Chi squared for proportional data.

Plasma cytokines

Undiluted plasma samples were used to analyse an 11-plex panel of cytokines by magnetic Luminex® immunoassays. Only one of the cytokines, IL-18 was detectable in all samples tested, one in 40% of samples, IFN-γ; the rest were detected in less than 20% of samples, Table 3.5. In descending order of percentage of results falling within the standard curve range of the assay, the cytokines from most to least detectable were IL-18, IFN-γ, IL-2, IL-4, IL-8, TNF-α, GM-CSF, IL-5, IL-1β, IL-6 and IL-10. Since 9 of the 11 cytokines were not consistently detectable, cytokine analysis was not extended to the full cohort.

Table 3.5 Plasma cytokine assay ranges
The total number of plasma samples was 89, from 32 study participants. 25 of these provided samples at each of the three sampling timepoints.

Cytokine	Standard curve range (pg/ml)	Samples within standard curve range of assay (N) (Total = 89)	% within standard curve range of assay	Sensitivity level (pg/ml)
IL-18	10.12 - 2460	89	100%	1.93
IFN-γ	58.48 - 14210	36	40.4%	0.4
IL-2	29.63 - 7200	17	19.1%	1.8
IL-4	14.61 - 3550	17	19.1%	9.3
IL-8	5.19 - 1260	14	15.7%	1.8
TNF-α	9.71 - 2360	11	12.4%	1.2
GM-CSF	12.22 - 2970	9	10.1%	4.1
IL-5	6.63 - 1610	3	3.4%	0.5
IL-1β	19.51 - 4740	1	1.1%	0.8
IL-6	4.73 - 1150	0	0%	1.7
IL-10	4.77 - 1160	0	0%	1.6

IL-18

Plasma IL-18 was observed to increase with advancing gestation with a statistically significant difference detected in both term groups (term no intervention p=0.004, term with intervention p<0.0001) in the longitudinal cohort, Figure 3.22 B. This upwards trajectory with advancing gestation was seen across the three pregnancy outcome groups, Figure 3.22 C.

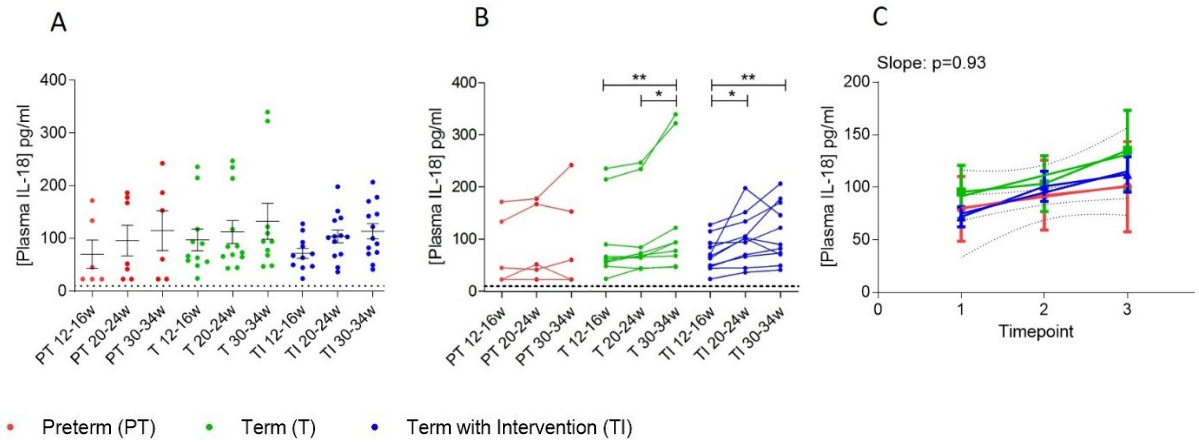


Figure 3.22 Plasma IL-18 across pregnancy and comparisons between preterm and term pregnancies
Plasma IL-18 concentrations did not differ significantly between different pregnancy outcome groups, preterm (PT), term (T) and term intervention (TI) in samples from the whole study population (A) (N=32) or samples from the longitudinal cohort (B) (N=25), timepoint 1: 12-16 weeks, timepoint 2: 20-24 weeks, timepoint 3: 30-34 weeks). In the longitudinal cohort, there was a significant increase in plasma IL-18 with advancing gestation in the term and term intervention groups. The dotted line marks the concentration of the lowest standard on the assay's standard curve. Statistical analysis was by the one-way ANOVA with Dunnett's multiple comparisons test and a linear regression, (* p<0.05, ** p<0.01) with the p value comparing the slopes of the three pregnancy outcome groups. All plasma samples to detect IL-18 were undiluted. The results are expressed as mean ±SEM.

Other cytokines

The concentrations of plasma cytokines IFN-γ, IL-2, IL-4, IL, IL-8, TNF-α, GM-CSF, IL-5, IL-1β, IL-6 and IL-10 across pregnancy in the pregnancy outcome groups in samples from the whole study population, preterm (PT), term (T) and term intervention (TI) are shown in Figure 3.23. No significant differences between the pregnancy outcome groups or across as a function of gestational age was observed for any of these cytokines ($p>0.05$). Similarly, analysis of the longitudinal cohort did not demonstrate any gestation related changes (data not shown).

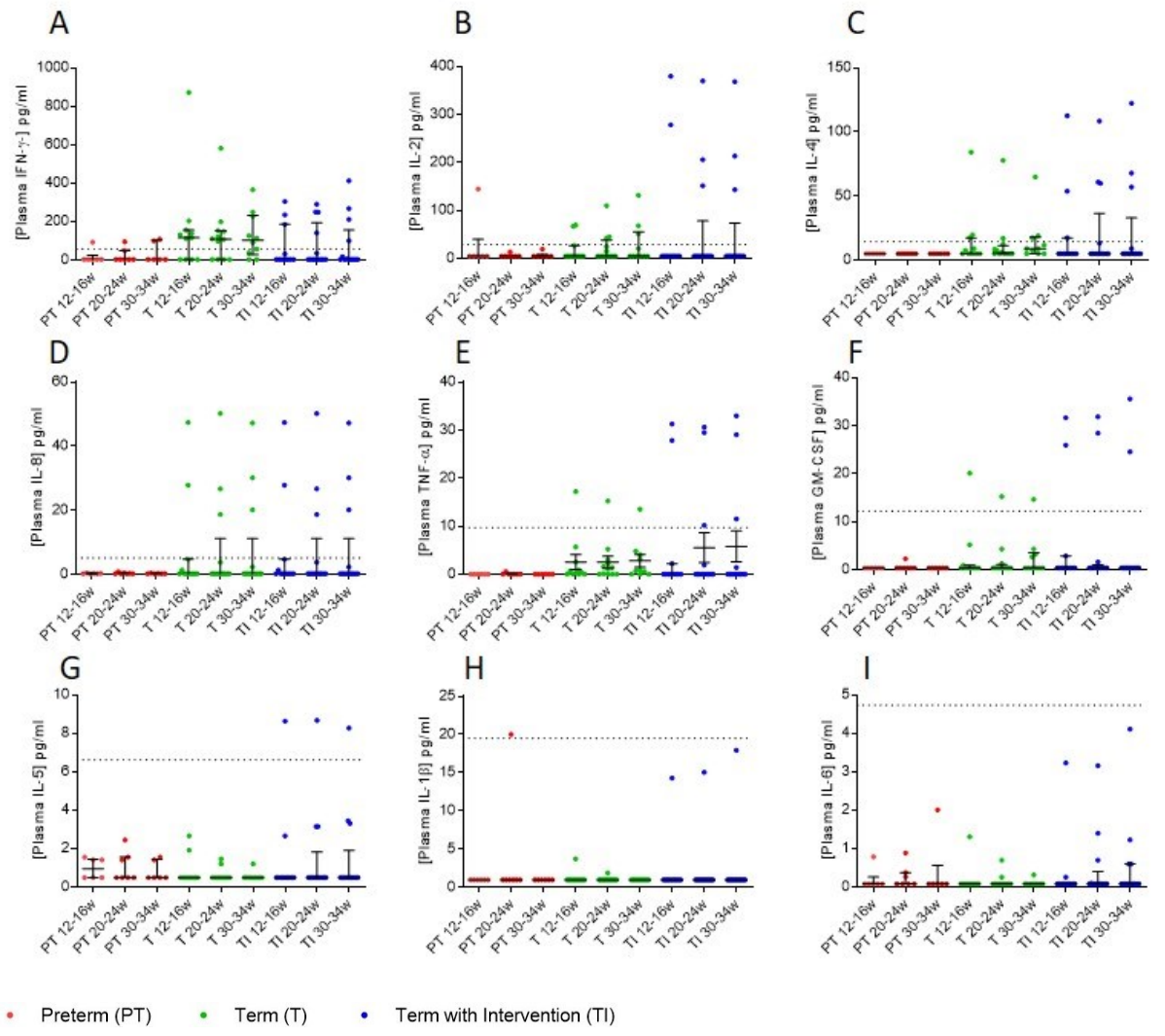


Figure 3.23 Plasma cytokines across pregnancy and comparisons between preterm and term pregnancies
Plasma cytokines at three gestation time points in three different pregnancy outcome groups, preterm (PT) N=14, term (T) N=30 and term intervention (TI) N=26 are shown. The dotted line on each graph indicates the lowest standard of each respective analyte on the multiplex assay. The number of concentrations measured below the lowest standard ranged between 60-100% for individual analytes. Of the concentrations that could be detected, there was no difference between the pregnancy outcome groups or across gestation. Statistical analysis was by the Kruskal-Wallis test with Dunn's multiple comparisons test. The horizontal dotted lines mark the concentration of the lowest standard on the assay. The results are expressed as median and interquartile range.

IL-18 is pro-inflammatory and induces cell-mediated immunity following microbial infection. It is produced by macrophages and monocytes, and stimulates NK and T cells, releasing IFN-γ, which then activates more cells of the innate immune response. IL-18 concentrations increased with advancing gestation in the longitudinal cohort, but other Th1 cytokines IFN-γ, IL-2 and TNF-α were not detectable in many samples, therefore the same gestational rise was not seen, Figure 3.24 A-D. However, IL-18 did positively correlate with Th1 cytokines, IFN-γ, TNF-α and IL-2 ($p<0.0001$, $p=0.01$ and $p=0.0009$ respectively), Figure 3.24 E-G, whilst IFN-γ also positively correlated with IL-2 ($p<0.0001$), Figure 3.24H. In the presence of IL-2, the concentrations of IL-18, IFN-γ and TNF-α were significantly higher ($p<0.01$, $p<0.0001$, $p<0.0001$ respectively), Figure 3.24 I-K. The ratio of IFN-γ:IL4 was greater if IL-2 was detectable ($p<0.0001$) Figure 3.24 L. This supports the hypothesis that advancing gestation is associated with a shift to a Th1 response, via IL-18.

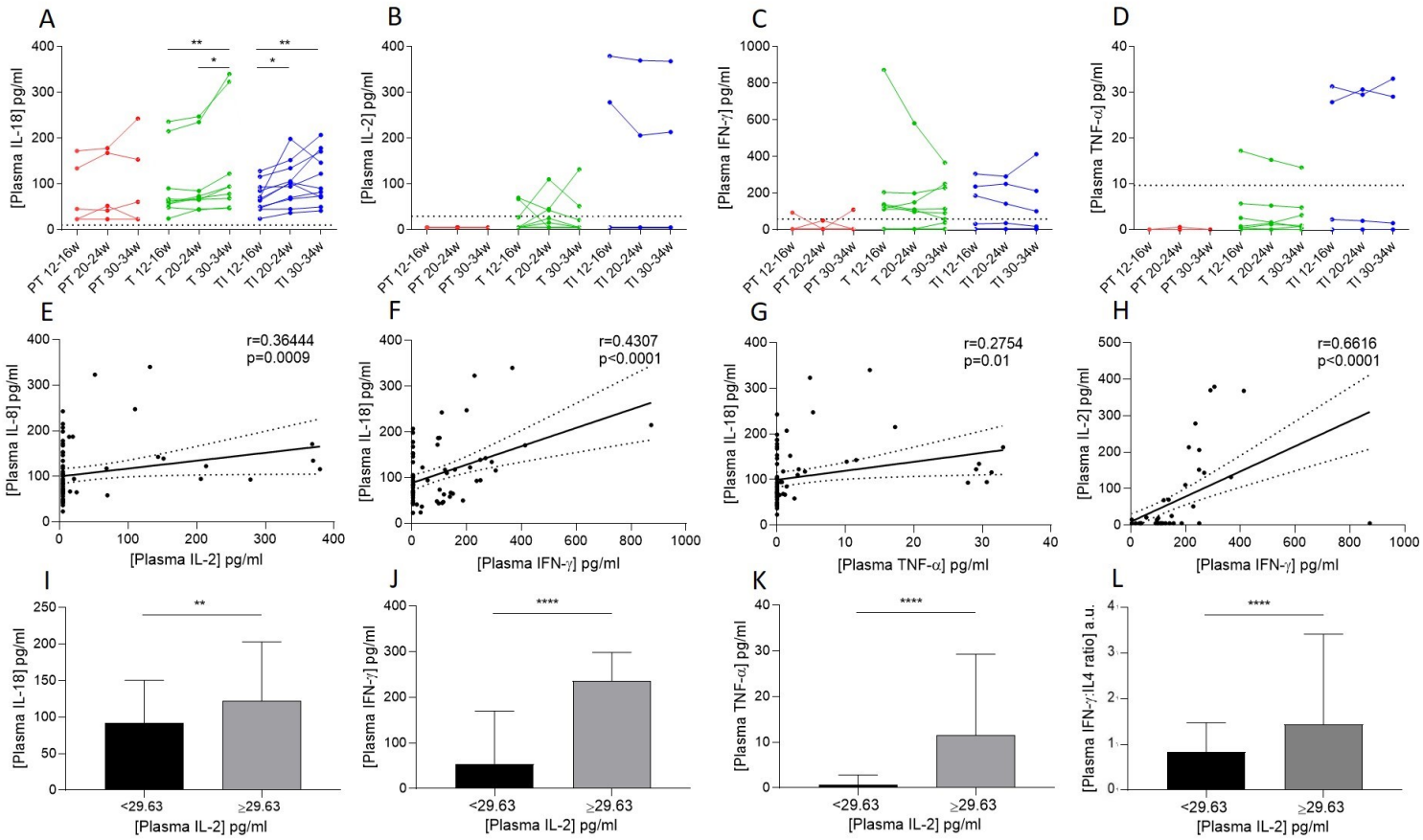


Figure 3.24 Plasma IL-18 and other pro-inflammatory cytokines
Plasma IL-18 increased with gestational age in longitudinal collected plasma samples (A) and was the most detectable plasma cytokine. Plasma IL-2, IFN-γ and TNF-α plasma cytokines were undetectable in many samples (B-D). However, IL-2, IFN-γ and TNF-α all positively correlated with increasing IL-18 (E-G). N=25 in the longitudinal cohort (A-D). Significantly higher concentrations of IL-18, IFN-γ and TNF-α were measured when IL-2 was greater than the lowest standard on the assay (29.63 pg/ml), compared to levels below the lowest standard (I-K). Incorporating the IFN-γ:IL-4 ratio this also increased when IL-2 was greater than 29.63 pg/ml. This supports a shift to a TH1 response with advancing gestation. N=71 plasma samples (E-L). Statistical analyses were performed with Friedman one-way ANOVA, Spearman correlation and Mann-Whitney U tests. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

As described in section 3.5.4, NK CD38 median fluorescence intensity (MFI) increased with advancing gestation, but as shown in Figure 3.25 A-C it negatively correlated with the Th1 cytokines, IL-18, IL-2 and IFN-γ in plasma (p=0.05, p=0.03 and p=0.06). Cytotoxic T cell activity as measured by CD25 MFI, however positively correlated with plasma IL-18 (p=0.03), Figure 3.25 D. Although reaching statistical significance, these correlations were weak. IL-18 is mainly produced by monocytes, but there was no correlation between the percentages or activation of peripheral monocytes and plasma IL-18 (p>0.05). IL-18 potentiates the cytotoxic activity of NK cells, but there was no correlation between peripheral NK cells and plasma IL-18. There was a positive correlation between monocytes and IL-2 in keeping with IL-2 being a strong monocyte activator, Figure 3.25 E.

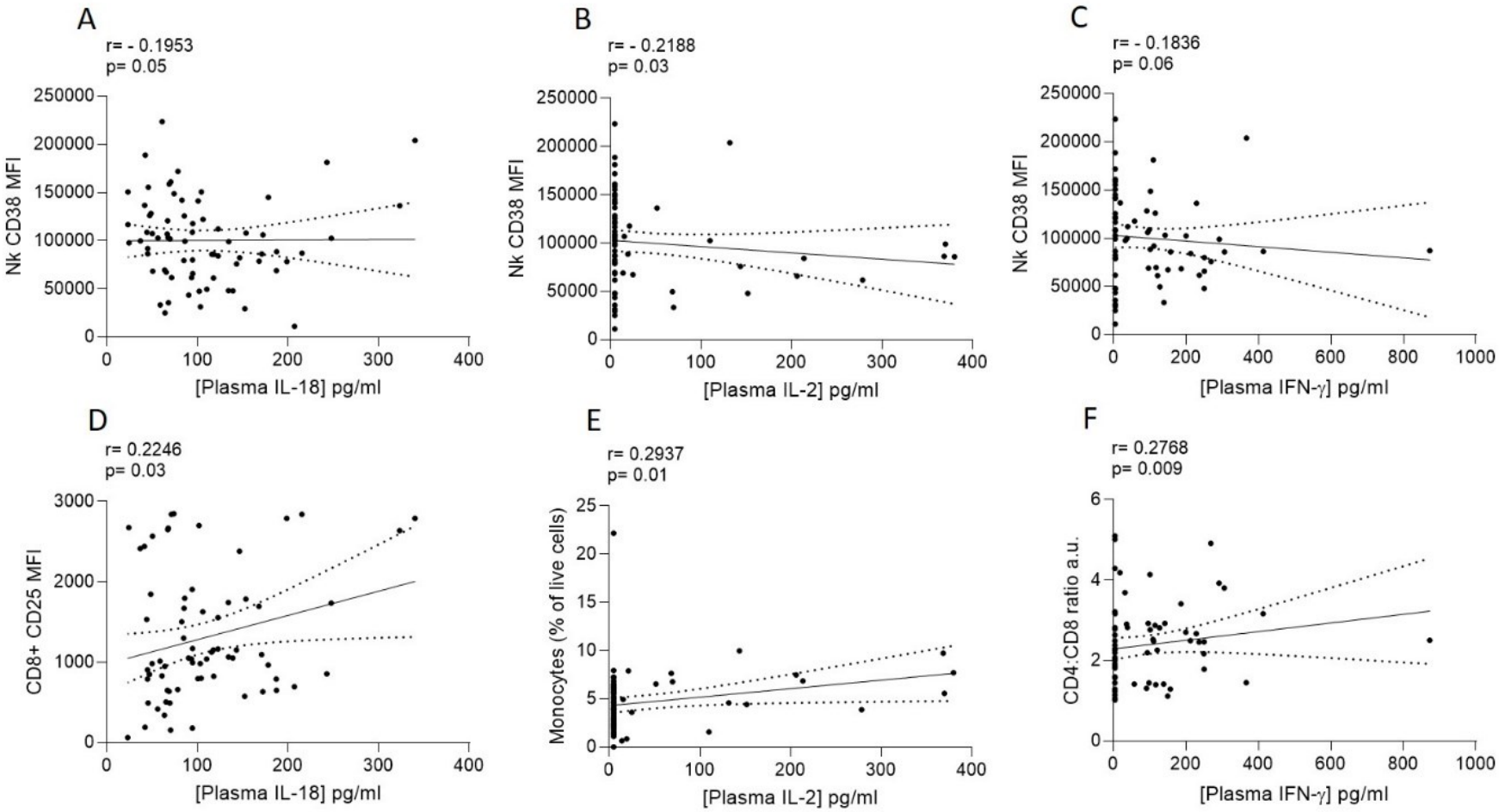


Figure 3.25 Correlations between plasma cytokines and NK cell and B cell activation, monocytes, CD4+ and CD8+ cells

Plasma IL-18, IL2 and IFN-γ negatively correlated with NK CD38 median fluorescence intensity (MFI), (A-C), whilst plasma IL-18 positively correlated with CD8+ CD25 MFI (D). Although there was no observed increase in plasma IL-2 or monocytes with advancing gestation, they did positively correlate with each other (E). Additionally, plasma IFN-γ positively correlated with the CD4:CD8 ratio (F). Statistical analysis was by Spearman correlation. N=71 samples.

Plasma complement

Plasma C5, C5a and MBL analytes were measured on the same multiplex panel, and C3b was measured on a single plex. As per the manufacturer's recommendation and my own titration optimisation experiments, plasma was diluted 1:200 for the C5, C5a and MBL plate and 1:400 for analysis of C3b. The assay range and standard curve range for each analyte are shown in Table 3.6.

Table 3.6 Plasma complement assay ranges
The total number of plasma samples was 89, from 32 study participants. 25 women provided samples at each of the three sampling timepoints.

Complement analyte	Standard curve range by manufacturer	Sensitivity level	Samples within standard curve range of assay (N) (Total = 89)	% within standard curve range of assay
C5	2.74-2000 ng/ml	1.04	89	100%
C5a	4.12-3000 pg/ml	0.0051	19	21%
MBL	0.14-100 ng/ml	0.04	88	99%
C3b	8.23-6000 ng/ml	3.639	89	100%

Plasma C5, C5a, MBL and C3b concentrations were compared between groups (preterm, term and term with intervention) and within groups across the three sampling timepoints, 12^{+0} - 16^{+6}, 20^{+0} - 24^{+6} and 30^{+0} - 34^{+6} weeks gestation. In total samples were collected for 32 individuals, and the longitudinal cohort included 25 individuals. There were 7 study participants who did not have longitudinal sampling at all three timepoints for two reasons, firstly there was one woman who delivered preterm before the third sampling timepoint and secondly six women failed to attend the scheduled clinic appointment. There were no significant differences in C5, C3b or MBL concentrations between the three pregnancy outcome groups, or across pregnancy ($p>0.05$). Only 21% of the results for C5a were within the range of the standard curve. The trajectories for all analytes were similar with advancing gestation, Figures 3.26.

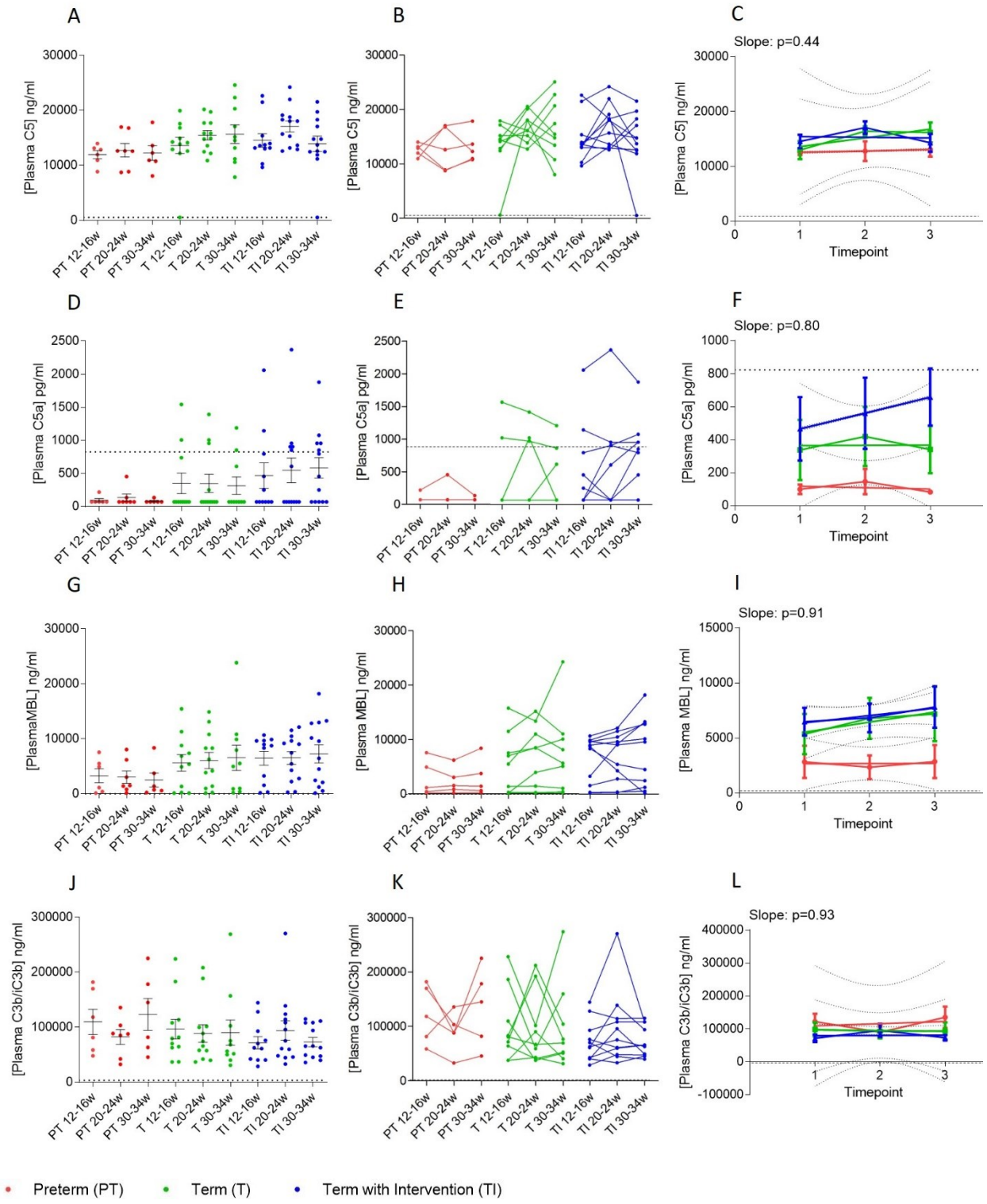


Figure 3.26 Plasma complement across pregnancy and comparisons between preterm and term pregnancies
Plasma C5, C5a, MBL and C3b concentrations did not differ significantly between different pregnancy outcome groups, preterm (PT), term (T) and term intervention (TI) or with advancing gestation in all samples (A, D, G and J) (N=32) or samples from the longitudinal cohort (B, E, H, K, C, F, I and L) (N=25). The trajectory of plasma C5, C5a, MBL and C3b were similar across the three pregnancy outcome groups (C, F, I and L). Statistical analysis was by the one-way ANOVA (all samples) or Friedman test (longitudinal cohort) with Dunnett's multiple comparisons test and a linear regression. Plasma samples were diluted 1:400 for C3b, and 1:20 for C5, C5a and MBL. The results are expressed as mean ±SEM (A,D,G,J).

3.5.7 Cervical shortening and the peripheral immune response

Peripheral blood mononuclear cells

In order to assess if there was a peripheral immune signature in women who subsequently undergo cervical shortening, the study participants with PBMC results were separated into two groups and compared; those who had cervical shortening during their pregnancy, (identified as a cervical length ≤25mm) and those who had normal cervical length measurements throughout their pregnancy, >25mm. PBMCs were collected from 78 women. Twenty-seven had cervical shortening during the pregnancy, and fifty-one maintained a normal cervical length. In those who experienced cervical shortening, at the 12^{+0} - 16^{+6} week timepoint, there were 16 women who had PBMC collected prior to cervical shortening and at the 20-24 week timepoint, there were 13 women who had PBMC collected prior to cervical shortening. There were 51 women with no cervical shortening. This is summarised in Figure 3.27. Their clinical and demographical characteristics are described in Table 3.7.

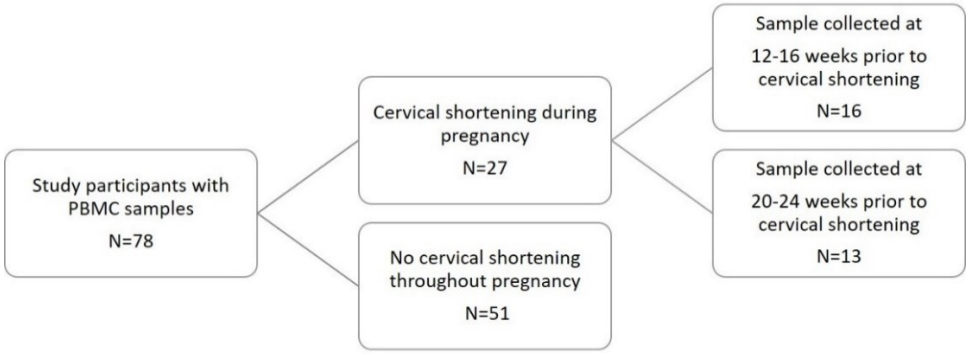


Figure 3.27 The selection of study participants to compare PBMC immune responses in women who develop cervical shortening compared to women who do not

No difference in the age or ethnicity was observed between women with or without cervical shortening, however BMI was significantly higher in those experiencing cervical shortening (p=0.02). Women with cervical shortening delivered at a significantly earlier gestation compared to women without cervical shortening (p<0.05). The median gestational age at delivery for those with cervical shortening at 12-16 or 20-24 weeks was 38^{+0} and 37^{+6} weeks^{+days} respectively compared to 39^{+1} for the no shortening group, therefore cervical shortening was associated with earlier delivery. At the point of PBMC sampling, cervical lengths in the cervical shortening group were significantly less than those in the no cervical shortening group (p=0.02), even though women in the shortening group had not yet shortened below 25mm at this stage. Taking into account the shortest cervical length during at any point during the pregnancy, the median shortest cervical length in the groups experiencing cervical shortening was 22mm, compared to 32mm in the group with no cervical shortening, (p<0.0001).

Table 3.7 Clinical and demographical characteristics of the study population, comparing PBMC in women with and without cervical shortening

	A	B	C	
	Cervical shortening (sampling at 12-16 weeks)	Cervical shortening (sampling at 20-24 weeks)	No cervical shortening (sampling at 12-16 and 20-24 weeks)	p value (column A vs B and column B vs C)
N	16	13	51	
Gestation at delivery (weeks) median and IQ range	38+0 (37+0-40+0)	37+6 (37+0-38+0)	39+1 (37+5-40+0)	A vs C: t test p=0.02 * B vs C: t test p=0.04 *
Early preterm </=33+6	1	1	2	Preterm vs Term A vs C: χ^2=0.01 * B vs C: χ^2=0.23
Late preterm >/=34+0	2	2	5	
Term cohort	4	5	33	
Term intervention cohort	9	5	11	
Age median and IQ range	33 (31-39)	34 (31-38)	33 (31-37)	A vs C: t test p=0.27 B vs C: t test p=0.22
BMI median and IQ range	27 (23-29)	27 (23-30)	24 (21-27)	A vs C: t test p=0.02 * B vs C: t test p=0.02 *
Ethnicity White N (%) Black N (%) Other N (%)	 10 (62.5%) 4 (25%) 2 (12.5%)	 5 (38%) 5 (38%) 3 (24%)	 31 (60%) 10 (20%) 10 (20%)	A vs B: χ^2=0.77 B vs C: χ^2=0.28
Cervical length at point of PBMC sampling median and IQ range (mm) 12-16 weeks 20-24 weeks	 33 (30-39) NA	 NA 30 (29-34)	 35 (31-40) 35 (32-39)	A vs C: t test p=0.02 * B vs C: t test p=0.02 *
Shortest cervical length during pregnancy median and IQ range (mm)	22 (19-24)	22 (18-23)	32 (29-35)	A vs C: t test p<0.0001 **** B vs C: test p<0.0001 ****
Cervical cerclage	9 (56%) 3 cerclages in situ at time of sampling	6 (46%) 6 cerclages in situ at time of sampling	12 (23%)	
History indicated cervical cerclage and material	5 (Mersilene 2, Nylon 2, Unknown 1)	3 (Mersilene 1, Nylon 1, Unknown 1)	12	
USS indicated cervical cerclage and material	4 (Mersilene 3, Nylon 1)	3 (Mersilene 1, Nylon 2)	0	
Progesterone only	2	1	0	

BMI=body mass index, USS=ultrasound. Data presented as median (interquartile range (IQ)) or number (%). P values: One-way ANOVA for multiple comparisons or Chi squared for proportional data.

Monocytes

The proportion of monocytes (as a percentage of live cells), and their subsets, classical, intermediate and non-classical monocytes (as a percentage of monocytes) were similar in women who did or did not develop cervical shortening when sampled at 12^{+0} - 16^{+6} or 20^{+0} - 24^{+6} weeks gestation ($p>0.05$). The monocyte effector status as measured by the HLA-DR and PD-1 MFI also did not show any differences, Figure 3.28.

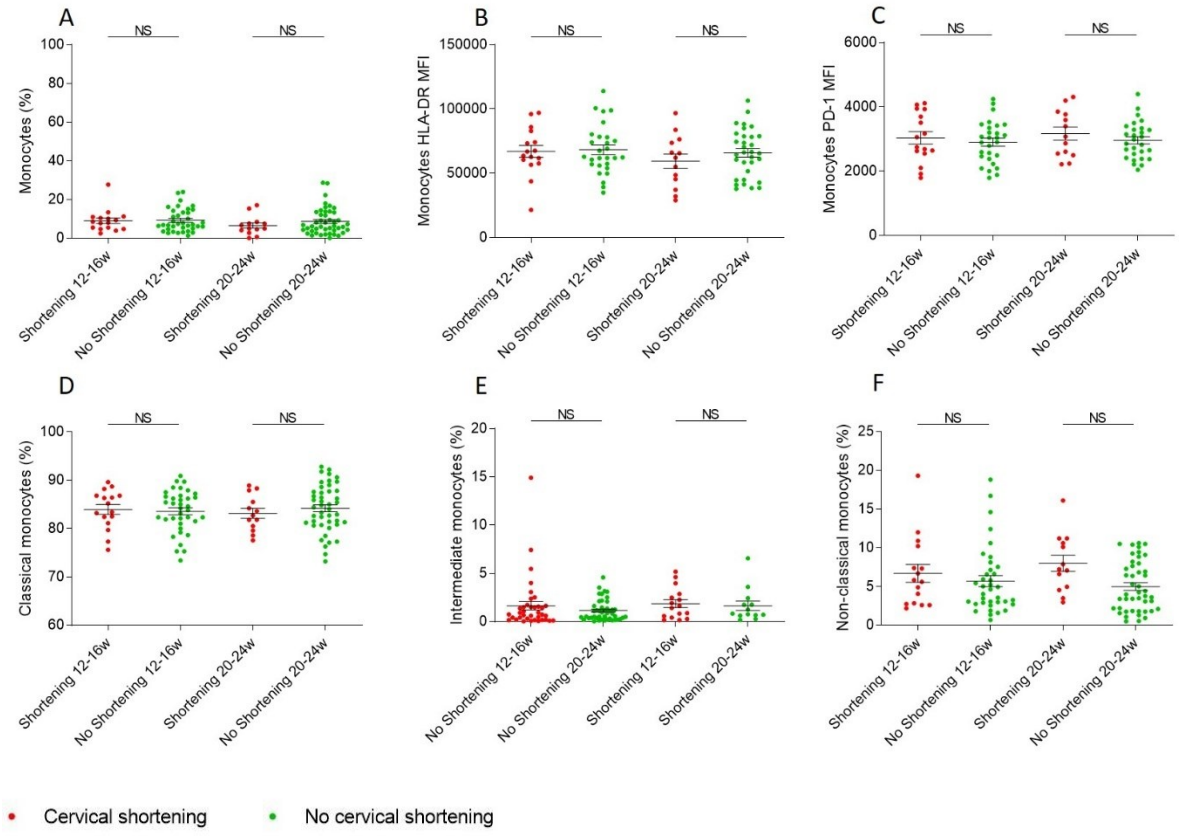


Figure 3.28 Comparison of monocytes, subsets and their effector status in women who do or do not develop cervical shortening

There was no significant difference in the proportion of monocytes, their subsets (classical, intermediate and non-classical monocytes) or in the monocytes HLA-DR and PD-1 median fluorescence intensity (MFI) in women who do or do not develop cervical shortening at 12-16 weeks or 20-24 weeks gestation, prior to cervical shortening. (Shortening 12-16 weeks N=16, Shortening 20-24 weeks N=13, No shortening at 12-16 or 20-24 weeks N=51). Statistical analysis was by an unpaired t-test. The results are expressed as mean ±SEM.

Lymphocytes and CD3+ T cells

The proportion of lymphocytes as a percentage of gated live cells and CD3+ lymphocytes (T cells) were similar in women who did and did not develop cervical shortening when sampled at 12^{+0} - 16^{+6} or 20^{+0} - 24^{+6} weeks gestation (p>0.05), Figure 3.29.

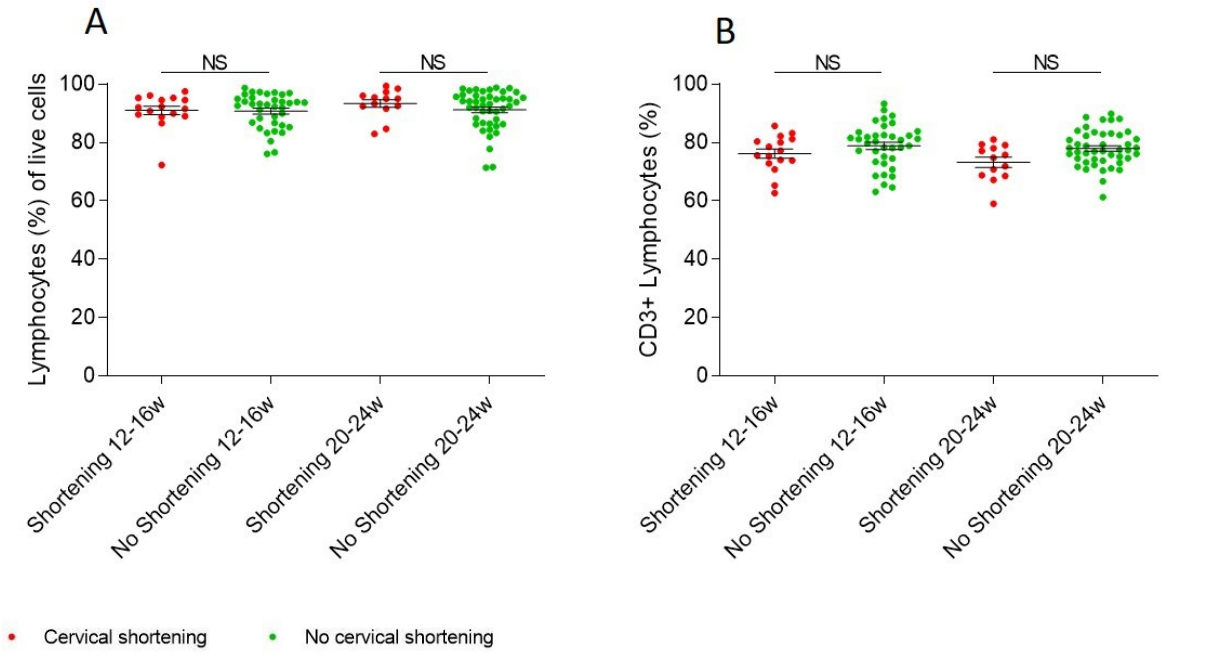


Figure 3.29 Comparison of peripheral lymphocytes in women who do or do not develop cervical shortening
There was no significant difference in the proportion of lymphocytes, (as a percentage of live cells) (A) or CD3+ lymphocytes (B) in women who do or do not develop cervical shortening at 12-16 weeks or 20-24 weeks gestation, prior to cervical shortening. (Shortening 12-16 weeks N=16, Shortening 20-24 weeks N=13, No shortening at 12-16 or 20-24 weeks N=51). Statistical analysis was by an unpaired t test. The results are expressed as mean ±SEM.

CD4+ T cells

The proportion of CD4+ T cells (as a percentage of CD3+ cells) were similar in women who did and did not develop cervical shortening when sampled at 12^{+0} - 16^{+6} or 20^{+0} - 24^{+6} weeks gestation (p>0.05). The CD4+ CD25, CD38, HLA-DR and PD-1 MFI also did not show any differences (p>0.05), Figure 3.30.

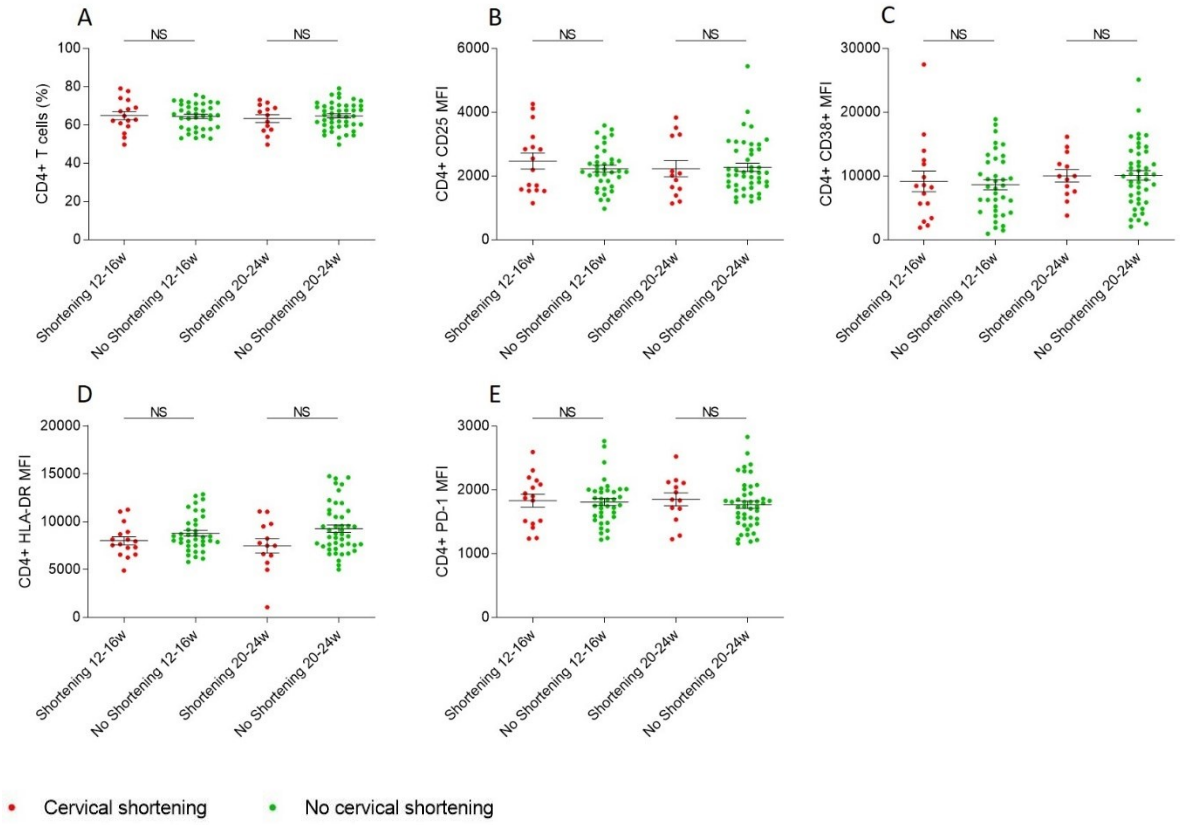


Figure 3.30 Comparison of CD4+ T cells and their effector status in women who do or do not develop cervical shortening

There was no significant difference in the proportion of CD4+ T cells (A) or in the CD4+ T cells CD25 (B), CD38 (C), HLA-DR (D) and PD-1 (E) median fluorescence intensity (MFI) in women who do or do not develop cervical shortening at 12-16 weeks or 20-24 weeks gestation, prior to cervical shortening. (Shortening 12-16 weeks N=16, Shortening 20-24 weeks N=13, No shortening at 12-16 or 20-24 weeks N=51). Statistical analysis was by an unpaired t test. The results are expressed as mean ±SEM.

CD8+ T cells

There was no difference in the proportion of CD8+ T cells (as a percentage of CD3+ cells) in women who did and did not develop cervical shortening when sampled at 12^{+0} - 16^{+6} or 20^{+0} - 24^{+6} weeks gestation ($p>0.05$). The CD8+ CD25, CD38, HLA-DR and PD-1 MFI were also similar ($p>0.05$), Figure 3.31.

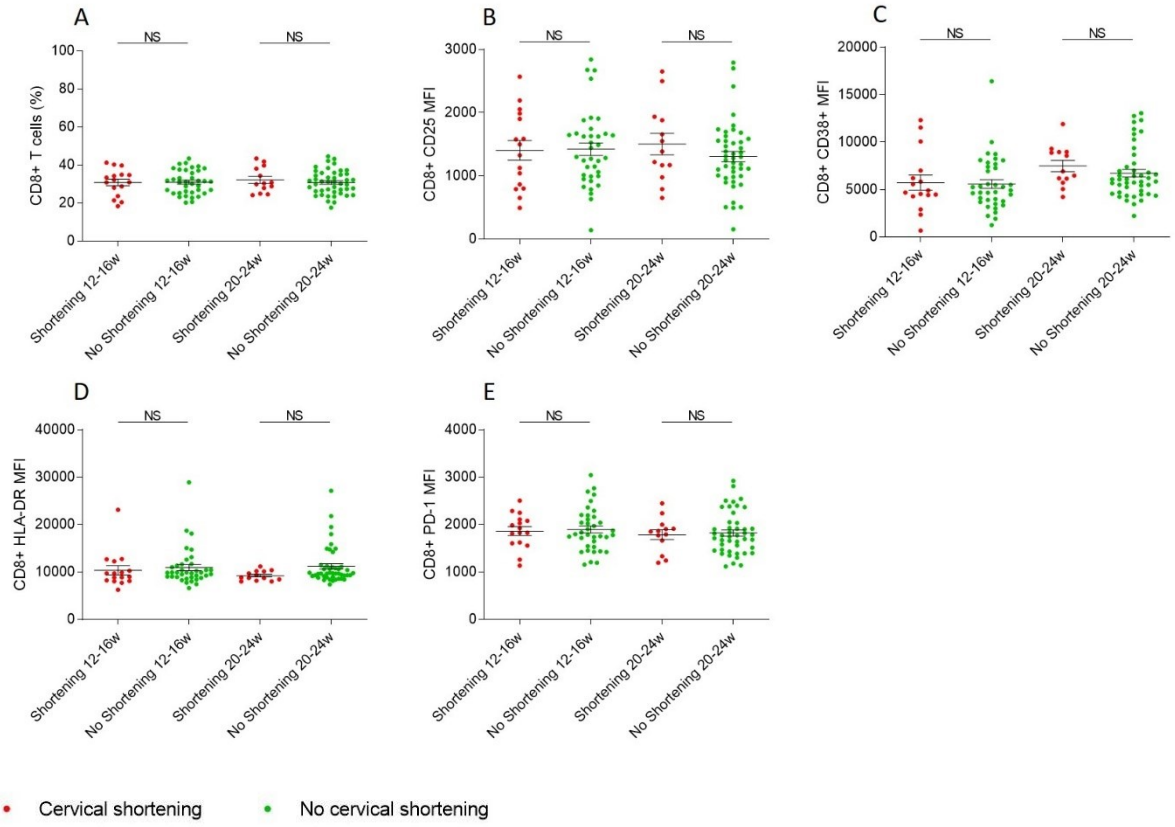


Figure 3.31 Comparison of C84+ T cells and their effector status in women who do or do not develop cervical shortening

There was no significant difference in the proportion of CD8+ T cells (A) or in the CD8+ T cells CD25 (B), CD38 (C), HLA-DR (D) and PD-1 (E) median fluorescence intensity (MFI) in women who do or do not develop cervical shortening at 12-16 weeks or 20-24 weeks gestation, prior to cervical shortening. (Shortening 12-16 weeks N=16, Shortening 20-24 weeks N=13, No shortening at 12-16 or 20-24 weeks N=51). Statistical analysis was by an unpaired t test. The results are expressed as mean ±SEM.

Treg and γδ T cells

There were no differences in the proportion of Treg cells (as a percentage of CD4+ cells) or the proportion of γδ T cells (as a percentage of total lymphocytes) in women who did and did not develop cervical shortening when sampled at 12^{+0} - 16^{+6} or 20^{+0} - 24^{+6} weeks gestation (p>0.05). The Treg CD25, CD38 and HLA-DR MFI also did not show any differences (p>0.05), Figure 3.32.

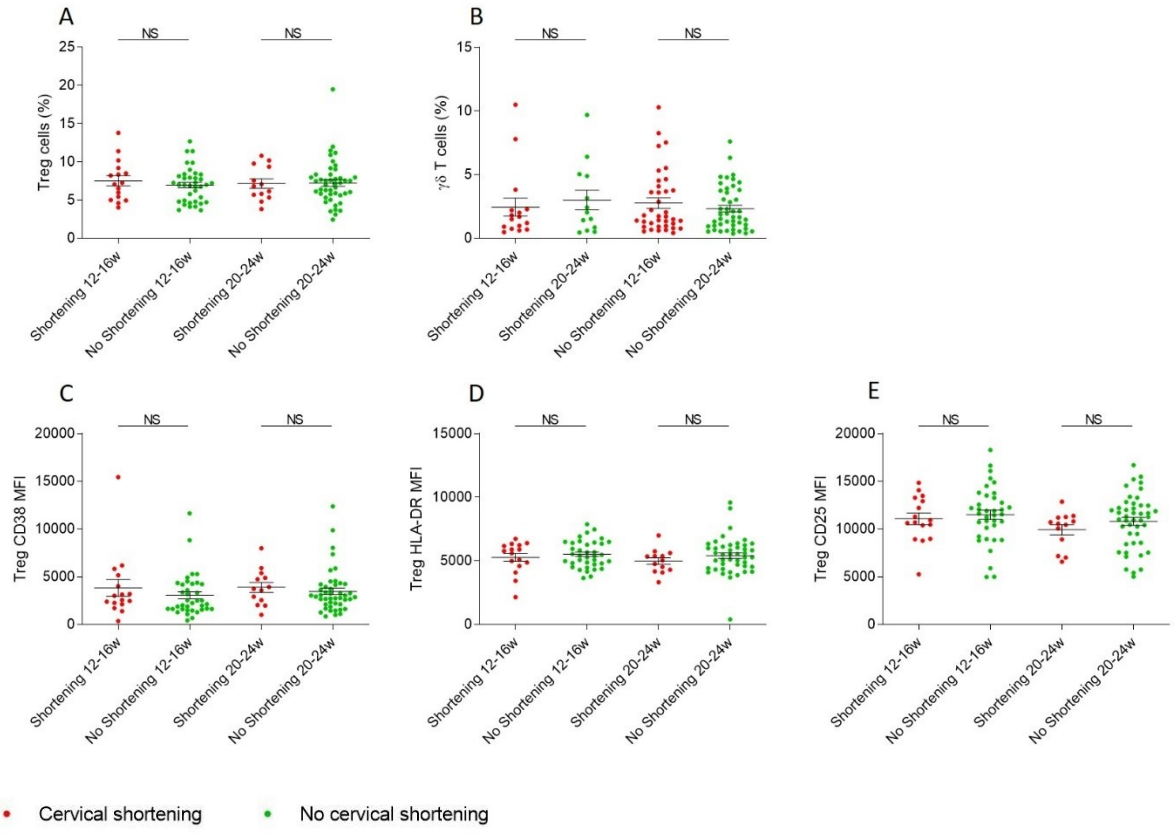


Figure 3.32 Comparison of Treg cells and their effector status, and γδ T cells in women who do or do not develop cervical shortening

There was no significant difference in the proportion of Treg cells (A) or γδ T cells (B), or in the Treg cells CD25 (C), CD38 (D) and HLA-DR (E) median fluorescence intensity (MFI) in women who do or do not develop cervical shortening at 12-16 weeks or 20-24 weeks gestation, prior to cervical shortening. (Shortening 12-16 weeks N=16, Shortening 20-24 weeks N=13, No shortening at 12-16 or 20-24 weeks N=51). Statistical analysis was by an unpaired t test. The results are expressed as mean ±SEM.

B cells

There was no difference in the proportion of B cells (as a percentage of CD3- cells) in women who did and did not develop cervical shortening when sampled at 12^{+0} - 16^{+6} or 20^{+0} - 24^{+6} weeks gestation ($p>0.05$). The B cell IgD and PD-1 MFI also did not show any differences ($p>0.05$), Figure 3.33.

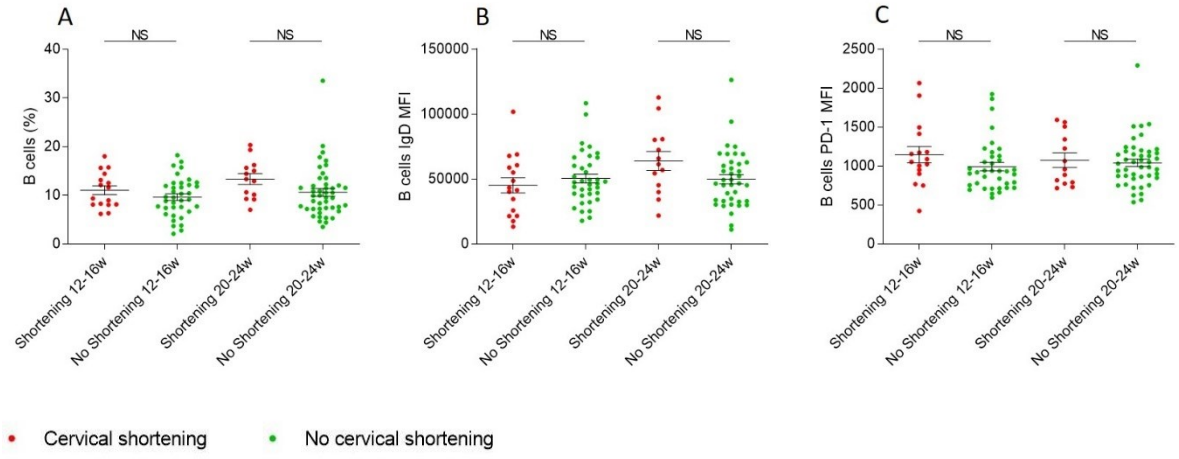


Figure 3.33 Comparison of B cells and their effector status in women who do or do not develop cervical shortening

There was no significant difference in the proportion of B cells (A) or in the B cells IgD (B) and PD-1 (C) median fluorescence intensity (MFI) in women who do or do not develop cervical shortening at 12-16 weeks or 20-24 weeks gestation, prior to cervical shortening. (Shortening 12-16 weeks N=16, Shortening 20-24 weeks N=13, No shortening at 12-16 or 20-24 weeks N=51). Statistical analysis was by an unpaired t test. The results are expressed as mean ±SEM.

NK cells and NK T cells

There was no difference in the proportion of NK cells (as a percentage of CD3- cells), or proportion of CD4+ NK T cells and CD8+ NK T cells (as a percentage of CD3- cells) in women who did and did not develop cervical shortening when sampled at 12^{+0} - 16^{+6} or 20^{+0} - 24^{+6} weeks gestation ($p>0.05$). The NK CD38 MFI also did not show any differences ($p>0.05$), Figure 3.34.

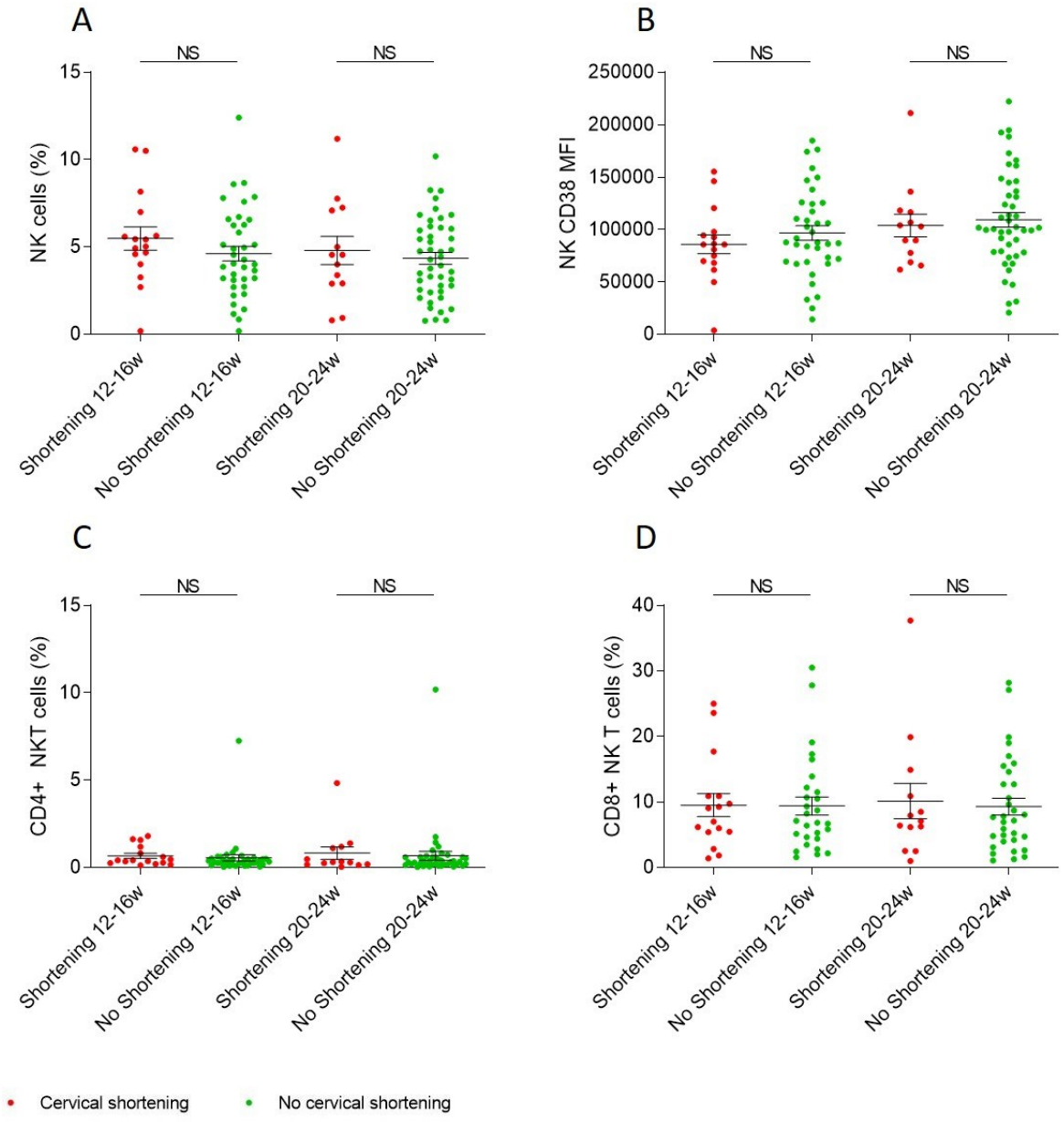


Figure 3.34 Comparison of NK cells, NK cell effector status, CD4+ NK T cells and CD8+ NK T cells in women who do or do not develop cervical shortening

There was no significant difference in the proportion of NK cells (A), CD4+ NK T cells (C) and CD8+ NK T cells (D), or in the NK CD38 (B) median fluorescence intensity (MFI) in women who do or do not develop cervical shortening at 12-16 weeks or 20-24 weeks gestation, prior to the cervical shortening. (Shortening 12-16 weeks N=16, Shortening 20-24 weeks N=13, No shortening at 12-16 or 20-24 weeks N=51). Statistical analysis was by an unpaired t test. The results are expressed as mean ±SEM.

According to PBMC subsets and markers of PBMC activity, there was no peripheral immune response prior to cervical shortening. Therefore, next examined were if there were any differences in women already with cervical shortening, compared to those with normal cervical length.

At the 12^{+0} - 16^{+6} week sampling timepoint, there were 6 women with cervical lengths ≤25mm, (median cervical length was 24mm) and at the 20^{+0} - 24^{+6} week sampling timepoint, there were 8 women with cervical lengths ≤25mm, (median cervical length was 23mm). PBMC subsets and markers of PBMC activity were compared to 48 women with cervical length >25mm at the 12^{+0} -16^{+6} week timepoint, (median cervical length was 35mm) and 42 women with cervical length >25mm at the 20^{+0} - 24^{+6} week sampling timepoint, (median cervical length was 34mm). Women with normal cervical lengths with a cervical cerclage in situ were excluded from these analyses. Table 3.8 demonstrates that the presence of cervical shortening was not reflected in the proportion of PBMC cell subsets or markers of PBMC activity ($p>0.05$).

Table 3.8 PBMC and markers of PBMC activity in women with and without cervical shortening

	Cervical shortening ≤25mm at 12-16 weeks N=6	Normal cervical length at 12-16 weeks N=8	p-value	Cervical shortening ≤25mm at 20-24 weeks N=48	Normal cervical length at 20-24 weeks N=42	p-value
PBMC subsets (%)						
Lymphocytes (% live cells)	88.13 (8.26)	90.84 (6.04)	0.51	90.99 (6.03)	91.10 (6.98)	0.83
Monocytes (% live cells)	11.87 (8.26)	9.16 (6.04)	0.51	8.64 (6.52)	8.73 (7.11)	0.96
B cells (% of lymphocytes)	8.42 (2.87)	10.16 (3.84)	0.29	10.43 (3.72)	10.92 (4.30)	0.76
Lymphocytes CD3+ (% of lymphocytes)	78.18 (5.79)	78.04 (6.73)	0.96	75.58 (4.67)	77.35 (5.60)	0.41
CD4+ T cells (% of CD3+)	63.57 (7.88)	64.49 (7.48)	0.78	61.86 (6.71)	64.33 (7.55)	0.39
CD8+ T cells (% of CD3+)	32.32 (7.83)	30/95 (6.72)	0.65	33.83 (6.37)	31.21 (6.92)	0.33
Treg cells (% of CD3+ CD4+)	7.88 (2.12)	7.20 (2.43)	0.36	7.63 (2.96)	7.98 (2.24)	0.40
NK cells (% of lymphocytes)	4.36 (2.77)	4.91 (2.65)	0.21	4.77 (2.72)	4.51 (2.32)	0.78
Classical monocytes (% of monocytes)	82.02 (4.14)	83.73 (4.36)	0.37	81.19 (4.04)	84.12 (4.84)	0.11
Intermediate monocytes (% of monocytes)	0.94 (0.46)	1.76 (2.52)	0.75	1.04 (1.33)	1.43 (1.41)	0.23
Non-classical monocytes (% of monocytes)	5.62 (3.54)	6.32 (4.48)	0.97	4.30 (2.12)	4.99 (3.42)	0.84
γδ T cells (% of lymphocytes)	1.30 (0.46)	2.75 (2.65)	0.41	1.29 (0.50)	2.29 (1.74)	0.29
CD4+ NK T cells (% of CD3+)	0.48 (0.30)	0.57 (1.03)	0.67	0.43 (0.33)	0.64 (1.57)	0.62
CD8+ NK T cells (% of CD3+)	10.80 (8.02)	8.61 (6.73)	0.55	8.66 (3.92)	8.33 (6.92)	0.33
Markers of PBMC activity (median fluorescence intensity)						
CD4+ CD25	2373 (530)	2303 (670)	0.32	2361 (599)	2205 (758)	0.58
CD4+ CD38	8867 (3731)	8772 (5451)	0.09	7603 (4748)	8571 (4462)	0.26
CD4+ HLA-DR	8492 (2203)	8584 (1845)	0.91	8138 (1449)	8913 (2591)	0.42
CD4+ PD-1	1716 (335)	1803 (364)	0.58	1736 (321)	1782 (396)	0.76
CD8+ CD25	1447 (373)	1400 (626)	0.86	1384 (320)	1266 (430)	0.47
CD8+ CD38	5492 (3718)	5701 (3010)	0.76	5655 (3285)	5576 (2650)	0.49
CD8+ HLA-DR	11321 (1846)	10781 (1113)	0.08	10612 (2265)	10831 (2766)	0.75
CD8+ PD-1	1899 (440)	1900 (436)	0.99	1862 (524)	1858 (452)	0.98
Treg CD25	11650 (1581)	11284 (2888)	0.76	10692 (2076)	10997 (2335)	0.74
Treg CD38	3330 (1037)	3322 (2773)	0.32	2935 (1508)	3189 (1619)	0.54
Treg HLA-DR	5652 (1289)	5411 (1122)	0.26	5623 (901)	5396 (1501)	0.68
NK CD38	103557 (38864)	94986 (41063)	0.63	117872 (40490)	114040 (40553)	0.81
B cells IgD	54284 (30272)	49320 (21590)	0.61	46132 (18120)	51208 (22285)	0.55
B cells PD-1	939 (250)	1037 (366)	0.74	999 (216)	1013 (284)	0.90

Mean % (standard deviation). Statistical analysis was Wilcoxon matched-pairs signed rank test for non-parametric data and a paired t-test for parametric data.

Plasma cytokines and complement

In order to assess the plasma cytokines and complement levels prior to cervical shortening study participants with plasma cytokines and complement results were separated into two groups; those who had cervical shortening during their pregnancy, identified as a cervical length ≤25mm were compared to study participants who had normal cervical length measurements throughout their pregnancy, >25mm. As described earlier, plasma cytokines and complement levels were determine in a total of 32 study participants, Figure 3.35. Eleven women had cervical shortening during the pregnancy and 21 women maintained a normal cervical length during the pregnancy. There were six women who had plasma cytokines and complement measurements at the 12^{+0} - 16^{+6} week timepoint prior to cervical shortening. There were 5 women who had plasma cytokines and complement measurements at the 20^{+0} - 24^{+6} week timepoint prior to cervical shortening. Clinical and demographical characteristics of these patient groups are presented in Table 3.9.

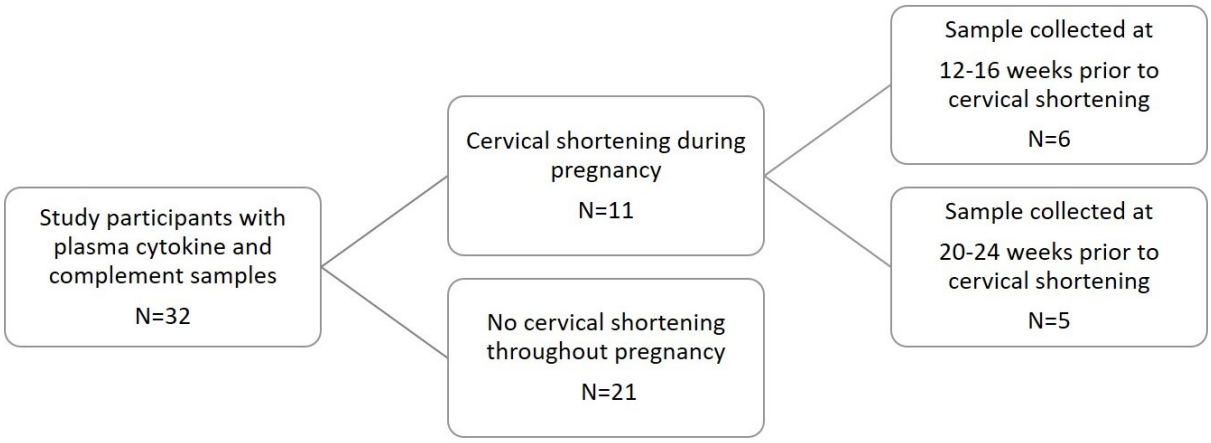


Figure 3.35 The selection of study participants to compare plasma cytokine and complement responses in women who develop cervical shortening compared to women who do not.

There were no differences in the age, BMI or ethnicity between the women who had cervical shortening and those who did not. The gestation at delivery was significantly lower in the group sampled at 20^{+0} - 24^{+6} weeks who developed cervical shortening compared to those who maintained a normal cervical length (p=0.04). The median gestational age at delivery in the cervical shortening group at 12^{+6} - 16^{+6} weeks was 38+3, in cervical shortening group at 20^{+0} - 24^{+6} weeks was 37^{+6}, and in the no cervical shortening group was 38^{+1}. Taking into account the shortest cervical length during at any point during the pregnancy, the median shortest cervical length in the groups experiencing cervical shortening was 21mm, compared to 33mm in the group with no cervical shortening, ($p<0.0001$).

Table 3.9 Clinical and demographical characteristics of the study population, comparing plasma cytokines and complement in women with and without cervical shortening

	A	B	C	
	Cervical shortening (sampling at 12-16 weeks)	Cervical shortening (sampling at 20-24 weeks)	No cervical shortening (sampling at 12-16 and 20-24 weeks)	p value (column A vs C and column B vs C)
N (%)	6	5	21	
Gestation at delivery (weeks) median and IQ range	38+3 (36+0-39+0)	37+6 (33+0-38+3)	38+6 (38+0-39+3)	A vs C: t test p=0.24 B vs C: t test p=0.04*
Early preterm </=33+6	1	1	1	A vs C: χ^2=0.27 B vs C: χ^2=0.31
Late preterm >/=34+0	0	1	2	
Term cohort	1	1	11	
Term intervention cohort	4	2	7	
Age median and IQ range	35 (31-42)	33 (26-37)	36 (29-37)	A vs C: t test p=0.32 B vs C: t test p=0.41
BMI median and IQ range	25 (22-27)	26 (26-30)	24 (20-27)	A vs C: t test p=0.54 B vs C: t test p=0.06
Ethnicity White N (%) Black N (%) Other N (%)	 3 (50%) 2 (33%) 1 (17%)	 0 (0%) 3 (60%) 2 (40%)	 11 (52%) 5 (24%) 5 (24%)	A vs C: χ^2=0.87 B vs C: χ^2=0.09
Cervical length median and IQ range (mm) 12-16 weeks 20-24 weeks	 30 (29-41) NA	 NA 30 (30-36)	 35 (30-41) 35 (30-39)	A vs C: t test p=0.37 B vs C: t test p=0.25
Shortest cervical length during pregnancy, median and IQ range (mm)	21 (17-24)	21 (17-24)	33 (28-35)	A vs C: test p<0.0001 **** B vs C: test p<0.0001 ****
Cervical cerclage	5 (83%) 1 cervical cerclage in situ at time of sampling	4 (80%) 3 cervical cerclage in situ at time of sampling	7 (33%)	
History indicated cervical cerclage and material	2 (Nylon 1, material not known 1)	2 (Nylon 1, material not known 1)	7 (Mersilene 2, Nylon 5)	
USS indicated cervical cerclage and material	3 (Mersilene 2, Nylon 1)	2 (Mersilene 1, Nylon 1)	0	
Progesterone only	0	0	1	

BMI=body mass index, USS=ultrasound. Data presented as median (interquartile range (IQ)) or number (%). P values: One-way ANOVA for multiple comparisons or Chi squared for proportional data.

Of the panel of cytokines measured, Table 3.5, only IL-18 was detected within the standard curve range of the assay in all samples. The next most commonly detected cytokine was IFN-γ with 40% of samples detected within the standard curve range of the assay. Concentrations below the lowest standard were generated by extrapolation. From the available data, no differences in the concentration of any of the cytokines in the multiplex panel, (IL-8, IFN-γ, IL-2, IL-4, IL-8, TNF-α, GM-CSF, IL-5, IL-1β and IL-6) were observed in women who did or did not have cervical shortening during their pregnancy, when sampled before the shortening event at 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks gestation ($p>0.05$), Figure 3.36. The results for IL-10 are not presented as all the values were below the minimum level of detection of the assay and concentrations were too low for extrapolation.

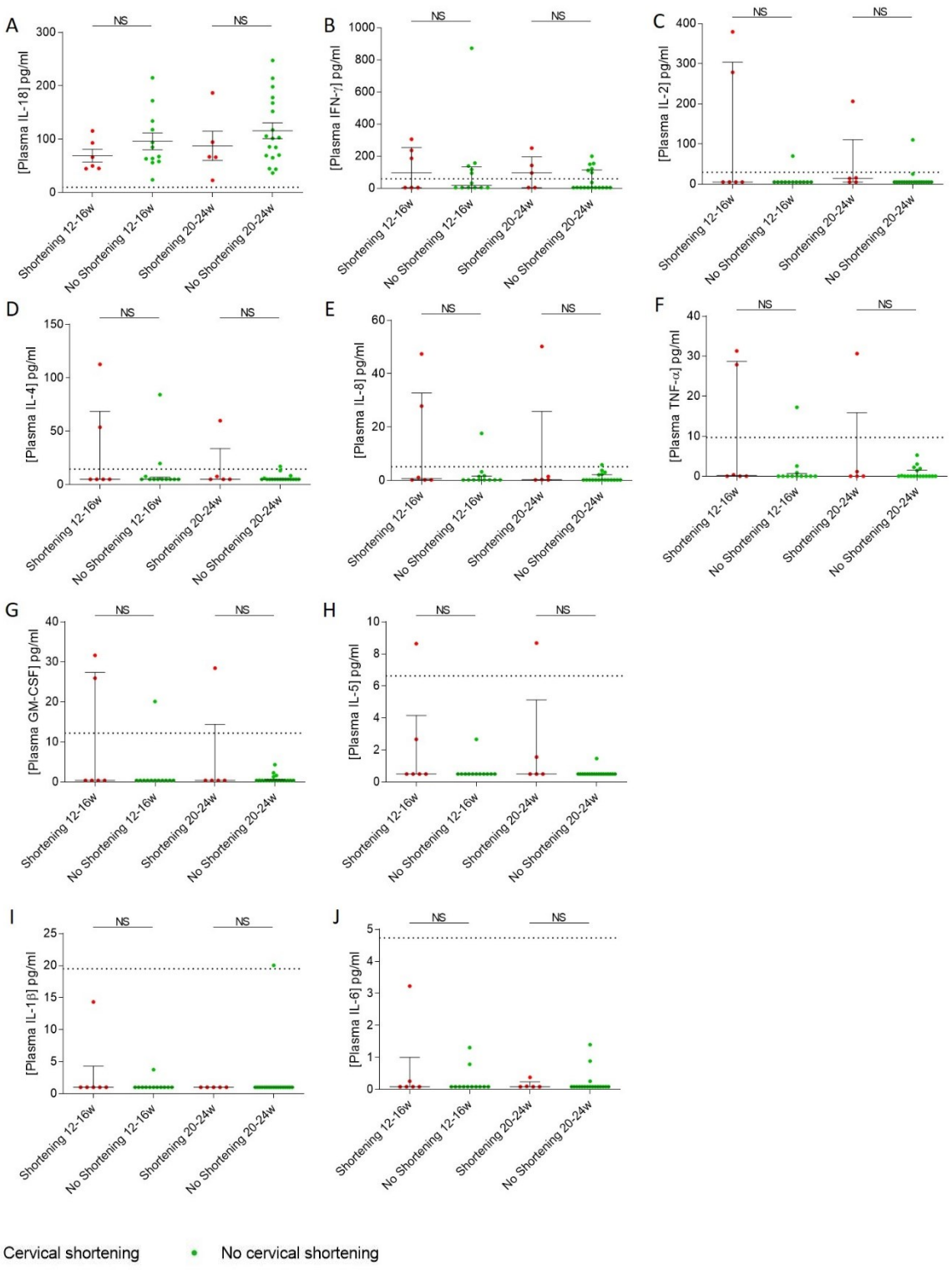


Figure 3.36 Comparison of plasma cytokines in women who do or do not develop cervical shortening
There was no significant difference in the concentration of IL-18, IFN-γ, IL-2, IL-4, IL-8, TNF-α, GM-CSF, IL-5, IL-1β or IL-6 in women who do or do not develop cervical shortening when sampled at 12-16 weeks or 20-24 weeks gestation, prior to cervical shortening. The dotted lines show the concentration of the lowest standard on the standard curve. Values below the lowest standard were generated by extrapolation. Statistical analysis was by an unpaired t test (IL-18) or Mann Whitney U test (all other cytokines). (Shortening 12-16 weeks N=6, Shortening 20-24 weeks N=5, No shortening at 12-16 or 20-24 weeks N=21). The results are expressed as mean ± SEM (A), and median and interquartile range (B-J).

The plasma concentration of complement proteins C5, C5a, C3b and MBL were compared between women who developed a shortened cervix and women who did not, at 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks gestation, prior to cervical shortening. There was no difference in the production of the complement proteins between women who underwent cervical shortening and those who did not ($p>0.05$), Figure 3.37.

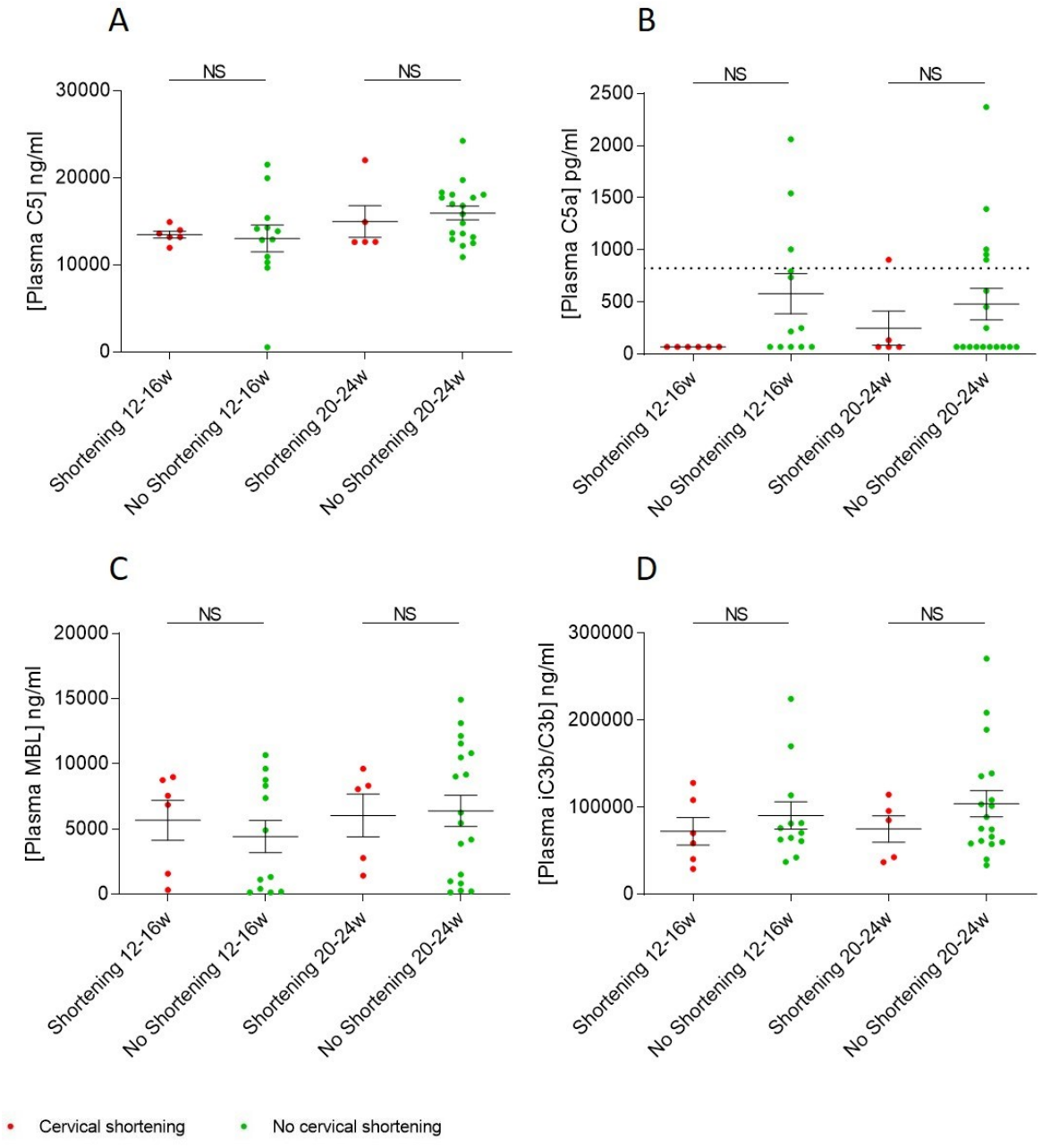


Figure 3.37 Comparison of plasma complement in women who do or do not develop cervical shortening
There was no significant difference in the concentration of plasma C5, C5a, C3b or MBL in women who do or do not develop cervical shortening when sampled at 12-16 weeks or 20-24 weeks gestation, prior to cervical shortening. Statistical analysis was by an unpaired t test. Plasma was diluted 1:200 for the C5, C5a and MBL multiplex assays, and 1:400 for the C3b assay. The dotted line on the graph for C5a is the concentration for the lowest standard on the assay's standard curve. (Shortening 12-16 weeks N=6, Shortening 20-24 weeks N=5, No shortening at 12-16 or 20-24 weeks N=21). The results are expressed as mean ± SEM.

Measurement of plasma cytokines and complement did not show differences in the peripheral immune response prior to cervical shortening ($p>0.05$). However, there were insufficient numbers to assess for any differences in women already with cervical shortening, compared to those with normal cervical length.

3.5.8 Cervical cerclage and the peripheral immune response

Peripheral blood mononuclear

In the absence of peripheral immune response prior to cervical shortening, the next question to ask was if the intervention to manage cervical shortening induced a peripheral immune response. Of the 78 women who had PBMC sampling, there were 26 women who had a cervical cerclage. Sampling was performed before and after cervical cerclage in 19 of these women. The median gestation pre cerclage was 13^{+5} weeks and the median gestation post cerclage was 22^{+2} weeks. The median gestation at delivery was 38^{+1} weeks with a range of 30^{+6} to 41^{+0} weeks. 3 of the 19 women had an early preterm delivery before 34 weeks, and 2 further had a late preterm delivery between 34^{+0} - 36^{+6} weeks. Nine were history indicated cerclages and ten were ultrasound indicated cerclages. The median number of days before cerclage insertion was 9 (range 1-32) and the median number of days post cerclage insertion was 49 (range 11-113).

PBMC subsets, monocytes, (including classical monocytes, intermediate monocytes, non-classical monocytes), CD3+ lymphocytes, CD4+ T cells, CD8+ T cells, Treg cells, γδ T cells, B cells, NK cells, CD4+ NK T cells, and CD8+ NK T cells did not show any significant proportional differences before and after cervical cerclage ($p>0.05$), Table 3.10. CD8+ CD38 MFI increased following cervical cerclage, ($p<0.01$). However, this was not related to the cerclage material or whether the cerclage was history indicated or ultrasound indicated, Figure 3.38. CD8+ CD38 MFI has previously been shown to increase with advancing gestation and is the likely explanation in this case. All other markers of PBMC activation did not reveal any significant differences per and post cervical cerclage (CD4+ CD25, CD4+ CD38, CD4+ HLA-DR, CD4+ PD-1, CD8+ CD25, CD8+ HLA-R, CD8+ PD-1, Treg CD25, Treg CD38, Treg HLA-DR, NK CD38, B cells IgD and B cells PD-1, Table 3.11. Of the 19 cerclages, 9 were with Mersilene and 10 were with Nylon. The cerclage material also did not affect the fold change in PBMC subsets or their markers of activity, (data not shown).

Table 3.10 PBMC and markers of PBMC activity pre and post cervical cerclage

	Pre cervical cerclage N=19	Post cervical cerclage N=19	p-value (Wilcoxon signed rank test)
PBMC subset (%)			
Lymphocytes (% live cells)	91.61 (5.70)	92.15 (4.85)	0.51
Monocytes (% live cells)	8.23 (5.89)	7.85 (4.85)	0.59
B cells (% of lymphocytes)	9.57 (3.44)	9.88 (4.39)	0.61
Lymphocytes CD3+ (% of lymphocytes)	78.6 (5.79)	78.39 (5.57)	0.85
CD4+ T cells (% of CD3+)	65.82 (8.44)	65.76 (7.75)	0.95
CD8+ T cells (% of CD3+)	30.27 (7.89)	30.00 (7.09)	0.74
Treg cells (% of CD3+ CD4+)	7.85 (2.58)	8.37 (3.46)	0.14
NK cells (% of lymphocytes)	5.05 (2.50)	4.20 (2.16)	0.14
Classical monocytes (% of monocytes)	82.35 (3.92)	81.80 (3.53)	0.57
Intermediate monocytes (% of monocytes)	1.29 (1.2)	0.90 (1.05)	0.06
Non-classical monocytes (% of monocytes)	7.01 (4.43)	6.24 (3.4)	0.09
γδ T cells (% of lymphocytes)	1.39 (0.58)	2.47 (2.56)	0.29
CD4+ NK T cells (% of CD3+)	0.39 (0.22)	0.37 (0.19)	0.80
CD8+ NK T cells (% of CD3+)	8.95 (5.43)	8.40 (4.52)	0.76
Markers of PBMC activity (median fluorescence intensity)			
CD4+ CD25	2284 (833)	2319 (683)	0.76
CD4+ CD38	11359 (5932)	12264 (6942)	0.19
CD4+ HLA-DR	8693 (2112)	9640 (2142)	0.89
CD4+ PD-1	1702 (323)	1724 (312)	0.71
CD8+ CD25	1518 (566)	1361 (579)	0.49
CD8+ CD38	6026 (3082)	6934 (2711)	** <0.01
CD8+ HLA-DR	11127 (3451)	10989 (3088)	0.73
CD8+ PD-1	1802 (388)	1762 (386)	0.422
Treg CD25	10867 (2719)	10122 (2611)	0.37
Treg CD38	4038 (3658)	3951 (2600)	0.32
Treg HLA-DR	5837 (1316)	5411 (1048)	0.08
NK CD38	98025 (47575)	116915 (51751)	0.11
B cells IgD	47973 (25831)	44213 (25628)	0.61
B cells PD-1	1094 (364)	1060 (227)	0.56

Mean % (standard deviation). Statistical analysis was Wilcoxon matched-pairs signed rank test for non-parametric data and a paired t-test for parametric data.

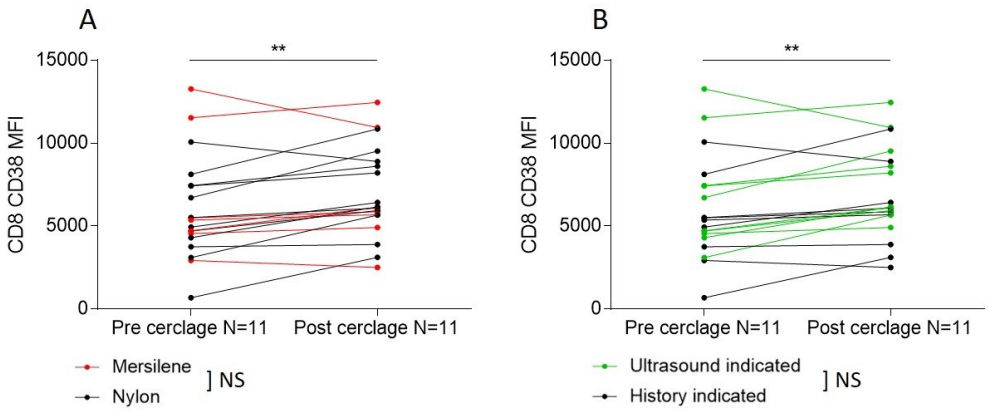


Figure 3.38 CD8 CD38 median fluorescence intensity pre and post cervical cerclage
CD8 T cells CD38 median fluorescence intensity increased significantly post cervical cerclage, but there was no difference in the fold change between women who had a Mersilene or Nylon cerclage (A), or between women who had an ultrasound or a history indicated cerclage (B). Statistical analysis was by a paired t-test.

Plasma cytokines and complement

There were 32 women in the whole cohort with plasma cytokines and complement results. 16 women had a cervical cerclage and 11 of them had plasma cytokines and complement results available for analysis pre and post cervical cerclage. The median gestation pre cerclage was 13^{+5} weeks and the median gestation post cerclage was 22^{+4} weeks. The median gestation at delivery was 38+4 weeks with a range of 31^{+4} to 40^{+4} weeks. 1 of the 11 women had an early preterm delivery before 34 weeks, and 2 further had a late preterm delivery between 34^{+0} - 36^{+6} weeks. There was no significant difference in cervical length pre and post cerclage. Seven of the cerclages were history indicated and 4 were ultrasound indicated cerclages. The median number of days before cerclage insertion was 7 (range 1-32) and the median number of days post cerclage insertion was 39 (range 11-63).

There was an increase in pro-inflammatory IL-18 post cervical cerclage, although due to small numbers, this did not reach significance (p=0.06). The increase was likely to be related to the increase in gestational age between pre cerclage and post cerclage sample, rather than due to the effect of cerclage. There was no difference in the remaining cytokine analytes before and following cervical cerclage IFN-γ, IL-2, IL4-, IL-8, TNF-α, GM-CSF, IL-5, IL-1β, IL-6 (p>0.05). Many cytokines were below the lowest concentration on the standard curve which therefore was a limitation to this analysis. No significant differences were seen in C5, C5a, MBL and C3b following cervical cerclage (Table 3.11).

Four of the 11 women with paired sampling pre and post cervical cerclage had a Mersilene cerclage and the remaining 7 had a Nylon cerclage. The study numbers were small, but there was no significant difference in the fold change of the plasma cytokines and complement before and after the cervical cerclage, based upon the cerclage material (data not shown).

Table 3.11 Plasma cytokines and complement analytes pre and post cervical cerclage

	Pre cervical cerclage N=11	Post cervical cerclage N=11	p-value (Wilcoxon signed rank test)
Plasma cytokine			
IL-18	75.64 (38.41)	100.3 (56.98)	0.06
IFN-γ	48.02 (101.5)	42.65 (92.29)	0.50
IL-2	39.95 (112.9)	38.16 (110)	0.99
IL-4	15.92 (32.3)	15.16 (31.08)	0.50
IL-8	3.24 (8.29)	2.95 (7.96)	0.25
TNF-α	3.09 (9.39)	2.89 (8.85)	0.50
GM-CSF	3.46 (9.38)	3.37 (9.45)	0.99
IL-5	0.79 (0.70)	0.82 (0.81)	0.99
IL-1β	2.21 (4.02)	2.28 (4.24)	0.99
IL-6	0.44 (0.95)	0.56 (0.97)	0.50
Plasma complement			
C5	13941 (3150)	15433 (4398)	0.41
C5a	397.2 (641.3)	443.3 (705.6)	0.63
MBL	5178 (3774)	4981 (3675)	0.81
C3b	87336 (46732)	86761 (68610)	0.52

Mean pg/ml (standard deviation) for all analytes except C5a, MBL and C3b (ng/ml). Statistical analysis was Wilcoxon matched-pairs signed rank test for non-parametric data and a paired t-test for parametric data.

3.6 Discussion

The peripheral maternal immune system is required to be responsive and dynamic during pregnancy to mount a protective immune response to invading pathogens, whilst providing tolerance to the fetal allograft.

Cellular innate immune response

Increased activation of the innate immune response has been reported during pregnancy and some studies suggest that this is in part a result of an increase in peripheral monocytes [270-272]. However, many of these studies included women with pregnancy induced hypertension, pre-eclampsia and diabetes, which may have introduced some bias. Other studies report that it is the activation of monocytes rather than the cell population that increases [273]. By migrating to the decidua, monocytes mature into decidual macrophages and display the immunosuppressive M2 phenotype which is important for feto-maternal immune tolerance [274]. Macrophages are key antigen presenting cells and are involved in regulating local immune homeostasis. In the current study, the peripheral monocyte count and activation as measured by HLA-DR were stable across pregnancy at the three sampling timepoints. This is in keeping with studies by Kraus et al and Luppi et al, which have similar longitudinal sampling timepoints [272, 273].

NK cells are CD16+ $CD56^{bright}$ or CD16+ $CD56^{dim}$, with peripheral NK cells being predominantly CD16+ $CD56^{dim}$ which have a more cytotoxic phenotype. The reduction in NK cells with advancing gestation is in keeping with a reduction seen in the latter stages of pregnancy by Kraus et al [272]. There is a likely physiological explanation for this decline in NK cells. A key part of placentation involves decidual NK cells remodelling spiral arteries creating a low resistance system maternal-placental circulation [275]. NK cells are required for trophoblast invasion and placentation, and once it is complete their circulating numbers fall.

NK cell activity as measured by the CD38 MFI increased irrespective of preterm or term pregnancy. The temporal increase coincides with the pro-inflammatory shift that occurs as pregnancy advances and labour nears. This is in keeping with Le Gars et al's findings that pregnancy was associated with increased NK CD38 activity [276]. CD38 is highly expressed on NK cells, potentiating the ability of lymphocytes to adhere to endothelial cells in the process of extravasation[276, 277]. Decidual NK cells express even higher levels of CD38 compared to peripheral NK cells. It is plausible that there is NK cell migration from the periphery to the decidua, but the origin of decidual NK cells and if there is an increase in decidual NK cells during labour is unclear at present. NK cells are primarily part of the

innate immune response as they lack antigen specific receptors, but the support they provide to the adaptive immune response is well recognised.

NK CD38 MFI negatively correlated with Th1 plasma cytokines, IL-18, IL-2 and IFN-γ. A potential explanation is that NK cells may help to regulate Th1 cytokine activity, preventing an uncontrolled Th1 response as pregnancy advances.

Cellular adaptive immune response

The population of B cells and their activity was stable across pregnancy in this study. The effect of oestrogen on reduced B cell lymphopoiesis in pregnancy is recognised [278]. Increased oestrogen in females has been attributed to higher autoreactivity of B cells and therefore increased prevalence of autoimmune diseases in women [279]. A reduction in B cells in pregnancy or their activity may explain the improvement of symptoms of some autoimmune diseases in pregnancy. The absence of a reduction in the B cell population in this study may be because a relative decrease occurs at an earlier gestation than the first sampling timepoint of 12-16 weeks and then persists during pregnancy. B cell IgD MFI was used as a measure of B cell activation, whilst PD-1 MFI measured the immunosuppressive activity of B cells. B cells expressing PD-1 influence the development of naïve B cells, modulate T cell responses and down-regulate aberrantly active lymphocytes[280] [281] [282].

I presented an increase in CD4+ T cells in term (uncomplicated) pregnancies. CD4 expressing T helper cells have many subtypes and drawing a conclusion from this finding is not straightforward. There is a dominant pro-inflammatory Th1 profile in early pregnancy to allow for implantation, and in late pregnancy to allow for labour. Between this period, an anti-inflammatory Th2 response dominates allowing for fetal growth. The cytokine profiling may have helped to distinguish which subset CD4+ T cells expanded during term (uncomplicated) pregnancies, but in unstimulated cells, plasma detection of cytokines was limited. The balance between a Th1/Th2 response is hormonally influenced by progesterone and oestrogen [283, 284], and in part explains immune adaptions during pregnancy.

The proportion of CD8+ T cells was stable across gestation which is in keeping with other studies [261, 285]. There was a gestational increase in both CD4+ CD38 MFI and CD8+ CD38 MFI. This increased activation of CD4+ and CD8+ T cells represents physiological time dependent immune adaptation, consistent with an immunological clock. CD38 is involved in cell adhesion, signal transduction and calcium signalling. An increase in CD4+ and CD8+ T cell activity may contribute to the sterile inflammatory response that occurs as labour approaches.

Treg cells are a subset of CD4+ T cells and are key to the maternal immune system not recognising semi-allogenic fetal antigens. Earlier human studies, characterising Treg cells by CD4 and CD25

expression have shown that there is an increase in Treg cells in early pregnancy peaking at the end of the first trimester which coincides with placentation [286]. This expansion of the Treg pool which is absent in pre-eclampsia and has been implicated its pathogenesis [287]. The immune tolerance conferred by Treg cells appears to be more important in early gestation and implantation, rather than later in pregnancy [288]. It is thought that Treg cells at the feto-maternal interface are likely to originate from the thymus and are commonly referred to as natural Treg cells which may explain why the peripheral Treg population did not show a gestational increase in this study [289]. Peripheral Treg cells are derived from naïve peripheral CD4+ T cells and are referred to as induced Treg cells. There is no cell marker that differentiates between natural and induced Treg cells at present.

There are some more recent studies however that actually report an overall reduction in peripheral Treg cells during pregnancy and that selective expansion of a certain subset of Treg occurs instead to promote immune tolerance. The disparity originates from the Treg gating strategy used and the identification of a distinct $CD4^{dim}$, $CD25^{high}$, $CD127^{low}$, FOXP3+, HLA-DR+ population [290]. Mjösberg et al argue that gating on CD4+, CD25+ alone, will include Treg cells that do not have suppressive activity. There are also likely to be differences in the immune suppressive capacity of Treg cells in the periphery and those at the maternal-fetal interface [291].

This study found differential activation of peripheral Treg cells in women who had preterm and term deliveries, as measured by the trajectory of Treg HLA-DR MFI, (Figure 3.19 D). Treg cell activation appeared to increase across gestation in the term group, but there appeared to be an inability of Treg cells to do the same in women who eventually had preterm deliveries, although the difference in Treg HLA-DR MFI did not reach statistical significance, (Figure 3.11 D). HLA-DR+ Treg cells have the most suppressive activity of all Treg cells. However, within the CD4+ CD25+ $CD127^{low}$ pool of Treg cells, is a further subset which are FOXP3+ HLA-DR+. Kisielewicz et al showed that women who had preterm deliveries had significantly lower CD4+, $CD127^{low}$, CD25 Treg pool compared to healthy pregnancies and HLA-DR expression of the FOXP3+ HLA-DR+ was also reduced in women who had preterm deliveries [292]. A limitation of results presented is that HLA-DR+ Treg cells represent a subset of Treg cells, therefore the suppressive function of all Treg cells is not measured by the gating strategy used.

γδ T cells comprise <1% of peripherally circulating lymphocytes and are not well studied in pregnancy. They may still contribute to immune adaptations in pregnancy as an increased proportion has been reported in cases of unexplained recurrent spontaneous first trimester miscarriages [293]. NK T cells are also present in very low abundance in the peripheral circulation, constituting 0.1% of T cells. Similar to γδ T cells an increase in NK T cell activity has been associated with pregnancy loss, but this is reported in murine studies [294, 295].

Plasma cytokines

IL-18 was the only consistently detectable plasma cytokine in this study. IL-18 belongs to the same group of pro-inflammatory IL-1 cytokines which include IL-1α and IL-1β, which are synthesised as precursor proteins. IL-18 is mainly produced by monocytes and macrophages in response to pathogen-associated molecular patterns (PAMPs) recognised by pattern recognition receptors (PPRs), although there was no correlation between monocytes and their activation with plasma IL-18. IL-18 is closely linked to IFN-γ which has strong phagocytic and cytotoxic activity and IL-18 also potentiates the cytotoxic activity of NK cells [(296)]. However, there was no correlation with NK cells and their activation with IL-18.

In the longitudinal cohort, the gestational increase in plasma IL-18, with a positive correlation with IL-2, IFN-γ and TNF-α is in keeping with a bias towards a Th1 cell response. The increase in plasma IL-18 with advancing pregnancy may have a priming effect by preparing the maternal immune response for an infectious insult. If there was an infectious insult during pregnancy, IL-18 may prime an inflammatory response to induce labour to prevent systemic infection which could have deleterious effects if pregnancy were to continue.

Ida et al found that serum IL-18 concentrations were higher in pregnant compared to non-pregnant controls. Blood was collected from women at each trimester of pregnancy, but longitudinal sampling was not performed, therefore a gestational rise was not observed [(297)].

IL-2 was found to positively correlate with the ratio of IFN-γ:CD4 cells. Th1 cells secrete IFN-γ promoting more undifferentiated Th0 cells to differentiate into Th1 cells, thereby promoting the Th1 cell response.

Reflecting upon the study design in this thesis, firstly, the timing of blood sampling was considered for the analysis of plasma cytokines. Where possible, all participants had blood collected in the morning. Cytokine secretion demonstrates a diurnal rhythm and peak levels of pro-inflammatory cytokines such as IL-6, TNF-α and IFN-γ are seen early in the morning [298]. Exercise has also been shown to temporarily increase cytokine levels, e.g. myocytes release IL-6 following exercise [299]. This was a variable which was not controlled for, but it is unlikely the pregnant participants were undertaking strenuous exercise prior to sampling.

Collecting blood in EDTA tubes have shown consistent cytokine recovery [300]. Samples were centrifuged and plasma was separated and frozen at -80℃ within 30 minutes of collection which limited variation in cytokine concentrations detected due to degradation, absorption or cellular production. Samples also only underwent one free thaw cycle to limit the effect on cytokine recovery. Experiments were performed within the 2 years of sample collection as recommended by De Jaeger et al recommended to limit the effect of cytokine degradation [300].

In the literature, there are similarities and discordances in studies assessing maternal peripheral cytokines during term pregnancy. Holtan et al reported gestational increases in IFN-γ, IL-1β, IL-6, IL-8 and IL-12 [301]. Serum cytokines were measured in 16 women by multiple cytokine assays, with a median number of 18 samples collected per study participant from the first trimester to delivery. Curry et al also noted increasing IL-12 and IFN-γ concentrations, and decreasing IL-2 and GM-CSF levels [302]. These were measured in plasma samples by multiplex flow cytometry. In contrast, Kraus et al and Denny et al reported decreasing IFN-γ with advancing pregnancy [303, 304]. Kraus et al's study was most similar in study design to this thesis with cytokines measured longitudinally at similar gestational timepoints, however serum samples were used. Denny et al's study involved stimulating whole blood with phytohaemaglutinin or lipopolysaccharide prior to detecting cytokine concentrations. Heterogeneity between studies and individual participant variability may explain some of these differences.

Higher serum IL-6 and IL-12 were found in women with preterm compared to term deliveries, although samples were collected shortly following birth [305]. At the onset of preterm and term labour, Jarocki et al reported an increase in serum IL-6 [306]. Tency measured cytokines in serum on a 30-plex Multiplex assay. Only 8 of the 30 were detectable in more than 50% of serum samples: epidermal growth factor (EGF), hepatocyte growth factor (HGF), IL-12, eotaxin, macrophage inflammatory protein (MIP)-1β, monocyte chemoattractant protein (MCP)-1, interferon-γ induced protein (IP)-10 and soluble IL-2 receptor. There were no differences in serum cytokine concentrations in women with term or preterm

labour [307]. There are no studies measuring peripheral cytokines longitudinally in term and preterm pregnancies to allow for direct comparisons.

Plasma complement

This current study reports that the concentrations of C5, C5a, MBL and C3b were stable across pregnancy, and that there were no significant differences between term and preterm pregnancies. Whilst a basal level of complement activation is required to maintain pregnancy, many adverse pregnancy outcomes have been associated with dysregulation of complement activation, including preterm labour, miscarriage, fetal growth restriction and hypertensive disorders of pregnancy [308, 309]. A well-regulated, primed complement system is key to protection against pathogens and maintaining host defence. It has a key role during placentation to achieve successful implantation via trophoblast invasion with the balance of the pro-invasion activity of C1q and the regulatory activity of MBL [310]. Many believe that it is this early balance of complement activity during placentation which is key to achieving a healthy pregnancy [311]. During placentation there is an increase in apoptotic cells with cellular debris and DNA fragments that are cleared by the complement system. This current study may not have sampled at a sufficiently early gestation to detect an early increase in complement activity.

It is widely thought that pregnancy is associated with increased complement activation in the periphery [198, 312, 313]. He et al measured plasma complement across healthy term pregnancies and found increased concentrations of C3a and C3c. However C5b-9 concentrations were unchanged which suggests that complement activation in normal pregnancy does not extend to the final common pathway [313]. It is important to also note that activation of the complement pathways may not be reflected in high circulating levels of complement proteins as concomitant consumption can lead to unchanged or perhaps even reduced concentrations.

With regards to adverse pregnancy outcomes, increased peripheral Factor Bb (alternative pathway) in early pregnancy has been associated with preterm birth [207, 314], and plasma C3a measured at the end of the first trimester has been found to be significantly higher in women with spontaneous preterm labour and preterm prelabour rupture of membranes (PPROM) [208]. In the mouse model, complement activation via C5a and its receptor led to local macrophage recruitment and matrix metalloprotease-9 production, culminating in cervical ripening and preterm birth [209].

A deficiency in MBL is associated with adverse pregnancy outcomes, including recurrent miscarriage and preterm birth [315-317]. A potential genetic susceptibility to preterm birth with the single nucleotide polymorphism in the MBL2 gene resulting in reduced MBL activity has been explored [318-320]. This may

partly explain wide variability in MBL plasma concentrations in this current study population and in others [313].

The current study does not show gestational changes in the peripheral complement activation during pregnancy across the sampling timepoints, and samples were not collected at the time of labour. But others have reported an increase in plasma C3b-9 and in the fetal membranes during parturition [321]. Furthermore, complement activation is likely to be at a local level during parturition. Murine studies have shown that complement activation is required for cervical remodelling, by the involvement of complement proteins in metalloproteinase activity, collagen degradation and macrophage chemotaxis [209, 322].

Cook et al found that there were microRNA biomarkers in peripheral blood which were predictive of cervical shortening and preterm delivery [266]. MicroRNAs are non-coding nucleotide molecules regulating messenger RNA stability and transcription. Up to 80% of human genes are believed to be regulated by microRNAs [323]. Cook et al identified has-miR-150-5p to be predictive of cervical shortening and preterm birth. It is regulated by NFKB, a key inflammatory transcription factor and a known target are MMPs which are involved in cervical ripening [324]. Despite this, we did not find a peripheral immune signature associated with cervical shortening.

Strengths and limitations

The collection of longitudinal samples is labour intensive and dependent upon the commitment of study participants. However, sample collection at two additional timepoints may help to answer several more important questions. Assessing the peripheral immune response at an earlier timepoint than 12-16 weeks may have revealed pregnancy immune adaptations related to implantation, and a later timepoint at the onset of parturition may have revealed differences in preterm and term labour.

The conclusions that could be drawn from the plasma cytokine data was limited due to the majority of concentrations being too low to be detected with suitable accuracy. This also demonstrates that there is no peripheral cytokine signature associated with preterm birth. For future evaluation of plasma cytokines, cell stimulation could be considered.

The study comprehensively phenotypes peripheral blood mononuclear cells, plasma complement and plasma cytokines in a well-defined study population. Components of the innate and adaptive immune responses and cellular and humoral immunity are collectively investigated, developing our understanding of the mechanistic peripheral immune adaptions to pregnancy. This current study also makes use of an advanced form of spectral flow cytometry to simultaneously evaluate many cell subtypes.

In summary, temporal systemic immune adaptions were identified in both healthy pregnancy and in women who subsequently delivered preterm. There was not a strong immune peripheral signature that discriminated between women who delivered preterm, underwent cervical shortening or cerclage placement compared to women who had uncomplicated pregnancies. Local immune changes will be explored in the next chapter.

CHAPTER 4: THE LOCAL IMMUNE RESPONSE IN HEALTHY PREGNANCY AND IN WOMEN WHO DELIVER PRETERM

4.1 Chapter summary

Hypothesis

- A successful term pregnancy is dependent on a highly regulated local cervico-vaginal immune response.
- There is immune dysregulation in inflammation induced preterm birth.

Aims

- To describe gestational age-related changes in cervico-vaginal fluid (CVF) cytokine, complement and immunoglobulins through healthy pregnancy.
- To describe changes in CVF cytokine, complement and immunoglobulins in women who deliver preterm.
- To describe the local CVF immune response in women who have had previous cervical excisional treatment compared to women who have a history of MTL/PTB.
- To describe the local CVF immune responses in women who have a short cervix.
- To describe changes in the local CVF immune response following intervention with cerclage and progesterone.

Methods

Cervico-vaginal fluid (CVF) was sampled from the posterior fornix of the cervix using a Liquid Amies swab (BBL™ CultureSwab™, Becton, Dickinson and Company) from study participants at three timepoints during pregnancy 12^{+0} - 16^{+6}, 20^{+0} - 24^{+6} and 30^{+0} - 34^{+6} weeks gestation. The cervico-vaginal fluid solution was centrifuged, and the resulting supernatant was used to measure concentrations of cytokine, complement and immunoglobulin analytes. CVF cytokines (IL-18, IFN-γ, IL-2, IL-4, IL-8, TNF-α, GM-CSF, IL-5, IL-1β, IL-6 and IL-10), CVF complement (C3b, MBL, C5 and C5a) and CVF immunoglobulins (IgG1-4, IgA and IgM) were measured by magnetic Luminex® assays. Detailed metadata was collected from each study participant, including gestational age at delivery, treatment with progesterone and/or cervical cerclage and cervical length measurement by transvaginal ultrasound. The transvaginal ultrasound was performed following CVF collection. Study participants were divided into 3 pregnancy outcome groups: term pregnancy, preterm pregnancy, term pregnancy with intervention (progesterone and/or cervical cerclage). Statistical differences between groups were analysed using the t-test or one-way ANOVA test if data was parametric, or Mann Whitney U test or Kruskal-Wallis test for non-parametric data, with Tukey's or Dunn's multiple comparisons test.

Results

Cervico-vaginal fluid immune mediators were stable across healthy pregnancy. In contrast, there was a local pro-inflammatory immune response early to mid-gestation in women who delivered preterm. In early pregnancy, there was a subtle pro-inflammatory signature in women with a history of previous MTL and/or PTB, in contrast to women with a history of cervical excisional treatment. Cervical shortening was associated with local inflammation, with activation of both the innate and adaptive immune response. Cervical cerclage with Mersilene resulted in a significantly increased local pro-inflammatory milieu compared to Nylon, regardless of the indication for cerclage and delivery outcome.

Conclusions

Local innate and adaptive immune activation precedes spontaneous preterm birth in a significant proportion of women. It is plausible that women with a prior history of PTB/MTL have a primed immune response, and that inflammation drives some but not all cases of cervical shortening. Whilst Nylon is immunologically inert, Mersilene augments both the innate and adaptive immune response and is associated with a higher preterm birth rate, and therefore should not be used.

4.2 Introduction

The leading cause of preterm labour is inflammation and infection, with the primary route of infection believed to be ascending from the vagina to the uterus. The cervix provides a structural and immunological barrier to ascending microbes. Collagen fibres in the extracellular matrix provide tensile strength, whilst the squamous epithelial lining produces a mucus layer which prevents microbial adherence and epithelial invasion. Mucus proteins and anti-microbials such as lysozyme, defensins and immunoglobulins constitute some of the innate and adaptive defenses. A healthy lower reproductive tract has a low pH between 4-4.5 which prevents the proliferation of pathogenic organisms.

In physiological term labour, there is a gradual decline in collagen concentration and solubility in the cervix as the cervix undergoes structural remodelling [325]. IL-6, IL-8, GM-CSF and TNF-α increase locally and induce cervical leukocyte invasion, and the secretion of matrix metalloproteinases, namely MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9 [326-329]. There is also a key role for cervical fibroblasts [330]. Activated fibroblasts also secrete IL-6, IL-8, MMP-1 and MMP-3. Prostaglandins are used in clinical practice to induce cervical ripening and labour and there is known to be decreased local prostaglandin degradation in term and preterm labour [331].

The free radical nitric oxide is also involved in cervical remodelling by acting on collagen fibres [332, 333]. Another factor is corticotropin-releasing hormone (CRH) which regulates the hypothalamic-pituitary-adrenal axis and has been shown to stimulate IL-8 production in cervical fibroblasts [334].

IL-10 is an anti-inflammatory cytokine and has been shown to cause a decrease in the production of pro-inflammatory cytokines, IL-8, IL-6, TNF-α and IL-1β, MMPs and prostaglandin E2 in fetal membranes [335-338]. Interestingly, polymorphisms in single nucleotide polymorphisms in genes encoding cytokines, including anti-inflammatory IL-10, pro-inflammatory IL-4 and IL-6 and complement protein MBL have been described to influence the susceptibility of preterm birth [317].

Cervical cerclage is used to prevent or halt cervical shortening and is thought to reduce the risk of preterm delivery by mechanically supporting the cervix, protecting the mucus plug, and preventing ascending infection. The procedure is invasive and has the potential to cause tissue trauma and thus inflammation. Additionally, there is much interest in the choice of suture material to perform the

procedure with emerging evidence to support an enhanced inflammatory response following the insertion of a braided Mersilene cerclage [49, 339].

Less well studied is the mucosal immunity conferred by complement and immunoglobulins in the context of pregnancy. Complement plays an important role in host defence mechanisms, including bacteriolysis, immune adherence and enhancing phagocytosis. Mucosal complement activity is known to increase in infection at the mucosal surfaces in mastitis, conjunctivitis, inflammatory bowel disease and inflammatory nephritides [340-343]. The role of complement on genital tract mucosal surfaces requires exploration. Murine studies suggest local complement activation in infection induced preterm labour in murine models, but as yet there are no reported human studies assessing local complement activation [209, 322].

The protective role of immunoglobulins at mucosal surfaces is highlighted by the IgA, IgG, IgM in cervico-vaginal fluid from HIV-1 positive women inhibiting viral transcytosis across human epithelial cells [344, 345]. An increase in cervico-vaginal fluid IgA and IgG has also been observed around the time of ovulation [346, 347]. The role of local cervico-vaginal fluid immunoglobulins is relatively unexplored in pregnancy.

This Chapter explores the local innate and adaptive immune response in cervico-vaginal fluid longitudinally in term and preterm pregnancies. The study of complement and immunoglobulin activity in cervico-vaginal fluid in pregnancy is novel. The study population is large and well phenotyped allowing the comparison of the local immune response according to the risk factor of preterm birth, (mechanically driven previous excisional cervical treatment or presumed inflammation mediated in those with previous MTL/PTB), and the local immune response to cervical shortening and cervical cerclage.

4.3 Study design

To study the local cervico-vaginal immune changes in women with preterm and term pregnancies, women with risk factors for preterm birth were prospectively recruited from the preterm birth prevention clinics at Queen Charlotte's and Chelsea Hospital, St Mary's Hospital, Chelsea and Westminster Hospital, University College Hospital and Royal Infirmary of Edinburgh. Exclusion criteria were multiple pregnancies, women who had sexual activity within 72 hours of sampling, vaginal bleeding and women who were HIV or hepatitis B positive.

Women were followed up longitudinally through pregnancy. Swabs were taken to sample the cervico-vaginal fluid (CVF) in the posterior fornix of the vagina at three timepoints, 12^{+0} - 16^{+6} weeks, 20^{+0} -24^{+6} weeks and 30^{+0} - 34^{+6} weeks. The cervical length was obtained by a transvaginal ultrasound at the same timepoints on the same day as CVF sampling. Study participants were divided into 3 outcome groups retrospectively: a) women who had spontaneous preterm deliveries (preterm, PT); b) women who delivered at term without intervention (term, T); c) women who delivered at term following an elective history or ultrasound indicated cerclage and/or progesterone (term intervention, TI). Detailed metadata was collected from all study participants from the hospital notes and the electronic patient database, Cerner Millennium Powerchart®. As described in the Materials and Methods chapter, section 2.2.5, CVF cytokines, complement and immunoglobulins were measured by magnetic Luminex® assays.

4.4 Statistical analysis

Statistical analysis was performed using Graphpad Prism 8.4.1. Differences between two groups were analysed using the t-test if the data was normally distributed, or the Mann Whitney U test for data which was not normally distributed. Differences between three groups were analysed using the one-way ANOVA or the Kruskal-Wallis test depending on the distribution of the data together with the appropriate post hoc comparisons test, Dunnett's or Dunn's. The Fisher's exact or Chi square test was used to test for proportional differences. For all tests, the level of statistical significance was taken as a p value ≤0.5.

4.5 Results

4.5.1 Study population characteristics

133 women were prospectively recruited to this study whilst attending the preterm birth prevention clinics. Referral criteria included previous preterm birth, previous mid-trimester miscarriage and previous excisional cervical treatment. Based upon the pregnancy outcome, they were divided into 3 groups, women who had a preterm delivery before 37 weeks gestation N=37 (28%), women who delivered at term not requiring any intervention to prevent preterm birth N=56 (42%), and the third group were women who delivered at term but required an intervention to prevent preterm birth (cervical cerclage and/or progesterone) N=40 (30%). The median gestational age at delivery in each cohort were 33^{+3}, 39^{+5} and 38^{+3} weeks respectively (Table 4.1).

Between the three pregnancy outcome groups, there were no significant differences in maternal age, BMI or ethnicity. Women were divided into 3 categories for ethnicity, White, Black and Other. Table 4.2 contains further detail for 28 women in the 'Other' category. The largest proportion were of South Asian origin 12/28 (43%). The remaining included women of Middle Eastern origin 4/28 (14%), South East Asian origin 1/28 (4%) and mixed origins 11/28 (39%). This represents the multi-ethnicities of residents in Central and West London where most study participants were recruited.

Most cervical cerclages performed in the preterm group were ultrasound indicated, 13/21 (62%), whilst the majority in the term with intervention group were history indicated, 24/38 (63%). The cerclage material used in each case was also recorded in 99% of cases. The choice of cerclage was based on the clinician's choice or following randomisation to C-STITCH. Of women who received Mersilene N=26), the preterm birth rate was 50%, and of women who received Nylon (N=29), the preterm birth rate was 24%, which was statistically significant, (Fisher's exact test $p<0.05$).

The most common risk factor in the three pregnancy outcome groups was significantly different; previous preterm birth was a risk factor in 62% of the study participants in the preterm birth group, cervical excisional treatment was a risk factor in 64% of study participants who had a term delivery (without intervention) and previous mid trimester miscarriage was a risk factor for 55% of study participants who delivered at term but with an intervention (cervical cerclage +/- progesterone).

Table 4.1 Clinical and demographical characteristics of the study population N=133

	Preterm (PT) (<37 weeks)	Term (T) no intervention	Term intervention (TI)	p value (PT vs T vs TI)
N (%)	37 (28%)	56 (42%)	40 (30%)	
Gestation at delivery (weeks) median and IQ range	33^{+3} (31^{+5} - 36^{+0})	39^{+5} (39^{+0} - 40^{+5})	38^{+3} (38^{+0} - 39^{+0})	One-way ANOVA p<0.0001 **** PT vs T p<0.0001 **** PT vs TI p<0.0001 ****
Early preterm </=33+6	21 (57%)	NA	NA	
Late preterm >/=34+0	16 (43%)	NA	NA	
Age median and IQ range	31 (28-38)	33 (30-36)	33 (31-36)	One-way ANOVA p=0.93
BMI median and IQ range kg/m^2	24 (22-28)	22 (20-27)	26 (23-28)	One-way ANOVA p=0.20
Ethnicity White N (%) Black N (%) Other N (%)	 18 (48%) 9 (24%) 10 (28%)	 40 (72%) 7 (12%) 9 (16%)	 19 (48%) 12 (30%) 9 (22%)	χ^2 =0.09
Parity Nulliparous Multiparous	 11 (30%) 26 (70%)	 31 (55%) 25 (45%)	 14 (35%) 26 (65%)	
Cervical cerclage	21 (57%)	0	38 (95%)	
History indicated cervical cerclage and material	8/21 (38%) (Mersilene 5, Nylon 3)	NA	24/38 (63%) (Mersilene 11, Nylon 11, Abdominal 1, Unknown 1)	
USS indicated cervical cerclage and material	13/21 (62%) (Mersilene 8, Nylon 4, Unknown 1)	NA	14/38 (37%) (Mersilene 2, Nylon 11, Abdominal 1)	
Progesterone only	3	NA	2	
Risk factor for PTB: (by individual) Cervical treatment Cervical treatment + MTL Cervical treatment + PTB MTL MTL + PTB PTB 3x 1^{st} trimester miscarriage Short cervix at anomaly	 6 1 7 7 1 15 0 0	 34 2 0 5 2 12 1 0	 8 3 1 13 6 8 0 1	
Risk factor for PTB [(a)] Cervical treatment Previous PTB Previous MTL	 14 (38%) 23 (62%) 9 (24%)	 36 (64%) 14 (25%) 9 (16%)	 12 (30%) 15 (38%) 22 (55%)	χ^2 p<0.0001 ****

BMI=body mass index, USS=ultrasound, PTB=preterm birth, MTL=mid trimester loss, misc=miscarriage. Data presented as median (interquartile range (IQ)) or number (%). P values: One way ANOVA for multiple comparisons or Chi squared for proportional data.
[(a)]Some study participants will have more than one risk factor for PTB.

Table 4.2 Further classification of 'Other' ethnicity category

	Preterm (PT) (<37 weeks)	Term (T) no intervention	Term intervention (TI)
South East Asian (e.g. Chinese, Japanese, Mongolian, Korean)	0	0	1
South Asian (e.g. Indian, Pakistan, Bangladesh)	6	3	3
Middle East	1	1	2
Mixed European, South East Asian	2	1	2
Mixed European, South Asian	1	3	1
Mixed non-European, South East Asian	0	1	0

4.5.2 Standardisation techniques for multiple Luminex® assay

The concentrations of the cytokine, complement and immunoglobulin analytes were determined by the magnitude of emitted fluorescence signal which was in direct proportion to the amount of analyte bound to the analyte-specific capture binding antibody binding to the specific analyte. This was calculated by the Bio-Plex® 200 software. Standard concentrations were provided by the manufacturer and analyte detections were compared to the standard ranges. Prior to performing the analysis for the study cohort, the interpatient and interassay variation was explored.

It was important to standardise the results obtained from the Luminex® assays based upon the potential for differing protein quantities in individual CVF swabs. The Bradford protein assay was used to measure the concentration of the total cellular protein in each sample in duplicates, (pg/ml). The concentration of protein in each sample was calculated per analyte. The concentration of protein per sample = the concentration of protein (Bradford assay) x volume of sample used on assay. From this, the concentration of analyte in pg/ml/µg of protein was calculated by dividing the raw observed concentrations of the analytes measured by Luminex by the concentration of protein by the Bradford assay. Proportionally, the raw and normalised concentrations for the analytes did not show any differences, therefore the raw concentrations are presented in this chapter.

Due to the high number of CVF samples for analysis by Luminex® assays, experiments were performed on multiple 96 well plates. The standards and controls were compared across the different cytokine (N=12), complement (N=12) and immunoglobulin (N=13) plates.

The mean average of co-efficient of variation of each standard (accounting for each analyte) and a control sample per type of multiplex plate is shown in Table 4.3. A co-efficient of variation of <5% for control samples was deemed an acceptable level of variation across plates. Cytokines analytes IFN-γ, IL-10, IL-2, IL-5, TNF-α, GM-CSF, IL-1β, IL-18, IL-4 and IL-6 were analysed together on a multiplex plate. Complement analytes C5, C5a and MBL were analysed together on a multiplex plate. The mean

average of co-efficient of variation was calculated for a single analyte for the single plex plates IL-8 and C3b. Overall, the level of variation between plates was low, (Table 4.3). The variation was greater for the most diluted standards which were more prone for introducing variation across different plates. The similarity and lack of variation between the standard curves across plates is demonstrated in graphically in Figures 4.1, 4.2, 4.3.

Table 4.3 Co-efficient of variation for each standard on each type of Luminex plate
S1-S7 represents each standard, S1 being the most concentrated.

Luminex plate	S1	S2	S3	S4	S5	S6	S7	S8	Control
Cytokines (except IL-8)	0.98%	1.31%	1.55%	2.06%	1.76%	1.72%	3.57%	4.13%	4.56%
IL-8	0.31%	0.39%	1.13%	2.26%	2.18%	0.63%	-	-	1.52%
C5, C5a, MBL	0.14%	2.14%	2.87%	6.02%	9.46%	13.54%	12.37%	-	4.32%
C3b	0.74%	1.87%	3.25%	2.83%	2.93%	6.09%	10.64%	-	3.26%
Immunoglobulins	0.81%	1.38%	2.40%	2.50%	3.43%	7.45%	3.62%	-	4.44%

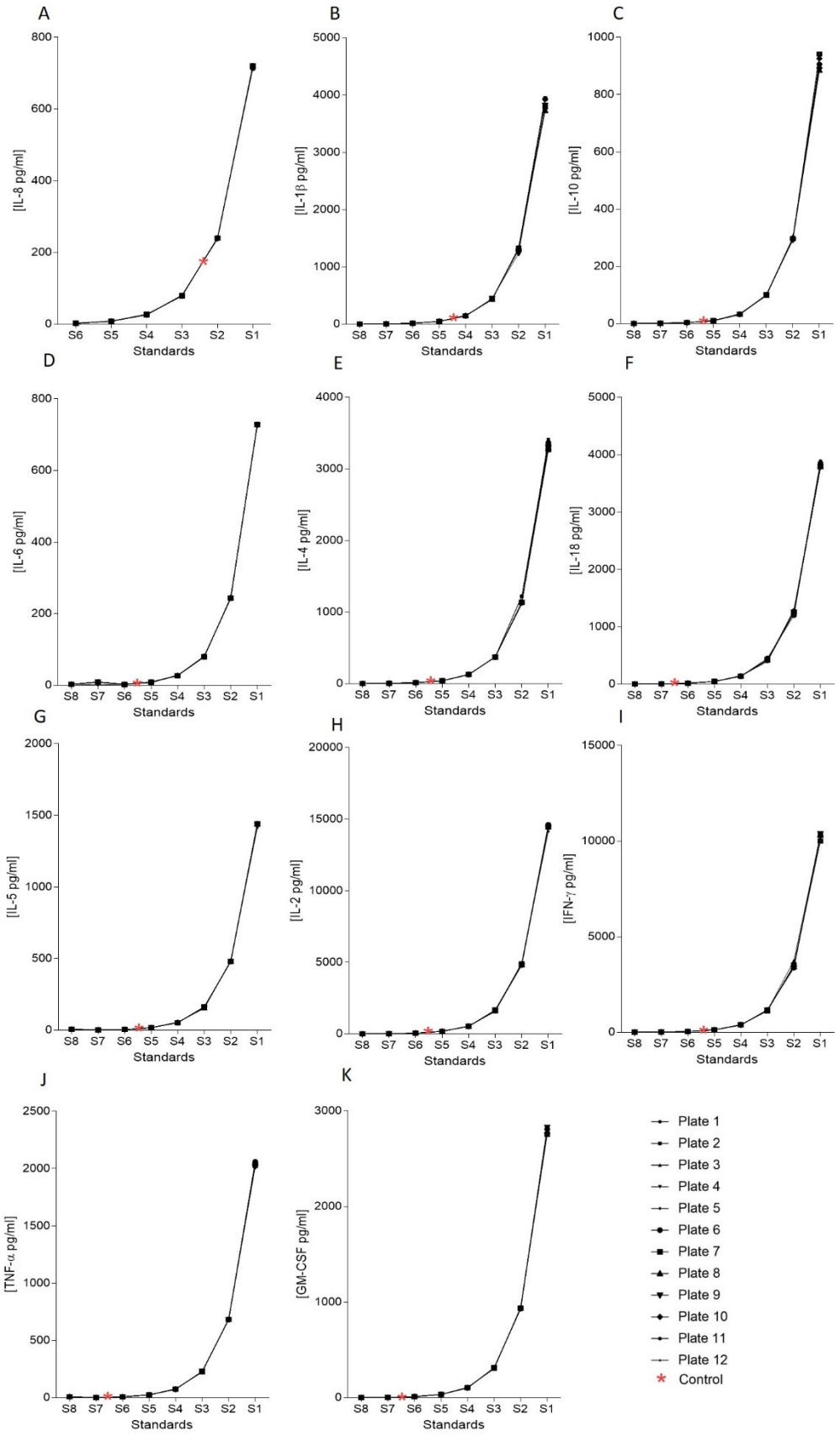


Figure 4.1 Standard curves for each cytokine analyte
Standard curves generated from Luminex® assay standards and control samples for IL-8, IL-1β, IL-10, IL-6, IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α and GM-CSF over multiple assay plates.

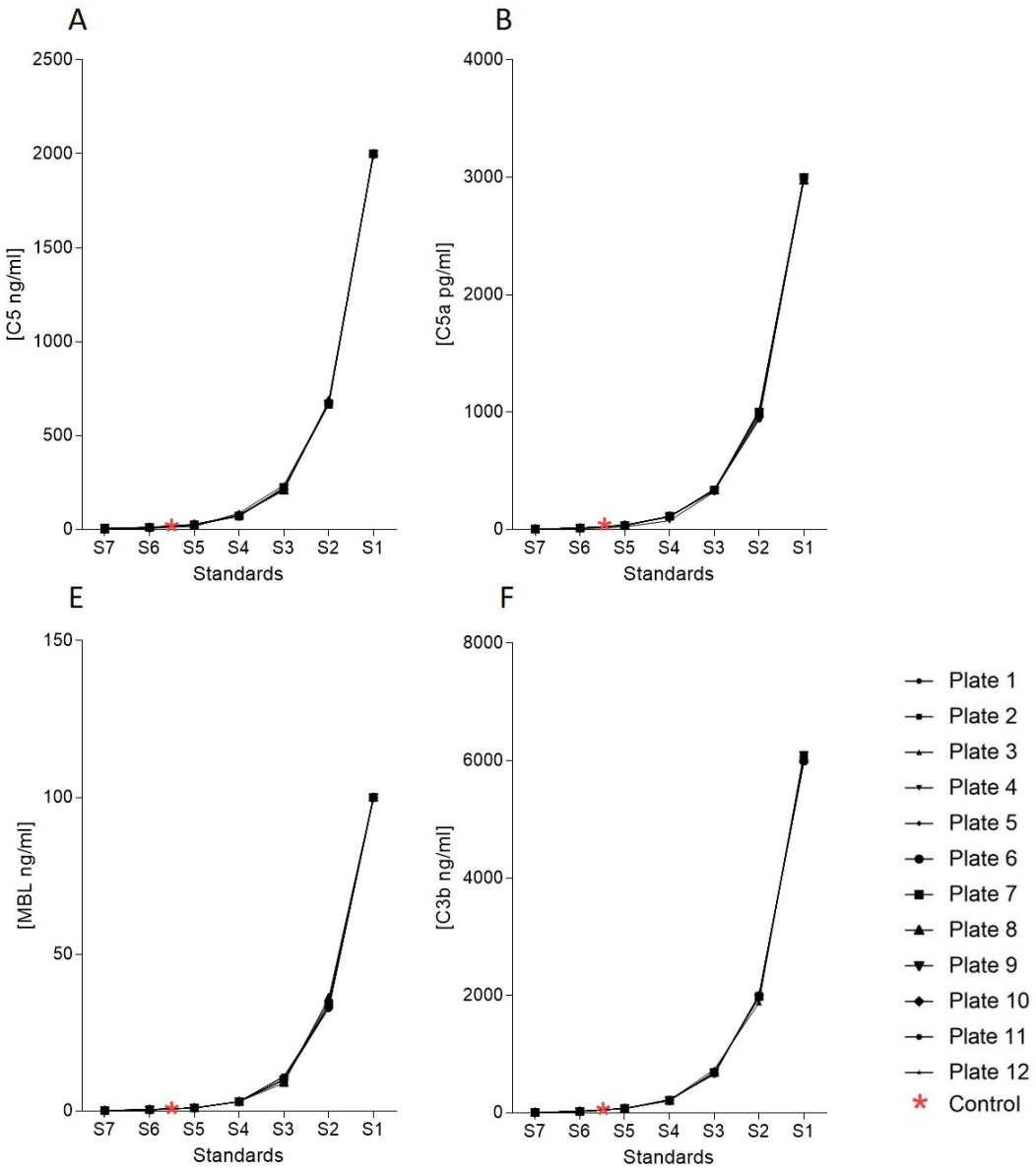


Figure 4.2 Standard curves for each complement analyte
Standard curves generated from Luminex® assay standards and control samples C5, C5a, MBL and C3b over multiple assay plates.

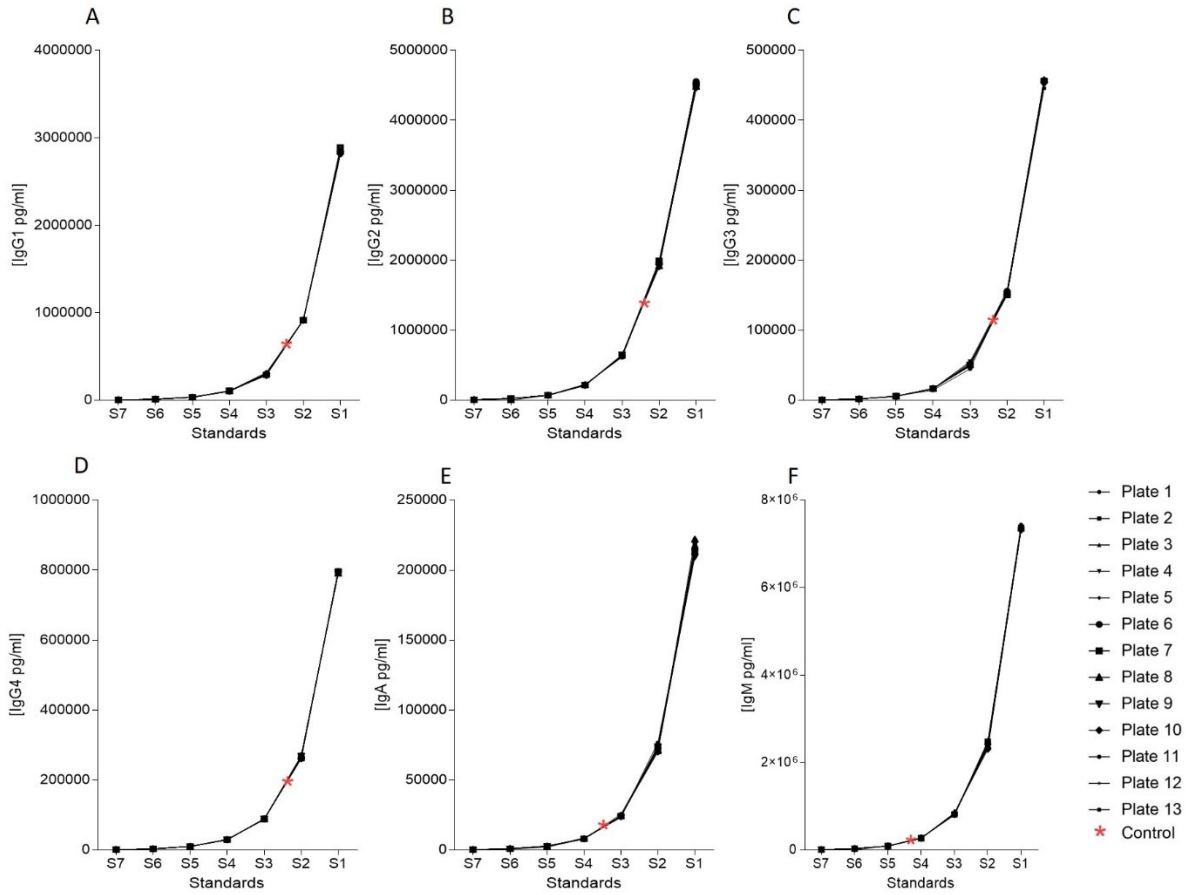


Figure 4.3 Standard curves for each immunoglobulin analyte
Standard curves generated from Luminex® assay standards and control samples for IgG1-4, IgA and IgM d over 13 assay plates.

385 swabs were collected from 133 study participants. The IL-8 assay was performed using a 1:10 dilution. All samples had detectable IL-8 within the standard curve range of the assay. All other cytokines were measured in undiluted cervico-vaginal fluid samples. IL1-β was the next most detectable cytokine in cervico-vaginal fluid, and 92% of samples had concentrations within the standard curve range of the assay. In descending order of percentage of concentrations falling within the standard curve range of the assay, the remaining cytokines from the most to the least detectable were IL-10, IL-6, IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α, GM-CSF (Table 4.4 A). Concentrations of GM-CSF were greater than the lowest standard concentration in only 21% of samples. C5a was the most detectable complement analyte; detectable in 97% of samples (Table 4.4 B). Almost all the immunoglobulin analytes were within the range of the standard curve, and this was above 95% for IgG1-3, IgA and IgM (Table 4.4 C).

Table 4.4 Cervico-vaginal fluid cytokine, complement and immunoglobulin assay ranges
The total number of CVF samples was 385, from 133 study participants.

A.

Cytokine	Standard curve range (pg/ml)	Samples within standard curve range of assay (N) (Total = 385)	% within standard curve range of assay	Sensitivity level (pg/ml)
IL-8	2.96 - 720	385	100%	1.8
IL-1β	1.81 - 3950	356	92%	0.8
IL-10	0.41 - 900	295	77%	1.6
IL-6	0.33 - 730	246	64%	1.7
IL-4	1.55 - 3380	235	61%	9.3
IL-18	1.73 - 3790	209	54%	1.93
IL-5	0.66 - 1440	209	54%	0.5
IL-2	6.62 - 14470	204	53%	1.8
IFN-γ	4.7 - 10290	185	48%	0.4
TNF-α	0.94 - 2050	165	43%	1.2
GM-CSF	1.28 - 2800	80	21%	4.1

B.

Complement analyte	Standard curve range	Samples within standard curve range of assay (N) (Total = 385)	% within standard curve range of assay	Sensitivity
C5 ng/ml	2.74-2000	264	69%	1.04
C5a pg/ml	4.12-3000	373	97%	0.0051
MBL ng/ml	0.14-100	286	74%	0.04
C3b ng/ml	8.23-6000	249	65%	3.639

C.

Immunoglobulin analyte	Standard curve range	Samples within standard curve range of assay (N) (Total N=385)	% within standard curve range of assay
IgG1 pg/ml	3784.5-137945000	382	99%
IgG2 pg/ml	7922.63-5775600	377	98%
IgG3 pg/ml	620.16-150700	378	98%
IgG4 pg/ml	1092.73-79600	363	94%
IgA pg/ml	297.53-10845000	385	100%
IgM pg/ml	1092.73-7324600	384	99%

There were no sensitivities provided by Procarta, the supplier of the immunoglobulin plates.

4.5.3 Cervico-vaginal immune response across gestation and by pregnancy outcome

The clinical and demographical characteristics for all study participants are in Table 4.1. There were 133 women in this cohort. 37 women delivered before 37 weeks gestation. 56 women were in the term (uncomplicated), and 40 women were in the term with intervention group. They delivered at term gestation but received a cervical cerclage and/or progesterone. The three sampling timepoints were 12^{+0} - 16^{+6}, 20^{+0} - 24^{+6} and 30^{+0} - 34^{+6} weeks gestation.

In the preterm group, 28 women were sampled at all three timepoints. 6 women were sampled at the first two timepoints only because they delivered before sampling at the third timepoint. There was one woman who was only sampled at the 12^{+0} - 16^{+6} week timepoint, one woman who was only sampled at 20^{+0} - 24^{+6} weeks and one woman who was only sampled at 20^{+0} - 24^{+6} and 30^{+0} - 34^{+6} weeks.

In the term (uncomplicated) group, 51 women were sampled at all three timepoints. 4 women were sampled at the latter two timepoints only, and 1 woman was sampled at the first and third timepoints only. In the term with intervention group 37 women were sampled at all three timepoints. Three women were sampled at only 2 timepoints. Women who did not attend appointments at the required timepoints were not sampled.

The term with intervention group contained a heterogenous mix of women. Some of whom were highly likely to deliver preterm if they had not received a cervical cerclage, and some who were more phenotypically similar to the women in the term (uncomplicated) group.

There was no gestational age effect in cervico-vaginal fluid concentrations of IL-8, IL-1β, IL-10, IL-6, IL-4, IL-18, IL-5, IL-2, TNF-α or GM-CSF in either of the two term groups, Figures 4.4 and 4.5. IFN-γ decreased with advancing gestation, but only in the term with intervention group (p=0.01). In this analysis of the whole study population considering all three sampling timepoints, there was an increase in IL-8 (p=0.05) between 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks in the preterm group, but the statistical significance was lost with Dunn's post hoc comparisons test.

Cytokines were compared at each of the sampling timepoints according to pregnancy outcome, (preterm vs term vs term with intervention at 12^{+0} -16^{+6} weeks, preterm vs term vs term with intervention at 20^{+0} - 24^{+6} weeks, and preterm vs term vs term with intervention at 30^{+0} - 34^{+6} weeks). IL-8 was significant higher in the preterm compared to the term (uncomplicated group) at the 20^{+0} - 24^{+6} week timepoint, (p=0.04), Figure 4.4 A. Other analytes showing significant differences in women with preterm and term deliveries were IL-6 which was significantly higher at 20^{+0} - 24^{+6} weeks in preterm compared to term with intervention group, (p=0.04) Figure 4.4 C, IL-2 was significantly higher at 20^{+0} - 24^{+6} weeks in the preterm group compared to both term groups, (p=0.03), Figure 4.5 D, IFN-γ was significantly higher at 20^{+0} - 24^{+6} week in preterm compared to term with intervention group (p=0.05) and higher at 30^{+0} - 34^{+6} weeks in term compared to term with intervention group, (p=0.05), Figure 4.5 E, and GM-CSF was significantly higher at 20^{+0} - 24^{+6} weeks in preterm compared to term group, (p=0.04), Figure 4.5 G. Analytes showing significantly higher concentrations in women with term (no intervention) compared to term with intervention were IL-18, IL-5 and IFN-γ (p=0.04), Figure 4.5 B, C and E. The highest and lowest values on the standard curve for each analyte are shown on Figures 4.4 and 4.5.

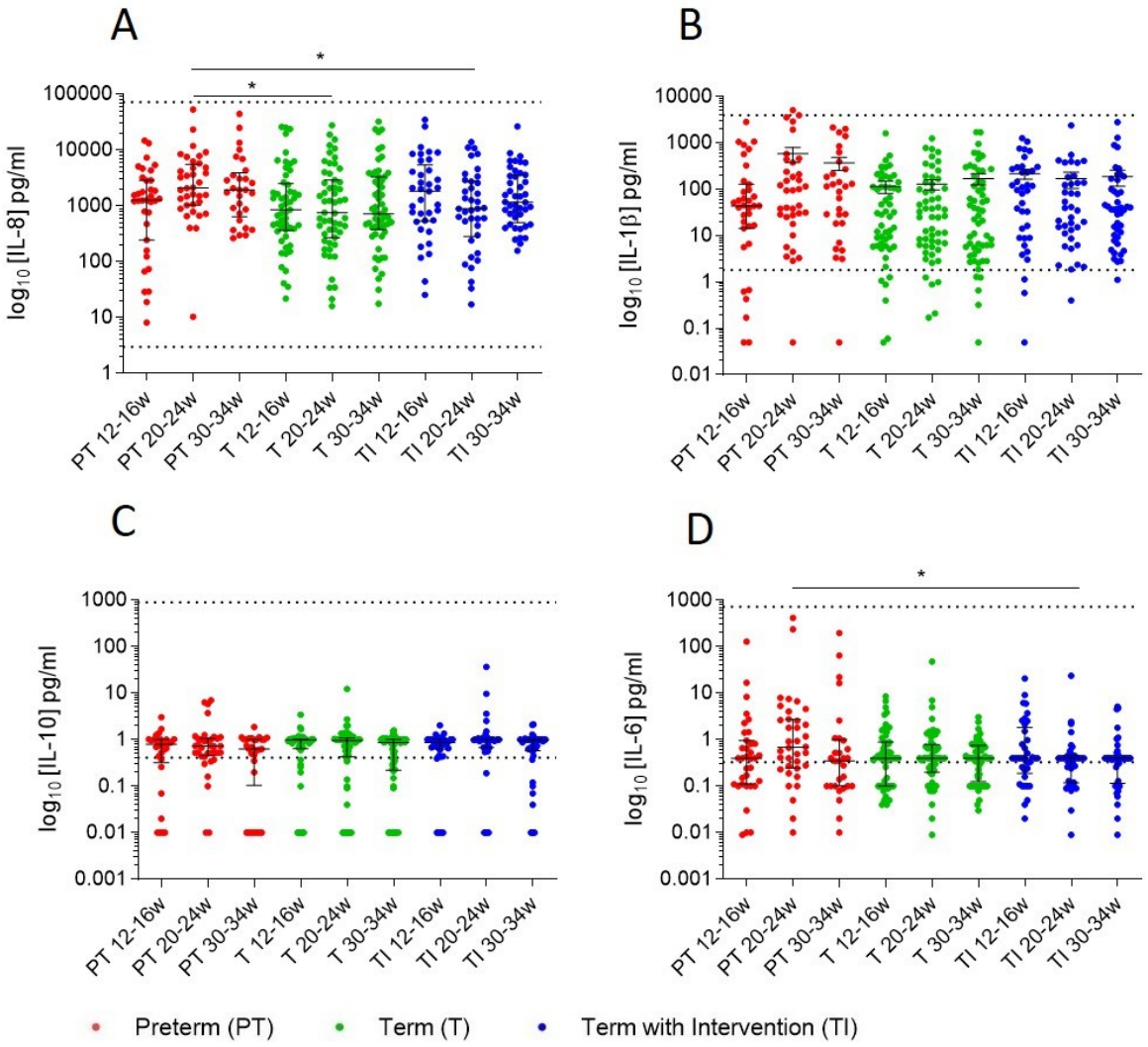


Figure 4.4 Cervico-vaginal fluid cytokines IL-8, IL-1β, IL-10 and IL-6 in preterm, term and term with intervention pregnancies and across pregnancy, in the whole study population

There were no gestational differences across the three sampling timepoints with IL-8, IL-1β, IL-10 or IL-6. IL-8 was significantly higher in the preterm compared to both term groups at the 20-24 week timepoint ($p<0.05$) (A). IL-6 was significantly higher in the preterm group compared to the term with intervention group at the 20-24 week timepoint ($p<0.05$) (D). IL-1β and IL-10 concentrations did not differ significantly between the 3 pregnancy outcome groups at the three sampling timepoints.

Statistical analysis assessing the differences across the 3 pregnancy outcome groups was by the Kruskal-Wallis test with Dunn's multiple comparisons test, preterm (PT) N=37, term (T) N=56, term with intervention (TI) N=40. Statistical analysis assessing the differences across pregnancy gestation, across the three sampling time points 12-16, 20-24 and 30-34 weeks was also by Kruskal-Wallis test with Dunn's multiple comparisons test.

(* $p<0.05$). Dotted lines on A-D mark the concentration of the lowest and highest standards on the assay. The assay for IL-8 was performed using a 1:10 dilution of CVF. The results are expressed as median and interquartile range.

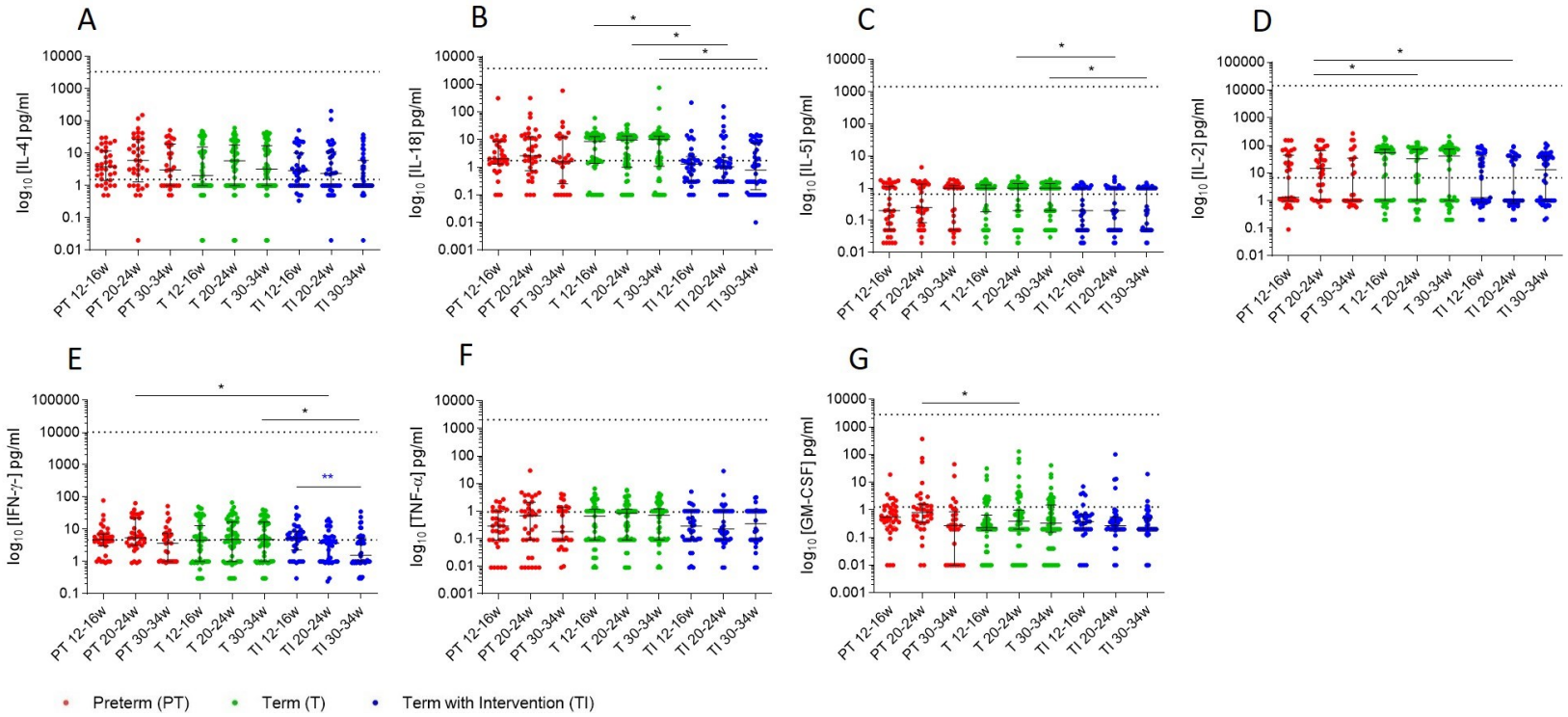


Figure 4.5 Cervico-vaginal fluid cytokines IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α, GM-CSF in preterm, term and term with intervention pregnancies and across pregnancy in the whole study population

IL-18 was significantly higher in the term group compared to the term with intervention group at all sampling timepoints ($p<0.05$) (B). IL-5 showed the same pattern but was significantly higher at the latter two sampling timepoints ($p<0.05$) (C) and IFN-γ at the latest sampling timepoint ($p<0.05$) (E). IL-2 and GM-CSF were significantly higher in the preterm group compared to the term group at 20-24 weeks ($p<0.05$) (D and G), whilst IFN-γ was higher in the preterm compared to the term with intervention group at 20-24 weeks ($p<0.05$) (E). IL-4 and TNF-α did not show differences between the preterm, term and term with intervention groups (A and F). There was a significant decrease in IFN-γ with advancing gestation ($p<0.05$) (E). The other analytes did not show any change over pregnancy.

Statistical analysis assessing the differences across the 3 pregnancy outcome groups was by the Kruskal-Wallis test with Dunn's multiple comparisons test, preterm (PT) N=37, term (T) N=56, term with intervention (TI) N=40. Statistical analysis assessing the differences across pregnancy gestation, across the three sampling time points 12-16, 20-24 and 30-34 weeks was also by Kruskal-Wallis test with Dunn's multiple comparisons test. Dotted lines on A-G mark the concentration of the lowest and highest standards on the assay. (* $p<0.05$, ** $p<0.01$). The results are expressed as median and interquartile range.

In this analysis of the whole study population, comparing across the three sampling timepoints there was no statistically significant gestational change in concentrations of C5, C5a, C3b and MBL in preterm and term pregnancies (p>0.05), Figure 4.6.

C3b was significantly higher in women who had preterm compared to term deliveries at the 20^{+0} - 24^{+6} week sampling timepoint, (p=0.04), Figure 4.6 D. There were no differences according to the preterm, term and term with intervention pregnancies for C5, C5a and MBL, when comparing each at the three sampling timepoints (p>0.05). There was no significant gestational change in the concentrations of C5, C5a, MBL or C3b (p>0.05), Figure 4.6 A-D.

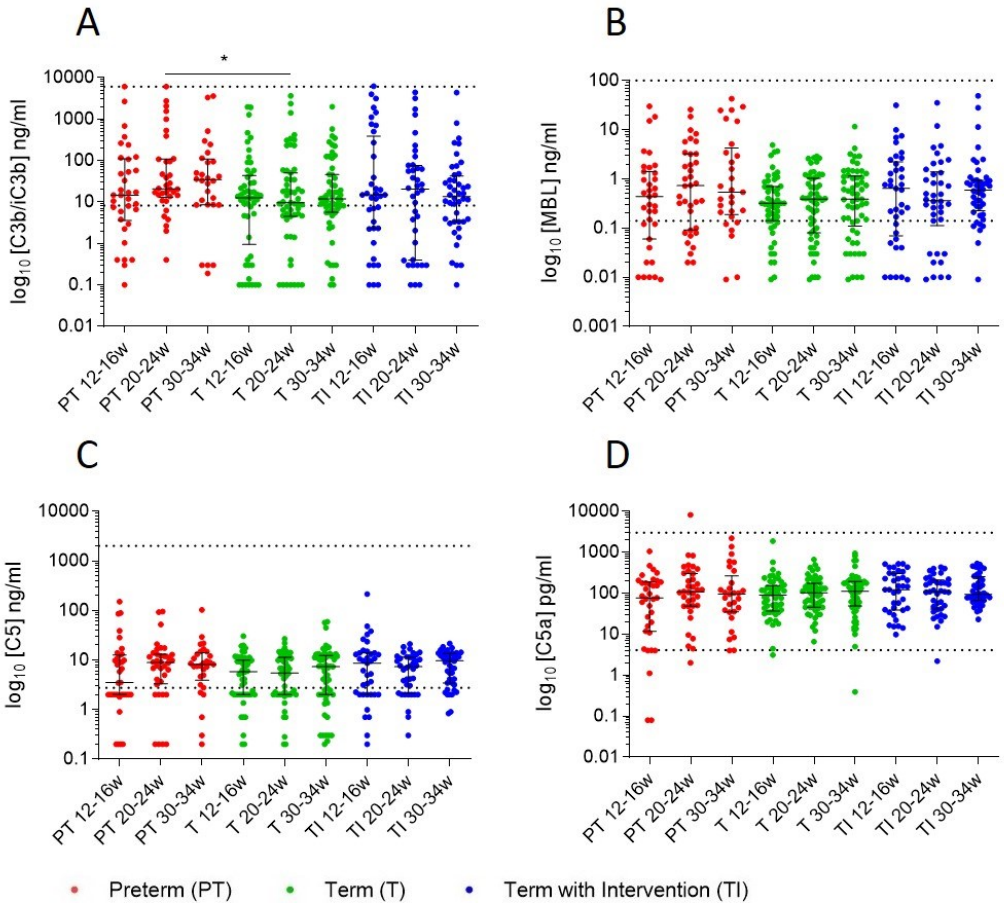


Figure 4.6 Cervico-vaginal fluid complement in preterm, term and term with intervention pregnancies and across pregnancy in the whole study population
C3b was significantly higher at 20-24 weeks in the preterm group compared to the term group (p<0.05). There were no differences in MBL, C5 or C5a according to the three pregnancy outcome groups, preterm (PT), term (T) and term with intervention (TI). There were no differences in these analytes with advancing gestation.
Statistical analysis assessing the differences across the 3 pregnancy outcome groups was by Kruskal-Wallis test with Dunn's multiple comparisons test, preterm (PT) N=37, term (T) N=56, term with intervention (TI) N=40. Statistical analysis assessing the differences across pregnancy gestation, across the three sampling time points 12-16, 20-24 and 30-34 weeks was also by Kruskal-Wallis test with Dunn's multiple comparisons test. Dotted lines on A-D mark the concentration of the lowest and highest standards on the assay. (* p<0.05, ** p<0.01). The results are expressed as median and interquartile range.

The following immunoglobulins were measured, IgG1-4, IgA and IgM in cervico-vaginal fluid, Figure 4.7. The most abundant immunoglobulin was IgG1. The only immunoglobulin to show a gestational change was IgA, which increased with advancing gestation, however this was only in the term with intervention outcome group, ($p=0.04$), Figure 4.7 E. Concentrations of IgG1-4 and IgM did not significantly change across pregnancy in the three sampling timepoints ($p>0.05$).

IgM was significantly more abundant in women with preterm deliveries compared to term deliveries at both the 20^{+0} - 24^{+6} week and 30^{+0} - 34^{+6} week sampling timepoints ($p=0.03$), Figure 4.7F. There were no other differences in IgG1-4 and IgA between preterm and term deliveries ($p>0.05$).

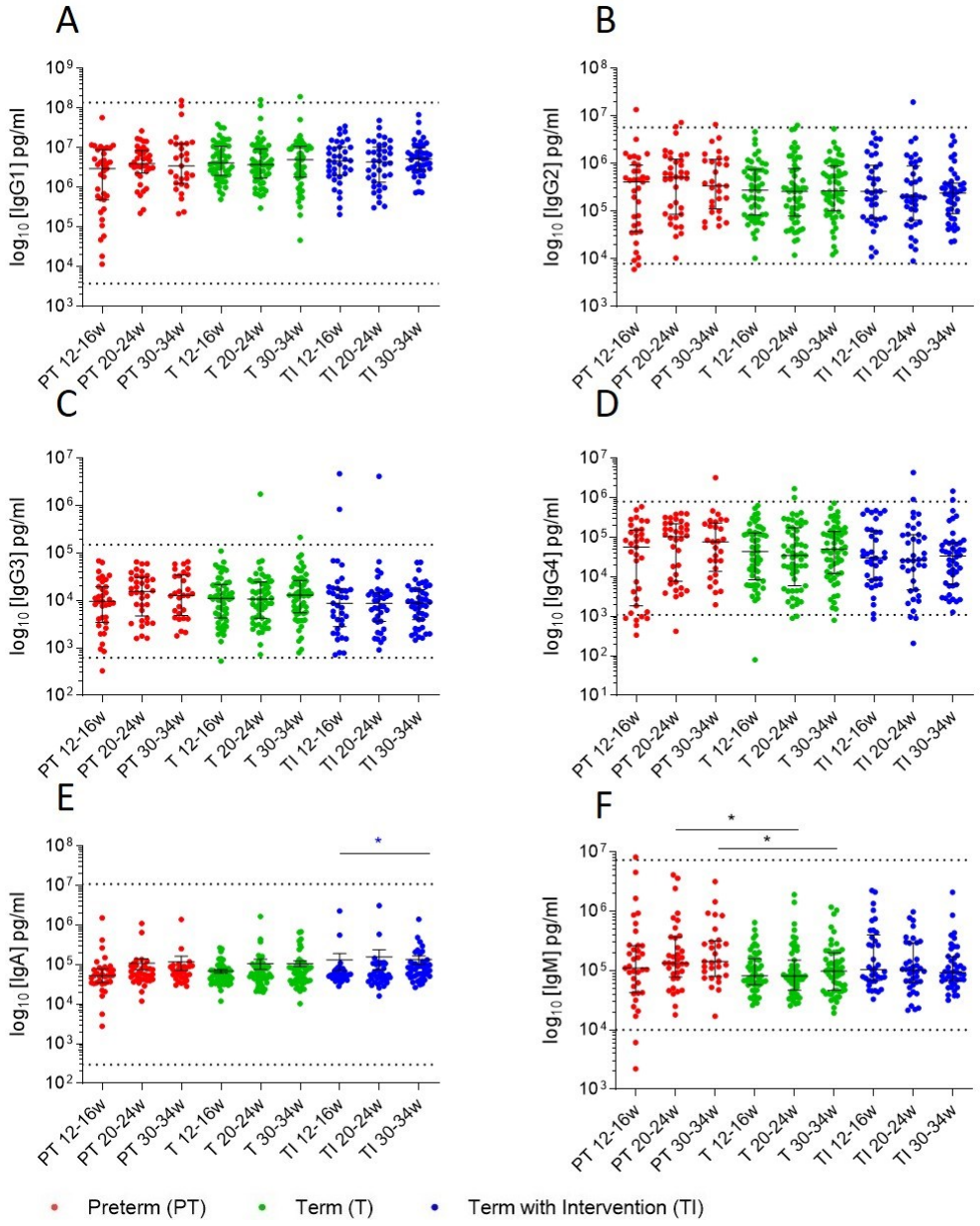


Figure 4.7 Cervico-vaginal fluid immunoglobulins in preterm, term and term with intervention pregnancies and across pregnancy, in the whole study population

IgM was significantly higher in the women who delivered preterm compared to those who delivered at term. This was statistically significant at 20-24 weeks and 30-34 weeks (F). There was no difference in CVF IgG1-4 and IgA according to the three pregnancy outcome groups. Only IgA showed a change with gestation, with an increase across pregnancy in the term intervention group (E), $p<0.05$.

Statistical analysis assessing the differences across the 3 pregnancy outcome groups was by Kruskal-Wallis test with Dunn's multiple comparisons test, preterm (PT) N=37, term (T) N=56, term with intervention (TI) N=40. Statistical analysis assessing the differences across pregnancy gestation, across the three sampling time points 12-16, 20-24 and 30-34 weeks was also by Kruskal-Wallis test with Dunn's multiple comparisons test. Dotted lines on A-D mark the concentration of the lowest and highest standards on the assay. (* $p<0.05$). Dotted lines on A-F mark the concentration of the lowest and highest standards on the assay. The results are expressed as median and interquartile range.

4.5.4 Cervico-vaginal immune response across gestation (longitudinal cohort)

In longitudinally sampled women across all three pregnancy outcome groups, there were 28 women in the preterm group, 51 women in the term group and 37 women in the term intervention group with samples taken at each of the three sampling time points 12^{+0} - 16^{+6}, 20^{+0} - 24^{+6} and 30^{+0} - 34^{+6} weeks. The median gestational age at delivery for the preterm group was 34^{+1}, for the term group was 39^{+5} and for the term intervention group was 38^{+3}.

In this longitudinal analysis, across three timepoints in pregnancy there were no significant changes in cervico-vaginal IL-8, IL-1β, IL-10, IL-6, IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α or GM-CSF in the two term groups ($p>0.05$), Figures 4.8 and 4.9. In the preterm group, IL-18 increased from 12^{+0} - 16^{+6} weeks to 30^{+0} - 34^{+6} weeks ($p=0.04$), Figure 4.9 B. In this longitudinal analysis, there was an increase IL-8, IL-1β, IL-6 and IL-2 ($p<0.05$) between 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks in the preterm group, but the statistical significance was lost with Dunnett's post hoc test. There is a variety in temporal responses which is more marked in the preterm and the term with intervention groups. There are women who largely follow an increasing pro-inflammatory trajectory and women who have a more stable local immune response in both groups. This shows that there is a variation between study participants which may reflect different mechanisms and aetiological factors for preterm birth.

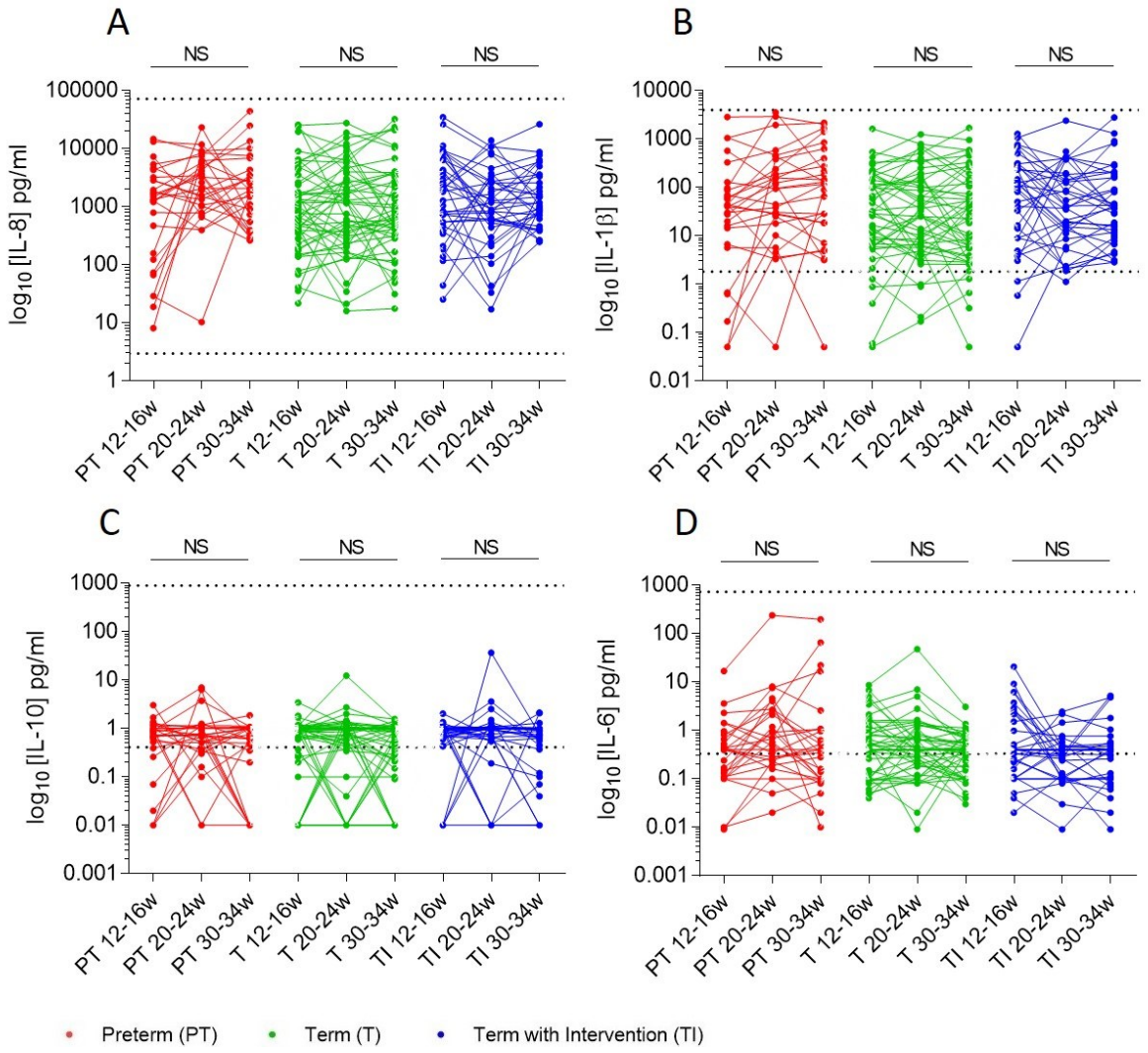


Figure 4.8 Cervico-vaginal fluid cytokines IL-8, IL-1β, IL-10 and IL-6 in preterm, term and term with intervention pregnancies and across pregnancy, in the longitudinal cohort

IL-8, IL-1β, IL-10 and IL-6 did not show any gestational changes in the three pregnancy outcome groups preterm (PT) N=28, term (T) N=51, term with intervention (TI) N=37, comparing across three sampling time points 12-16, 20-24 and 30-34 weeks .

Statistical analysis assessing the gestational effect across the three sampling time points 12-16, 20-24 and 30-34 weeks was by the one way ANOVA (Friedman) with Dunnett's multiple comparisons test for each of the three pregnancy outcome groups, preterm (PT), term (T) and term with intervention (TI). (NS=non-significant). Dotted lines on A-D mark the concentration of the lowest and highest standards on the assay. The assay for IL-8 was performed using a 1:10 dilution of CVF.

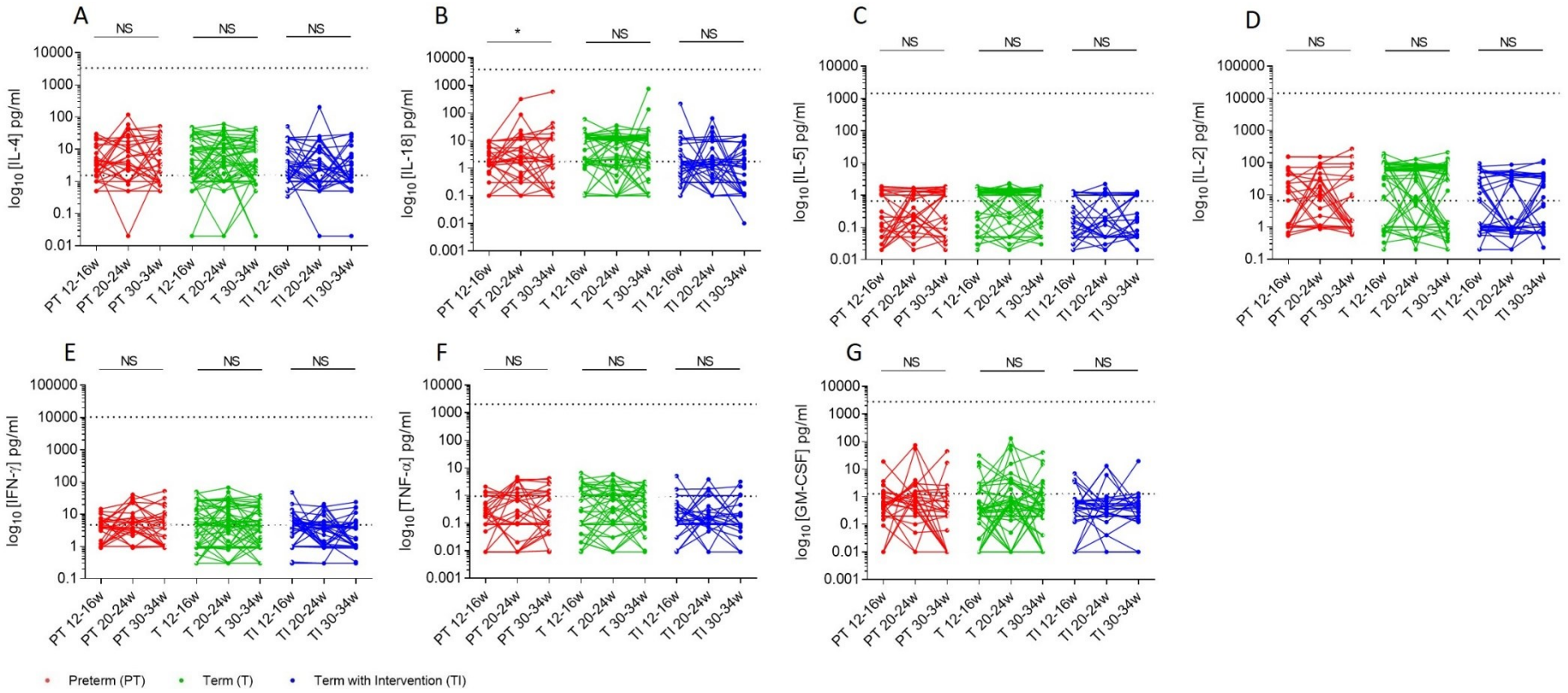


Figure 4.9 Cervico-vaginal fluid cytokines IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α, GM-CSF in preterm, term and term with intervention pregnancies and across pregnancy, in the longitudinal cohort

IL-4, IL-5, IL-2, IFN-γ, TNF-α and GM-CSF did not show any gestational changes in the three pregnancy outcome groups preterm (PT) N=28, term (T) N=51, term with intervention (TI) N=37, comparing across three sampling time points 12-16, 20-24 and 30-34 weeks (A, D-H). However, IL-18 did increase with advancing gestation in the preterm group (B). Statistical analysis assessing the gestational effect across the three sampling time points was by the one way ANOVA (Friedman) with Dunnett's multiple comparisons test for each of the three pregnancy outcome groups, preterm (PT), term (T) and term with intervention (TI). (NS=non-significant). Dotted lines on A-H mark the concentration of the lowest and highest standards on the assay.

There was no gestational change in cervico-vaginal fluid concentrations of C5, C5a, MBL or C3b in both term groups, in the longitudinal analysis (p>0.05), Figure 4.10. However, C5a and C3b increased significantly from 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks in the preterm group (p=0.02 and p=0.03 respectively), Figure 4.10 B and D. Similarly, to the cytokine results, there is much individual variation particularly in the preterm and term with intervention groups.

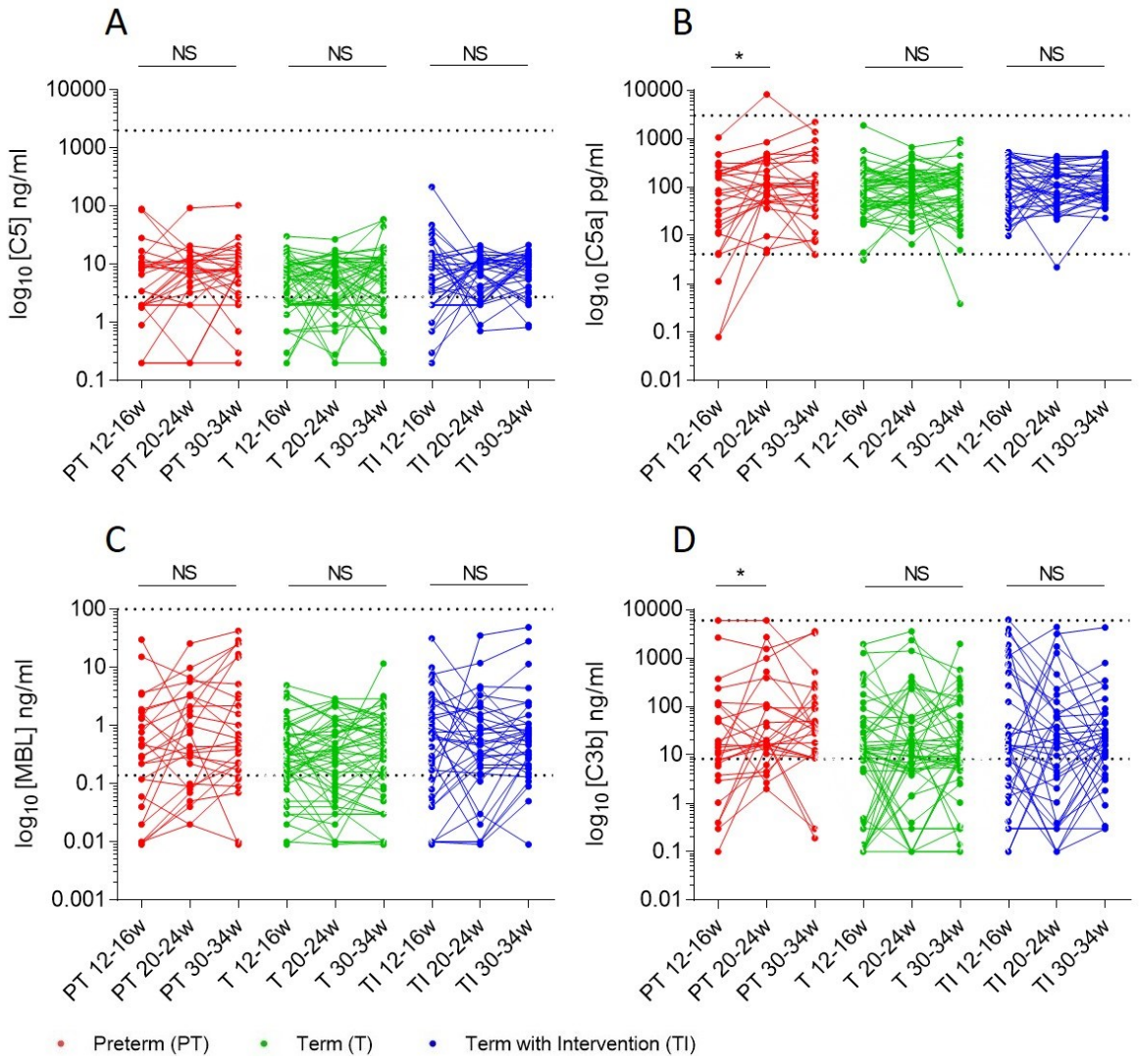


Figure 4.10 Cervico-vaginal fluid complement in preterm, term and term with intervention pregnancies and across pregnancy, in the longitudinal cohort

C3b and C5a increased significantly between the first two time points 12-16 and 20-24 weeks in the preterm group only, (A and D). Whilst MBL and C5 did not show any significant gestational changes in any of the pregnancy outcome groups, (B and C).

Statistical analysis assessing the gestational effect across the three sampling time points 12-16, 20-24 and 30-34 weeks was by the one-way ANOVA (Friedman) with Dunnett's multiple comparisons test for each of the three pregnancy outcome groups, preterm (PT) N=28, term (T) N=51, term with intervention N=37, (* p<0.05, NS=non-significant). Dotted lines on A-D mark the concentration of the lowest and highest standards on the assay.

IgG1-4, IgA and IgM did not show any gestational changes in the term and term with intervention groups in the longitudinal analysis (p>0.05). In the women who delivered preterm, IgG1, IgG2 and IgG4 increased significantly between 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks, and 12^{+0} - 16^{+6} and 30^{+0} - 34^{+6} weeks gestation (p<0.05) and IgG3 increased significantly between the first and second sampling timepoints (p=0.05), Figure 4.11.

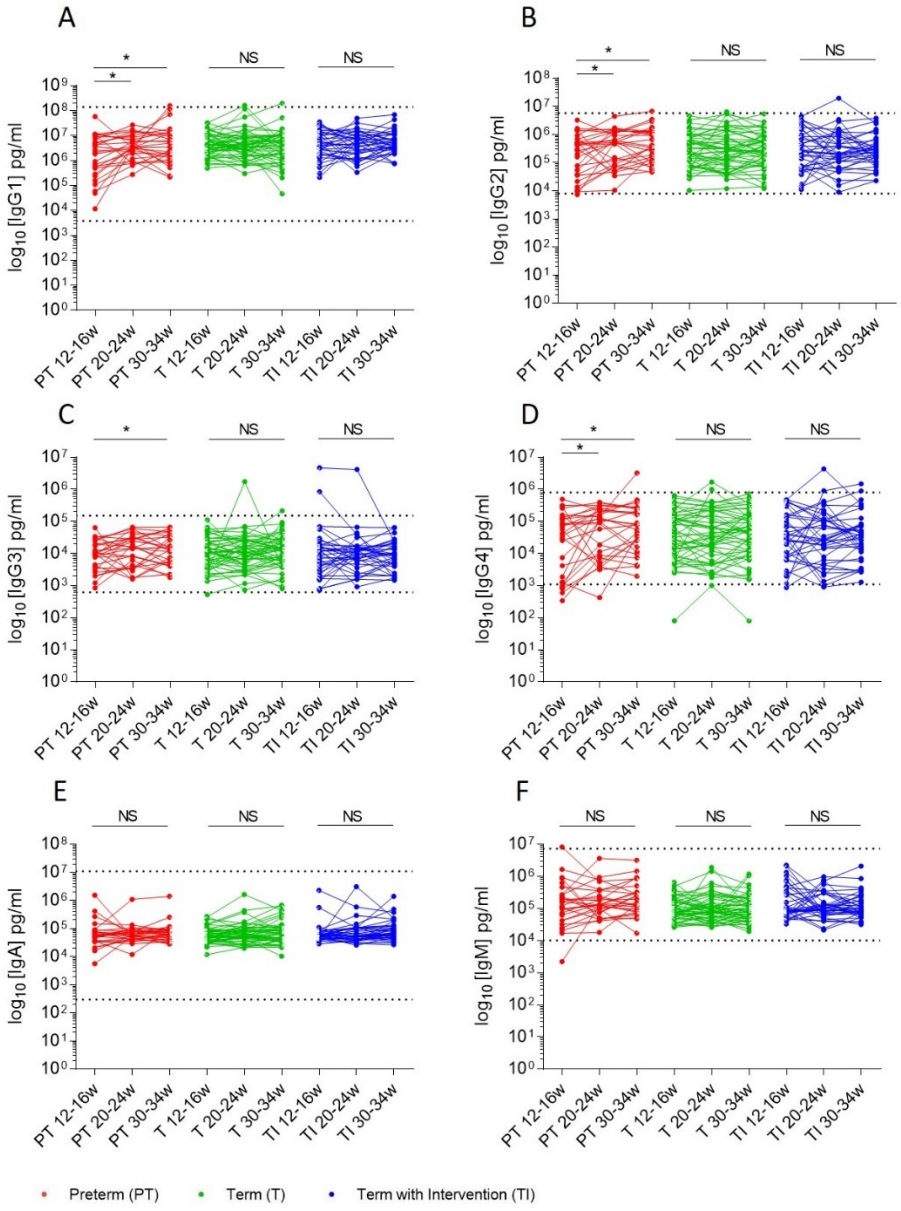


Figure 4.11 Cervico-vaginal fluid immunoglobulins in preterm, term and term with intervention pregnancies and across pregnancy, in the longitudinal cohort

IgG1, IgG2, IgG3 and 4 increased significantly with advancing gestation in the preterm group, but not in the two term groups (A-D). There were no other gestation related changes in IgA and IgM did not demonstrate gestational changes in any of the three pregnancy outcome groups, (E and F). Statistical analysis assessing the gestational effect across the three sampling time points 12-16, 20-24 and 30-34 weeks was by the one-way ANOVA (Friedman) with Dunnett's multiple comparisons test for each of the three pregnancy outcome groups, preterm (PT) N=28, term (T) N=51, term with intervention (TI) N=37, (* p<0.05, NS=non-significant). Dotted lines on A-F mark the concentration of the lowest and highest standards on the assay.

The early-mid gestation inflammatory response in women who deliver preterm

From the analysis of the gestational effect across the three timepoints in the longitudinal cohort, it was apparent that there was a pro-inflammatory early-mid gestation inflammatory response in the preterm group, between 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks. The gestational effect on the local immune response between these first two sampling timepoints was explored in the three pregnancy outcome groups, preterm (N=34), term (N=51), term with intervention (N=37). The median gestational age at delivery for each group were, 33^{+3}, 39^{+5} and 38^{+3} respectively. Compared to the previous longitudinal analysis across all three sampling timepoints, the paired analysis across the first two sampling timepoints included 6 women who delivered preterm before the third sampling timepoint.

There was a significant increase between 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks in IL-8, IL-1β, IL-6 and IL-2 in the preterm group (p=0.05, p=0.04, p=0.003, p=0.04). There was no gestational change across these two timepoints in the women who delivered at term gestation (p>0.05), Figure 4.12.

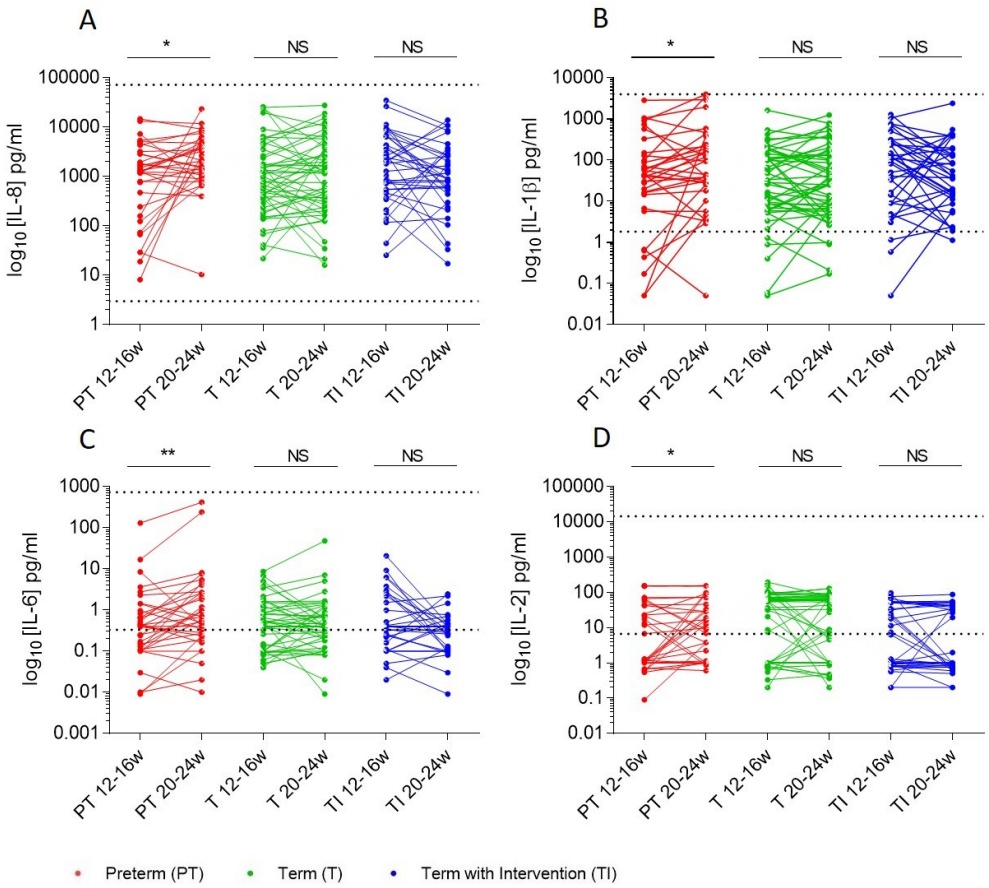


Figure 4.12 Cervico-vaginal fluid IL-8, IL-1β, IL-6 and IL-2 increase early to mid-gestation in preterm deliveries
An increase in cervico-vaginal fluid IL-8, IL-1β, IL-6 and IL-2, was observed between sampling timepoints 12-16 and 20-24 weeks in women who had preterm deliveries <37 weeks, in paired samples N=34. No difference was seen in women who delivered at term, N=51, or at term with intervention, N=37.
Statistical analysis assessing the gestational effect across the two sampling time points 12-16 and 20-24 weeks was by Wilcoxon matched-pairs signed rank test, (* p<0.05, ** p<0.01). Dotted lines on A-D mark the concentration of the lowest and highest standards on the assay.

There was a significant increase between 12^{+0} - 16^{+6} weeks and 20^{+0} - 24^{+6} weeks in C5a, MBL and C3b in the preterm group (p=0.002, p=0.05 and p=0.05 respectively), but no change in C5 (p>0.05). There was no gestational change for C5, C5a, MBL or C3b across the two timepoints in the women who delivered at term gestation (p>0.05), Figure 4.13.

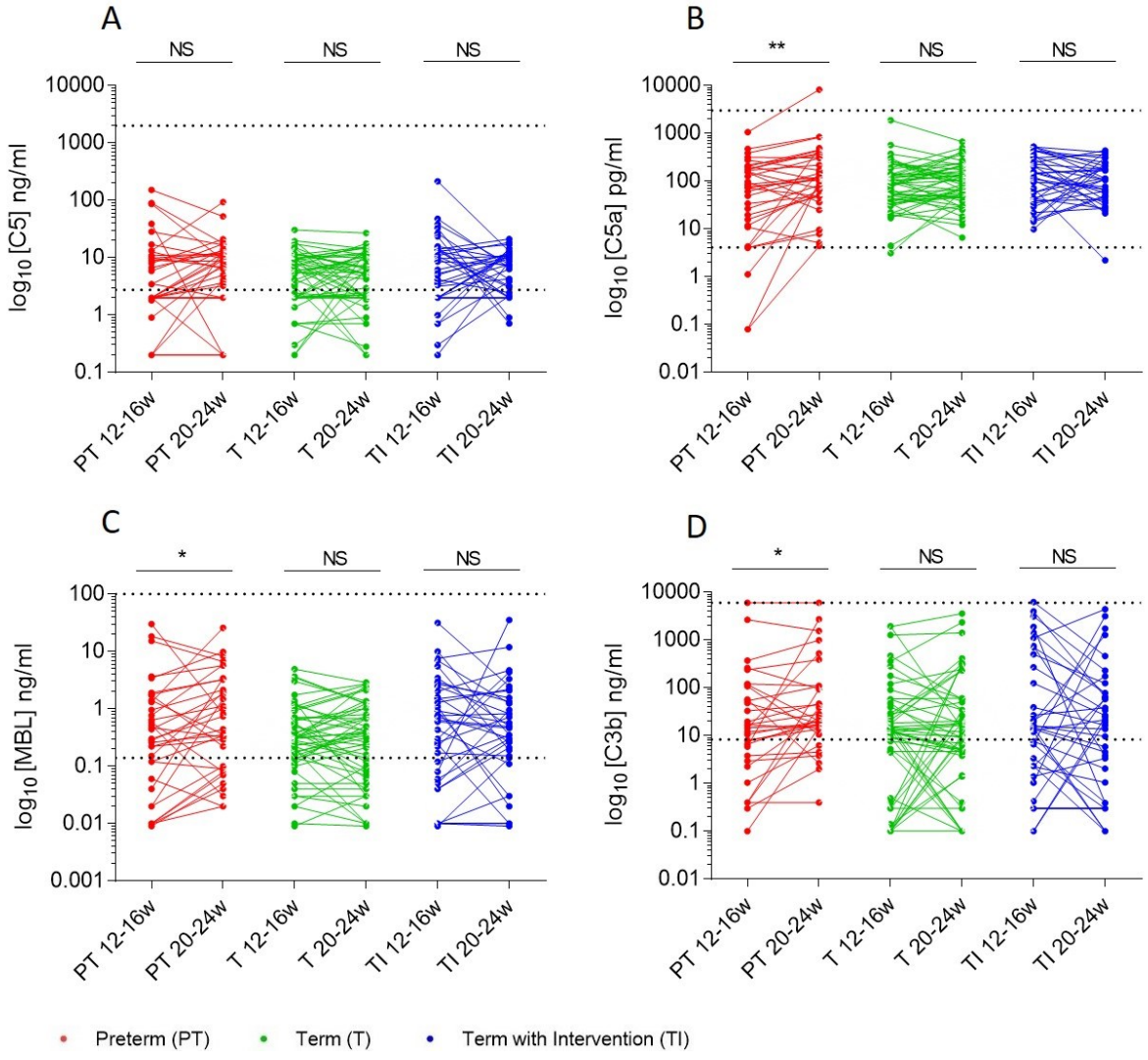


Figure 4.13 Cervico-vaginal fluid C5a, MBL and C3b increase early to mid-gestation in preterm deliveries
C5a, MBL and C3b increased significantly between 12-16 and 20-24 weeks in women who delivered preterm, N=34. No difference was seen in women who delivered at term, N=51, or at term with intervention, N=37. Statistical analysis assessing the gestational effect across the two sampling time points 12-16 and 20-24 weeks was by Wilcoxon matched-pairs signed rank test, (* p<0.05, ** p<0.01). Dotted lines on A-D mark the concentration of the lowest and highest standards on the assay.

There was a significant increase between 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks in IgG1 and IgG3 in the preterm group (both p=0.04), however there was no change in IgG2, IgG4, IgA or IgM (p>0.05). The median IgM concentration was higher at each of the three timepoints compared to women delivering at term, Figure 4.7, and may explain why no further increase was seen with advancing gestation. There was no gestational change for IgG1-4, IgA or IgM across these two timepoints in the women who delivered at term gestation (p>0.05), Figure 4.14.

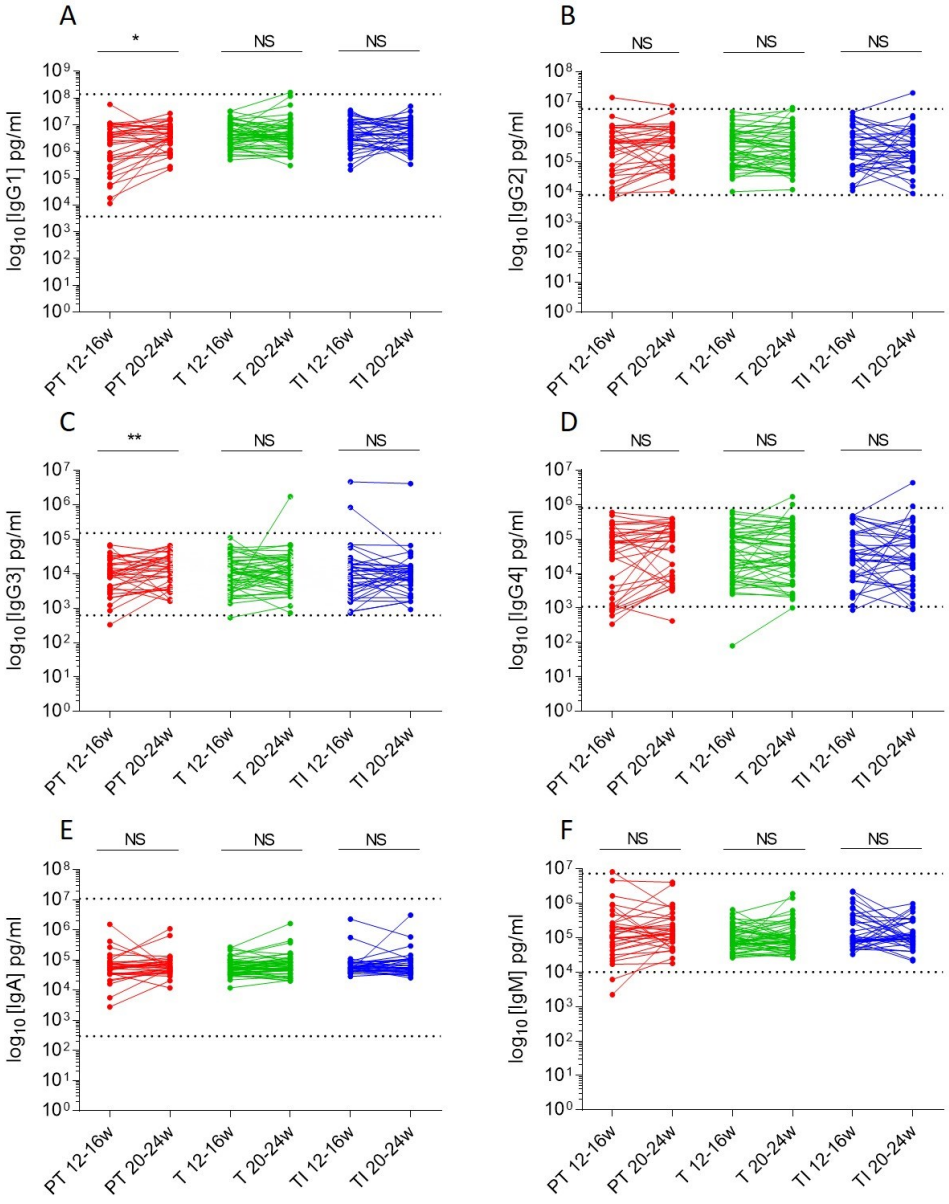


Figure 4.14 Cervico-vaginal fluid IgG1 and IgG3 increase early to mid-gestation in preterm deliveries
C3b, MBL, C5a, IgG1 and IgG3 increased significantly between 12-16 and 20-24 weeks in women who delivered preterm, N=34. No difference was seen in women who delivered at term, N=51, or at term with intervention, N=37.
Statistical analysis assessing the gestational effect across the two sampling time points 12-16 and 20-24 weeks was by Wilcoxon matched-pairs signed rank test, (* p<0.05, ** p<0.01). Dotted lines on A-F mark the concentration of the lowest and highest standards on the assay.

4.5.5 Cervico-vaginal immune response depending on the risk factor for preterm birth

As demonstrated at 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks there was an increased pro-inflammatory innate and adaptive immune response in a proportion of women who deliver preterm. This could be due to different pathological mechanisms. Of the women who delivered preterm and who had sampling at both 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks, six were referred to the preterm surveillance clinics due to previous excisional cervical treatment, and twenty-eight were referred due to previous mid-trimester loss and/or previous preterm birth. Women with previous mid-trimester loss and previous preterm birth were analysed together as their pathogenesis likely involves an abnormal inflammatory response, in contrast to mechanically driven cervical shortening in women with previous excisional cervical treatment.

Comparing the cytokine concentrations between 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks, there was a more profound pro-inflammatory shift in the women with a previous history of MTL and/or PTB, compared to the women who had a previous history of excisional cervical treatment. IL-8, IL-1β, IL-6 and IL-2 increased significantly between the two sampling timepoints in the women with previous history of MTL and/or PTB ($p=0.05$, $p=0.02$, $p=0.0005$ and $p=0.05$ respectively), Figures 4.15 and 4.16. There was also a significant increase in IFN-γ in the women with previous cervical treatment ($p=0.03$), Figure 4.16 E. Anti-inflammatory IL-10 decreased significantly in the women with previous MTL and/or PTB ($p=0.05$), Figure 4.15 C.

There was no significant change in IL-4, IL-18, IL-5, TNF-α or GM-CSF between 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks gestation in either the women with previous history of MTL and/or PTB or women with previous excisional cervical treatment ($p>0.05$), Figure 4.15.

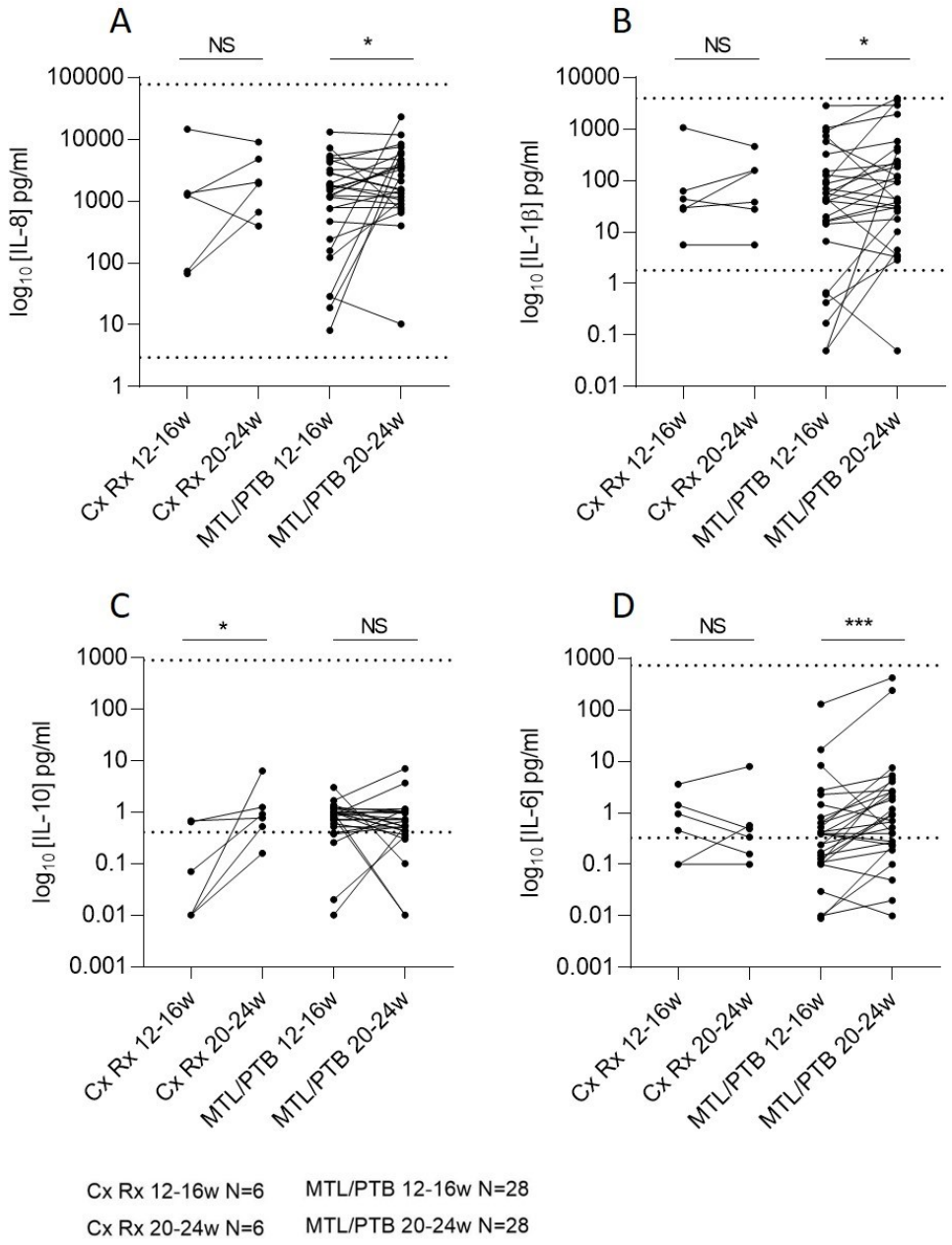


Figure 4.15 Comparison of cervico-vaginal fluid IL-8, IL-1β, IL-10 and IL-6 in women who deliver preterm according to their risk factor for preterm birth from early to mid-gestation

Pro-inflammatory IL-8, IL-1β and IL-6 increased significantly between 12-16 and 20-24 weeks in women who delivered preterm if their risk factor for preterm delivery was previous mid-trimester loss (MTL) or previous preterm birth (PTB), compared to if the risk factor was previous cervical treatment (Cx Rx), IL-8 ($p<0.05$), IL-1β ($p<0.05$), IL-6 ($p<0.001$). There was a significant increase in anti-inflammatory IL-10 in the group with previous cervical treatment, ($p<0.05$). Statistical analysis assessing the gestational effect across the two sampling time points 12-16 and 20-24 weeks was by Wilcoxon matched-pairs signed rank test, (* $p<0.05$, *** $p<0.001$). Dotted lines on A-D mark the concentration of the lowest and highest standards on the assay. Cx Rx N=6 and MTL/PTB N=28.

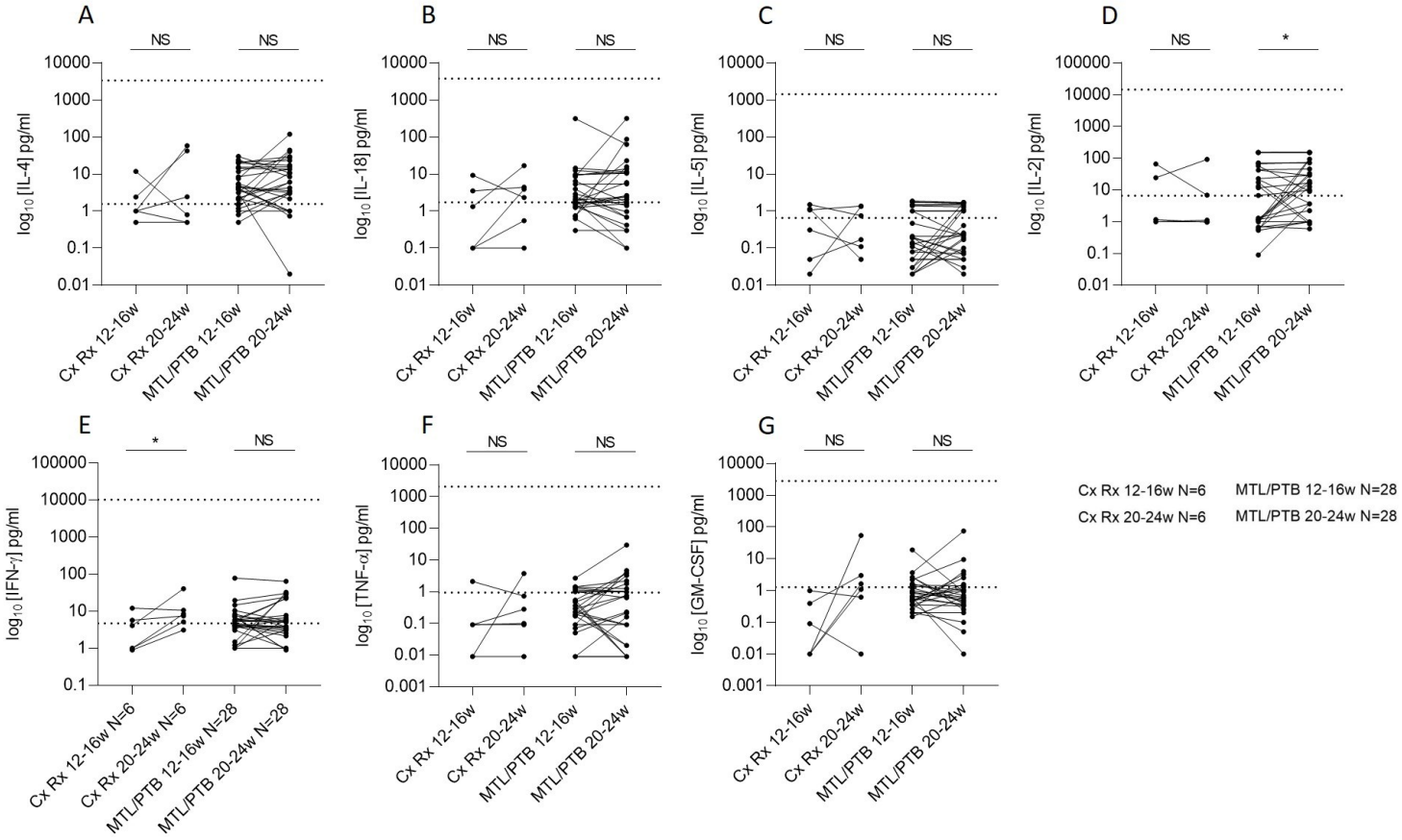


Figure 4.16 Comparison of remaining cervico-vaginal fluid cytokines in women who deliver preterm according to their risk factor for preterm birth from early to mid-gestation

Pro-inflammatory IL-2 increased significantly between 12-16 and 20-24 weeks in women who delivered preterm if their risk factor for preterm delivery was previous mid-trimester loss (MTL) or previous preterm birth (PTB), compared to if the risk factor was previous cervical treatment ($p<0.05$). IFN-γ increased significantly in the group with previous excisional cervical treatment, but interpretation of this is with caution as many concentrations were below the lowest standard of the assay. There was no significant change with IL-4, IL-18, IL-5, TNF-α or GM-CSF. Statistical analysis assessing the gestational effect across the two sampling time points 12-16 and 20-24 weeks was by Wilcoxon matched-pairs signed rank test, (* $p<0.05$). Dotted lines on A-G mark the concentration of the lowest and highest standards on the assay. Cx Rx N=6 and MTL/PTB N=28.

There was a more prominent increase in complement activation between early and mid-gestation in the women who had preterm deliveries who had previous MTL and/or previous PTB compared to previous excisional cervical treatment. C5a, MBL and C3b showed a significant increase between 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks in the women who had previous MTL and/or previous PTB (p=0.002, p=0.02 and p=0.03 respectively), Figure 4.17. There was no significant change with C5 (p>0.05).

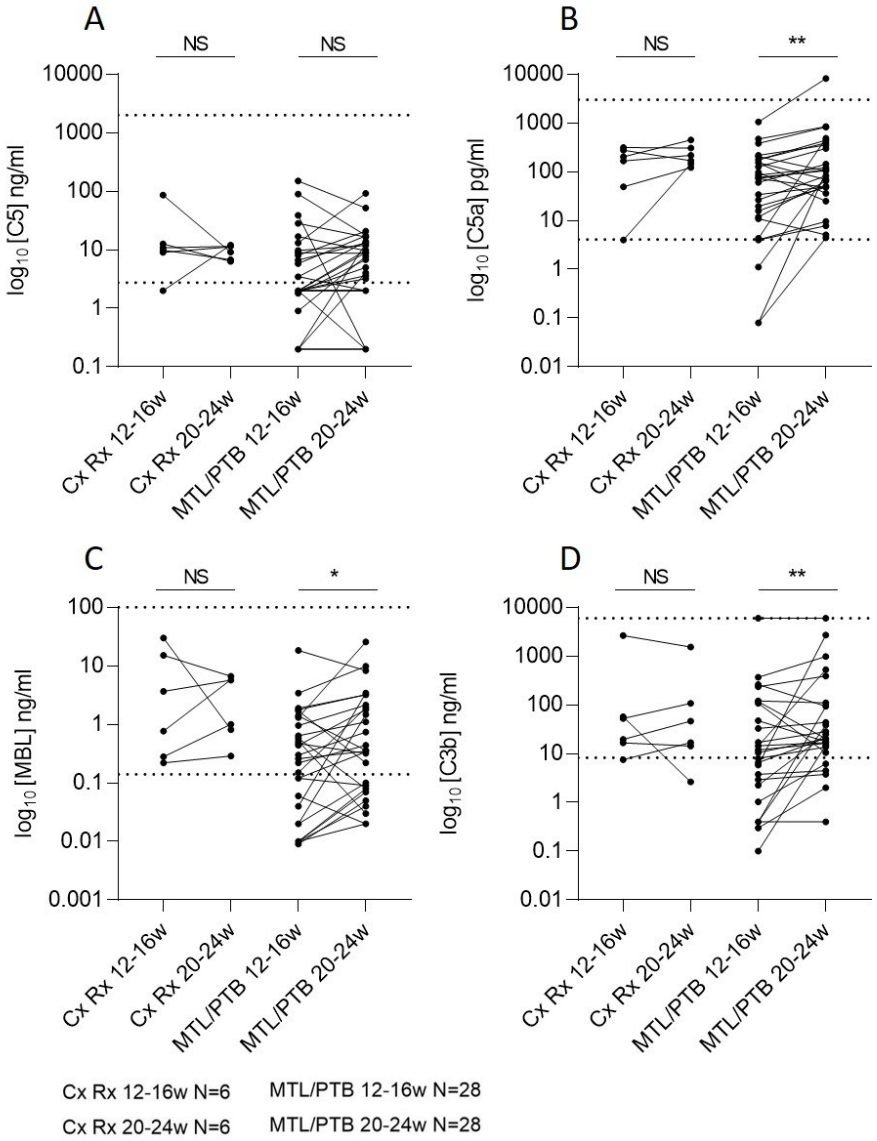


Figure 4.17 Comparison of cervico-vaginal fluid complement in women who deliver preterm according to their risk factor for preterm birth from early to mid-gestation

C5a, MBL and C3b increased significantly between 12-16 and 20-24 weeks in women who delivered preterm if their risk factor for preterm delivery was previous mid-trimester loss (MTL) or previous preterm birth (PTB), compared to if the risk factor was previous cervical treatment (Cx Rx),($p<0.05$). There was a significant increase in anti-inflammatory IL-10 in the group with previous cervical treatment, ($p<0.05$). Statistical analysis assessing the gestational effect across the two sampling time points 12-16 and 20-24 weeks was by Wilcoxon matched-pairs signed rank test, (* $p<0.05$, *** $p<0.001$). Dotted lines on A-D mark the concentration of the lowest and highest standards on the assay. Cx Rx N=6 and MTL/PTB N=28.

Similarly, activation of the adaptive immune response as measured by IgG1, IgG2, IgG3, IgG4, and IgA was seen only in women who had preterm deliveries who had previous MTL and/or previous PTB (p=0.004, p=0.04, p=0.03, p=0.02 and p=0.009 respectively). In contrast, concentrations remained stable in women with a history of cervical treatment (p>0.05), Figure 4.18.

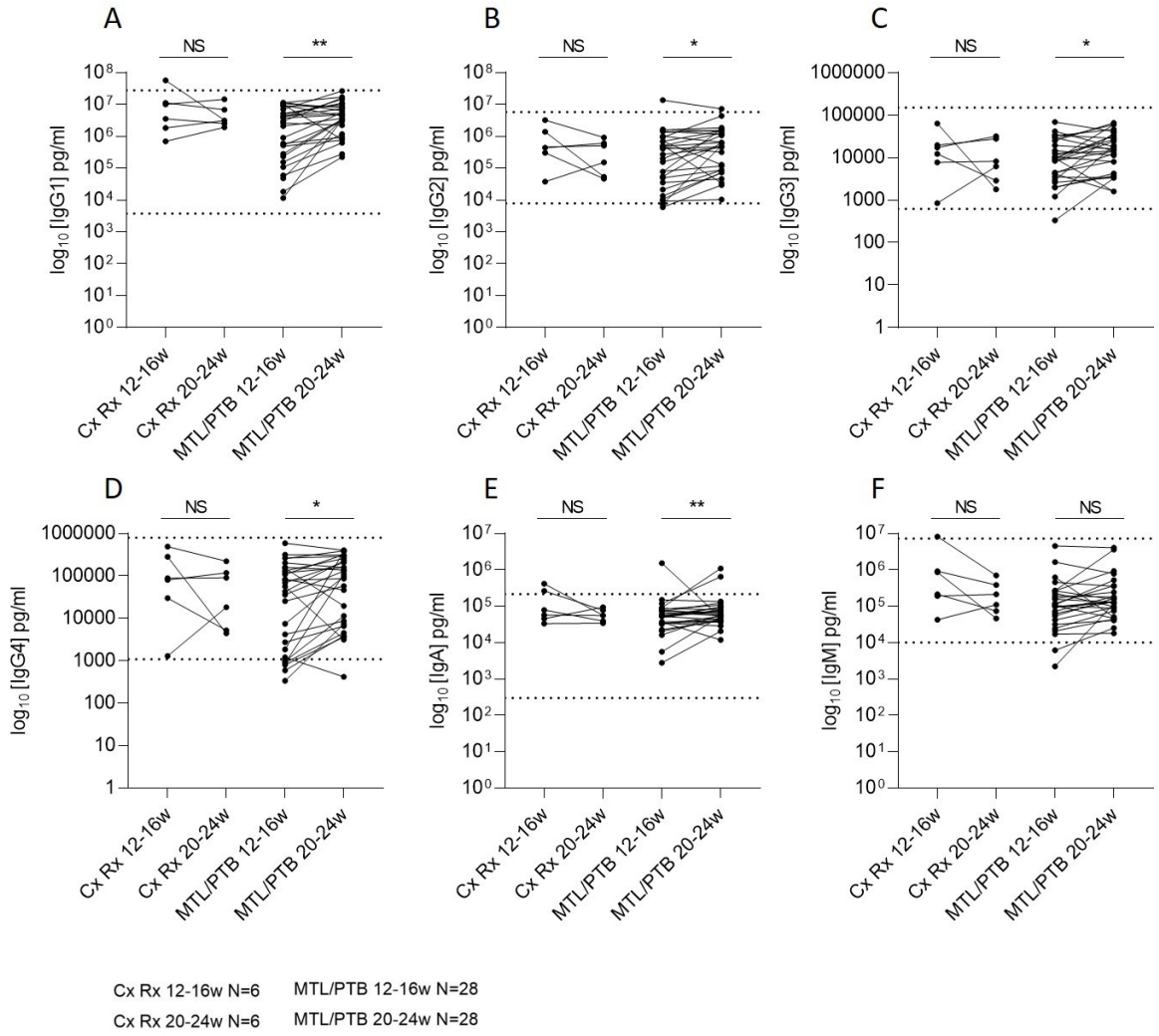


Figure 4.18 Comparison of cervico-vaginal fluid immunoglobulins in women who deliver preterm according to their risk factor for preterm birth from early to mid-gestation

IgG1, IgG2, IgG3, IgG4 and IgA increased significantly between 12-16 and 20-24 weeks in women who delivered preterm if their risk factor for preterm delivery was previous mid-trimester loss (MTL) or previous preterm birth (PTB), compared to if the risk factor was previous cervical treatment (Cx Rx),(p<0.05). There was no significant change in IgM according to the risk factor for preterm delivery. Statistical analysis assessing the gestational effect across the two sampling time points 12-16 and 20-24 weeks was by Wilcoxon matched-pairs signed rank test, (* p<0.05, *** p<0.001). Dotted lines on A-F mark the concentration of the lowest and highest standards on the assay. Cx Rx N=6 and MTL/PTB N=28.

We also studied if there was a higher basal inflammatory response in the women with previous MTL and/or PTB, compared to women with previous excisional cervical treatment in the whole study population.

For the whole study population, the reason for referral to the preterm birth prevention clinic included previous excisional cervical treatment, previous mid-trimester loss (MTL) and previous preterm birth (PTB). Many women had a combination of risk factors. For the women for whom the sole reason for referral for excisional cervical treatment, one could postulate that the mechanism for why they have may a preterm delivery is due to a mechanical, structural problem with the cervix. Cervical shortening is associated with local inflammation, but likely not to the same degree as women who have had a previous MTL and/or previous PTB who we hypothesise are more likely to have a degree of immune dysregulation, or aberrant microbial-immune interactions driving their risk for premature cervical remodeling and dilatation, uterine contractions and fetal membrane rupture. There were two women who were not categorised into either group. One was referred to the preterm birth prevention clinic for 3 previous first trimester miscarriages, and one had a shortened cervix at anomaly ultrasound. As their reasons for referral did not naturally fall into either major category they were excluded from the next analyses presented. Both women had term deliveries.

The study population was divided into two groups, the first consisted of women where their sole reason for referral to the preterm birth surveillance clinic was previous excisional cervical treatment. The second group consisted of women where their reasons for referral included previous MTL, previous PTB, and either of these could be combined with excisional cervical treatment. Preterm birth was significantly higher in the previous MTL and/or PTB group compared to the previous cervical treatment group, ($p<0.01$).

Study participants were compared at the first sampling timepoint only, 12^{+0} - 16^{+6} weeks, and those who already had a cervical cerclage and those with a cervical length ≤25mm were excluded. This allowed the local immune profile of the two main risk factor groups to be compared without bias. This resulted in a group of 40 women with previous excisional cervical treatment and 56 women with previous MTL +/or PTB. The clinical and demographical details of the two groups are described in Table 4.5.

The gestation at delivery was significantly earlier in the previous MTL +/or PTB group ($p<0.0001$). Most women in the previous excisional cervical treatment group were of White ethnicity (90%), whilst there were 45% white women, 25% black women and 30% of women who were classified into 'other' ethnicity category in the previous MTL and/or PTB group. As expected, there were significantly more multiparous women in the previous MTL and/or PTB group. There was a greater proportion of the

women in the previous MTL +/or PTB group who subsequently had a cervical cerclage 43%, vs 14% in the previous excisional cervical treatment group. There was no difference in history or ultrasound indicated cerclages, or cerclages performed with Mersilene or Nylon between the two groups.

Table 4.5 Clinical and demographical characteristics based upon reason for referral to preterm birth surveillance clinic. N=96

	Previous excisional cervical treatment (Cx Rx)	Previous mid-trimester loss or previous preterm birth (MTL/PTB)	p value
N (%)	40 (42%)	56 (58%)	
Gestation at delivery (weeks) median and IQ range	40+0 (39+0-41+0)	38+0 (34+5-39+0)	p<0.0001 ****
Early preterm </=33+6 weeks	2 (5%)	11 (20%)	
Late preterm >/=34+0 weeks	2 (5%)	10 (18%)	
Term <37 weeks	36 (90%)	35 (62%)	PT vs Term p<0.01 **
Age median and IQ range	33 (32-36)	32 (28-36)	p=0.52
BMI median and IQ range (kg/m^2)	23 (21-27)	24 (22-28)	p=0.39
Ethnicity White N (%) Black N (%) Other N (%)	 36 (90%) 3 (7%) 1 (3%)	 25 (45%) 14 (25%) 17 (30%)	χ^2 <0.0001 ****
Parity Nulliparous Multiparous	 31 (78%) 9 (22%)	 11 (20%) 45 (80%)	χ^2 <0.0001 ****
Cervical cerclage (later in pregnancy) Yes No	 5 (14%) 35 (86%)	 24 (43%) 32 (57%)	χ^2 <0.01 **
History indicated cervical cerclage and material	1 (25%) (nylon 1)	14 (58%) (mersilene 6, nylon 8)	Hx vs USS indicated cerclage χ^2 =0.11 Mersilene vs Nylon χ^2 =0.97
USS indicated cervical cerclage and material	4 (75%) (mersilene 2, nylon 2)	10 (42%) (mersilene 3, nylon 6, unknown 1)	
Progesterone only	1	2	
Risk factor for PTB: (by individual) Cervical treatment alone Cervical treatment + MTL Cervical treatment + PTB MTL MTL + PTB PTB	 40 (100%) 0 0 0 0 0	 0 3 (5%) 7 (13%) 16 (29%) 4 (7%) 26 (46%)	

BMI=body mass index, USS=ultrasound, PTB=preterm birth, MTL=mid trimester loss, misc=miscarriage. Data presented as median (interquartile range (IQ)) or number (%). P values: t-test for comparisons or Chi squared for proportional data.

At 12^{+0} - 16^{+6} weeks, there was a propensity for an increased inflammatory response in the study participants whose risk factor for preterm delivery was previous MTL and/or PTB as opposed to previous excisional cervical treatment. This is demonstrated by increased IL-8 in the former group (p=0.02), Figure 4.19 A. Following the pattern of an increased inflammatory response in women whose risk factor was previous MTL and/or PTB was a non-significant increase in IL-1β (p=0.07), Figure 4.19 B. IL-18 was significantly increased in the group with previous excisional cervical treatment (p=0.02), Figure 4.19 E. Other analytes assessed were IL-6, IL-10, IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α and GM-CSF. There were no significant differences between the two groups for these analytes (p>0.05), (data not shown).

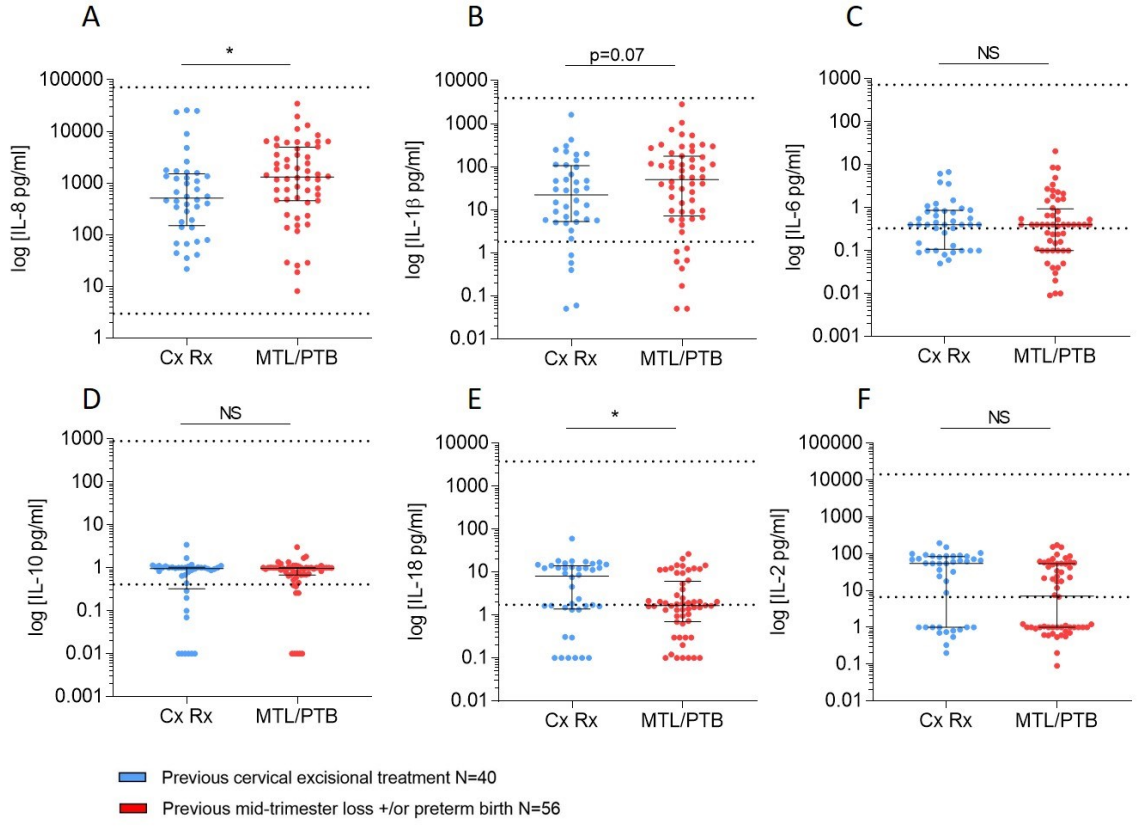


Figure 4.19 Comparison of cervico-vaginal fluid cytokines according to risk factor for preterm delivery
At 12-16 weeks, IL-8 was significantly higher whilst IL-18 was significantly lower when the risk factor for preterm delivery was previous cervical treatment group (Cx Rx) (N=40), compared to the risk factor of previous mid-trimester loss +/or previous preterm birth (MTL/PTB) (N=56). There was a non-significant increase for IL-1β if the risk factor was previous mid-trimester loss +/or previous preterm birth. There was no difference in the concentration of IL-6, IL-10 and IL-2 between the two groups according to risk factor for preterm delivery. Statistical analysis was by Mann Whitney test U, * p<0.05, NS=non-significant. The results are expressed as median and interquartile range.

At the 12^{+0} - 16^{+6} week timepoint, independent of subsequent preterm or term delivery, complement analytes C5, C5a, MBL and C3b were not significantly different between women with a history of cervical treatment of previous MTL and/or PTB (p>0.05), Figure 4.20.

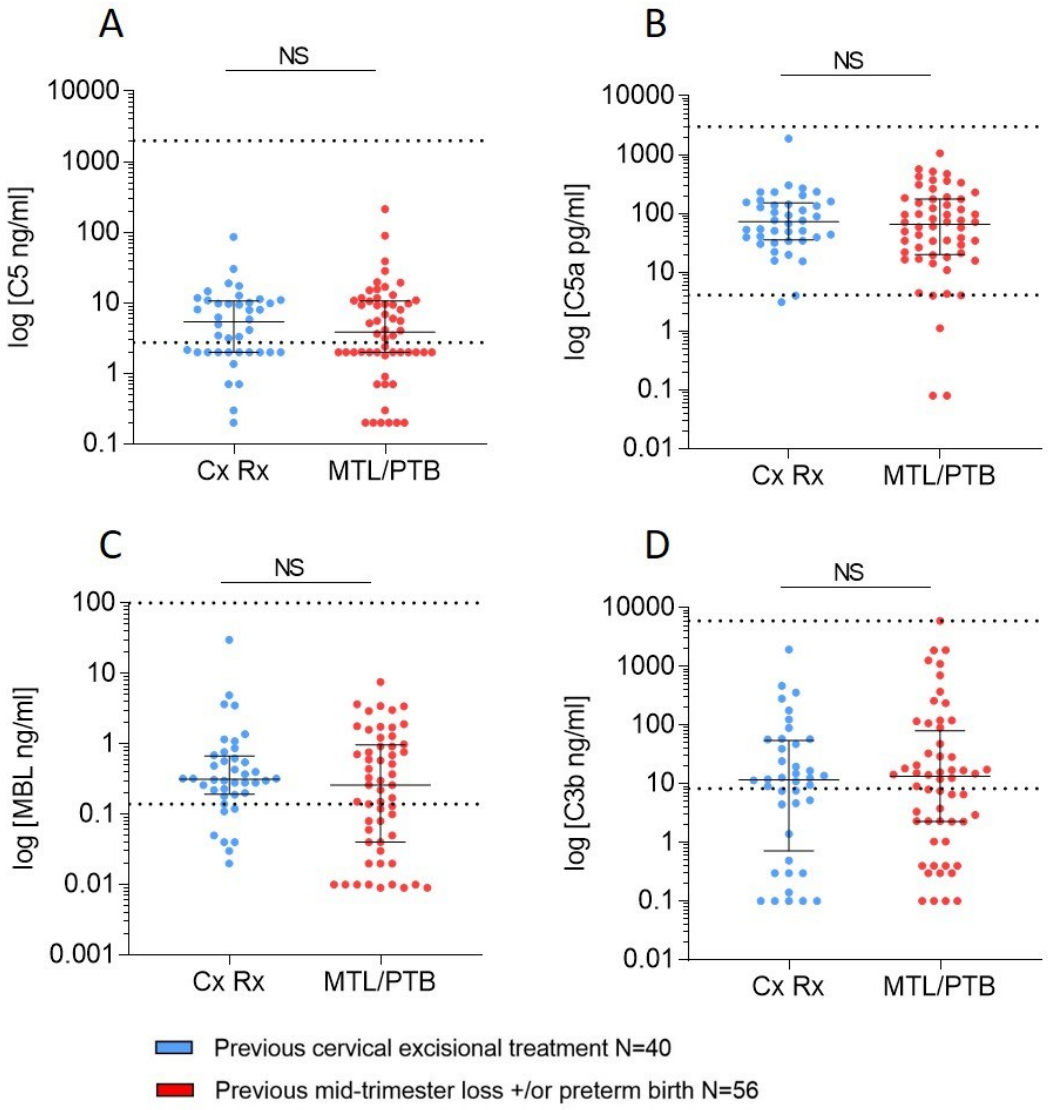


Figure 4.20 Comparison of cervico-vaginal fluid complement according to risk factor for preterm delivery
At 12-16 weeks, there was no difference in the concentrations of C5 C5a, MBL or C3b in cervico-vaginal fluid of women whose risk factor for preterm delivery was previous cervical treatment group (N=40), compared to the risk factor of previous mid-trimester loss +/or previous preterm birth (N=56). Statistical analysis was by Mann Whitney U test, NS = non-significant. The results are expressed as median and interquartile range.

At the 12^{+0} - 16^{+6} week timepoint, independent of preterm or term delivery IgG1-4, IgA and IgM were not significantly different between women with a history of cervical treatment of previous MTL and/or PTB (p>0.05), Figure 4.21. However, the median concentrations of IgG2 and IgG4 were both 1.8 times higher in women with a history of MTL/PTB.

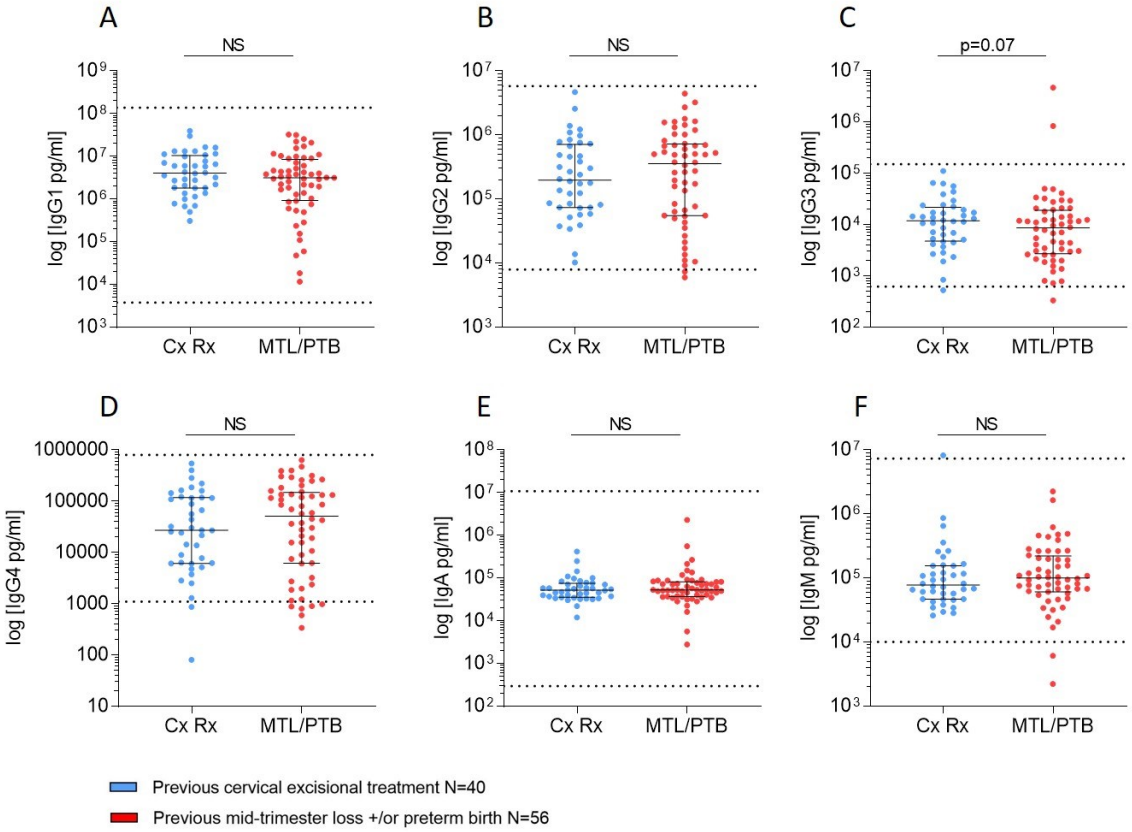


Figure 4.21 Comparison of cervico-vaginal fluid immunoglobulins according to risk factor for preterm delivery
At 12-16 weeks, IgG3 was non-significantly increased in cervico-vaginal fluid of women whose risk factor for preterm delivery was previous mid-trimester loss or previous preterm birth (N=56), rather than previous cervical excisional treatment (N=40). There was no difference in the concentrations of IgG1, IgG2, IgG4, IgA or IgM in cervico-vaginal fluid between the two groups. Statistical analysis was by Mann Whitney U test, NS = non-significant. The results are expressed as median and interquartile range.

In this section, a local pro-inflammatory immune response has been demonstrated early to mid-gestation in women who delivery preterm with a previous history of MTL and/or PTB, compared to a previous history of cervical treatment. This is seen to a lesser extent when women with term deliveries are included in the analysis.

4.5.6 Cervico-vaginal immune response in women who undergo cervical shortening

To investigate if there is an association between cervical shortening and cervico-vaginal fluid cytokines, complement and immunoglobulins profiles, the 133 study participants were divided into two groups based upon if the cervical length was ≤25mm or >25mm at the first sampling timepoint of between 12^{+0} - 16^{+6} weeks. Cervical length ≤25mm measured on transvaginal ultrasound before 24 weeks gestation is the universally accepted definition of a short cervix, conferring a high risk of preterm delivery [23]. Study participants were excluded if they already had a cervical cerclage in situ. This resulted in the comparison of 13 women with a cervical length ≤25mm to 96 women with normal cervical length >25mm at 12^{+0} - 16^{+6} weeks. Looking at this first sampling timepoint eliminated the effect of gestation and the presence of cervical suture, thus maximising testing the effect of cervical shortening only.

Clinical comparisons between those with and without cervical shortening and demographic details are shown in Table 4.6. The gestation at delivery was significantly earlier in the group with cervical shortening, median 36^{+0} compared to the group with normal cervical length, median 38^{+6} ($p<0.01$). There were twice as many preterm deliveries in the cervical shortening group compared to the normal cervical length group. There were no significant differences in age, BMI, ethnicity or parity between the two groups. As expected, those in the cervical shortening group were significantly more likely to receive a cervical cerclage, ($p<0.0001$). There were 14 (15%) women in the normal cervical length group at 12^{+0} - 16^{+6} weeks gestation who subsequently had a cervical length of <25mm, later in pregnancy.

Table 4.6 Clinical and demographical characteristics of study participants with cervical shortening and with normal cervical length, sampled between 12-16 weeks N=109

	Cervical shortening ≤25mm	Normal cervical length >25mm	p value
N (%)	13 (12%)	96 (88%)	
Gestation at delivery (weeks) median and IQ range	36^{+0} (31^{+0} - 39^{+0})	38^{+6} (36^{+5}- 40^{+1})	p<0.01 **
Cervical length mm (median and IQ range)	23 (19-25)	34 (31-39)	p<0.0001 ****
Early preterm </=33+6 weeks	6 (46%)	13 (13%)	PT vs Term p<0.05 *
Late preterm >/=34+0 weeks	1 (8%)	12 (13%)	
Term <37 weeks	6 (46%)	71 (74%)	
Age median and IQ range	31 (24-36)	33 (29-36)	p=0.15
BMI median and IQ range (kg/m^2)	22 (20-30)	24 (21-27)	p=0.29
Ethnicity White N (%) Black N (%) Other N (%)	 5 (38%) 4 (31%) 4 (31%)	 60 (62%) 17 (28%) 19 (20%)	χ^2 =0.25
Parity Nulliparous Multiparous	 5 (38%) 8 (62%)	 42 (43%) 54 (56%)	p=0.72
Cervical cerclage (later in pregnancy) Yes No	 11 (85%) 2 (15%)	 28 (29%) 68 (71%)	χ^2 <0.0001 ****
History indicated cervical cerclage and material	0	14 (50%) (Mersilene 7, Nylon 7)	Hx vs USS indicated cerclage χ^2 <0.01 Mersilene vs Nylon χ^2 =0.65
USS indicated cervical cerclage and material	11 (100%) (Mersilene 4, Nylon 7)	14 (50%) (Mersilene 5, Nylon 8, Unknown 1)	
Progesterone only	2	3	
Risk factor for PTB: (by individual) Cervical treatment alone Cervical treatment + MTL Cervical treatment + PTB MTL MTL + PTB PTB 3 x 1^{st} trimester miscarriage	 3 (24%) 1 (7%) 1 (7%) 2 (15%) 2 (15%) 4 (32%) 0	 40 (42%) 3 (3%) 7 (7%) 16 (17%) 4 (4%) 25 (26%) 1 (1%)	

BMI=body mass index, USS=ultrasound, PTB=preterm birth, MTL=mid trimester loss, misc=miscarriage. Data presented as median (interquartile range (IQ)) or number (%). P values: t-test for comparisons or Chi squared for proportional data.

Cytokines known to be involved in the process of cervical remodelling and shortening, IL-6 and IL-1β were significantly increased in the presence of cervical shortening, (p=0.04), Figure 4.22 B and C. Both analytes were nearly two times higher in the presence of cervical shortening. IL-10 is known to be anti-inflammatory and was found to be significantly higher in the presence of a normal length cervix compared to a shortened cervix at 12^{+0} - 16^{+6} weeks, (p=0.05), Figure 4.22 D. The expression of IL-8, IL-1β, IL-4, IL-18, IL-2, IFN-γ, TNF-α and GM-CSF was not influenced by cervical shortening (p>0.05), Figures 4.22 and 4.23.

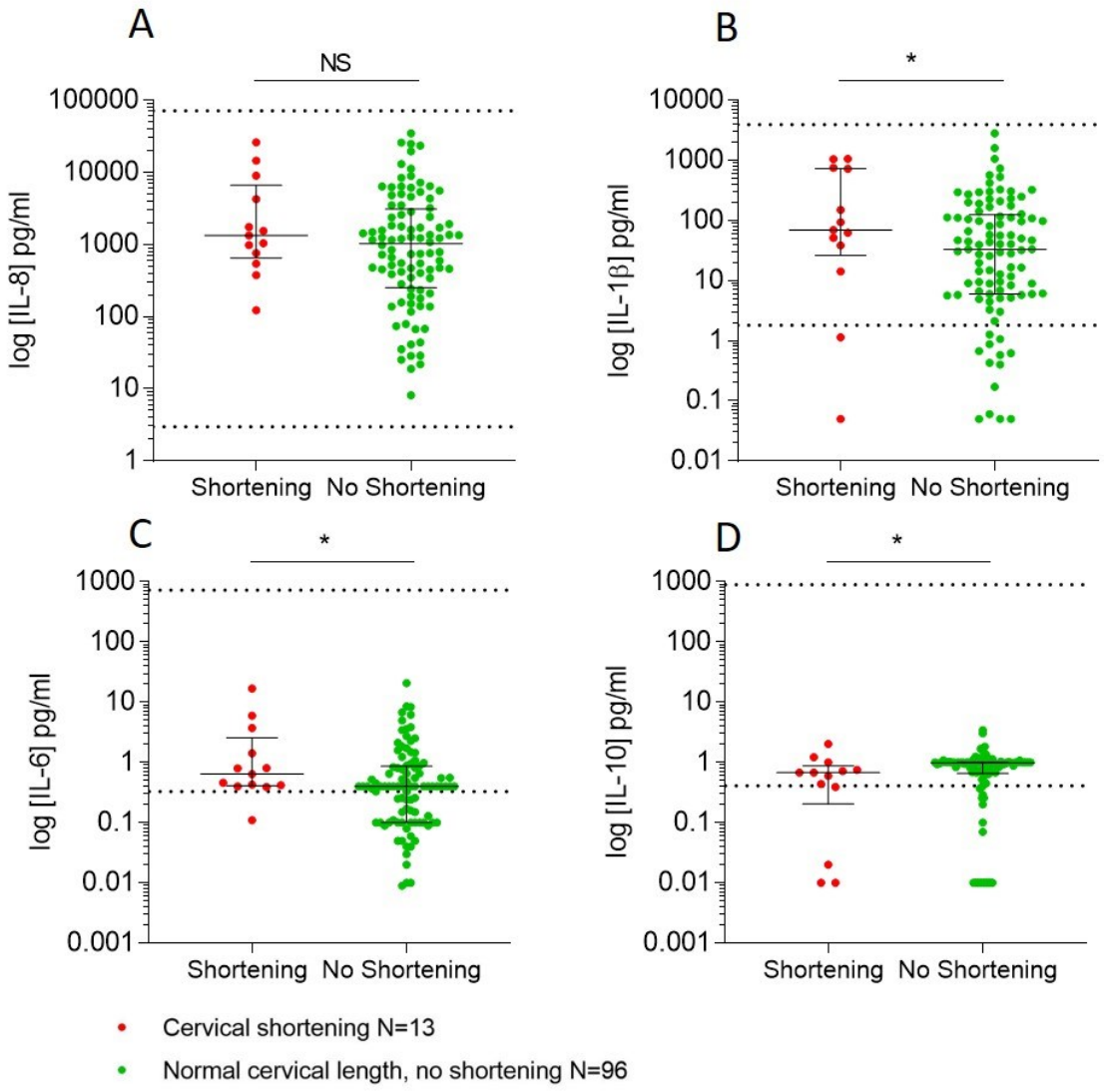


Figure 4.22 Cervico-vaginal cytokine expression in the presence of cervical shortening, or normal cervical length

Cervico-vaginal fluid cytokines were compared in women who had cervical shortening at the time of sampling between 12-16 weeks gestation, (cervical length ≤25mm, N=13) versus women who had a normal cervical length at the time of sampling, (cervical length <25mm, N=96). IL-1β and IL-6 were significantly higher in those with cervical shortening. There was a non-significant increase in IL-8 in those with shortening. IL-10 was significantly higher in those with a normal cervical length. The dotted lines mark the concentration of the lowest and highest standards on the assay's standard curve. Statistical analysis was by the Mann-Whitney test. *p<0.05, ** p<0.01, NS = non-significant. The results are expressed as median and interquartile range.

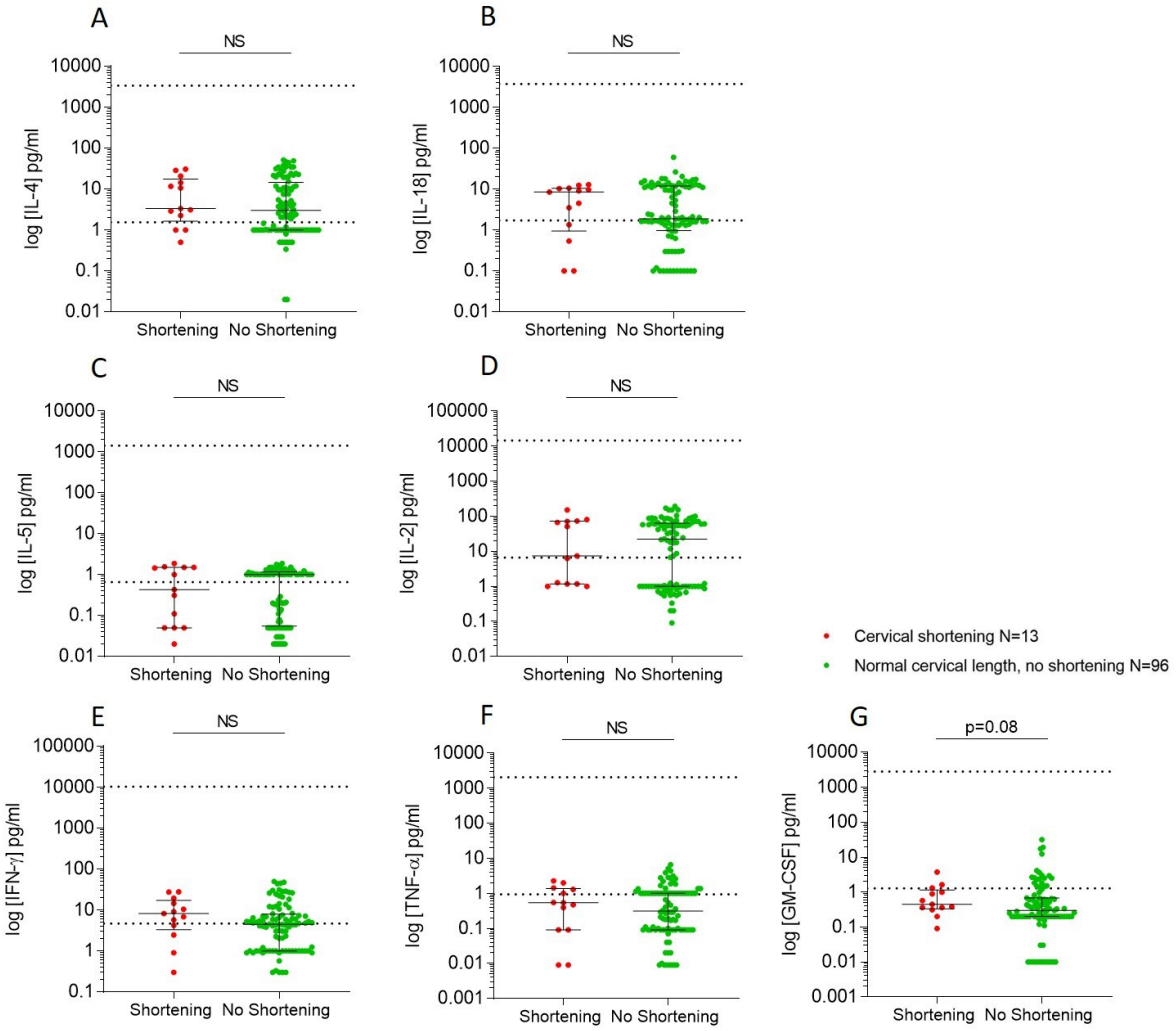


Figure 4.23 Cervico-vaginal cytokine expression in the presence of cervical shortening, or normal cervical length

Cervico-vaginal fluid cytokines were compared in women who had cervical shortening at the time of sampling (12-16 weeks gestation), (cervical length ≤25mm, N=13) versus women who had a normal cervical length at the time of sampling, (cervical length <25mm, N=96). There was no significant difference in the concentrations of IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α or GM-CSF in women with and without cervical shortening. The dotted lines mark the concentration of the lowest and highest standards on the assay's standard curve. Statistical analysis was by the Mann-Whitney U test, NS = non-significant. The results are expressed as median and interquartile range.

The contribution of complement analytes to cervical shortening have not been well studied, therefore it was novel to report that there was significantly increased expression of C5, C5a and MBL in cervico-vaginal fluid where the cervix was short ≤25mm, compared to a normal length of >25mm, (p=0.04, p=0.02 and p=0.03 respectively), Figure 4.24. C5, C5a and MBL concentrations were twice as high in those with cervical shortening compared to normal cervical length. There was no significant difference in C3b in those with cervical shortening or normal cervical length (p>0.05).

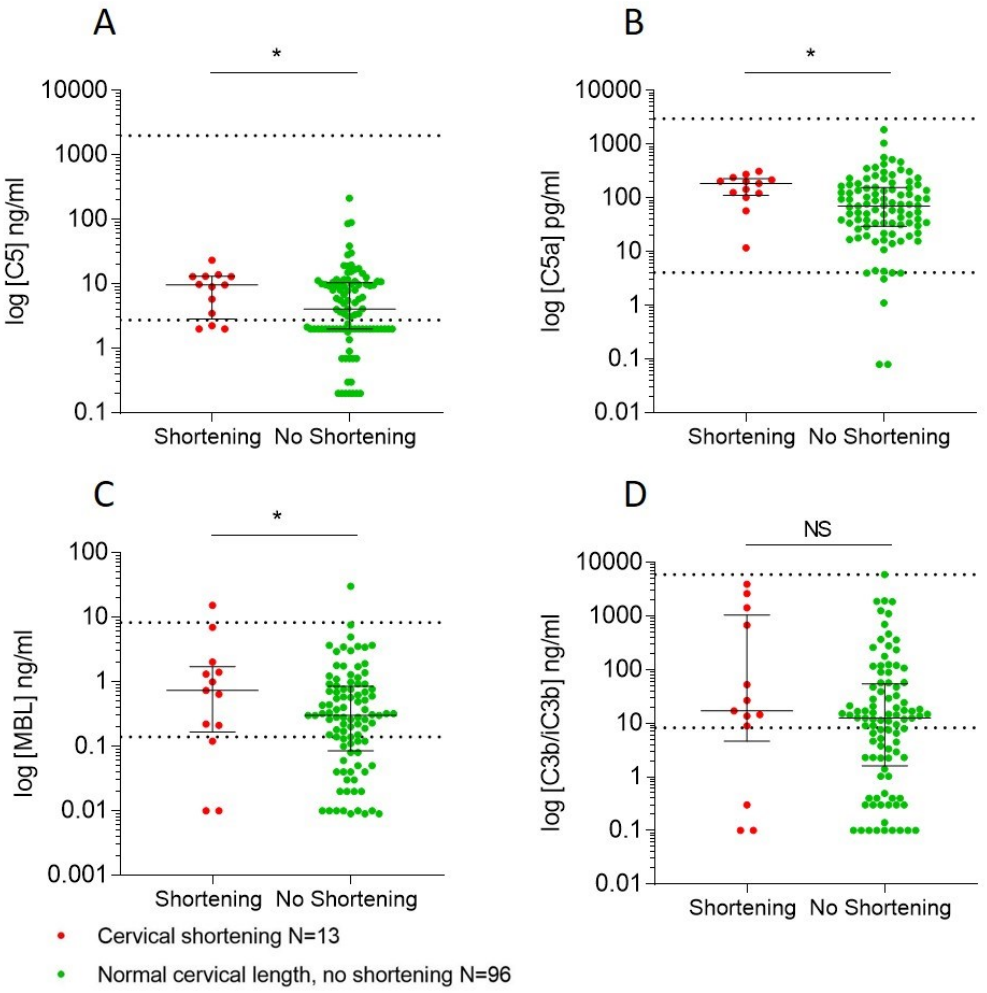


Figure 4.24 Cervico-vaginal complement expression in the presence of cervical shortening, or normal cervical length

Cervico-vaginal fluid complement analytes were compared in women who had cervical shortening at the time of sampling, (cervical length ≤25mm, N=13) versus women who had a normal cervical length at the time of sampling, (cervical length <25mm, N=96). C5, C5a and MBL were significantly higher in those with cervical shortening. There was no significant difference in C3b in those with or without cervical shortening. The dotted lines mark the concentration of the lowest and highest standards on the assay's standard curve. Statistical analysis was by the Mann-Whitney test. *p<0.05, ** p<0.01, NS = non-significant. The results are expressed as median and interquartile range.

The role of immunoglobulins in cervical shortening has not been widely explored, and in this cohort, the most abundant immunoglobulin in cervico-vaginal fluid, IgG1 and also IgM were significantly higher in women with cervical shortening, compared to women with normal cervical length (both p=0.03), Figure 4.25 A and F. IgG2-4, and IgA expression were not significantly between the two groups (p>0.05), Figure 4.25 B-E.

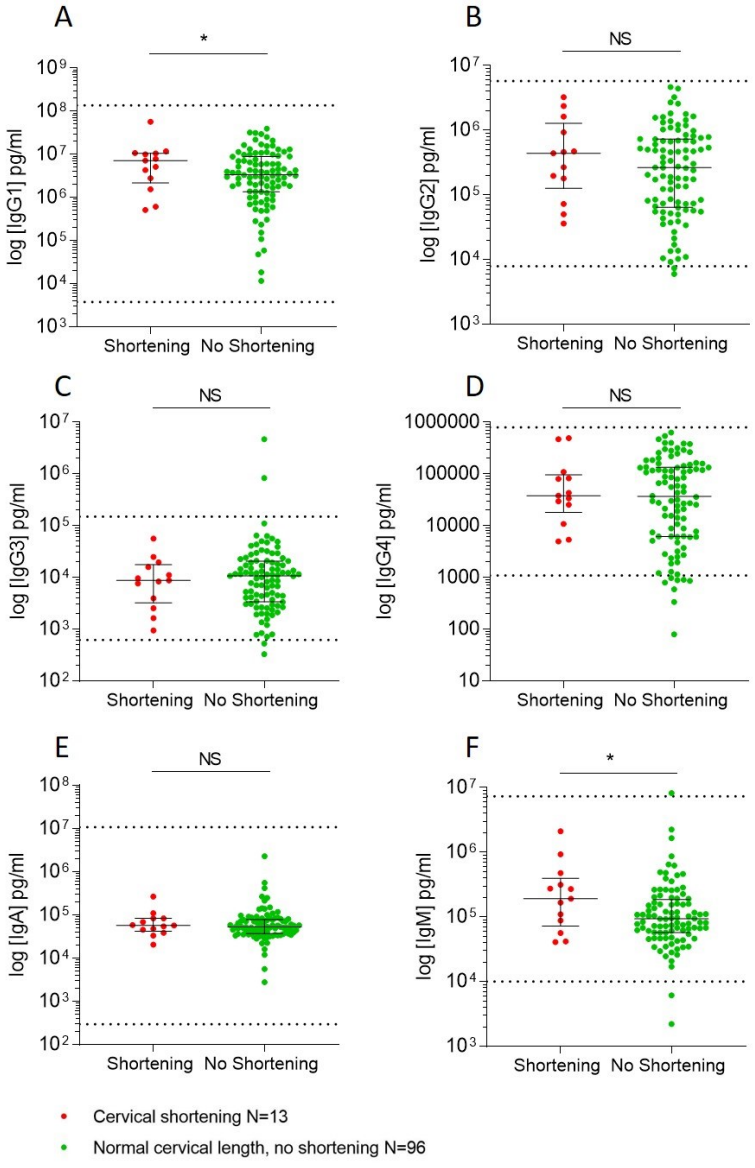


Figure 4.25 Cervico-vaginal immunoglobulin expression in the presence of cervical shortening, or normal cervical length

Cervico-vaginal fluid immunoglobulins were compared in women who had cervical shortening at the time of sampling, (cervical length ≤25mm, N=13) versus women who had a normal cervical length at the time of sampling, (cervical length <25mm, N=96). IgG1 and IgM were significantly higher in those with cervical shortening. There was a non-significant increase in IgG2, IgG3 and IgG4 in those with cervical shortening. The dotted lines mark the concentration of the lowest and highest standards on the assay's standard curve. Statistical analysis was by the Mann-Whitney test. *p<0.05, ** p<0.01, NS non-significant. The results are expressed as median and interquartile range.

Following on, the next analysis performed was to assess if there was a difference in the cervico-vaginal immune response in women who will develop cervical shortening ≤25mm compared to women who maintain a normal cervical length >25mm during pregnancy. At the first sampling timepoint of 12^{+0} - 16^{+6} weeks, excluding women who already had a cervical cerclage in situ, there were 25 women who would later develop cervical shortening. Their cervico-vaginal immune milieu was compared to 71 women who maintained a normal cervical length throughout pregnancy. The median cervical length at the time of sampling in the group who would later develop cervical shortening was 31mm (IQ range 29-36mm), and the median cervical length at the time of sampling in the group who maintain a normal cervical length in pregnancy was 35mm (IQ range 33-40mm). There was a significantly higher proportion of women in the group that subsequently developed cervical shortening for whom their risk factor for preterm delivery was previous MTL and/or PTB (76%), rather than previous cervical treatment (24%), compared to the group that maintain a normal cervical length in pregnancy ($p<0.05$), (53% of which had previous MTL and/or PTB, and 47% had previous cervical treatment).

Table 4.7 shows that the cervico-vaginal immune milieu is no different in women who will later develop cervical shortening ≤25mm, compared to women who maintain a normal cervical length throughout pregnancy, when sampling occurs at 12^{+0} - 16^{+6} weeks gestation. However, the median duration from sampling to actual cervical shortening was 9 weeks and 2 days (range: 1 week and 5 days to 18 weeks and 4 days).

Table 4.7 Cervico-vaginal fluid cytokines, complement and immunoglobulins in women who later develop cervical shortening, compared to women who maintain a normal cervical length

	Sampling at 12^{+0} - 16^{+6} weeks gestation		
CVF analytes	Women who will develop cervical shortening during pregnancy N=25	Women who maintain normal cervical length during pregnancy N=71	p-value
IL-8 pg/ml	789.4 (7144)	1194 (6204)	0.37
IL-1β pg/ml	44.86 (144.4)	33.27 (394.8)	0.21
IL-10 pg/ml	1 (0.56)	0.98 (0.52)	0.27
IL-6 pg/ml	0.4 (2.02)	0.4 (2.77)	0.30
IL-4 pg/ml	4.04 (13.99)	2.54 (12.6)	0.11
IL-18 pg/ml	1.68 (5.29)	2.02 (5.14)	0.12
IL-5 pg/ml	0.19 (0.54)	1.0 (0.57)	0.20
IL-2 pg/ml	17.97 (29.20)	21.95 (28.21)	0.31
IFN-γ pg/ml	4.3 (8.0)	4.4 (8.2)	0.24
TNF-α pg/ml	0.18 (1.24)	0.37 (1.12)	0.09
GM-CSF pg/ml	0.41 (3.79)	0.26 (4.38)	0.13
C5 ng/ml	4.97 (18.52)	4.08 (26.79)	0.30
C5a pg/ml	60.53 (120.5)	62.10 (163.1)	0.13
MBL ng/ml	0.28 (1.66)	0.31 (3.60)	0.12
C3b ng/ml	11.90 (1204)	12.47 (413.9)	0.38
IgG1 pg/ml	4015017 (6749639)	3190136 (8107852)	0.21
IgG2 pg/ml	303700 (891310)	253779 (835610)	0.16
IgG3 pg/ml	7840 (939157)	10912 (98752)	0.25
IgG4 pg/ml	45180 (103538)	35044 (138731)	0.18
IgA pg/ml	74945 (21177)	51937 (273566)	0.12
IgM pg/ml	103726 (329772)	93969 (997772)	0.18

Statistical analysis was by Wilcoxon matched-pairs signed. Median concentration (standard deviation).

This study was not powered to perform paired analysis in women who were sampled before cervical shortening and at the point of cervical shortening as there were only 9 women who had samples collected at the appropriate timepoints who did not already have a cervical cerclage in situ. Therefore, what we can conclude is there is a local inflammatory immune response in women with cervical shortening compared to those with normal cervical length. From this data set it appears that there is no sign of inflammation nine weeks ahead of cervical shortening, however we cannot ascertain if inflammation immediately precedes shortening.

4.5.7 Cervico-vaginal immune response pre and post cervical cerclage

Of the 133 women in the study population, 59 (44%) had a cervical cerclage during their pregnancy. 19 (32%) of those women were excluded from the following analysis to compare the cervico-vaginal immune response pre and post cervical cerclage. 17 were excluded as did not have a vaginal swab collected prior to cervical cerclage insertion and 2 did not have a vaginal swab collected after cervical cerclage insertion.

There were 40 women who had vaginal swabs collected pre and post cervical cerclage allowing the comparison of the cervico-vaginal immune response before and after the clinical intervention. Clinical and demographical data for these women are shown in Table 4.8.

Nearly two-thirds of the 40 women were multiparous. The median gestation of sampling before cervical cerclage was 13^{+5} weeks, and the median number of days before cervical cerclage was 7 days. The median gestation of sampling after cervical cerclage was 22^{+2} weeks, and the median number of days after the cervical cerclage was 50 days. 26 of the 40 women (65%) had a term delivery, after 37 weeks. The number of history and ultrasound indicated cerclages were roughly equal at 19 and 21 respectively, but more than two-thirds were Nylon cerclages, whilst the remaining one-third were Mersilene cerclages. The cervical length before cervical cerclage (median 28mm) was significantly less compared to after cervical cerclage (median 33mm) ($p<0.05$).

Table 4.8 Study population of 40 study participants allowing comparisons before and after cervical cerclage

Age	33 (29-36)
BMI (kg/m^2)	25.8 (23-29)
Ethnicity	
- White	18 (45%)
- Black	13 (32.5%)
- Other	9 (22.5%)
Parity	
- Nulliparous	14 (35%)
- Multiparous	26 (65%)
Gestation at sampling point before cerclage	13^{+5} (13^{+1} - 15^{+0})
Days before cerclage	7 (3-16)
Gestation at sampling point after cerclage	22^{+2} (21^{+2} - 23^{+4})
Days after cerclage	50 (33-61)
Gestation of delivery	38^{+0} (32^{+2}-39^{+0})
Preterm delivery <34 weeks	9 (22.5%)
Preterm delivery 34+0-36+6 weeks	5 (12.5%)
Term delivery	26 (65%)
Risk factor for PTB[(a)]	
- Cervical treatment	7 (17.5%)
- Cervical treatment and MTL	4 (10%)
- Cervical treatment and previous PTB	2 (5%)
- MTL	12 (30%)
- MTL and PTB	4 (10%)
- PTB	10 (25%)
- Short cervix at anomaly ultrasound	1 (2.5%)
Indication for cervical cerclage	
- History indicated	19 (47.5%)
- Ultrasound indicated	21 (52.5%)
Cervical cerclage material	
- Mersilene	14 (35%)
- Nylon	25 (62.5%)
- Unknown	1 (0.5%)
Cerclage material by indication for cerclage	
- History indicated (Mersilene vs Nylon)	7 vs 12
- Ultrasound indicated (Mersilene vs Nylon)	7 vs 13
Cervical length at sampling point before cerclage (mm)	28 (25-32) (2 missing data)
Cervical length at sampling point after cerclage (mm)	33 (26-37) (7 missing data)
Use of progesterone	21 (52.5%)

BMI=body mass index, USS=ultrasound, PTB=preterm birth, MTL=mid trimester loss, misc=miscarriage. Data presented as median (interquartile range (IQ)) or number (%). [(a)]Some study participants will have more than one risk factor for PTB.

Cytokines IL-8, IL-1β, IL-10, IL-6, IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α and GM-CSF did not show any significant differences in expression in cervico-vaginal fluid before and after cervical cerclage insertion, ($p>0.05$) Figure 4.26.

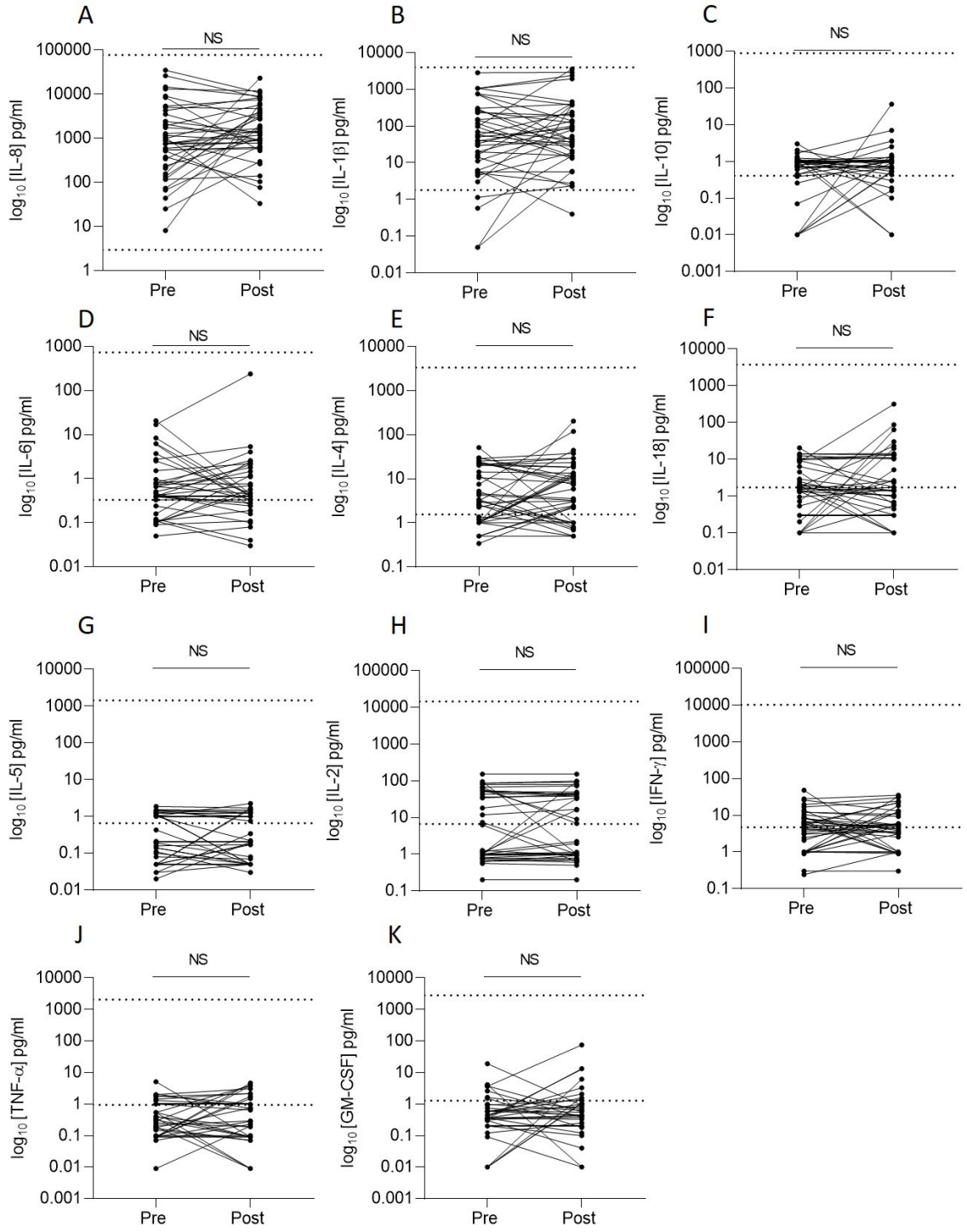


Figure 4.26 Cervico-vaginal fluid cytokines pre and post cervical cerclage
There was no significant change in expression of cervico-vaginal fluid cytokines pre and post cervical cerclage insertion. The dotted lines mark the range of the standard curve for the assays. Statistical analysis was by Wilcoxon matched-pairs signed rank test, NS = non-significant. N=40 paired samples.

Complement analytes in cervico-vaginal fluid C5a and MBL increased significantly following cervical cerclage (p=0.03 and p=0.04), whilst C5 and C3b concentrations were stable (p>0.05), Figure 4.27.

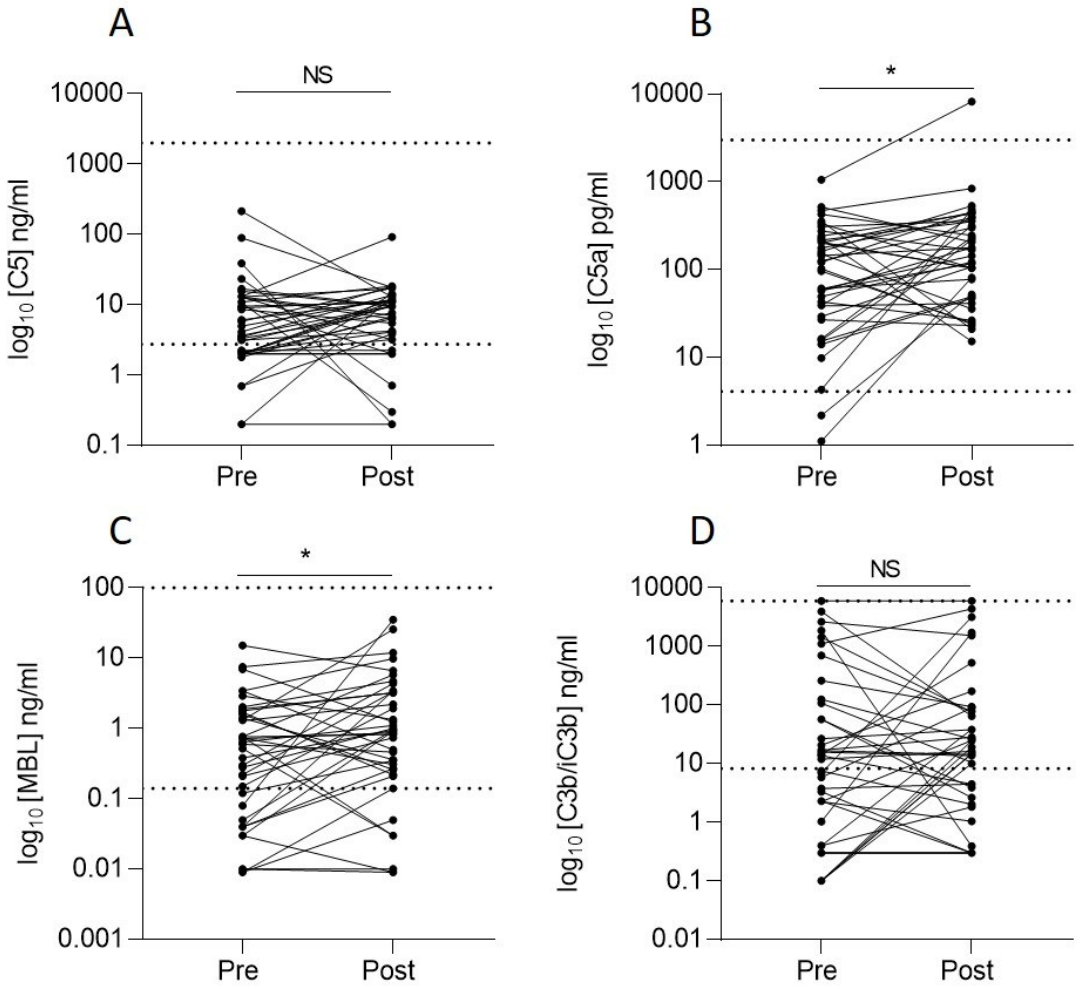


Figure 4.27 Cervico-vaginal fluid complement pre and post cervical cerclage
C5a and MBL increased significantly in cervico-vaginal fluid following cervical cerclage, (B and C). There was no significant change in expression of C5 and C3b (A and D) pre and post cervical cerclage insertion. The dotted lines mark the range of the standard curve for the assays. Statistical analysis was by Wilcoxon matched-pairs signed rank test, * p<0.05, NS = non-significant. N=40 paired samples.

The concentrations of immunoglobulins IgG1-4, IgA and IgM in cervico-vaginal fluid did not alter significantly following cervical cerclage insertion ($p>0.05$), Figure 4.28.

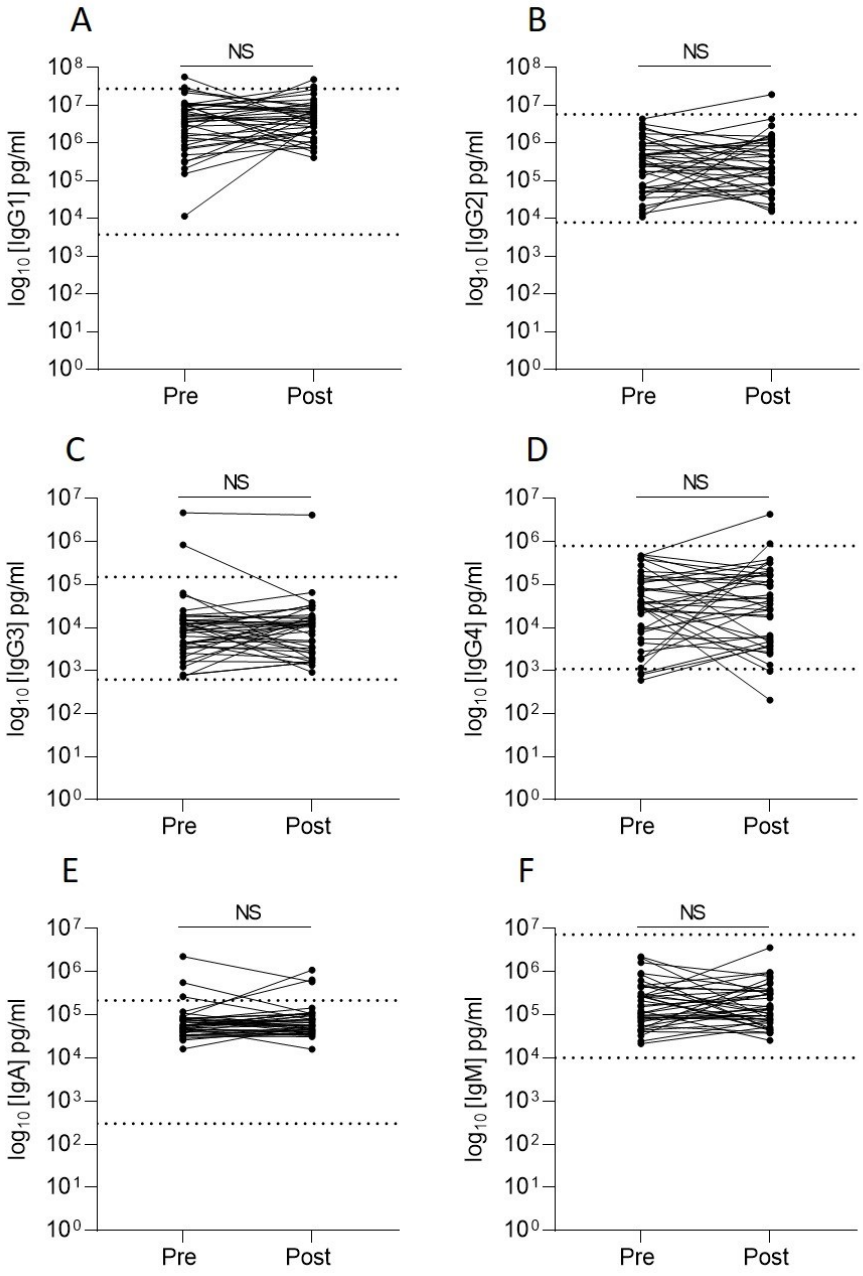


Figure 4.28 Cervico-vaginal fluid immunoglobulins pre and post cervical cerclage
There was no significant change in IgG1-4, IgA and IgM following cervical cerclage. The dotted lines mark the range of the standard curve for the assays. Statistical analysis was by Wilcoxon matched-pairs signed rank test, NS = non-significant. N=40 paired samples.

The local immune response according to the indication of cervical cerclage

With the only detectable local immune response to cervical cerclage being an increase in C5a and MBL pre and post cervical cerclage, further interrogation was performed as follows. To test if there was a different local immune response depending upon whether the cervical cerclage was performed due to a previous history of cervical insufficiency, or ultrasound indicated (shortened cervical length in the current pregnancy), the fold change in cervico-vaginal fluid analytes before and after cervical cerclage were compared, Figure 4.29. There were 19 history indicated cervical cerclages and 21 ultrasound indicated cerclages. The median gestation at delivery for women who had a history and women who had an ultrasound indicated cerclage were 38^{+0} and 38^{+1} weeks respectively, with no significant difference between the two. There were equal proportions of women in each group who had Mersilene (braided) and Nylon (monofilament) material used for the cerclages.

The mean fold change of all cytokines, complement and immunoglobulin analytes increased following cervical cerclage. There was non-significant increase in the fold change IL-8, IL-10 and TNF-α in women who had ultrasound indicated cerclages, for cervical shortening ≤25mm, compared women who had history indicated cerclages ($p>0.05$), Figure 4.29 A.

There were no significant differences in the mean fold change of complement analytes, C5, C5a, MBL or C3b based upon the indication for the cervical cerclage ($p>0.05$), Figure 4.29 B.

There were no significant differences in the mean fold change of immunoglobulins following cervical cerclage in women who had history indicated compared to ultrasound indicated cerclages ($p>0.05$), Figure 4.29 C.

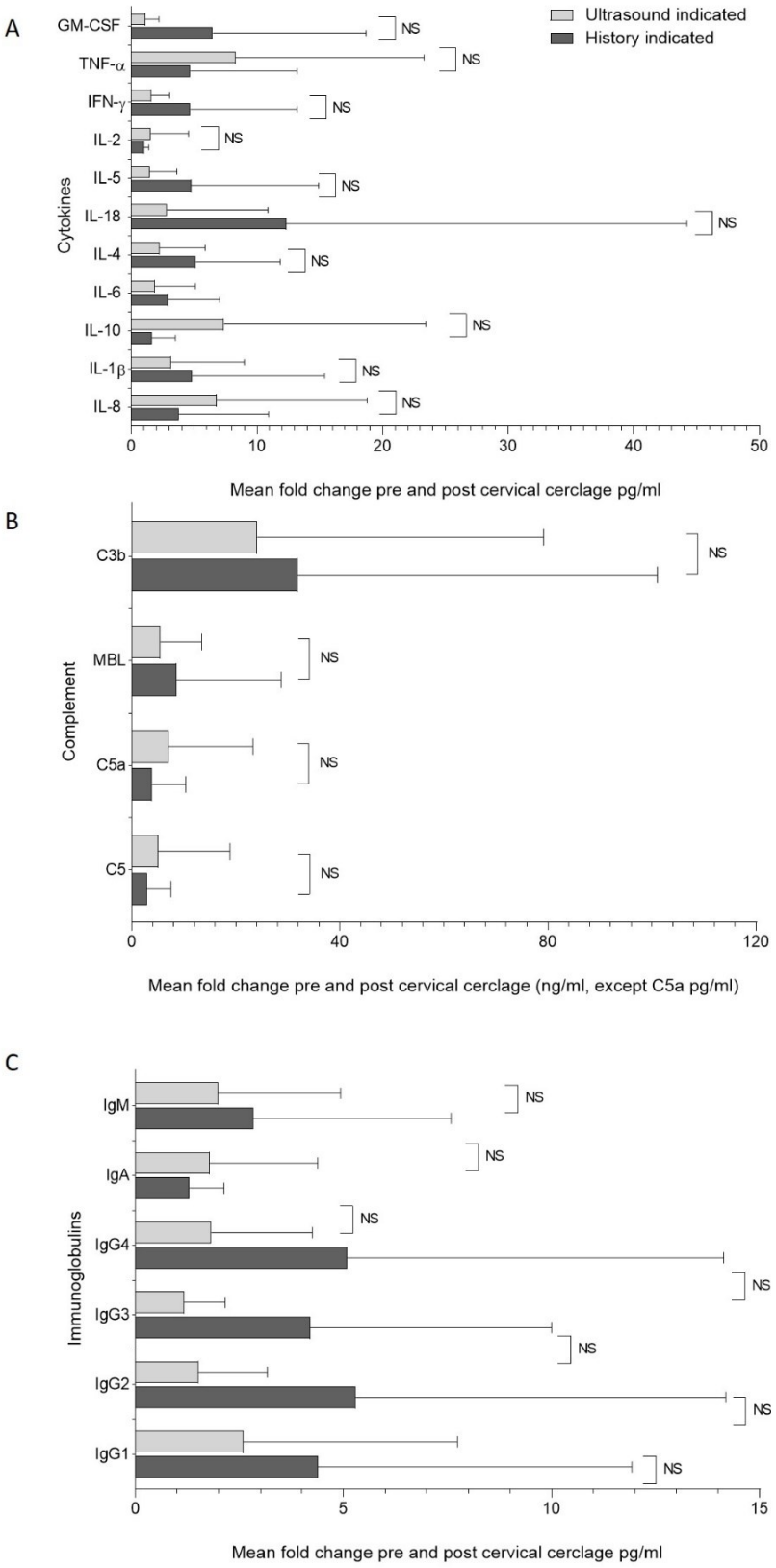


Figure 4.29 Local immune response pre and post cervical cerclage based on indication for cervical cerclage
There was no significant difference mean fold change in cervico-vaginal fluid cytokines, complement and immunoglobulins analytes, pre and post cervical cerclage comparing history and ultrasound indicated cerclages. Statistical analysis was by Mann-Whitney test. History indicated cerclages N=19 and ultrasound indicated cerclages N=21.

The local immune response according to of cervical cerclage material, independent of pregnancy outcome

To test if there was a different local immune response depending upon whether the cervical cerclage was performed using Mersilene (braided) or Nylon (monofilament) suture material, the fold change in cervico-vaginal fluid analytes before and after cervical cerclage according to cerclage material were compared. 14 cervical cerclages which were performed with Mersilene (braided) material and 25 were performed with Nylon (monofilament) material, with vaginal swab samples collected before and after the clinical procedure. The median gestation of delivery for women who had a Mersilene cerclage was 36^{+0} weeks, whilst it was 38^{+1} for women who had a Nylon cerclage. Of women who received Mersilene N=14), the preterm birth rate was 50%, and of women who received Nylon (N=25), the preterm birth rate was 24%, which was statistically significant, (Fisher's exact test $p<0.05$).

There was a significant increase in the mean fold change of IL-6 ($p=0.007$), IL-1β ($p<0.0001$) and IL-8 ($p=0.04$) from pre to post Mersilene cerclage compared to Nylon cerclage, Figure 4.30 A. Following a similar pattern, there was a non-significant increase for IL-2 for Mersilene compared to Nylon cerclages. There were minimal differences for the remaining cytokines, GM-CSF, TNF-α, IFN-γ, IL-5, IL-18, IL-4 and IL-10 ($p>0.05$).

All the complement analytes analysed, C5 ($p=0.009$), C5a ($p=0.002$), MBL ($p=0.005$) and C3b ($p=0.02$) showed a significant increase in the mean fold change from pre to post Mersilene cerclage compared to Nylon cerclage, Figure 4.30 B.

All the immunoglobulins analysed, IgG1 ($p=0.003$), IgG3 ($p=0.005$), IgA ($p=0.002$), IgM ($p=0.005$), IgG2 ($p=0.03$) and IgG4 ($p=0.04$) also showed a significant increase in the fold change from before to after Mersilene cerclage, compared to Nylon cerclage, Figure 4.30 C.

Mersilene cerclage induced both an innate and adaptive local immune response.

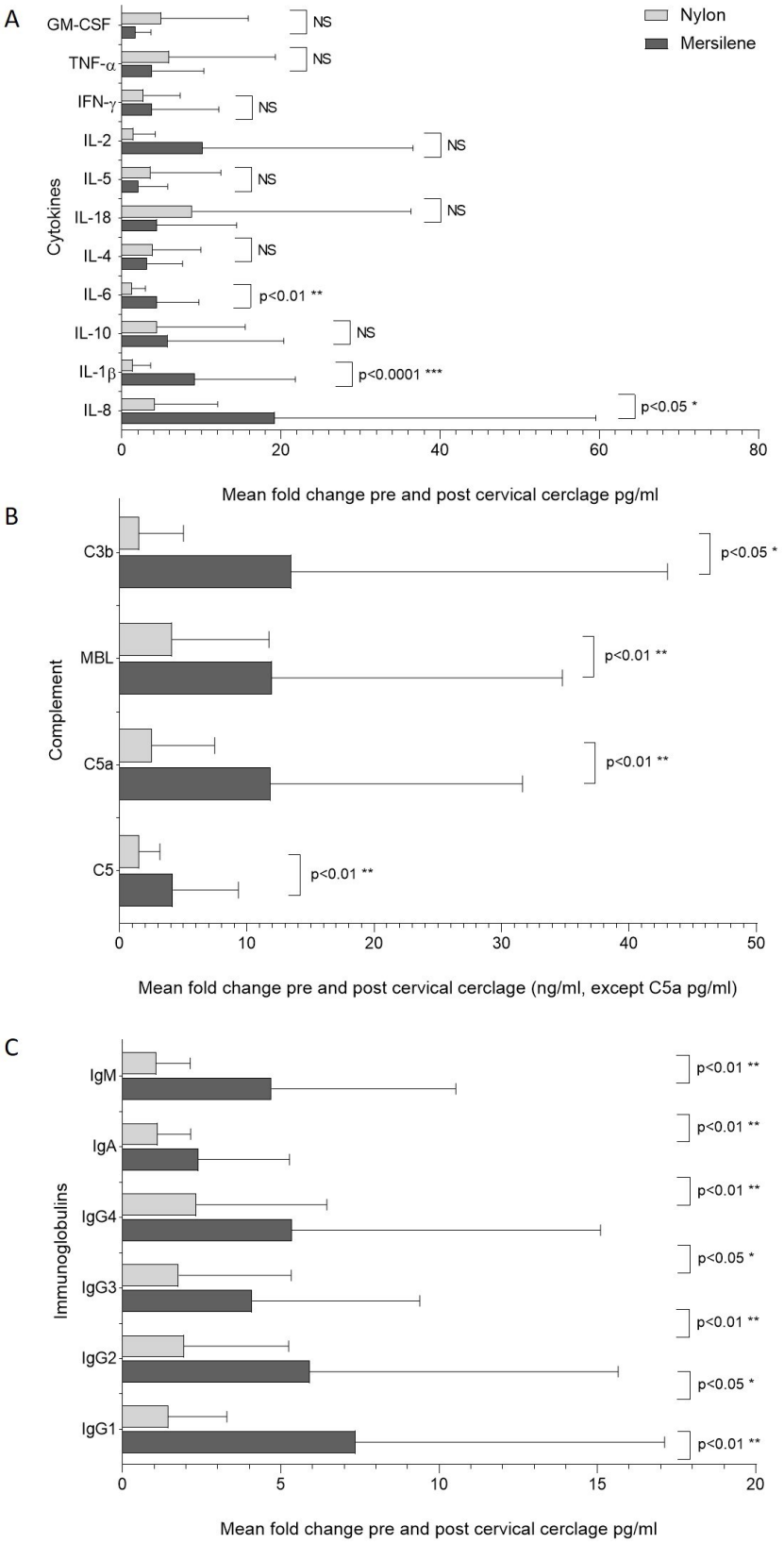


Figure 4.30 Local immune response pre and post cervical cerclage based on cerclage material
The mean fold increase in IL-6, IL-1β and IL-8 (A), C3b, MBL, C5a, C5 (B) and IgE, IgM, IgA, IgG1-4 (C) was significantly greater from pre to post cervical cerclage when comparing Mersilene and Nylon cerclages. Statistical analysis was by Mann-Whitney test, * p<0.05, ** p<0.01, NS = non-significant. Mersilene cerclages N=14 and Nylon cerclages N=25.

The local immune response according to of cervical cerclage material, in women who delivery preterm

There are existing studies exploring the difference between cervical cerclage material and their effect on the local immune response. The braided Mersilene has been found to be induce a pro-inflammatory immune response, compared to the monofilament Nylon. To explore if this was driving the pro-inflammatory shift seen in between the two sampling time points of 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks in the women that deliver preterm, the cohort of N=34 were divided into those women who had a Mersilene cerclage inserted between the two timepoints, Nylon cerclage inserted between the two timepoints, and no cervical cerclage. In the women who had preterm deliveries, there were 15 of who had cervical cerclages between the sampling time points, 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks. 10 were performed with Mersilene and 7 with Nylon; the remaining 17 did not have a cervical cerclage between the two sampling timepoints. Table 4.9 describes the clinical characteristics of women in these 3 comparison groups.

There were no differences in the gestational age at delivery, maternal age, maternal BMI, maternal ethnicity or parity between the three groups ($p>0.05$). Between the two cerclage groups, there were almost equal numbers of history and ultrasound indicated cerclages. There was also no significant difference in the risk factors for preterm delivery between the groups.

Table 4.9 Study participants with preterm deliveries, with or without cervical cerclage between sampling timepoints 12-16 and 20-24 weeks gestation N=34

	A	B	C	
	Preterm Mersilene cerclage	Preterm Nylon cerclage	Preterm No cerclage	p value (A vs B vs C)
N (%)	10	7	17	
Gestation at delivery (weeks) median and IQ range	32^{+3} (29^{+0} - 35^{+2})	36^{+0} (29^{+3} - 36^{+3})	33^{+3} (32^{+6} - 35^{+3})	One-way ANOVA p=0.26 (A vs B) Unpaired t-test p=0.15
Early preterm </=33+6	7 (70%)	3 (50%)	9 (53%)	χ^2 =0.39
Late preterm >/=34+0	3 (30%)	3 (50%)	8 (47%)	
Age median and IQ range	30 (26-37)	33 (30-42)	33 (30-39)	One-way ANOVA p=0.51
BMI median and IQ range (kg/m^2)	25 (23-31)	23 (22-27)	22 (21-27)	One-way ANOVA p=0.35
Ethnicity White N (%) Black N (%) Other N (%)	 4 (40%) 4 (40%) 2 (10%)	 3 (43%) 3 (43%) 1 (14%)	 10 (59%) 1 (6%) 6 (35%)	χ^2 =0.19
Parity Nulliparous Multiparous	 2 (20%) 8 (80%)	 5 (71%) 2 (29%)	 4 (24%) 13 (76%)	
Cervical cerclage inserted between 12-24 weeks	10 (100%)	7 (100%)	0 (0%)	
History indicated cervical cerclage	4 (40%)	3 (43%)	NA	
USS indicated cervical cerclage	6 (60%)	4 (57%)	NA	
Risk factor for PTB: (by pt) Cervical treatment Cervical treatment + MTL Cervical treatment + PTB MTL MTL + PTB PTB	 1 0 3 2 1 3	 2 1 0 3 0 1	 3 0 4 2 0 8	
Risk factor for PTB [(a)] Cervical treatment Previous PTB Previous MTL	 4 (40%) 7 (70%) 3 (30%)	 3 (43%) 1 (14%) 4 (57%)	 7 (41%) 12 (71%) 2 (11%)	χ^2 p=0.12

BMI=body mass index, USS=ultrasound, PTB=preterm birth, MTL=mid trimester loss, misc=miscarriage. Data presented as median (interquartile range (IQ)) or number (%). P values: One way ANOVA for multiple comparisons or Chi squared for proportional data.
[(a)]Some study participants will have more than one risk factor for PTB.

In women who delivered preterm, with regards to the cervico-vaginal fluid cytokines, IL-8, IL-1β and IL-6 increased between sampling timepoints 12^{+0} - 16^{+6} weeks and 20^{+0} - 24^{+6} weeks in the group who had a Mersilene cerclage (p=0.003, p=0.001 and p=0.001 respectively), Figure 4.31 A, B and D. IL-6 also increased in the group who did not have a cerclage at all (p=0.03), in women who delivered preterm. In women who had a Nylon cerclage, there was no increase in the local pro-inflammatory cytokine immune response (p>0.05), Figure 4.31. There was no change in the anti-inflammatory cytokine IL-10 (p>0.05), Figure 4.31 C. There was also no change in the less detectable cytokines in the CVF: IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α or GM-CSF (p>0.05) (data not shown).

Women who had a Mersilene compared to a Nylon cerclage was significantly more likely to have a rise in IL-8 and IL-1β (both p=0.003), Figure 4.31 A and B.

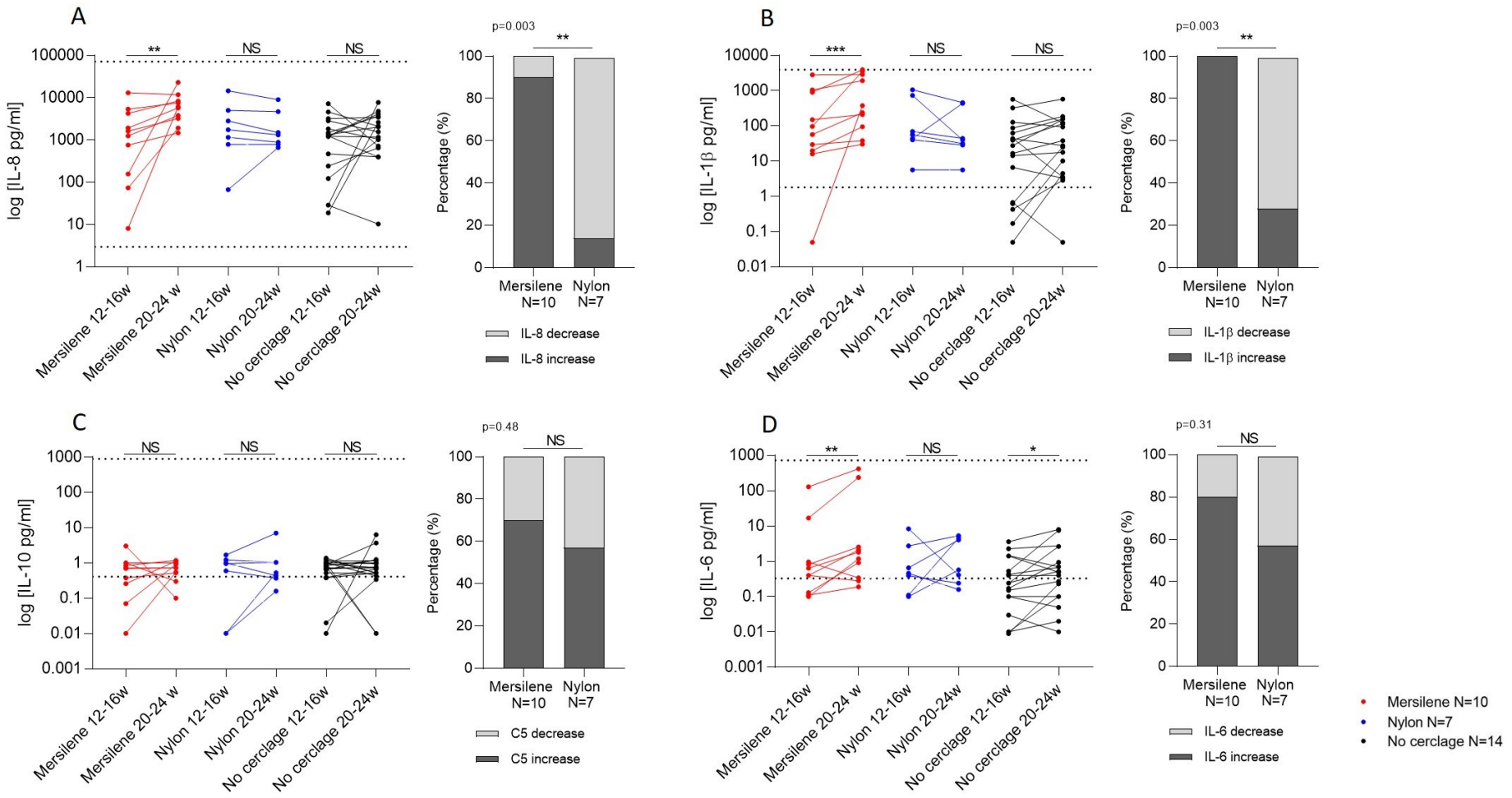


Figure 4.31 Early to mid-gestation cervico-vaginal fluid cytokines in women with preterm deliveries according to the insertion of a cervical cerclage
There was a significant increase in IL-8, IL-1β and IL-6 between 12-16 weeks and 20-24 weeks gestation in women who had a Mersilene cerclage. There was also a significant increase in IL-6 in women who did not have a cervical cerclage. There was no significant change in IL-8, IL-1β, IL-6 or IL-10 in women who had a Nylon cerclage (A). IL-8 and IL-1β were significantly more likely to increase in women who had a Mersilene cerclage. Statistical analysis was by Wilcoxon matched-pairs signed rank test and Fisher's exact test *p<0.05, **p<0.01. Mersilene cerclage N=10, nylon cerclage N=7 and no cerclage N=14.

Similarly complement analytes C5a and C3b increased significantly in the group who had a Mersilene cerclage and the group which did not have a cerclage, ($p=0.003$ and $p=0.05$), Figure 4.32 B and D, and MBL increased following a Mersilene cerclage ($p=0.05$), Figure 4.32 C, whilst women who had a Nylon cerclage did not exhibit this local pro-inflammatory complement response, ($p>0.05$) Figure 4.32. There was no significant difference in C5 pre and post Mersilene or Nylon cerclage, or in those without a cervical cerclage ($p>0.05$), Figure 4.32 A.

Women who had a Mersilene cerclage was significantly more likely to have a rise in C5a and C3b compared to women who had a Nylon cerclage, (both $p=0.05$), Figure 4.32 B and D.

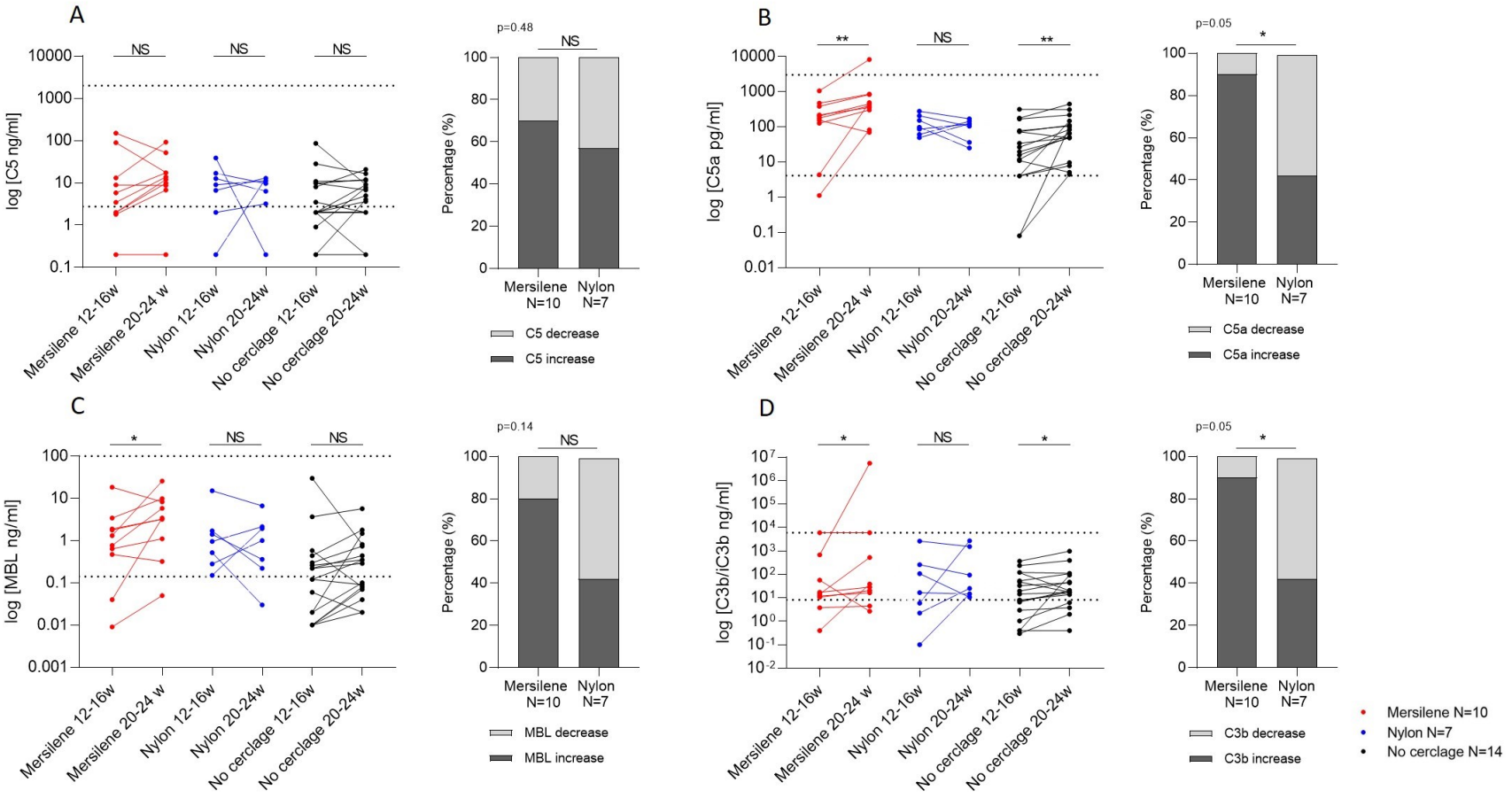


Figure 4.32 Early to mid-gestation cervico-vaginal fluid complement analytes in women with preterm deliveries according to the insertion of a cervical cerclage
There was a significant increase in C5a, MBL and C3b between 12-16 weeks and 20-24 weeks gestation in women who had a Mersilene cerclage. There was also a significant increase in C5a and C3b in women who did not have a cervical cerclage. There was no significant change in C5a, MBL or C3b in women who had a Nylon cerclage (A). There was no significant difference in C5 pre and post Mersilene or Nylon cerclage, or in those without a cervical cerclage.C5a and C3b were significantly more likely to increase in women who had a Mersilene cerclage, and there was non-significant trend for MBL to increase too. Statistical analysis was by Wilcoxon matched-pairs signed rank test and Fisher's exact test *p<0.05, **p<0.01. Mersilene cerclage N=10, nylon cerclage N=7 and no cerclage N=14.

There was no statistically significant augmentation of the adaptive immune response in women who had a Mersilene or Nylon cerclage between early and mid-pregnancy ($p>0.05$), Figure 4.33 and 4.34. However, there was a tendency for some women to show an increase in IgG1-4, IgA and IgM following Mersilene cerclage, and a tendency for some women to show a decrease following Nylon cerclage, regardless of preterm or term delivery.

The concentration of IgG1 increased significantly in women who did not have a cervical cerclage ($p<0.001$), Figure 4.33 A.

The concentrations of IgG1-4, IgA and IgM were no more likely to change whether a Mersilene or Nylon cerclage were inserted ($p>0.05$), Figure 4.33 and 4.34.

Overall, in the women who delivered preterm, Mersilene augmented the innate immune response, demonstrated in the changes to IL-8, IL-1β, IL-6, C5a, C3b and MBL, whilst Nylon was immunologically inert.

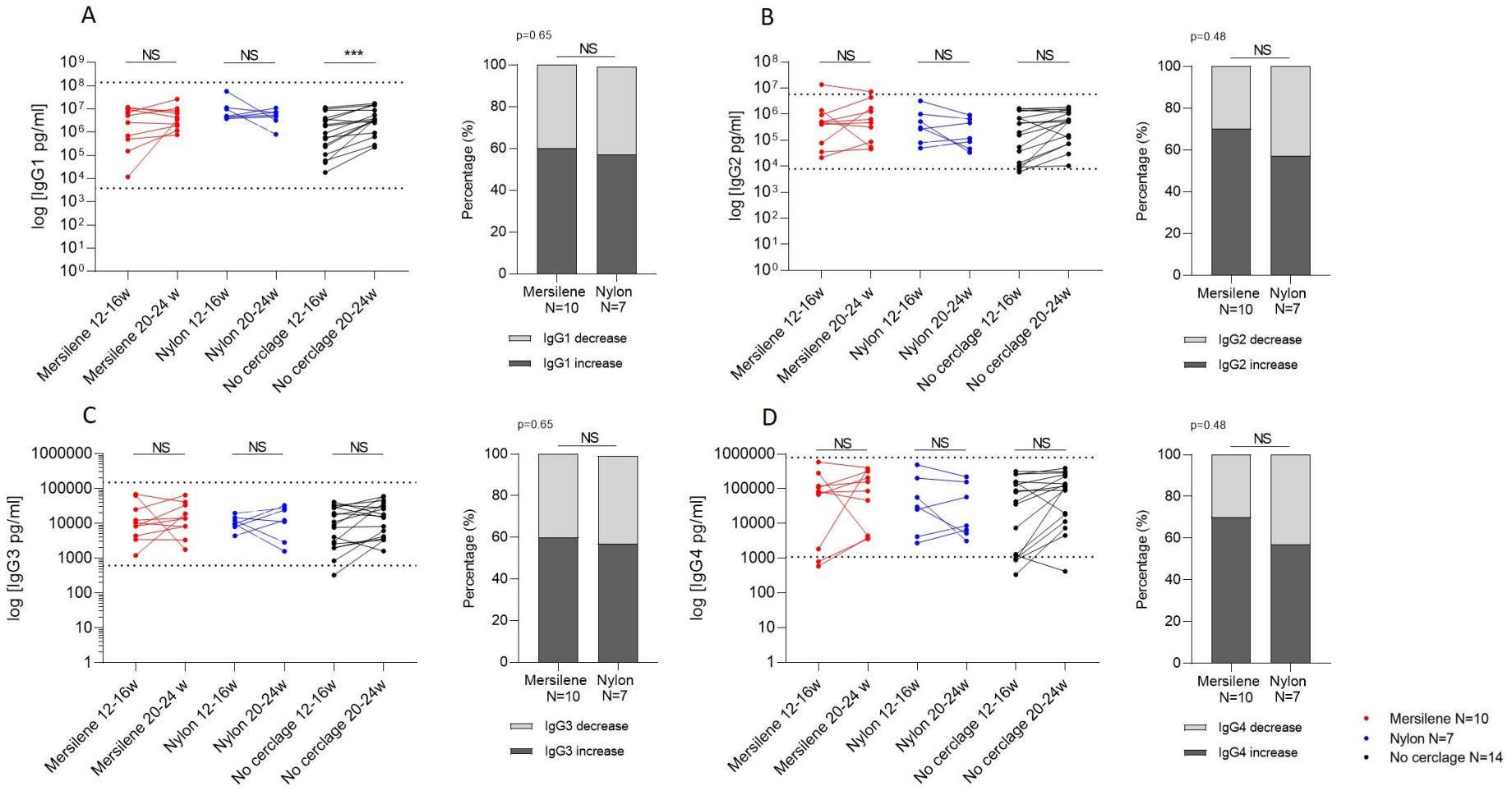


Figure 4.33 Early to mid-gestation cervico-vaginal fluid IgG1-IgG4 in women with preterm deliveries according to the insertion of a cervical cerclage

There was no significant change in IgG1, IgG2, IgG3 or IgG4 following the insertion of a Mersilene or a Nylon cerclage between 12-16 and 20-24 weeks gestation. There was a significant increase in IgG1 in the women who did not have a cervical cerclage ($p<0.001$), but no change in IgG2-4 in this group. The concentrations of IgG1-4 were no more likely to change based on receiving a Mersilene or a Nylon cerclage. Statistical analysis was by Wilcoxon matched-pairs signed rank test and Fisher's exact test ***$p<0.001$. Mersilene cerclage N=10, nylon cerclage N=7 and no cerclage N=14.

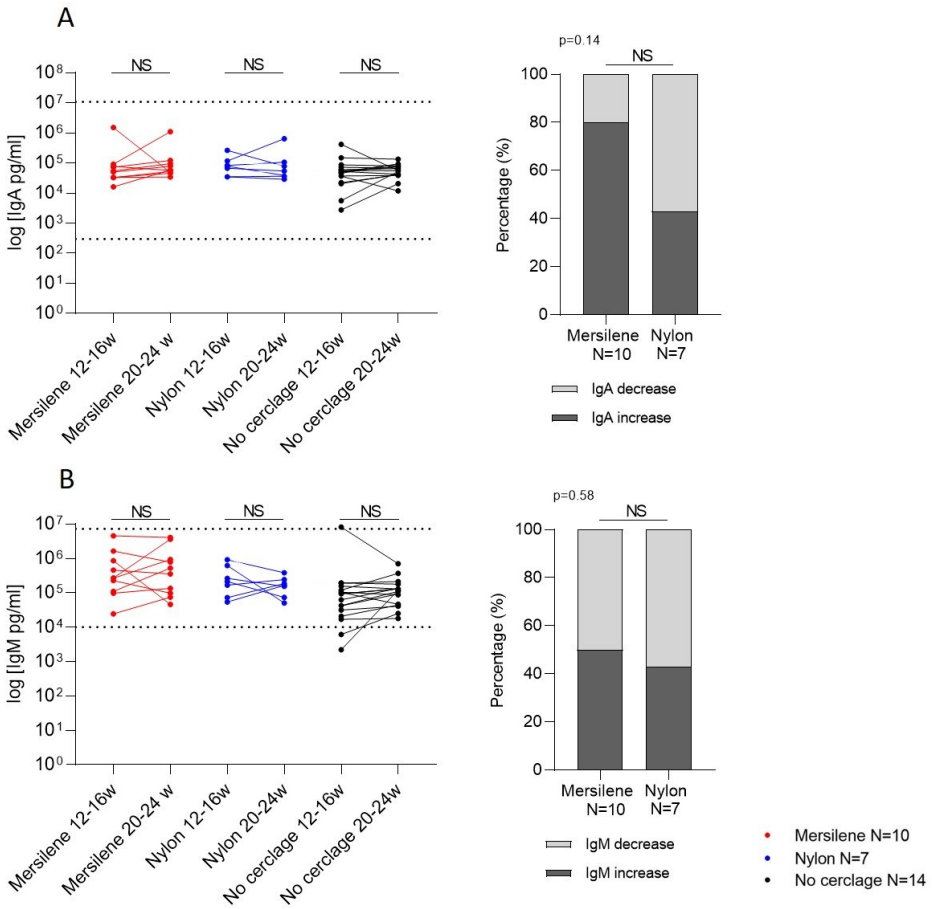


Figure 4.34 Early to mid-gestation cervico-vaginal fluid IgA or IgM in women with preterm deliveries according to the insertion of a cervical cerclage
There was no significant change in IgA or IgM following the insertion of a Mersilene or a Nylon cerclage, or in gestation matched women who did not receive a cerclage between 12-16 and 20-24 weeks gestation. The concentrations of IgA and IgM were no more likely to change based on receiving a Mersilene or a Nylon cerclage. Statistical analysis was by Wilcoxon matched-pairs signed rank test and Fisher's exact test ***p<0.001. Mersilene cerclage N=10, nylon cerclage N=7 and no cerclage N=14.

The inflammatory response to cervical cerclage material in women who deliver at term

CVF cytokines at 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks following Mersilene cerclage, Nylon cerclage or no cervical cerclage were also compared in women who had term deliveries. 5 women received a Mersilene cerclage and 14 women received a Nylon cerclage between the two sampling timepoints, whilst 53 women did not receive a cervical cerclage. Clinical and demographical details for the three groups are provided in Table 4.10. There was no difference in age, BMI, ethnicity or parity, and no difference in the indication for cerclage.

Table 4.10 Study participants with term deliveries, with or without cervical cerclage between sampling timepoints 12-16 and 20-24 weeks gestation N=71

	A	B	C	
	Term Mersilene cerclage	Term Nylon cerclage	Term No cerclage	p value (A vs B vs C)
N (%)	5	14	53	
Gestation at delivery (weeks) median and IQ range	39^{+0} (40^{+0} - 38^{+0})	38^{+2} (38^{+0} - 39^{+0})	39^{+5} (39^{+0} - 39^{+5})	One-way ANOVA p=0.10 (A vs B) Unpaired t-test p=0.18
Age median and IQ range	36 (32-39)	33 (27-35)	33 (29-37)	One-way ANOVA p=0.14
BMI median and IQ range (kg/m^2)	25 (22-34)	24 (22-31)	23 (20-27)	One-way ANOVA p=0.22
Ethnicity White N (%) Black N (%) Other N (%)	 4 (80%) 1 (20%) 0 (10%)	 5 (36%) 5 (36%) 4 (28%)	 38 (72%) 7 (13%) 8 (15%)	χ^2 =0.11
Parity Nulliparous Multiparous	 2 (40%) 3 (60%)	 1 (7%) 13 (93%)	 29 (55%) 24 (45%)	
Cervical cerclage inserted between 12-24 weeks	5 (100%)	14 (100%)	0 (0%)	
History indicated cervical cerclage	3 (60%)	10 (71%)	NA	χ^2 p=0.52
USS indicated cervical cerclage	2 (40%)	4 (29%)	NA	
Risk factor for PTB: (by pt) Cervical treatment Cervical treatment + MTL Cervical treatment + PTB MTL MTL + PTB PTB	 2 1 0 2 0 0	 2 0 0 3 4 5	 33 0 2 5 2 11	
Risk factor for PTB [(a)] Cervical treatment Previous PTB Previous MTL	 3 (60%) 3 (60%) 0 (0%)	 2 (43%) 7 (14%) 9 (57%)	 34 (64%) 7 (13%) 15 (28%)	χ^2 p<0.01 **

Despite the pro-inflammatory changes seen in IL-8, IL-1β and IL-6 women with preterm deliveries following Mersilene cerclage in Figure 4.31, the expression of IL-8, IL-1β and IL-6 did not change significantly post Mersilene cerclage in women who delivered at term gestation ($p>0.05$), Figure 4.35 A-C. What is apparent is the potential protective effect in a trend for a reduction in IL-1β and IL-6 with the Nylon cerclage in this group of women who delivered at term gestation, Figure 4.35 B and C. IL-10 concentrations did not alter significantly post Mersilene or Nylon cerclage insertion ($p>0.05$), Figure 4.35 D. There was no change in IL-8, IL-1β, IL-10 or IL-6 in the women who did not receive a cervical cerclage ($p>0.05$).

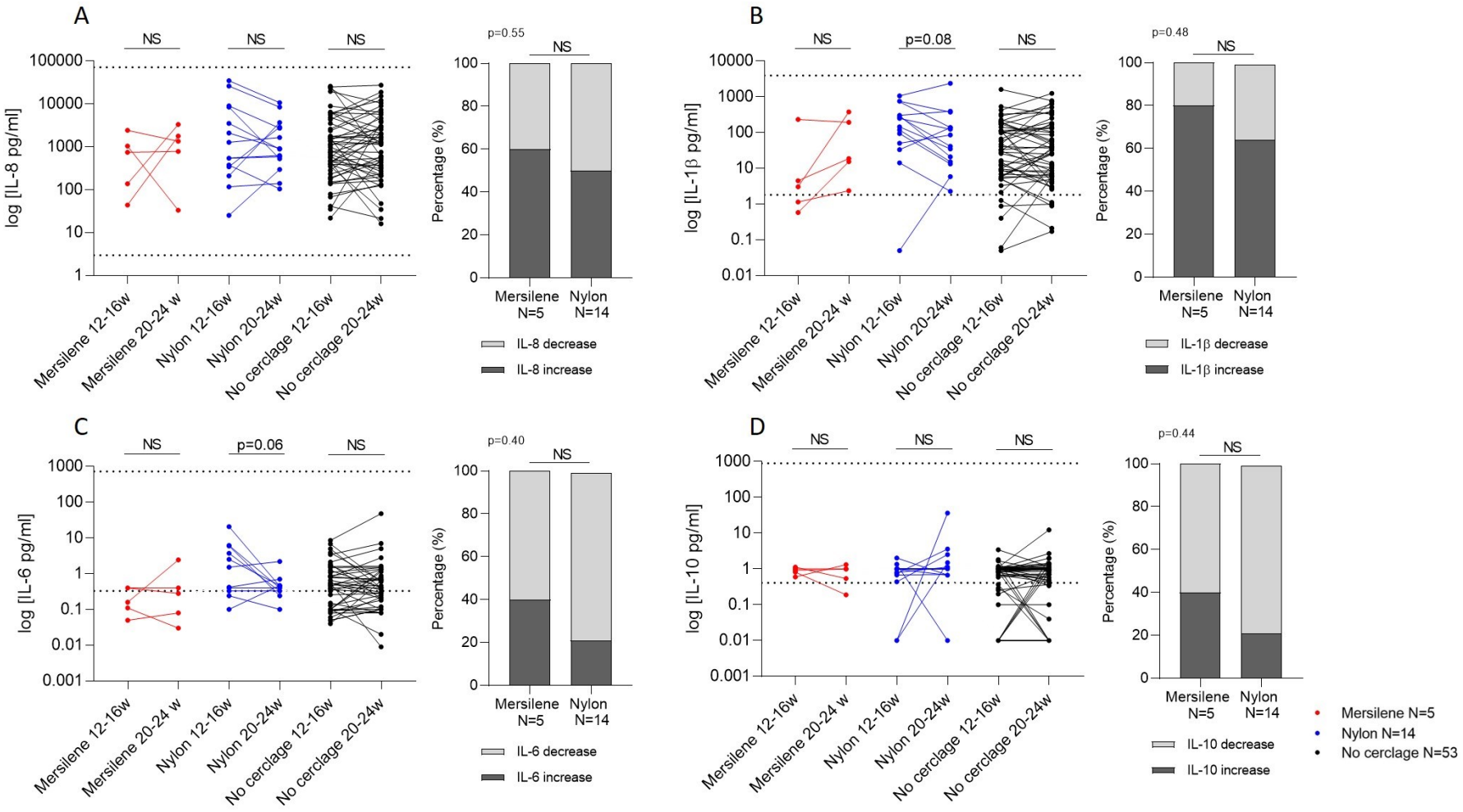


Figure 4.35 Early to mid-gestation cervico-vaginal fluid cytokines in women with term deliveries according to the insertion of a cervical cerclage
There was no change in IL-8, IL-1β, IL-10 and IL-6 between 12-16 weeks and 20-24 weeks gestation in women who had a Mersilene cerclage, Nylon cerclage or no cervical cerclage. Statistical analysis was by Wilcoxon matched-pairs signed rank test and Fisher's exact test, NS = non-significant. Mersilene cerclage N=5, nylon cerclage N=14 and no cerclage N=53.

There was a significant increase in C5a and a non-significant increase in MBL post Mersilene cerclage in this group of women who delivered at term, ($p=0.05$ and $p<0.06$ respectively), Figure 4.36 B and C. There was no change in C5 or C3b post Mersilene or Nylon cerclage ($p>0.05$), Figure 4.36 A and D. This is in contrast to the results presented in Figure 4.32 for women who delivered preterm, who had significantly higher concentrations of C5a, MBL and C3 following Mersilene cerclage insertion. There was no change in C5, C5a, MBL or C3b in the women who did not receive a cervical cerclage ($p>0.05$).

There was no significant difference in cervico-vaginal fluid IgG1-4, IgA or IgM in women who received a Mersilene or Nylon cerclage, or no cerclage between 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks ($p>0.05$), Figure 5.37 A-D and Figure 5.38 A-B. This is similar to the results for women who had preterm deliveries, presented in Figure 5.33 and 5.34. However, there was a tendency for an increase in immunoglobulins following Mersilene cerclage and a decrease following Nylon cerclage.

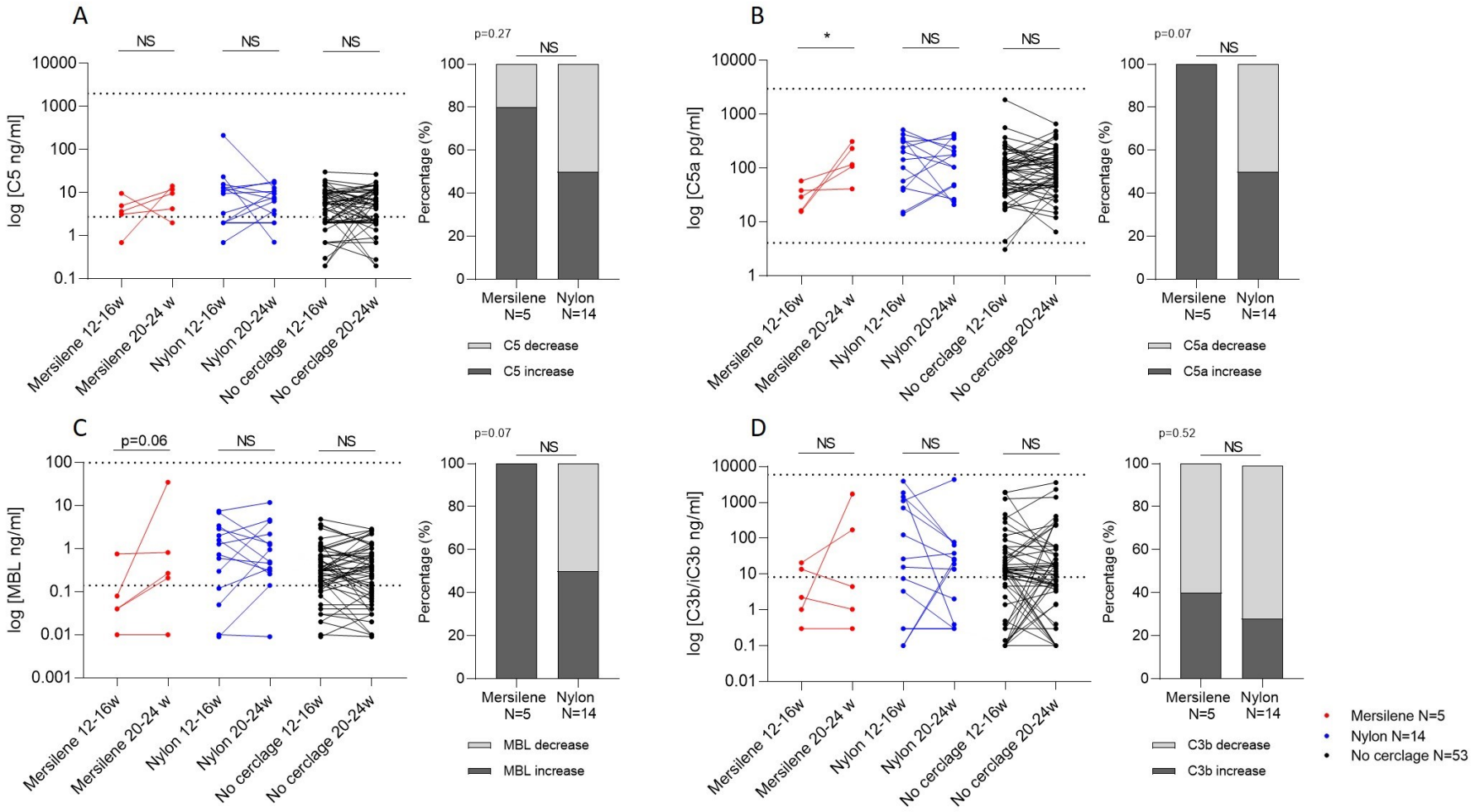


Figure 4.36 Early to mid-gestation cervico-vaginal fluid complement in women with term deliveries according to the insertion of a cervical cerclage
There was a significant increase in C5a and a non-significant increase in MBL in those who received a Mersilene cerclage. There was no change in C5, C5a, MBL or C3b in the women who had a Nylon cerclage or those who did not receive a cerclage between 12-16 weeks and 20-24 weeks gestation. Statistical analysis was by Wilcoxon matched-pairs signed rank test and Fisher's exact test, NS = non-significant. Mersilene cerclage N=5, nylon cerclage N=14 and no cerclage N=53.

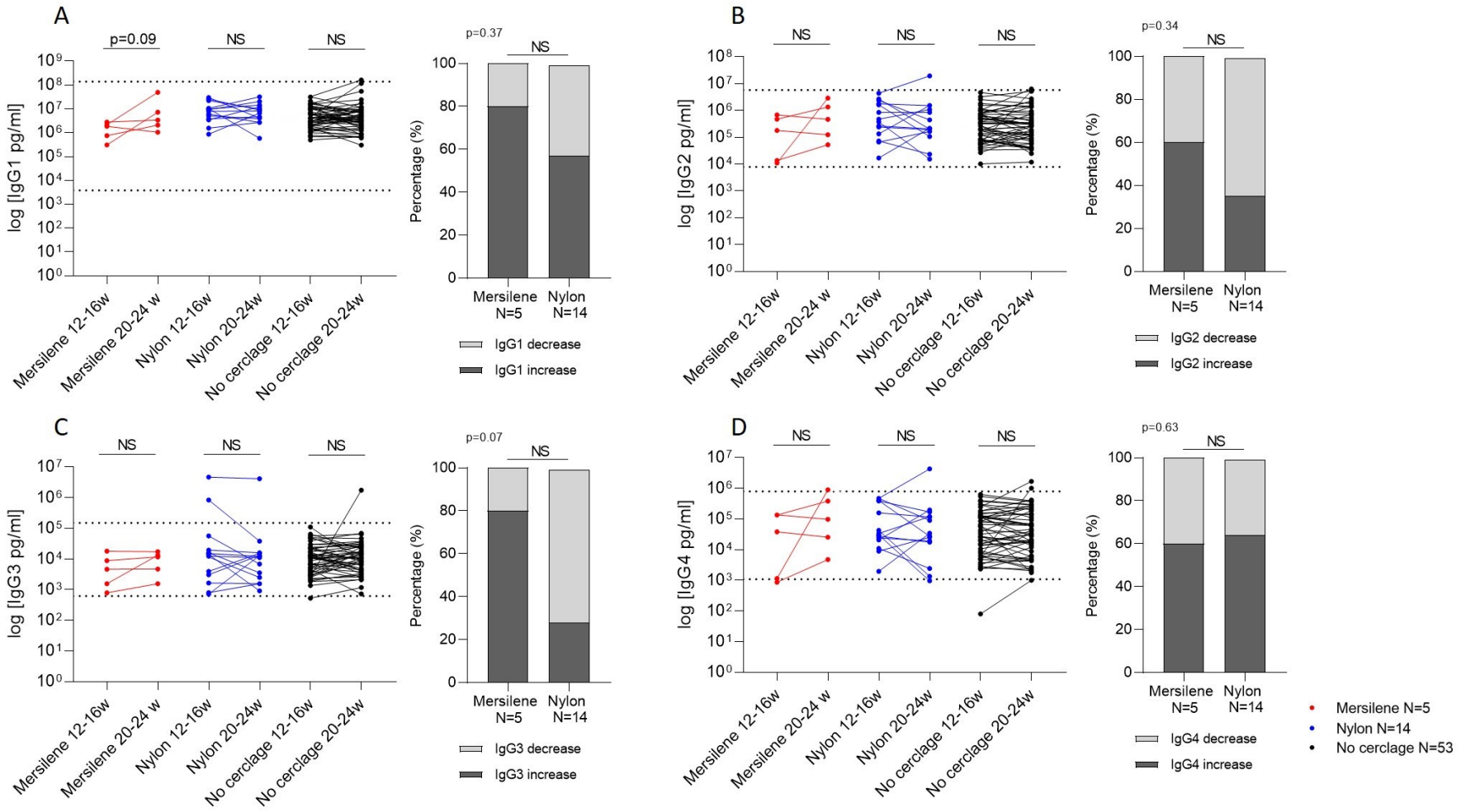


Figure 4.37 Early to mid-gestation cervico-vaginal fluid IgG1-4 in women with term deliveries according to the insertion of a cervical cerclage
There was no change in IgG1-4 between 12-16 weeks and 20-24 weeks gestation in women who had a Mersilene cerclage, Nylon cerclage or no cervical cerclage. Statistical analysis was by Wilcoxon matched-pairs signed rank test and Fisher's exact test, NS = non-significant. Mersilene cerclage N=5, nylon cerclage N=14 and no cerclage N=53.

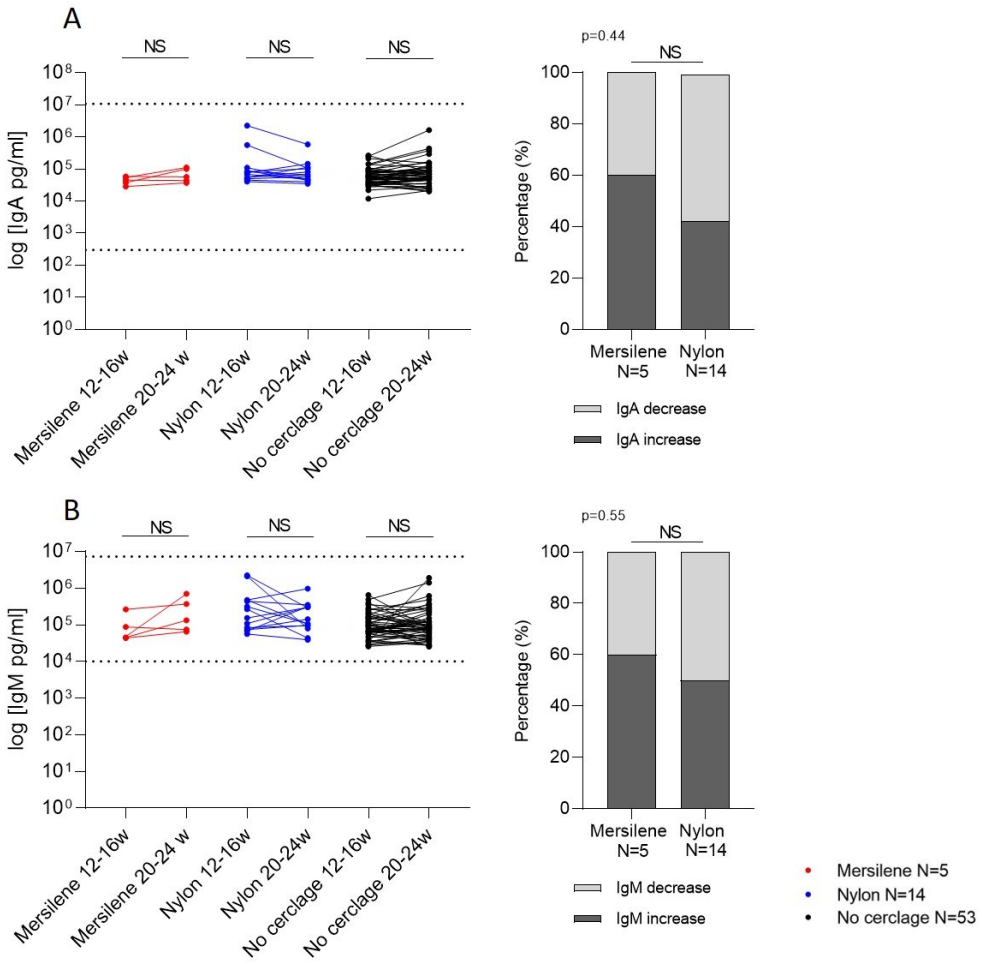


Figure 4.38 Early to mid-gestation cervico-vaginal fluid IgA and IgM in women with term deliveries according to the insertion of a cervical cerclage
There was no change in IgA or IgM between 12-16 weeks and 20-24 weeks gestation in women who had a Mersilene cerclage, Nylon cerclage or no cervical cerclage. Statistical analysis was by Wilcoxon matched-pairs signed rank test and Fisher's exact test, NS = non-significant. Mersilene cerclage N=5, nylon cerclage N=14 and no cerclage N=53.

Effect of progesterone

Progesterone can be prescribed following a cervical cerclage, but clinical practice can vary according to the clinician. In some study participants, it was administered vaginally as a pessary once a day. It can be used as an alternative treatment for cervical shortening to prevent preterm birth in those who choose not to have a cervical cerclage or where a cervical cerclage may be contraindicated.

In this study, of the 40 women who were sampled pre and post cervical cerclage, 21 women (52.5%) were prescribed progesterone following cervical cerclage, and the remaining 19 did not receive it (47.5%). For the women who received progesterone, 8 had a Mersilene cerclage (40%) and 12 had a Nylon cerclage (60%). For the women who did not receive progesterone, 6 (32%) had a Mersilene cerclage and 13 (68%) had a Nylon cerclage. The cerclage material was not recorded for one woman who received progesterone. There was no significant difference in the proportion of women who had a Mersilene or Nylon cerclage, who then received progesterone supplementation or not, Fisher's exact test $p=0.42$. This allowed the effect of progesterone on the local immune response to be assessed.

The mean fold change in local cytokines (IL-8, IL-1β, IL-10, IL-6, IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α and GM-CSF), Figure 4.39 A, complement (C5, C5a, MBL and C3b), Figure 4.39 B and immunoglobulins (IgG1-4, IgA and IgM), Figure 4.39 C were not significantly different in women who did or did not receive progesterone pessaries following the insertion of a cervical cerclage ($p>0.05$). The expected increase in anti-inflammatory IL-10 in women who received progesterone was not evident.

There was insufficient power to test if progesterone would mitigate the inflammatory response to the Mersilene cerclage, as only 8 women with a Mersilene cerclage received progesterone and 5 women with a Mersilene cerclage who did not receive progesterone.

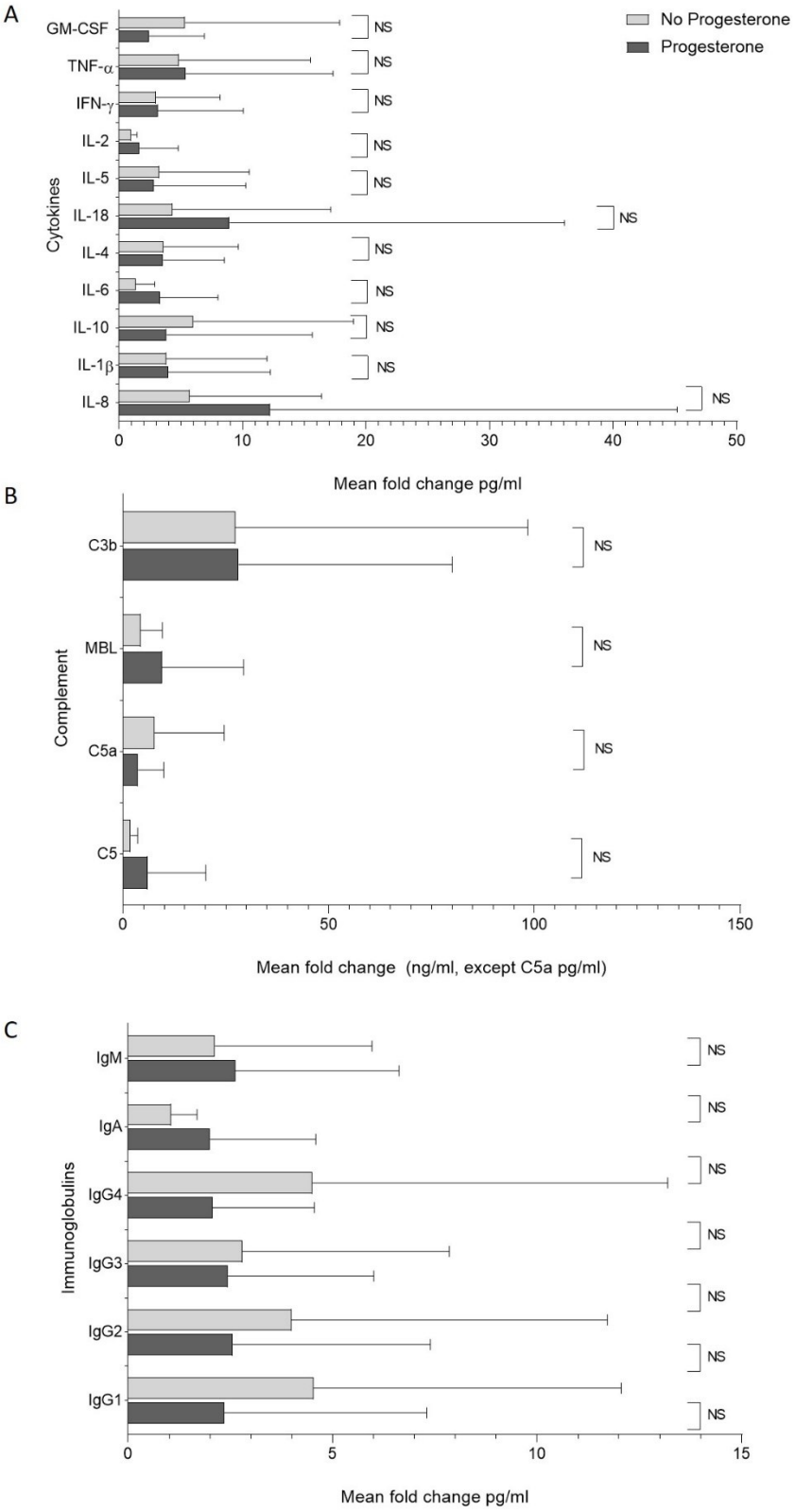


Figure 4.39 Local immune response to progesterone supplementation following cervical cerclage
There was no significant change in the concentrations of cytokines IL-8, IL-1β, IL-10, IL-6, IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α, GM-CSF; complement C5, C5a, MBL C3b; or immunoglobulins, IgG1-4, IgA, IgM in women who received and women who did not receive progesterone supplementation following cervical cerclage. Statistical analysis was by Mann-Whitney test. Progesterone supplementation N=19, No progesterone supplementation N=21.

4.6 Discussion

The key findings in this study are that in healthy term pregnancy, the local innate and adaptive immune responses as measured by cervico-vaginal fluid cytokines, complement and immunoglobulins were stable between 12^{+0} to 34^{+6} weeks. Women with a prior history of previous MTL and/or PTB showed a tendency for a primed immune response in early pregnancy compared to women with previous excisional cervical treatment. Cervical shortening and preterm delivery were independently associated with local immune activation. Although Nylon appears immunologically inert, Mersilene augmented the innate and adaptive immune response, and led to a dysregulated cascade of cytokine production and complement activation in women who subsequently delivered preterm.

The cervico-vaginal milieu in healthy pregnancy

This was a large cohort study evaluating mucosal immunity longitudinally during pregnancy. In healthy term pregnancy, without cervical shortening or cervical cerclage, the cytokine, complement and immunoglobulin profiles were stable across pregnancy. Blastocyst implantation is complete by the first sampling timepoint of 12^{+0}-16^{+6} weeks and labour did not commence for most of the women for another ten weeks explaining the absence of local inflammation. The stable mucosal immune response represents a good balance between appropriate immune modulation and a highly regulated immune system ready to manage any pathogenic insults, without collateral damage.

The cervico-vaginal milieu in women who deliver preterm

In contrast in women who delivered preterm, there was an increased local inflammatory response early to mid-gestation. Longitudinal profiling showed a gestational increase in IL-8, IL-1β, IL-6, IL-2, C5a, MBL, C3b, IgG1 and IgG3 between 12^{+0} - 16^{+6} and 20^{+0}-24^{+6} weeks. There appeared to be primed inflammatory immune response in women who deliver preterm with significantly higher cytokines, IL-8, IL-6, IL-2 and IFN-γ, complement C3b and MBL and IgM in cervico-vaginal fluid, compared to women who deliver at term.

Increased pro-inflammatory cervico-vaginal fluid cytokines have previously been shown to be associated with preterm delivery and chorioamnionitis, e.g IL-8 [348], IL-1β [349], IL-6 [350, 351] and TNF-α [348]. What is unique about this study is the ability to correlate local inflammation with cytokines, complement activation and immunoglobulins with cervical shortening and the background risk for preterm delivery in a longitudinally sampled cohort.

There is a basal level of complement activity to protect against pathogens and there are regulatory mechanisms in place to prevent uncontrolled complement activation. A dysregulated complement response either by excessive activation or insufficient regulation will overcome a pathogenic insult but can also cause tissue injury. Tissue injury itself can also stimulate complement activation. The mechanism driving the increasing complement activity early to mid-gestation in women who deliver preterm therefore could arise from a combination of pathogenic stimulation, tissue injury or internal immune dysregulation. It is plausible that the presence of a combination of these is sufficient to trigger preterm birth [352]. The early to mid-gestation increase in IgG1 and IgG3 in women who delivery preterm may again be related to increased complement activity as both are potent activators of the classical pathway.

The cervico-vaginal milieu depending on the risk factor for preterm birth

The primed inflammatory immune response in women who deliver preterm has been shown to have an association with the underlying risk factor for preterm birth. Mid-trimester loss is miscarriage that occurs between 14 and 23 weeks gestation. It has the same clinical manifestations as preterm birth, and similar underlying pathophysiological processes and some consider it to be a manifestation of extreme prematurity [14]. Grouping together the risk factors for previous preterm birth and previous mid-trimester miscarriage and comparing this to the other leading risk factor for preterm birth, excisional cervical treatment showed that independent of cervical shortening and cervical cerclage, these two groups of women have differing immune phenotypes, representing different aetiologies of preterm birth.

The increased local inflammation observed in women who had a history of previous MTL and/or PTB compared to previous excisional cervical treatment may relate to inflammation driven preterm birth in the former group, and mechanical cervical insufficiency driven preterm birth in the latter group. This study demonstrates that these two aetiologies have different immune manifestations. It is also plausible that there is a microbial influence on the local inflammatory response seen in women with previous MTL and/or PTB, which is explored in Chapter 5.

Increased IL-8, IL-1β and reduced IL-18 in those with previous MTL/PTB may point towards differences in the neutrophil: macrophage ratio in this extremely high-risk group. Studying local innate cell populations and their activation would from part of the next line of investigation.

The cervico-vaginal milieu in women who undergo cervical shortening

Supporting our existing knowledge concerning the pathways involved in cervical shortening, key cytokines IL-6 and IL-1β were found to be significantly increased in women with cervical shortening compared to those with normal cervical length [225]. This study was limited by the number of women who had cervical shortening but were sampled prior to the insertion of a cervical cerclage. This is a potentially why the increase in IL-8 was not significantly increased. IL-8 is a strong neutrophil attractant and C3b receptors are highly abundant on neutrophils and are involved in leukocyte adhesion. Therefore, an alternative explanation is that the absence of a significant increase in IL-8 and C3b in women with cervical shortening may be due to local inflammation driven by mediators from cervico-epithelial cells as opposed to an influx of neutrophils.

The novel finding is a role for complement activation in cervical shortening, with significantly increased C5, C5a and MBL concentrations in cervicovaginal fluid. Complement activation is typically known for its role in immune surveillance and inflammation, but in the context of cervical shortening, complement activation may be required for tissue repair [353]. Anaphylatoxins, C3a and C5a induce vasodilation and increase vascular permeability [354]. Receptors to C3a and C5a are not limited to immune cells, and have been detected on smooth muscle cells and endothelial cells [355, 356]. Gonzalez et al have described a possible role for complement activation in myometrial contractions in a murine study, with higher concentrations of C5a in the myometrium of mice with preterm labour when administered lipopolysaccharide [210]. A role for increased complement activity in cervical shortening has been described by Kim et al. C3a in amniotic fluid was found in significantly highly concentrations in women with subclinical intra-amniotic infection and cervical shortening [357].

IgG1 and IgM were significantly increased in women with cervical shortening, compared to those with normal cervical length. There was no significant difference with the other IgG subtypes, IgG2, IgG3 and IgG4. This is likely to represent differing effector functions of the immunoglobulins. As mentioned earlier IgG1, IgG3 as well as IgM are more potent activators of the classical complement pathway. IgG2 is the strongest responder to bacterial polysaccharides and IgG4 is the primary responder to allergens [188].

Inflammation can be cause or effect of cervical shortening. It poses the question if the local inflammation is microbial driven as cervical shortening provides an easier route for ascending bacteria from the vagina towards gestational tissues, or if activation of inflammatory pathways precedes cervical shortening. It was difficult to determine when local inflammation occurs relative to cervical

shortening in this study. To exclude the bias introduced by the insertion of a cervical cerclage, the number of paired samples before and at the point of cervical shortening was only nine. Therefore, there was insufficient power to demonstrate an inflammatory immune response as cervical shortening occurs.

The cervico-vaginal milieu in women following cervical cerclage

Without distinguishing between the type of cerclage material, the comparison of the local immune response pre and post cervical cerclage did not draw out any significant differences in cytokine, complement and immunoglobulin concentrations in cervico-vaginal fluid. One explanation is that the median number of days sampling occurred following cervical cerclage was 50 days, by which time the local inflammation that would likely have occurred as a result of the surgical intervention would likely be balanced by an immune tolerant response. However, when comparing the cerclage material, the braided Mersilene cerclage induced widespread innate and adaptive immune activation with significantly increased local cytokines, complement and immunoglobulin. This builds upon the findings reported by Kindinger et al regarding the local cytokine response that results following the insertion of a braided Mersilene cerclage [(49)].

Monsanto et al presented cervico-vaginal fluid cytokine concentrations in 28 women who underwent cervical cerclage for cervical insufficiency [(358)]. Compared to 19 gestation matched controls with normal cervical length, IL-1β, IL-6 and TNF-α were significantly higher in the women with cervical insufficiency before the insertion of a cervical cerclage. However, sampling one month post cerclage there was a reduction in all pro-inflammatory cytokines, and all cerclages were performed with a monofilament Prolene material which is similar to monofilament Nylon, supporting our findings that monofilament cerclage material is immunologically inert.

Evaluating the differences in Mersilene and Nylon cerclage in women who delivered preterm only, an augmentation in the innate rather than an adaptive immune response was observed. Mersilene cerclage resulted in increased local IL-1β, IL-8, IL-6, C5a, C3b and MBL, and increased local IgG1-4, IgA and IgM in some women but not all. This is plausible that this may be due to foreign material stimulating a non-specific innate immune response. In contrast, there was a tendency for the local immune mediators to decrease following Nylon cerclage.

The large majority of study participants with cervical shortening received a cervical cerclage, rather than progesterone treatment alone, therefore it was not possible to assess the local immune response

to progesterone treatment alone. Although this study can report that the known anti-inflammatory properties of progesterone following Mersilene cerclage does not sufficiently counteract the local inflammation resulting from Mersilene cerclage insertion. The results of the C-Stitch trial randomised controlled trial are highly anticipated, however findings here support the increasing evidence to recommend the use of Nylon suture in preference over Mersilene.

Study strengths and limitations

The key strength to this study is that the study population is very well characterised. The metadata obtained is very detailed and each study participants pregnancy journey is well described. Identifying the exact timeline of events to include the timing of cervical shortening and cervical cerclage allows immune phenotyping according to pathophysiological events, pregnancy interventions and environmental exposures.

Study recruitment was multicentered allowing for the recruitment of a diverse study population which confers a degree of generalisability to the study findings. A challenge to multicenter recruitment was ensuring the standardised procedure for sample collection and data recording was strictly followed, allowing for robust data analysis and correct comparisons. For example, strict exclusion criteria to not collect a cervico-vaginal fluid swab if there had been unprotected sexual intercourse or vaginal bleeding in the preceding 48 hours.

Using a combination of multiple Luminex® assay plates allowed the study of 20 cervico-vaginal fluid analytes from a single swab to form a comprehensive immune proteomic study. The separation of cervico-vaginal fluid into multiple tubes minimised the freeze/thaw cycles when performing the multiple assays at different times, which could have introduced bias by protein degradation.

There are many strengths to this study, but the findings need to be considered in the context of the study limitations. The practicality and costs involved in multiple timepoint sampling across pregnancy limited sampling to three timepoints during pregnancy. In the context of recruiting study participants from the preterm birth prevention clinic, for most women, their first study visit was from 12 weeks gestation onwards, and the first sampling timepoint covered 12^{+0} - 16^{+6} weeks gestation, and not at an earlier gestation. An advantage of recruiting women from all antenatal settings would mean sampling from an earlier timepoint and recruiting a truly low-risk study population. One-third of the study participants in the term cohort had previous MTL/PTB, therefore despite having an uncomplicated pregnancy, despite their normal pregnancy outcome at term, the local immune profile

may differ from a cohort with no significant past medical history or previous obstetric complications. It also seems appropriate at this time to recall that only 10% of women who deliver preterm have a prior history of preterm birth [359].

In order to characterise when local inflammation occurs relative to cervical shortening, the sampling timepoints would need to be individualised for each study participant. Ideally sample collection should take place before cervical shortening and at the point of cervical shortening, before a cervical cerclage is inserted.

Concluding remarks

Women with a prior history of previous MTL and/or PTB, and those who are destined to deliver preterm appear to have a primed local inflammatory immune response, compared to women with previous excisional cervical treatment. Later in pregnancy, the local immune response can be further potentiated by cervical shortening and the insertion of a Mersilene cervical cerclage, whilst a Nylon cerclage appears to be immunologically inert. The preterm birth rate in this study was higher in those who received a Mersilene cerclage. The additive effect of previous MTL and/or PTB, cervical shortening and Mersilene cerclage insertion resulting in excessive local inflammation may together be potent drivers for preterm birth. How these inflammatory responses are influenced by vaginal microbial communities will be explored in the next chapter.

CHAPTER 5: MICROBIAL-HOST IMMUNE RESPONSES IN HEALTHY PREGNANCY AND IN WOMEN WHO DELIVER PRETERM

5.1 Chapter summary

Hypothesis

- The local host immune milieu is influenced by the vaginal microbial composition.
- Dysregulated microbial driven immune activation leads to spontaneous preterm birth.

Aims

- To describe the vaginal microbial composition in the study population.
- To describe the local immune milieu in relation to the vaginal microbial composition.
- To describe the vaginal microbial composition and immune milieu according to the risk factor for preterm delivery.
- To describe the vaginal microbial composition and immune milieu in relation to cervical shortening.
- To describe the vaginal microbial composition following cervical cerclage.
- To describe the local immune response to vaginal microbial composition in women who deliver preterm and at term.
- To correlate the peripheral immune response with the vaginal microbial composition.

Methods

As described in Chapter 4, cervico-vaginal fluid (CVF) was sampled by a swab of the posterior fornix of the cervix using a Liquid Amies swab (BBL™ CultureSwab™, Becton, Dickinson and Company) from study participants at three timepoints during pregnancy: 12^{+0} - 16^{+6}, 20^{+0} - 24^{+6} and 30^{+0} - 34^{+6} weeks. Following delivery, study participants were divided into 3 pregnancy outcome groups: preterm pregnancy, term pregnancy without intervention, and term pregnancy with intervention (cervical cerclage and/or progesterone). Bacterial DNA was extracted from vaginal swabs and next generation sequencing of the 16S rRNA gene amplicons was performed to assess the vaginal microbial composition. This was correlated with the results of CVF cytokines, complement and immunoglobulins from the same samples presented in Chapter 4. Cross-sectional analysis of matched PBMC and plasma samples with vaginal microbial composition are also presented. Statistical differences between groups were analysed using the t-test or one-way ANOVA test if data was parametric, or Mann Whitney U test or Kruskal-Wallis test for non-parametric data, with Dunnett's or Dunn's multiple comparisons test. Statistical correlations were performed using Spearman rank correlation. For all tests, the level of statistical significance was taken as a p value ≤0.5.

Results

The vaginal microbial composition of the study population was categorised in two ways, at genera level into *Lactobacillus spp.* dominant and *Lactobacillus spp*. deplete, and at species level into six vaginal microbial groups, VMG 1 *L. crispatus*, VMG 2 *L. gasseri*, VMG 3 *L. iners*, VMG 4 Diverse species, VMG 5 *L. jensenii* and VMG 6 *Bifidobacterium* species. Independent of the gestation of sampling, *Lactobacillus* depletion was associated with a local pro-inflammatory signature compared to *Lactobacillus* dominance. VMG 3 *L. iners* was associated with significantly higher concentrations of local IL-8, IL1-β, IgG2, IgG3, IgG4 and IgM ($p<0.05$) compared to VMG 1 *L. crispatus*. VMG 4 Diverse species was associated with significantly higher concentrations of local IL-8, IL-1β, IL-6, C5, MBL, C3b, IgG1-4 and IgM ($p<0.05$) compared to VMG 1 *L. crispatus*.

VMG 3 *L. iners* in early pregnancy was associated with cervical shortening. In the presence of VMG 3 *L. iners*, IgM, C3b, C5, and C5a were significantly higher in women with cervical shortening, compared to those with normal cervical length ($p<0.05$). This resulted in an exaggerated local cytokine response with significantly higher concentrations of IL-8, IL-1β and IL-6 ($p<0.05$).

Preterm birth in the presence of VMG 3 *L. iners* and VMG 4 Diverse species was associated with increased microbial recognition by IgM and MBL, leading to dysregulated activation of the complement cascade via the classical and lectin pathways, which resulted in amplified cytokine production through chemotaxis of local immune cells. In contrast, preterm birth in the presence of VMG 1 *L. crispatus* was not associated with activation of the innate or adaptive immune response, suggesting a non-microbial immune mediated mechanism for preterm birth.

Conclusions

Microbial-driven inflammation involves a complex interplay between the innate and adaptive immune response, but if regulated, results in term delivery. Microbial-driven dysregulated complement activation leads to local immune cell infiltration and cytokine production and preterm birth.

5.2 Introduction

The leading cause of spontaneous preterm birth is infection and inflammation. Ascending bacteria from the cervix to gestational tissues is widely believed to be the pathophysiological mechanism by which this occurs [5]. Healthy pregnancy is associated with stable low diversity, *Lactobacillus* dominant vaginal microbiota [145, 146]. The vaginal microbiota is influenced by multiple factors including ethnicity, smoking status and hygiene practices, for example douching. Women of African descent are more likely to exhibit increased vaginal microbial diversity [145, 151, 360]. Independent of ethnicity, *Lactobacillus crispatus* has consistently been found to be protective against preterm delivery, whilst *Lactobacillus iners*, and bacteria associated with bacterial vaginosis, *Prevotella, Snethia, and Gardnerella* have been found in higher abundance in women who deliver preterm [147, 155-158].

Local inflammation is increased in *Lactobacillus* depletion [156, 244] but how this correlates with pregnancy outcome, term versus preterm delivery is not well understood. The host immune-microbial interactions are highly complex and may be population specific. Our knowledge so far concerning mucosal immunity and the microbiome is largely in the context of HIV infection. A dysbiotic vaginal microbial environment confers a greater susceptibility to HIV infection, with an augmented local immune response [243, 244]. Understanding host-microbial signatures characteristic of preterm and term birth will develop our knowledge of infection induced preterm birth and there is the potential to find population specific therapeutic targets.

In Chapter 4, I described local inflammation involving innate and adaptive pathways in mid-gestation in preterm delivery, in association with previous mid-trimester loss/previous preterm birth, cervical shortening and the insertion of a braided Mersilene cervical cerclage. This Chapter explores if there is a microbial driven element to local host inflammation.

Matched plasma cytokine, plasma complement, and peripheral blood mononuclear cell phenotyping was performed for a cross-section of the study population to also study the effect of the vaginal microbial composition on the host peripheral immune response.

5.3 Study design

To study the local cervico-vaginal immune changes and the vaginal microbiota in women with preterm and term pregnancies, women with risk factors for preterm birth were prospectively recruited from the preterm prevention clinics at Queen Charlotte's and Chelsea Hospital, St Mary's Hospital, Chelsea and Westminster Hospital, University College Hospital and Royal Infirmary of Edinburgh. Exclusion criteria were multiple pregnancies, women who had sexual activity within 72 hours of sampling, vaginal bleeding and women who were HIV or hepatitis B or C positive.

Women were followed up longitudinally through pregnancy. Swabs were taken to sample the cervico-vaginal fluid (CVF) in the posterior fornix at three timepoints, 12^{+0} - 16^{+6} weeks, 20^{+0} - 24^{+6} weeks and 30^{+0} - 34^{+6} weeks. The cervical length was obtained by a transvaginal ultrasound at the same timepoints on the same day as CVF sampling. Detailed metadata was collected from all study participants from the hospital notes and the electronic patient database, Cerner Millennium Powerchart®.

Bacterial DNA was extracted from the vaginal swabs and 16S rRNA gene sequencing was performed on the V1-V2 hypervariable regions. Supernatant from the same vaginal swab were used to measure CVF cytokines, complement and immunoglobulins by magnetic Luminex® assays as described in Chapter 4.

5.4 Statistical analysis

Examination of statistical differences between vaginal microbiota was performed at bacterial genera and species levels using the Statistical Analysis of Metagenomic Profiles software package (STAMP). Hierarchical clustering analysis using Ward linkage of species sequence data was used with a clustering density threshold of 0.75 with the 20 most abundant bacterial species displayed. Samples were classified into 6 vaginal microbial groups (VMG) according to Ward hierarchical clustering. Measures of microbial diversity: inverse Simpson index, Shannon index and richness (species observed) were calculated with Mothur and R using the Vegan package.

Statistical analysis was performed using Graphpad Prism 8.4.1. Differences between two groups were analysed using the t-test, or the Mann Whitney test depending upon the distribution of the data. Differences between three groups were analysed using the one-way ANOVA or the Kruskal-Wallis test depending on the distribution of the data. The appropriate post hoc comparisons test was used, Dunnett's or Dunn's. The Fisher's exact or Chi square test were used to test for proportional differences. Spearman correlation was used to correlate different CVF analytes. For all tests, the level of statistical significance was taken as a p value ≤ 0.5.

5.5 Results

5.5.1 Study population characteristics

The same cohort of 133 women from Chapter 4 are studied in this chapter. Their clinical and demographical characteristics are described in Chapter 4, Table 4.1. As per the previous chapter, the study participants were divided into 3 groups, women who had a preterm delivery before 37 weeks gestation N=37 (28%), women who delivered at term not requiring any intervention to prevent preterm birth N=56 (42%), and the third group were women who delivered at term but required an intervention to prevent preterm birth (cervical cerclage +/- progesterone) N=40 (30%).

5.5.2 Vaginal microbial composition, diversity and richness in the study population

385 vaginal swabs were analysed providing 15,597,988 high quality reads with an average read count of 29,209 per sample. After removing rare operational taxonomic units (OTUs) (<10 reads per sample), 235 taxa were identified in the samples. Read counts were subsampled down to 1333 which was the lowest read count obtained by sample. The coverage was 98%, demonstrating that subsampling did not alter the composition data. 385 vaginal swabs were extracted and sequenced on three runs at different time points during the study. This reflected the time taken to recruit study participants and follow them throughout their pregnancies. The laboratory work to extract DNA from the vaginal swab samples occurred in batches during the period of study recruitment and follow up. Run to run variation was accounted for by sequencing eight samples on both run 1 and 2, five samples on run 2 and 3 and five samples on run 1 and 3. Bacterial species abundance for paired samples were very similar between samples analysed across different runs, Figure 5.1, and the coefficient of variance (CV) approached 1 for all paired samples.

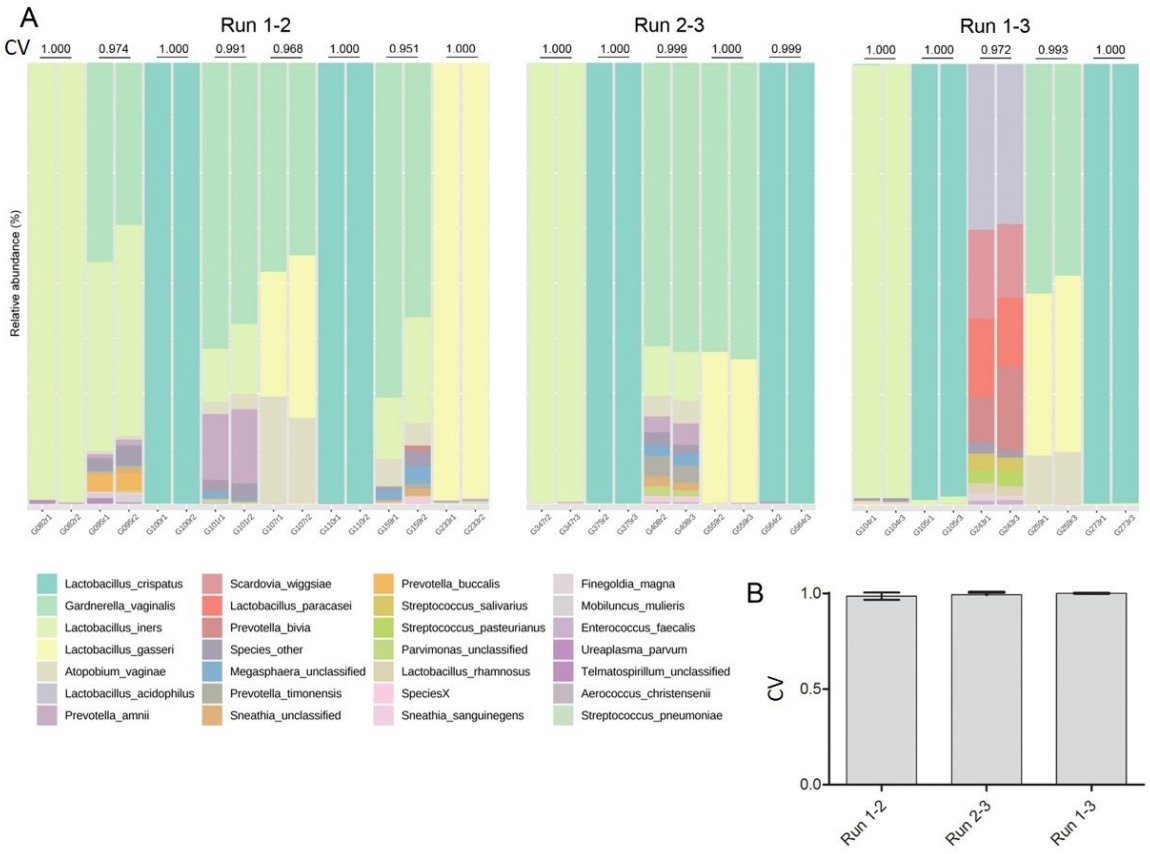


Figure 5.1 Accounting for variability between vaginal swab samples analysed on 3 separate 16S rRNA sequencing runs
Eight samples were analysed on run 1 and 2, five samples were analysed on run 2 and 3 and five samples were analysed on run 1 and 3. Bacterial species abundance for paired samples are compared (A). The co-efficient of variation (CV) approached 1 when comparing between runs (B), indicating that there was negligible difference across the three sequencing runs.

There were 385 vaginal swabs sampling the cervicovaginal fluid in 133 women longitudinally through pregnancy at three timepoints, 12^{+0} - 16^{+6} weeks, 20^{+0} - 24^{+6} weeks and 30^{+0} - 34^{+6} weeks. 16S rRNA sequencing was performed to species taxonomic level. At genera level, *Lactobacillus* dominant samples accounted for 76% of all samples (>75% *Lactobacillus* species), with the remaining 24% classified as *Lactobacillus* deplete (<75% *Lactobacillus* species), Figure 5.3 A. Using Ward hierarchical clustering analysis of 16S rRNA sequencing data, samples were classified into vaginal microbial groups (VMG): VMG 1 *Lactobacillus crispatus*, VMG 2 *Lactobacillus gasseri*, VMG 3 *Lactobacillus iners*, VMG 4 Diverse species, VMG 5 *Lactobacillus jensenii* and VMG 6 *Bifidobacterium* species, Figure 5.2. VMG 4 consisted largely of bacteria typical of bacterial vaginosis including *Gardnerella vaginalis*, *Atopobium vaginae* and *Prevotella* species and was deplete of *Lactobacillus* species. The most common VMG was VMG 1 *L. crispatus* (N=158, 41%), followed by VMG 2 *L. gasseri* (N=40, 10%), VMG 3 L. *iners* (N=99, 26%), VMG 4 Diverse species (N=48, 15%), VMG 5 *L. jenseii* (N=16, 4%) and lastly VMG 6 *Bifidobacterium* species (N=14, 4%), Figure 5.3B.

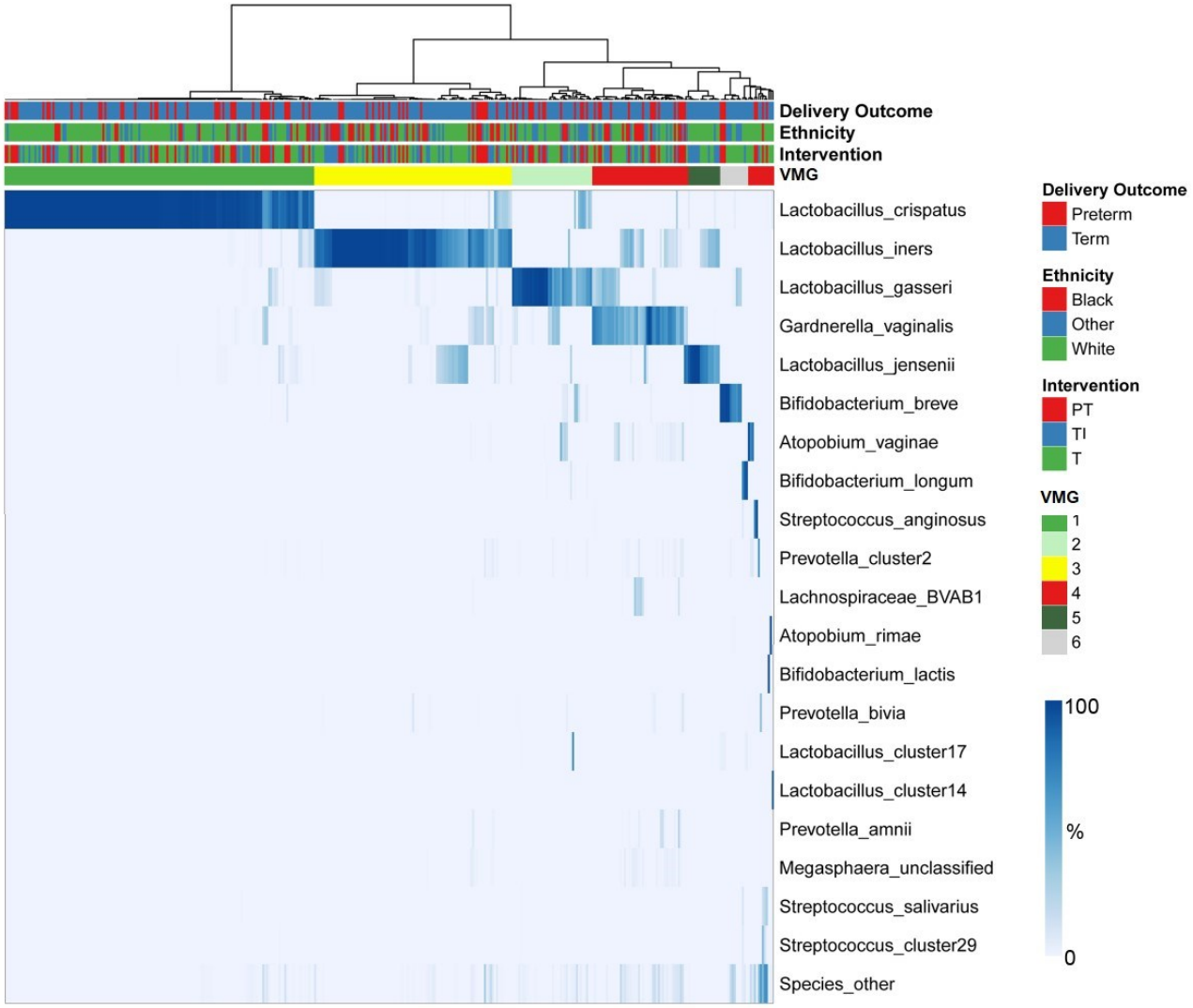


Figure 5.2 Bacterial species diversity of 385 vaginal swab samples

Ward hierarchical clustering analysis of the species sequence data identified 6 major clusters of samples which were classified into 6 vaginal microbial groups (VMG), VMG 1 (*L. crispatus* dominant), VMG 2 (*L. gasseri* dominant), VMG 3 (*L. iners* dominant), VMG 4 (Diverse species), VMG 5 (*L. jensenii* dominant) and VMG 6 (*Bifidobacterium* species). PT=preterm, T=term, TI=term with intervention.

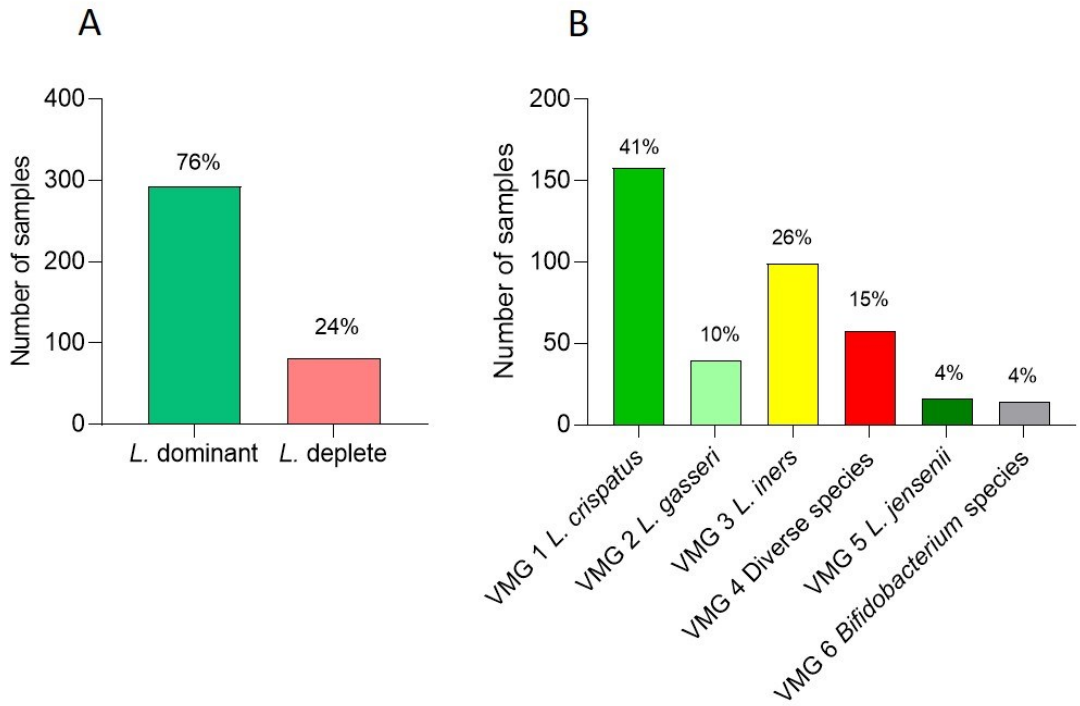


Figure 5.3 The frequency of *Lactobacillus* dominant or *Lactobacillus* deplete samples and assigned vaginal microbial groups as classified by Ward hierarchical clustering analysis and in 385 samples from 133 women in pregnancy

Lactobacillus dominant samples accounted for 76% of all samples (>75% *Lactobacillus* species), with the remaining 24% classified as *Lactobacillus* deplete (<75% *Lactobacillus* species) (A). In descending frequency, VMG 1 *L. crispatus* dominant accounted for the most samples (41%), followed by VMG 3 *L. iners* dominant (26%), VMG 4 Diverse species (15%), VMG 2 *L. gasseri* dominant (10%), VMG 5 *L. jensenii* dominant (4%) and lastly VMG 6 *Bifidobacterium* species (4%).

Lactobacillus deplete samples had significantly higher alpha diversity as measured by the mean inverse Simpson index and significantly higher richness as measured by the species observed compared to *Lactobacillus* dominant samples, Figure 5.4 A and B. Vaginal samples from VMG 1 *L. crispatus* showed the lowest diversity and richness, whilst the opposite was true for VMG 4 Diverse species, Table 5.1 and Figure 5.4 C and D. For the four most common VMGs, from low to high diversity, the vaginal microbial groups were, VMG 1 *L. crispatus*, VMG 3 *L. iners*, VMG 2 *L. gasseri*, and the most diverse was VMG 4 Diverse species.

The alpha diversity as measured by the inverse Simpson index was significantly higher in VMG 2 *L. gasseri*, VMG 3 *L. iners* and VMG 4 Diverse species compared to VMG 1 *L. crispatus* ($p<0.05$). The inverse Simpson index was significantly higher in VMG 4 Diverse species compared to both VMG 2 *L. gasseri* and VMG 3 *L. iners* ($p<0.01$). The richness was significantly higher in VMG 4 Diverse species compared to VMG 1 *L. crispatus*, VMG 2 *L. gasseri* and VMG 3 *L. iners* ($p<0.05$). Additionally, the richness was significantly higher in VMG 2 compared to VMG 1 ($p<0.05$). The average abundance of *Lactobacillus spp.* was lowest in VMG 4 Diverse species (19%) and VMG 6 *Bifidobacterium* species (8%).

There were 3 samples from the same individual who by the Ward hierarchical clustering algorithm were classified as VMG 1 *L. crispatus* but the abundance of Lactobacillus *spp.* was 61%, 61% and 60%, respectively at the three sampling timepoints.

Table 5.1 Bacterial diversity, richness and *Lactobacillus spp.* abundance for vaginal microbial groups

VMG	No. of samples	Sobs	Inverse Simpson Index	Shannon Index	Dominant *Lactobacillus spp.*	Average abundance of *Lactobacillus spp.* (%)
1	158	7 (1-62)	1.11 (1-2.4)	0.17 (0-1.58)	*L. crispatus*	98 (60-100)
2	40	12 (2-41)	1.6 (1-2.2)	0.6 (0.02-1.52)	*L. gasseri*	85 (47-99)
3	99	9 (1-57)	1.4 (1-3.4)	0.44 (0-1.99)	*L. iners*	95 (53-110)
4	58	17 (1-55)	2.4 (1-8.1)	1.03 (0-2.5)	NA	19 (0-54)
5	16	8 (4-22)	1.5 (1-2.3)	0.49 (0.03-0.97)	*L. jensenii*	99 (96-100)
6	14	11 (3-27)	1.7 (1-3.5)	0.6 (0.08-1.5)	NA	8 (0-35)

Sobs, Species Observed. Mean (range)

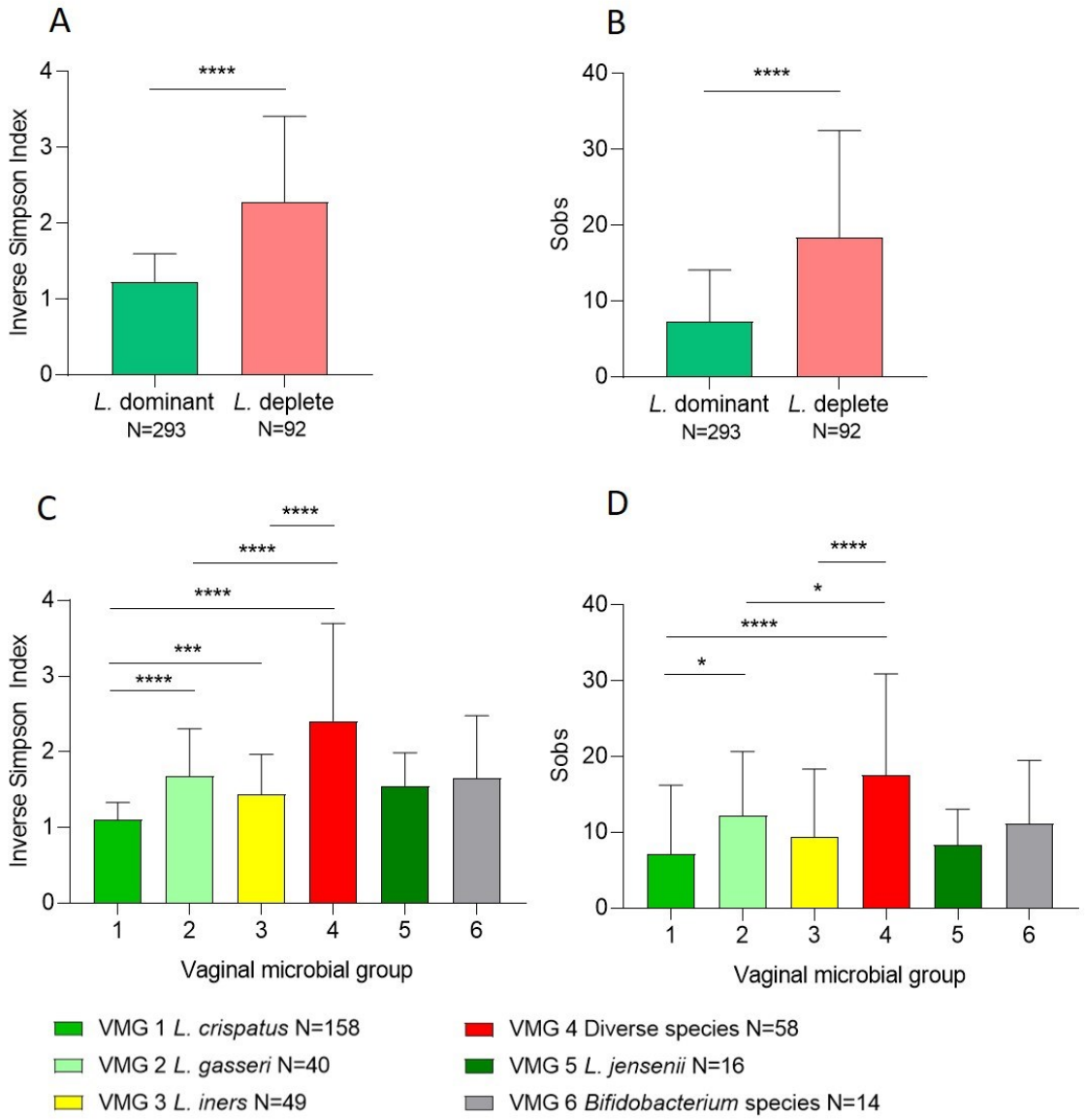


Figure 5.4 The diversity and species richness by vaginal microbial composition
Samples classified as *Lactobacillus* deplete displayed higher diversity as measured by the inverse Simpson index (A), and greater richness as measured by the species observed compared to *Lactobacillus* dominant samples (B). Samples classified as VMG 1 *L. crispatus* displayed the lowest alpha diversity, whilst VMG 4 Diverse species showed the highest diversity (C). The alpha diversity of VMG 4 was significantly greater than VMG 1, 2 and 3. Additionally the diversity of VMG 2 and 3 were significantly greater than VMG 1. VMG 4 Diverse species had the greatest richness as measured by the number of species observed (D). The richness of VMG 4 was significantly greater than VMG 1, VMG 2 and VMG 3. Statistical analysis was by Mann-Whitney U test, one-way ANOVA and Tukey's multiple comparisons test. (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001). The results are expressed as mean and one standard deviation. VMG 1 N=158, VMG 2 N=40, VMG 3 N=49, VMG 4 N=58, VMG 5 N=16 and V<G 6 N=14.

There were 385 samples collected in total. Where possible women were sampled at three timepoints during their pregnancy, 12^{+0} - 16^{+6} weeks, 20^{+0} - 24^{+6} weeks and 30^{+0} - 34^{+6} weeks. 126 samples were collected between 12^{+0} - 16^{+6} weeks, 133 samples were collected between 20^{+0} - 24^{+6} weeks and 126 samples were collected between 30^{+0} - 34^{+6} weeks.

5.5.3 Cervico-vaginal immune response and the vaginal microbial composition

The local cervico-vaginal immune response as measured by cytokines, complement and immunoglobulins in cervico-vaginal fluid were correlated with vaginal microbial diversity and richness as defined by the inverse Shannon index and the species observed. There was a significant positive correlation between vaginal microbial diversity and IL-8, IL-1β, C5, C5a, MBL, C3b, IgG1-4, IgA and IgM ($p<0.01$). IL-10 correlated negatively with vaginal microbial diversity, supporting its anti-inflammatory activity ($p<0.01$). There was a significant positive correlation between richness and IL-8, IL-1β, IL4, MBL, C3b, IgG2-4, IgA and IgM ($p<0.05$), (Table 5.2).

Table 5.2 Correlation between the cervico-vaginal immune response and vaginal microbial diversity and richness
Positive correlations with statistical significance are coloured in pink. Negative correlations with statistical significance are coloured in blue.

	Inverse Simpson		Sobs	
Analyte	Spearman r	p	Spearman r	p
Cytokines				
IL-8	0.16	<0.01 **	0.13	<0.01 **
IL-1β	0.38	<0.0001 ****	0.32	<0.0001 ****
IL-10	-0.14	<0.01 **	-0.06	0.27
IL-6	0.04	0.41	0.04	0.43
IL-4	0.07	0.16	0.10	<0.05 *
IL-18	-0.008	0.87	0.0004	0.99
IL-5	-0.02	0.68	0.008	0.87
IL-2	0.007	0.90	0.03	0.50
IFN-γ	-0.04	0.45	0.01	0.78
TNF-α	0.008	0.88	0.03	0.57
GM-CSF	-0.01	0.78	-0.03	0.56
Complement				
C5	0.14	<0.01 **	0.07	0.14
C5a	0.06	0.21	0.07	0.16
MBL	0.15	<0.01 **	0.12	<0.05 *
C3b	0.32	<0.0001 ****	0.26	<0.0001 ****
Immunoglobulins				
IgG1	0.20	<0.0001 ****	0.02	0.72
IgG2	0.31	<0.0001 ****	0.24	<0.0001 ****
IgG3	0.34	<0.0001 ****	0.32	<0.0001 ****
IgG4	0.24	<0.0001 ****	0.19	<0.0001 ****
IgA	0.07	0.16	0.07	0.19
IgM	0.28	<0.0001 ****	0.23	<0.0001 ****

Sobs = species observed.

Vaginal microbial composition and the local immune milieu at genera level

The association between the cervico-vaginal local immune response and vaginal composition was explored, firstly at genera level. Of the total 385 samples, 293 (76%) were *Lactobacillus* dominant and 92 (24%) were *Lactobacillus* deplete N=92. The classification into the two groups was based upon the percentage of *Lactobacillus* species in the sample: in *Lactobacillus* dominant samples, more than 75% species were *Lactobacillus*, and in *Lactobacillus* deplete samples, less than 75% species were *Lactobacillus*.

The four most detectable cytokines in cervico-vaginal fluid were IL-8, IL-6, IL-1β and IL-10. Independent of gestation, *Lactobacillus* depletion was associated was significantly higher concentrations of IL-8, IL-1β, IL-6 (p=0.04, p<0.0001 and p=0.0009 respectively), Figure 5.5 A-C, and significantly lower concentrations of IL-10 compared to *Lactobacillus* dominance (p=0.01), Figure 5.5D.

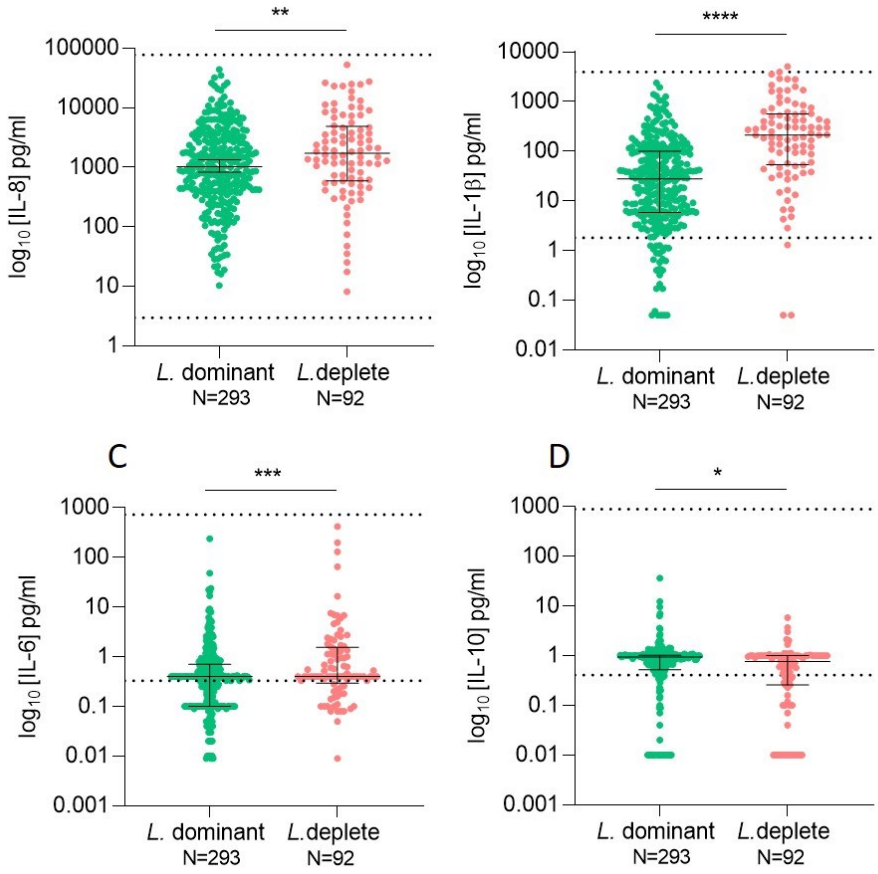


Figure 5.5 Cervico-vaginal cytokines, IL-8, IL-1β, IL-6 and IL-10 according to genera microbial classification, *Lactobacillus* dominance and *Lactobacillus* depletion, in all samples

Lactobacillus depletion was associated with significantly increased IL-8, IL-1β and IL-6 (A-C). IL-10 expression was increased in *Lactobacillus* dominant samples (D). Statistical analysis was by Mann Whitney U test, (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Abbreviations: *L.* dom = *Lactobacillus* dominant, *L.* deplete = *Lactobacillus* deplete. Dotted lines mark the concentrations of the highest and lowest standard on the assay. The results are expressed as median and interquartile range. *L.* dominant N=294 and *L.* deplete N=92.

Independent of the gestation of sampling, concentrations of the less abundant cytokines: IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α, GM-CSF in cervico-vaginal fluid were similar in *Lactobacillus* dominant and *Lactobacillus* deplete samples, (p>0.05) Figure 5.6.

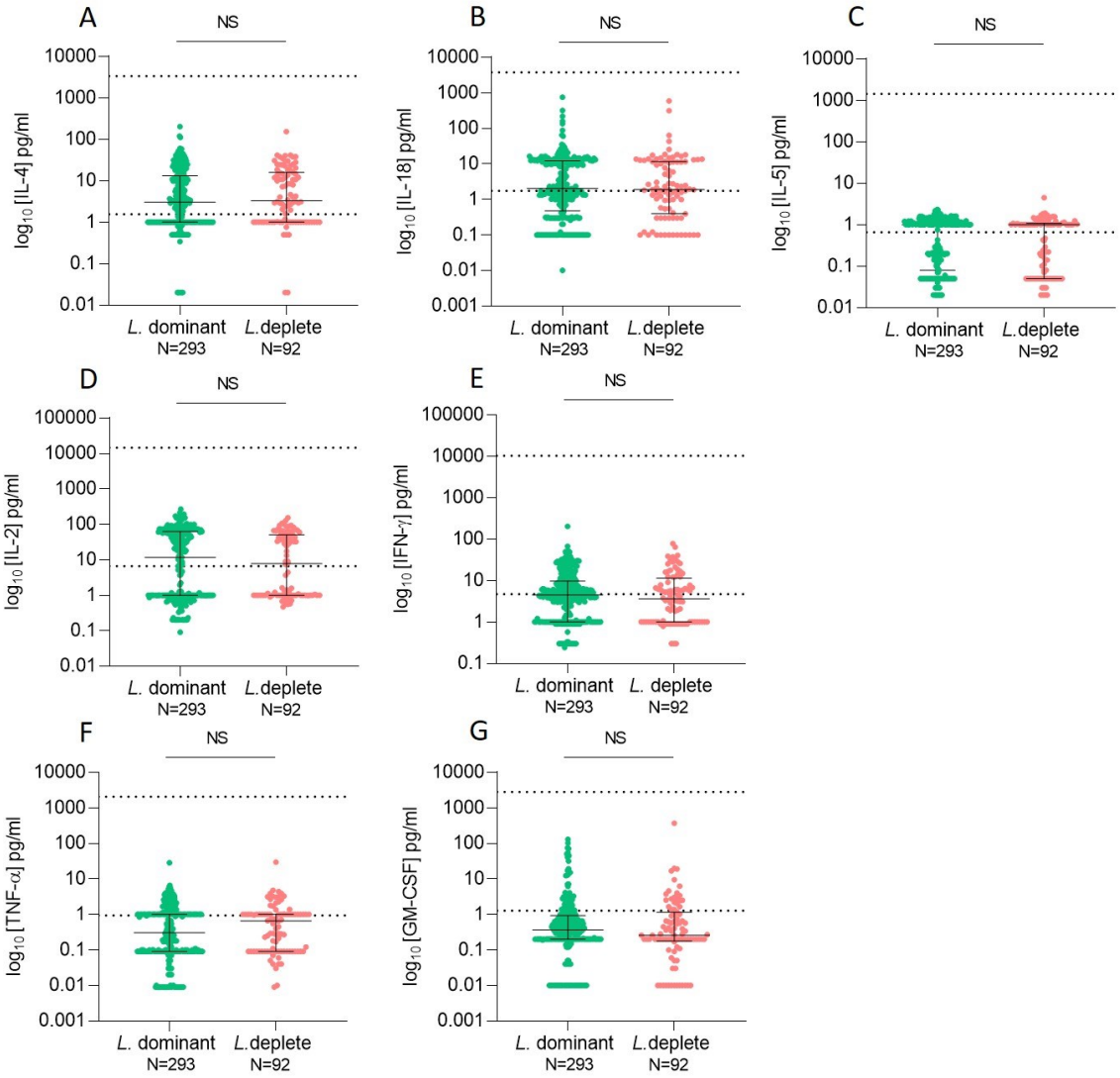


Figure 5.6 Cervico-vaginal cytokines according to genera microbial classification, *Lactobacillus* dominance and *Lactobacillus* depletion in all samples

There was no significant difference in IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α or GM-CSF concentrations in *Lactobacillus* dominant and *Lactobacillus* deplete samples. Statistical analysis was by Mann Whitney U test. Abbreviations: *L.* dom = *Lactobacillus* dominant, *L.* deplete = *Lactobacillus* deplete, NS = non-significant. Dotted lines mark the concentrations of the highest and lowest standard on the assay. The results are expressed as median and interquartile range. *L.* dominant N=294 and *L.* deplete N=92.

Grouping samples collected at all sampling timepoints, cervico-vaginal fluid C5, MBL and C3b concentrations were significantly higher in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples, (p=0.001, p<0.0001 and p<0.0001 respectively), Figure 5.7 A, C and D. There was a non-significant increase in C5a in *Lactobacillus* deplete samples (p=0.07), Figure 5.7 B.

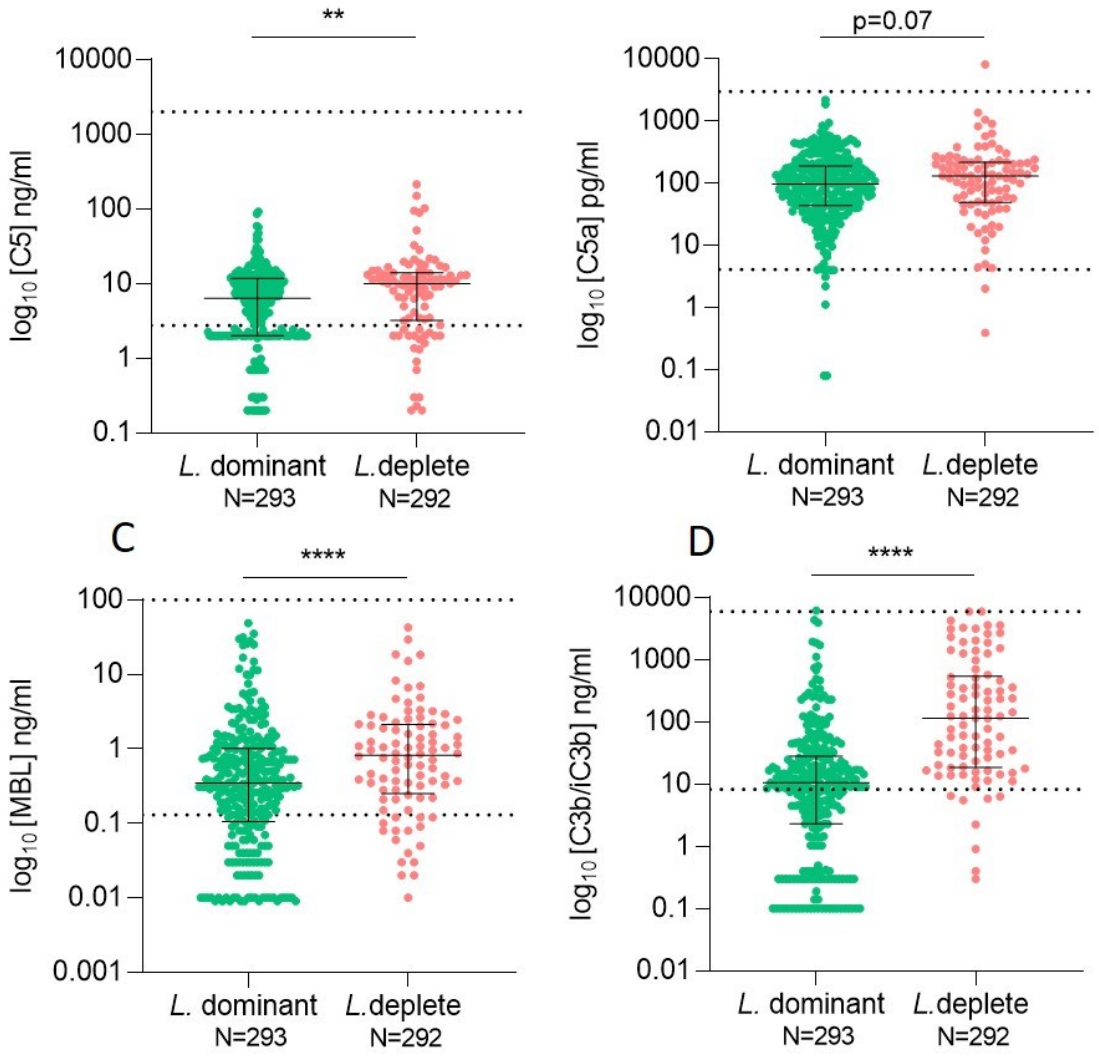


Figure 5.7 Cervico-vaginal complement according to genera microbial classification, *Lactobacillus* dominance and *Lactobacillus* depletion in all samples

Lactobacillus depletion was associated with significantly increased C5, MBL and C3b expression (A, C and D). There was non-significant increase in C5a expression according in *Lactobacillus* deplete samples (B). Statistical analysis was by Mann Whitney U test, (** p<0.01, **** p<0.0001). Abbreviations: *L.* dom = *Lactobacillus* dominant, *L.* deplete = *Lactobacillus* deplete). Dotted lines mark the concentrations of the highest and lowest standard on the assay. The results are expressed as median and interquartile range. *L.* dominant N=294 and *L.* deplete N=92.

All immunoglobulins, IgG1, IgG2, IgG3, IgG4, IgA and IgM were detected in significantly higher concentrations in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples, ($p<0.001$, $p<0.0001$, $p<0.0001$, $p<0.0001$, $p<0.05$ and $p<0.0001$ respectively), Figure 5.8 A-F.

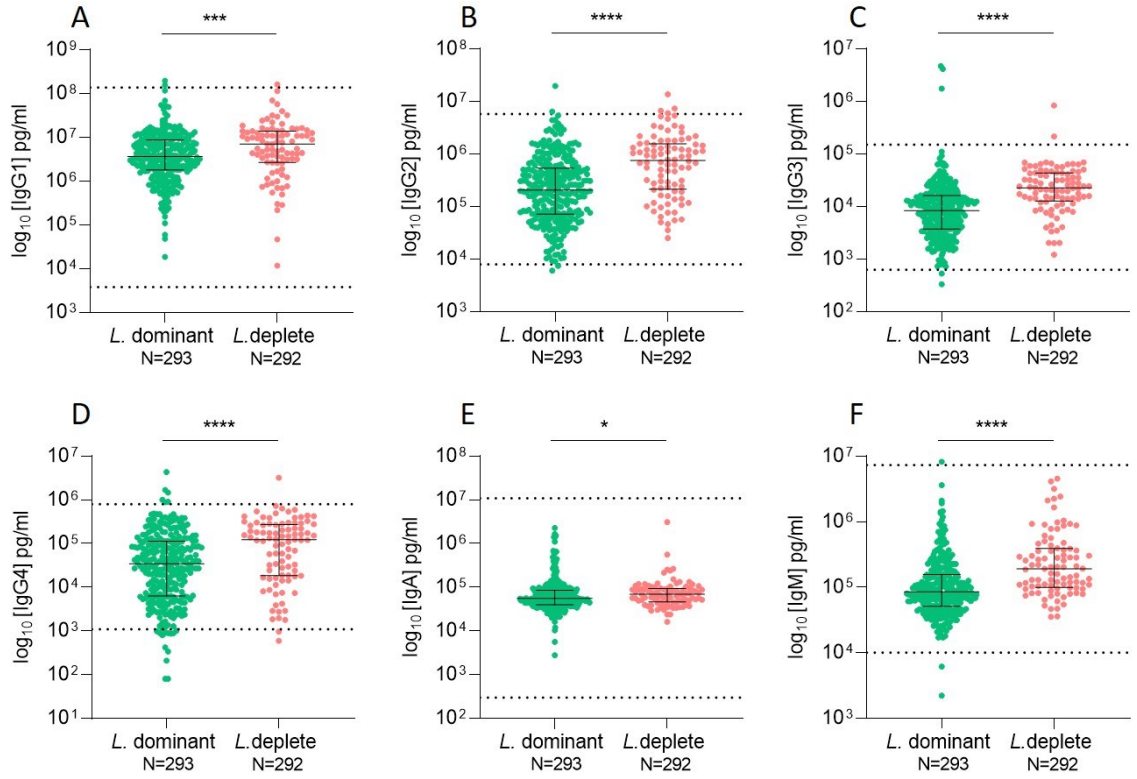


Figure 5.8 Cervico-vaginal immunoglobulins according to genera microbial classification, *Lactobacillus* dominance and *Lactobacillus* depletion in all samples

Lactobacillus depletion was associated with increased IgG1-IgG4, IgA and IgM expression Statistical analysis was by Mann Whitney I test, (* $p<0.05$, *** $p<0.001$, **** $p<0.0001$). Abbreviations: *L.* dom = *Lactobacillus* dominant, *L.* deplete = *Lactobacillus* deplete. Dotted lines mark the concentrations of the highest and lowest standard on the assay. The results are expressed as median and interquartile range. *L.* dominant N=294 and *L.* deplete N=92.

Vaginal microbial composition and the local immune milieu at species level

Ward hierarchial clustering analysis of the species sequence data identified 6 major clusters of samples which were classified into six vaginal microbial groups, Figure 5.2. The 385 samples were classified into the following: VMG 1 *L. crispatus* N=158 (41%), VMG 2 *L. gasseri* N=40 (10%), VMG 3 *L. iners* N=99 (26%), VMG 4 Diverse species N=58 (15%), VMG 5 *L. jensenii* N=16 (4%) and VMG 6 *Bifidobacterium spp* N=14 (4%). Comparisons were made for each individual cytokine, complement and immunoglobulin analyte across the four most common vaginal microbial groups, VMG 1-4.

The most abundant pro-inflammatory CVF cytokines, IL-8, IL-1β and IL-6 were significantly higher in VMG 4 Diverse species compared to VMG 1 *L. crispatus* (p=0.02, p<0.0001 and p=0.01 respectively), Figure 5.9 A-C. IL-8 and IL-1β were also significantly higher in VMG 3 *L. iners* compared to VMG 1 *L. crispatus* (both p=0.003). Anti-inflammatory IL-10 was significantly higher in VMG 1 *L. crispatus* compared to both VMG 3 *L. iners* and VMG 4 Diverse species (both p=0.05), Figure 5.9 D.

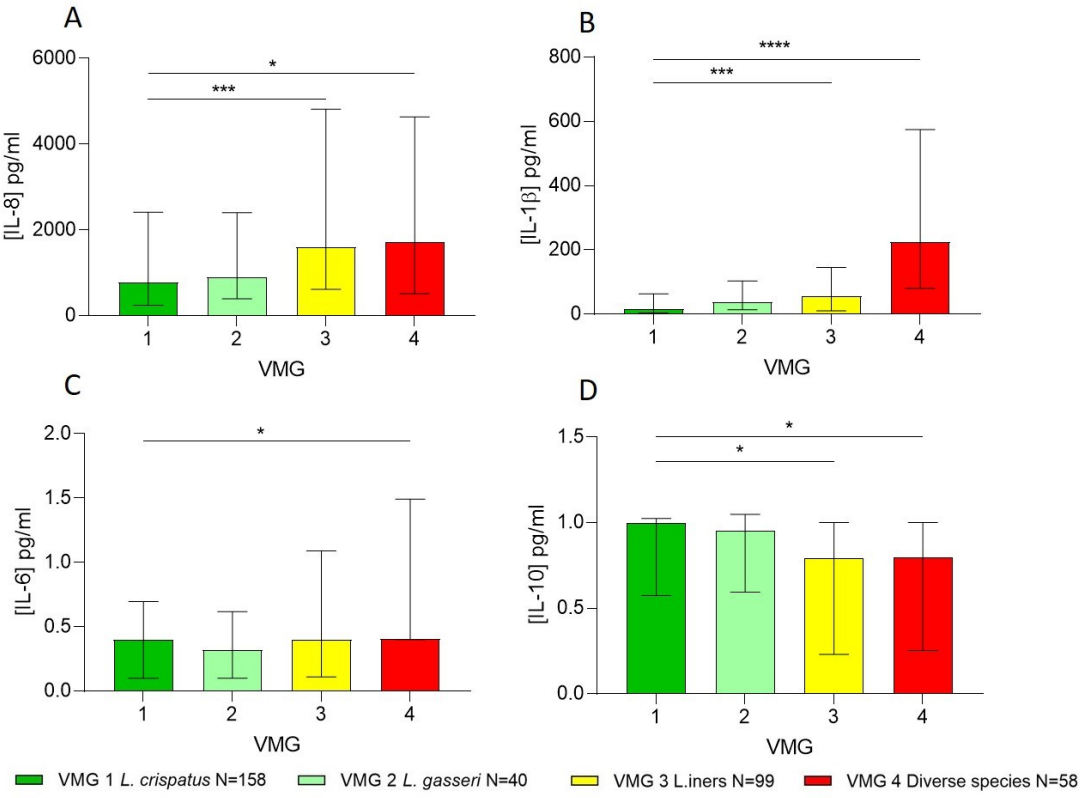


Figure 5.9 Significant differences in cervico-vaginal cytokines according to vaginal microbial groups
VMG 3 *L. iners* and VMG 4 Diverse species were associated with significantly increased IL-8 and IL-1β expression compared to VMG 1 *L. crispatus*, whilst VMG 1 *L. crispatus* was associated with significantly increased anti-inflammatory IL-10 compared to both VMG 3 *L. iners* and VMG 4 Diverse species. IL-6 was significantly higher in VMG 4 Diverse species compared to VMG 1 *L. crispatus*. Statistical analysis was by the Kruskal-Wallis test with Dunn's multiple comparisons test. (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001). The results are expressed as median and interquartile range. VMG 1 N=158, VMG 2 N=40, VMG 3 N=99, VMG 4=58.

For the less abundant CVF cytokines: IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α and GM-CSF, there were no differences in their expression according to the four most common vaginal microbial groups (p>0.05), Figure 5.10.

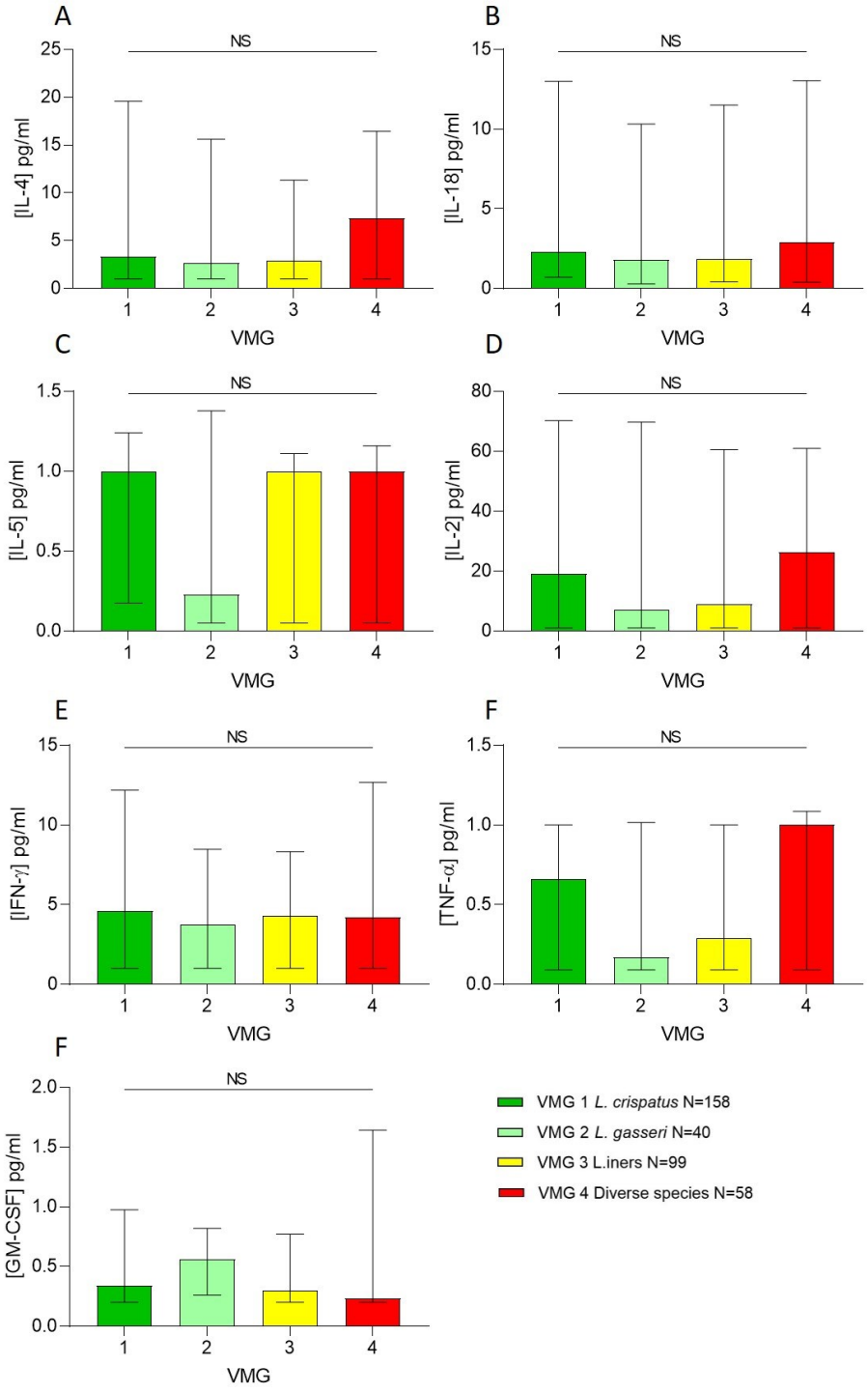


Figure 5.10 Cervico-vaginal cytokines according to vaginal microbial groups
IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α and GM-CSF did not show any differences in expression between the four main vaginal microbial groups. Statistical analysis was by the Kruskal-Wallis test with Dunn's multiple comparisons test. NS = non=significant. The results are expressed as median and interquartile range. VMG 1 N=158, VMG 2 N=40, VMG 3 N=99, VMG 4=58.

Regarding the complement analytes, VMG 4 Diverse species was associated with significantly higher local expression of C5, MBL and C3b compared to VMG 1 *L. crispatus* (p=0.004, p=0.002 and p<0.0001 respecitvely). There was no significant difference in C5a expression between VMG 1 and VMG 4 (p>0.05). C3b was also significantly lower in VMG 1 *L. crispatus* compared to VMG 2 *L. gasseri* (p<0.0001). Concentrations of C5, C5a, MBL or C3b were similar in VMG 1 *L. crispatus* and VMG 3 *L. iners,* (p>0.05) Figure 5.11.

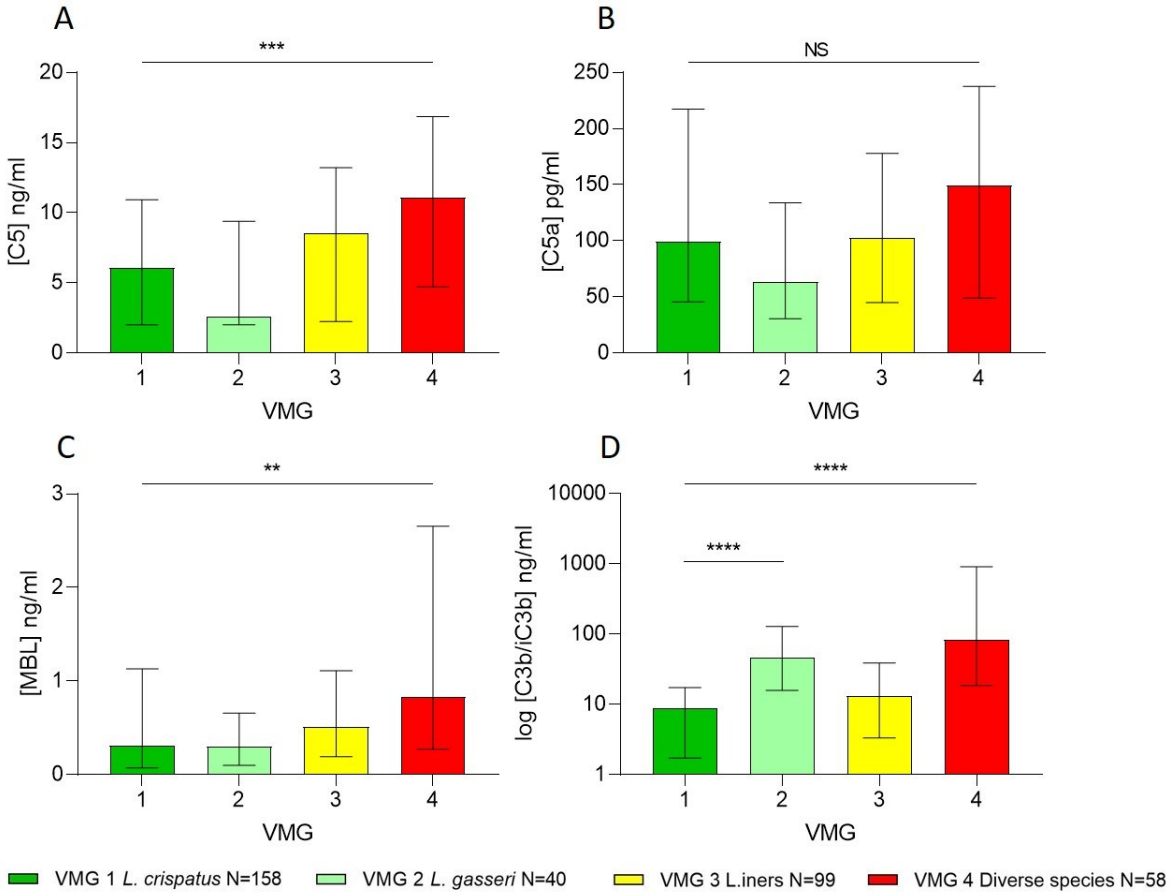


Figure 5.11 Cervico-vaginal complement according to vaginal microbial groups
C5, MBL and C3b expression were significantly increased in VMG 4 Diverse species compared to VMG 1 *L. crispatus*. Additionally, C3b was significantly lower in VMG 1 *L. crispatus* compared to VMG 2 *L. gasseri*. There was no difference in C5a across the four most common vaginal microbial groups. Statistical analysis was by the Kruskal-Wallis test with Dunn's multiple comparisons test. (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001). The results are expressed as median and interquartile range. VMG 1 N=158, VMG 2 N=40, VMG 3 N=99, VMG 4=58.

Following the same pattern for an increased local inflammatory response seen with the dysbiotic vaginal microbial groups with CVF cytokines and complement analytes, IgG1, IgG2, IgG3, IgG4 and IgM concentrations were significantly higher in VMG 4 Diverse species compared to VMG 1 *L. crispatus* (p=0.01 for IgG1, p<0.0001 for IgG2-4 and IgM). VMG 3 *L. iners* and *VMG 2 L. gasseri* concentrations were also significantly higher in IgG2, IgG3, IgG4 and IgM compared to VMG 1 *L. crispatus* (p<0.05), Figure 5.12.

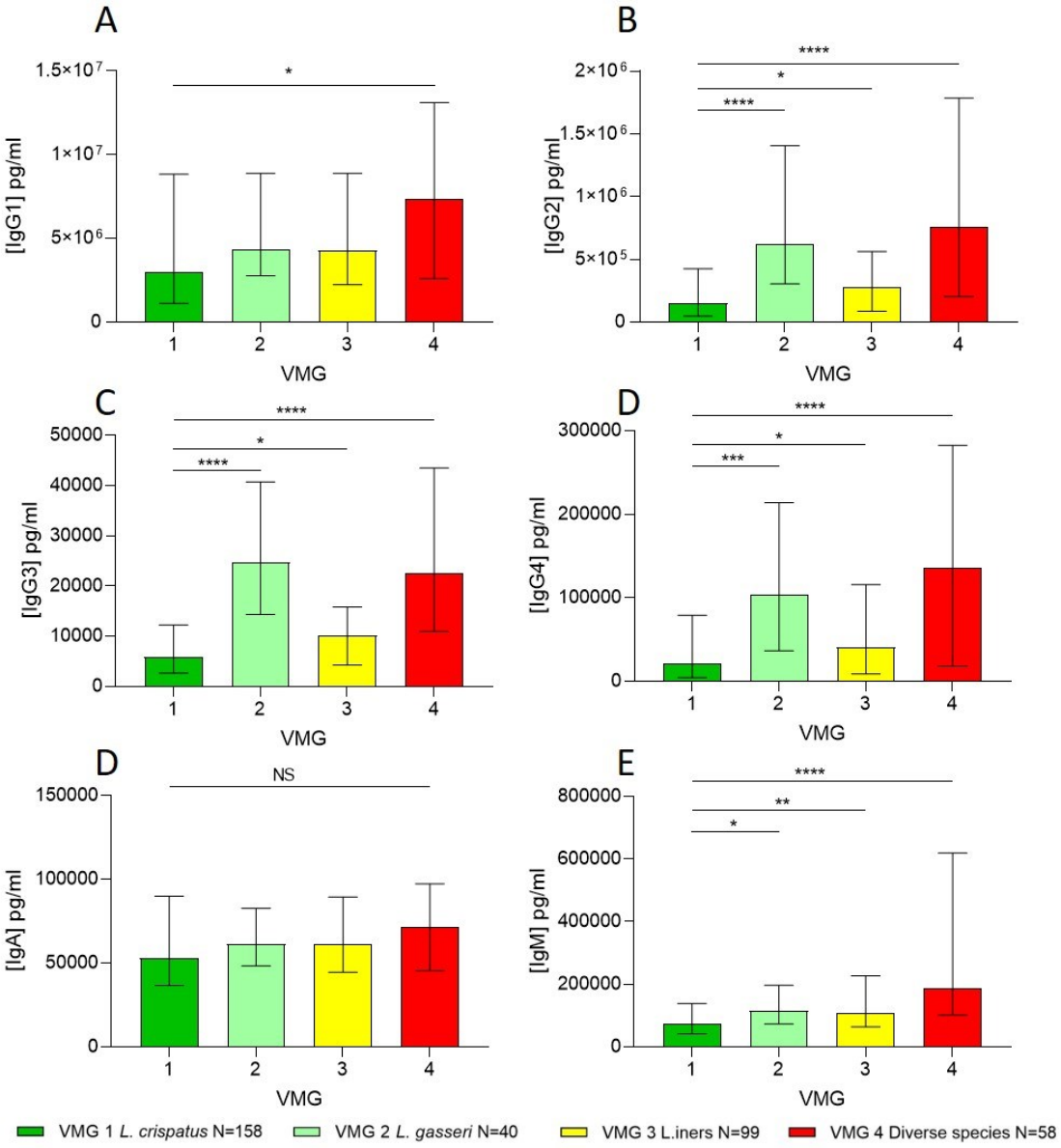


Figure 5.12 Cervico-vaginal immunoglobulins according to vaginal microbial groups
Independent of the gestation of sampling, the expression of IgG1, IgG2, IgG3, IgG4 and IgM were significantly higher in VMG 4 Diverse species compared to VMG 1 *L. crispatus*. IgG2, IgG3, IgG4 and IgM were significantly higher in VMG 3 *L. iners* and VMG 2 L. gasseri compared to VMG 1 *L. crispatus.* There was no difference in IgA expression across the four most common vaginal microbial groups. Statistical analysis was by the Kruskal-Wallis test with Dunn's multiple comparisons test. (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, NS=non-significant). The results are expressed as median and interquartile range. VMG 1 N=158, VMG 2 N=40, VMG 3 N=99, VMG 4=58.

Vaginal microbial composition local immune milieu at genera level by gestation of sampling

As mentioned earlier, samples were collected at three sampling timepoints, 12^{+0} - 16^{+6}, 20^{+0} - 24^{+6} and 30^{+0} - 34^{+-6} weeks. The relationship between the cytokines, complement and immunoglobulins with *Lactobacillus* dominance and *Lactobacillu*s depletion was compared at each sampling timepoint.

IL-8 was present in significantly higher concentrations at the second and third sampling timepoints in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples, (p=0.007 and p=0.03), and it was also non-significantly higher at the first sampling timepoint in *Lactobacillus* deplete samples. Figure 5.13 A. IL-1β was significantly higher in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples at all three sampling timepoints, (p<0.0001), Figure 5.13 B. IL-6 was significantly higher in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples at the first and second sampling timepoints, (p=0.04 and p=0.02), Figure 5.13 C. Anti-inflammatory IL-10 was significantly higher in *Lactobacillus* dominant compared to *Lactobacillus* deplete samples at the second sampling timepoint only, (p=0.004), Figure 5.13 D.

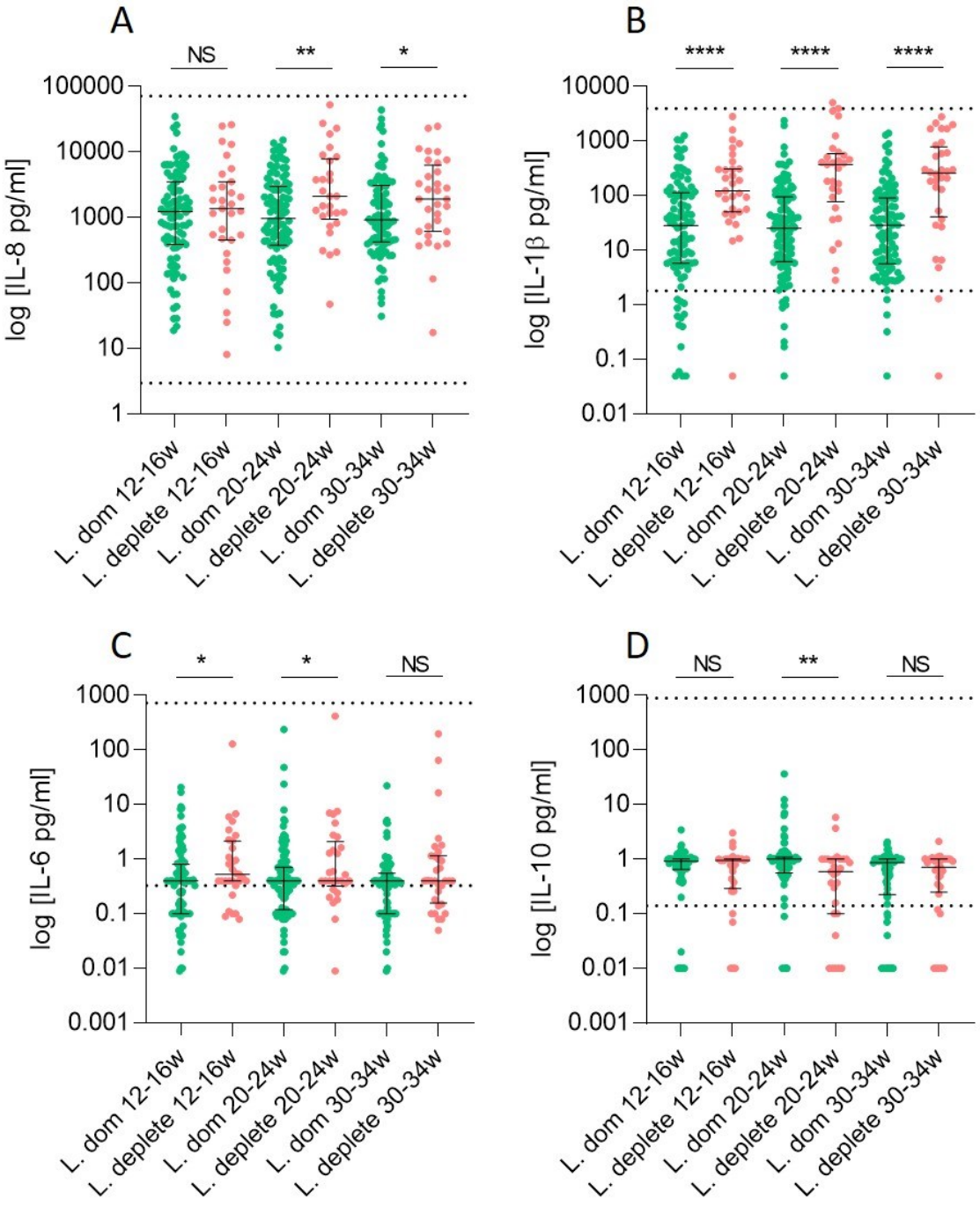


Figure 5.13 Cervico-vaginal cytokines according to genera microbial classification, *Lactobacillus* dominance and *Lactobacillus* depletion across pregnancy

Lactobacillus depletion was associated with increased IL-8, IL-1β and IL6 expression throughout pregnancy, at all three sampling timepoints, 12-16, 20-24 and 30-34 weeks gestation. IL-10 expression was increased in *Lactobacillus* dominant samples at the second sampling timepoint. Statistical analysis was by Mann Whitney U test, (* $p<0.05$, ** $p<0.01$,**** $p<0.0001$). Abbreviations: *L.* dom = *Lactobacillus* dominant, *L.* deplete = *Lactobacillus* deplete, w = weeks. *L.* dom at 12-16 weeks N=95, *L.* deplete at 12-16 weeks N=31, *L.* dom at 20-24 weeks N=104, *L.* deplete at 20-24 weeks N=29, *L.* dom at 30-34 weeks N=94, *L* deplete at 30-34 weeks N=32. The results are expressed as median and interquartile range.

IL-4 was significantly higher in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples at 30^{+0} - 34^{+6} weeks, (p=0.04), Figure 5.14 A and GM-CSF was significantly higher in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples at 20^{+0} - 24^{+6} weeks, (p=0.04), Figure 5.14 G. Consistent with the results independent of the gestation of sampling, there were no significant differences in the concentrations of IL-18, IL-5, IL-2, IFN-γ and TNF-α in *Lactobacillus* dominant and *Lactobacillus* depletion samples at each of the three sampling timepoints, 12^{+0} - 16^{+6}, 20^{+0} - 24^{+6} and 30^{+0} - 34^{+6} weeks, (p>0.05) Figure 5.14 B-F.

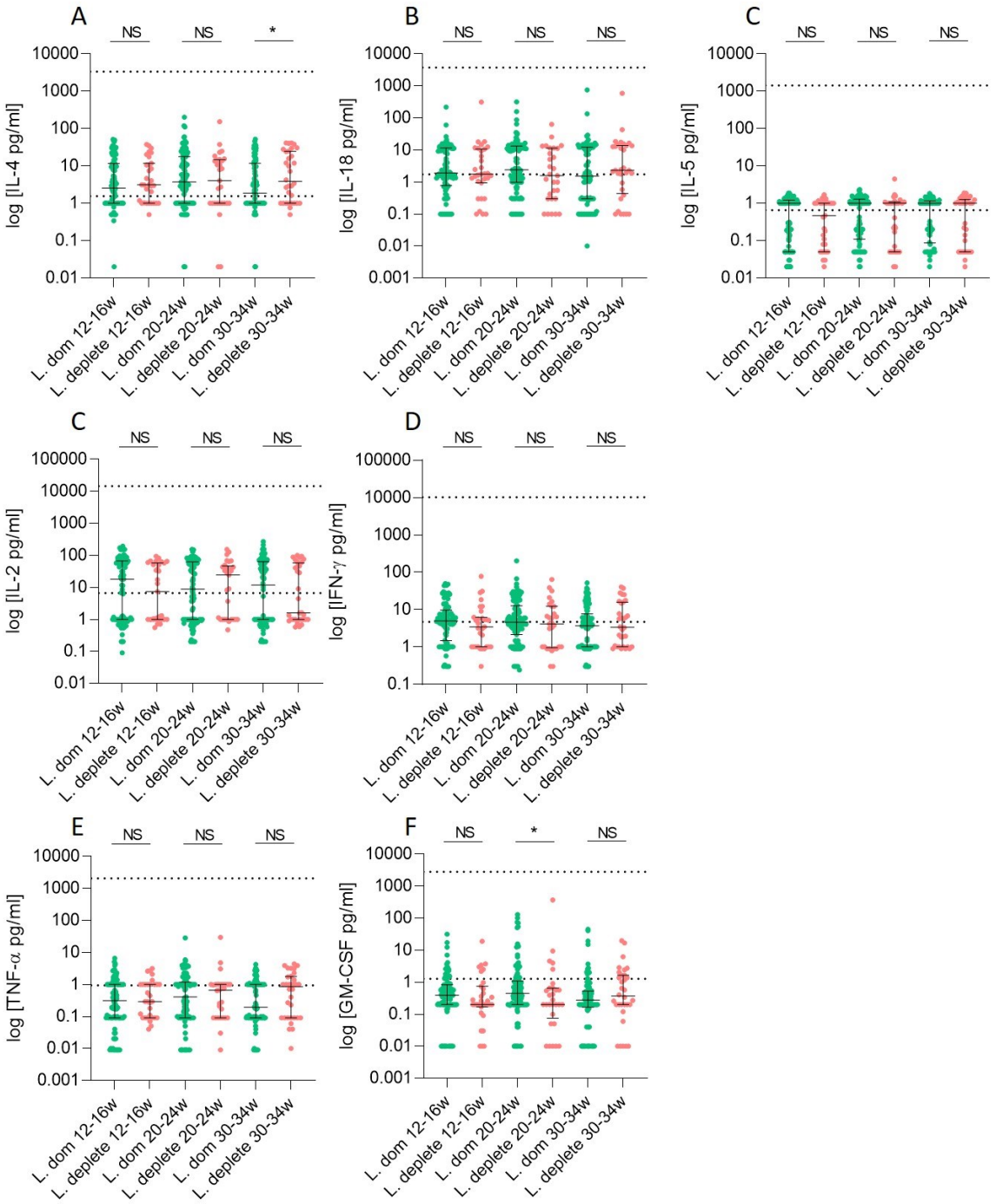


Figure 5.14 Cervico-vaginal cytokines according to genera microbial classification, *Lactobacillus* dominance and *Lactobacillus* depletion across pregnancy

Lactobacillus depletion was associated with increased IL-4 at the 30-34 weeks, and increased GM-CSF at 20-24 weeks. However, overall the *Lactobacillus* dominance and depletion had little effect on the expression on IL-18, IL-5, IL-2, IFN-γ and TNF-α. These were all the least detectable cytokines in cervico-vaginal fluid. Statistical analysis was by Mann Whitney U test, (* $p<0.05$, ** $p<0.01$,**** $p<0.0001$). Abbreviations: *L.* dom = *Lactobacillus* dominant, *L.* deplete = *Lactobacillus* deplete, w = weeks. *L.* dom at 12-16 weeks N=95, *L.* deplete at 12-16 weeks N=31, *L.* dom at 20-24 weeks N=104, *L.* deplete at 20-24 weeks N=29, *L.* dom at 30-34 weeks N=94, *L* deplete at 30-34 weeks N=32. The results are expressed as median and interquartile range.

Assessing the complement analytes in cervico-vaginal fluid, there was a highly significant pro-inflammatory response associated with *Lactobacillus* depletion compared to *Lactobacillus* dominance. C5 concentrations were significantly higher in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples at 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks (p=0.05 and p=0.001 respectively), with a non-significant increase at the 30^{+0} - 34^{+6} weeks (p=0.36), Figure 5.15 A. MBL and C3b concentrations were significantly higher across all sampling timepoints during pregnancy in *Lactobacillus* deplete samples (p<0.05 for MBL and p<0.0001 for C3b), Figure 5.15 C and D. There was no significant difference in C5a in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples across pregnancy, (p>0.05) Figure 5.15 B.

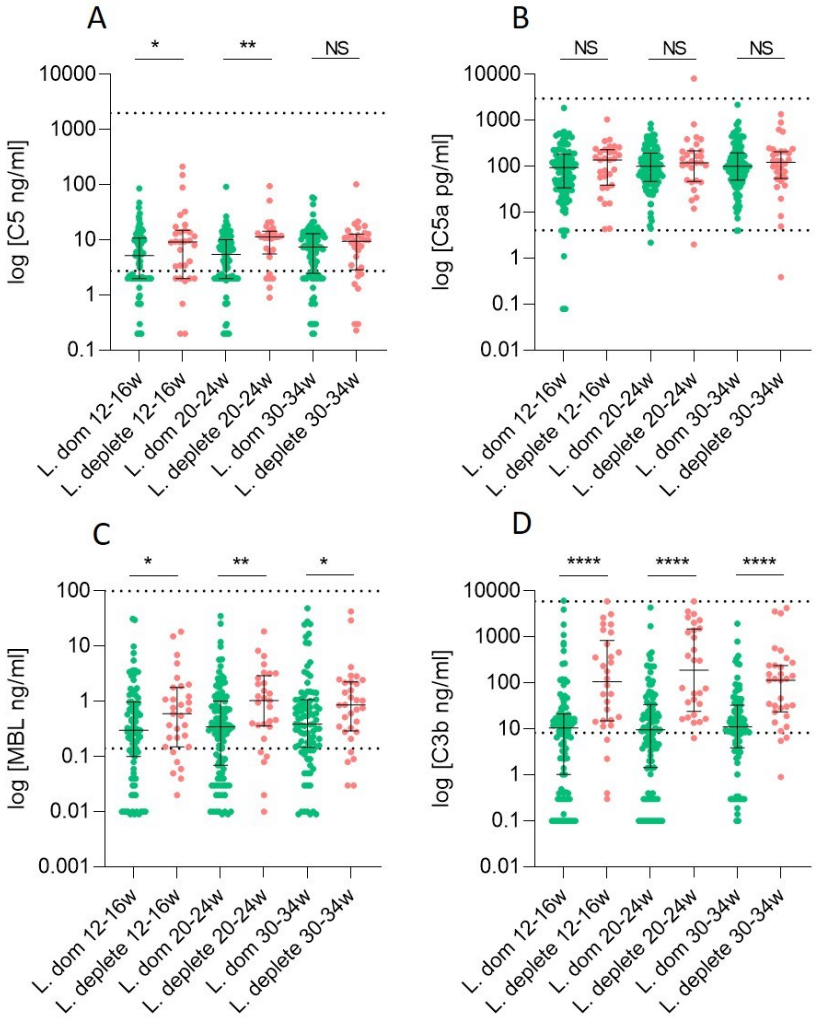


Figure 5.15 Cervico-vaginal complement according to genera microbial classification, *Lactobacillus* dominance and *Lactobacillus* depletion across pregnancy

Lactobacillus depletion was associated with increased C5, MBL and C3b expression at all three pregnancy sampling timepoints, 12-16, 20-24 and 30-34 weeks gestation. There was non-significant increase in C5a expression according by *Lactobacillus* dominance or depletion. Statistical analysis was by Mann Whitney U test, (* p<0.05, ** p<0.01,**** p<0.0001). Abbreviations: *L.* dom = *Lactobacillus* dominant, *L.* deplete = *Lactobacillus* deplete, w = weeks. *L.* dom at 12-16 weeks N=95, *L.* deplete at 12-16 weeks N=31, *L.* dom at 20-24 weeks N=104, *L.* deplete at 20-24 weeks N=29, *L.* dom at 30-34 weeks N=94, *L* deplete at 30-34 weeks N=32. The results are expressed as median and interquartile range.

For the immunoglobulins, IgG1 was significantly higher in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples at the first two sampling timepoints (p=0.02 and p=0.01), with a non-significant increase at the third sampling timepoint, (p=0.10) Figure 5.16 A. IgG2, IgG3, IgG4 and IgM were significantly higher in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples at all three sampling timepoint, (p<0.01, p<0.0001, p<0.05, p<0.001 respectively), Figure 5.16 B-D and F. IgA was significantly higher in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples, but just at the 20^{+0} - 24^{+6} week timepoint, (p=0.01), with non-significant increases at the first and third sampling timepoints, (p>0.05) Figure 5.16 E.

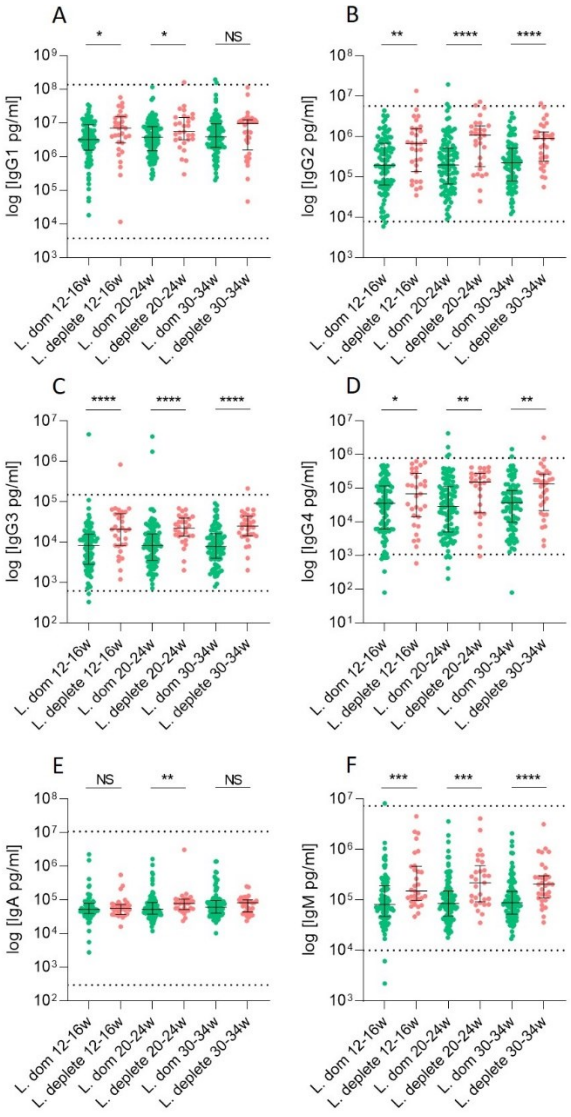


Figure 5.16 Cervico-vaginal immunoglobulins according to genera microbial classification, *Lactobacillus* dominance and *Lactobacillus* depletion across pregnancy

Lactobacillus depletion was associated with increased IgG1-IgG4 and IgM expression at all three pregnancy sampling timepoints, 12-16, 20-24 and 30-34 weeks gestation. *Lactobacillus* depletion was associated with increased IgA expression at the second sampling timepoint. Statistical analysis was by Mann Whitney U test, (* p<0.05, ** p<0.01,*** p<0.001, **** p<0.0001). Abbreviations: *L.* dom = *Lactobacillus* dominant, *L.* deplete = *Lactobacillus* deplete, w = weeks. *L.* dom at 12-16 weeks N=95, *L.* deplete at 12-16 weeks N=31, *L.* dom at 20-24 weeks N=104, *L.* deplete at 20-24 weeks N=29, *L.* dom at 30-34 weeks N=94, *L* deplete at 30-34 weeks N=32. The results are expressed as median and interquartile range.

Vaginal microbial composition at species level according to gestation of sampling

As previously described, the four main vaginal microbial groups were VMG 1 *L.* crispatus, VMG 2 *L.* gasseri, VMG 3 *L.* iners and VMG 4 Diverse species. Samples which were VMG 4 Diverse species showed significantly higher concentrations of IL-8 at 20^{+0} - 24^{+6} weeks (p=0.01), IL-1β at 12^{+0} - 16^{+6}, 20^{+0} - 24^{+6} and 30^{+0} - 34^{+6} weeks (p=0.002, p<0.0001 and p<0.0001 respectively), IL-6 at 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks (both p=0.05), compared to VMG 1 *L. crispatus*, Figure 5.17. Samples which were VMG 3 *L. iners* showed significantly higher concentrations of IL-8 at 20^{+0} - 24^{+6} weeks (p=0.002) and IL-1β at 20^{+0} - 24^{+6} (p=0.01), compared to VMG 1 *L. crispatus,* Figure 5.17. There were no significant differences between IL-8, IL-1β, IL-6 and IL-10 in VMG 2 *L. gasseri* and VMG 1 *L. crispatus* samples, (p>0.05) Figure 5.17.

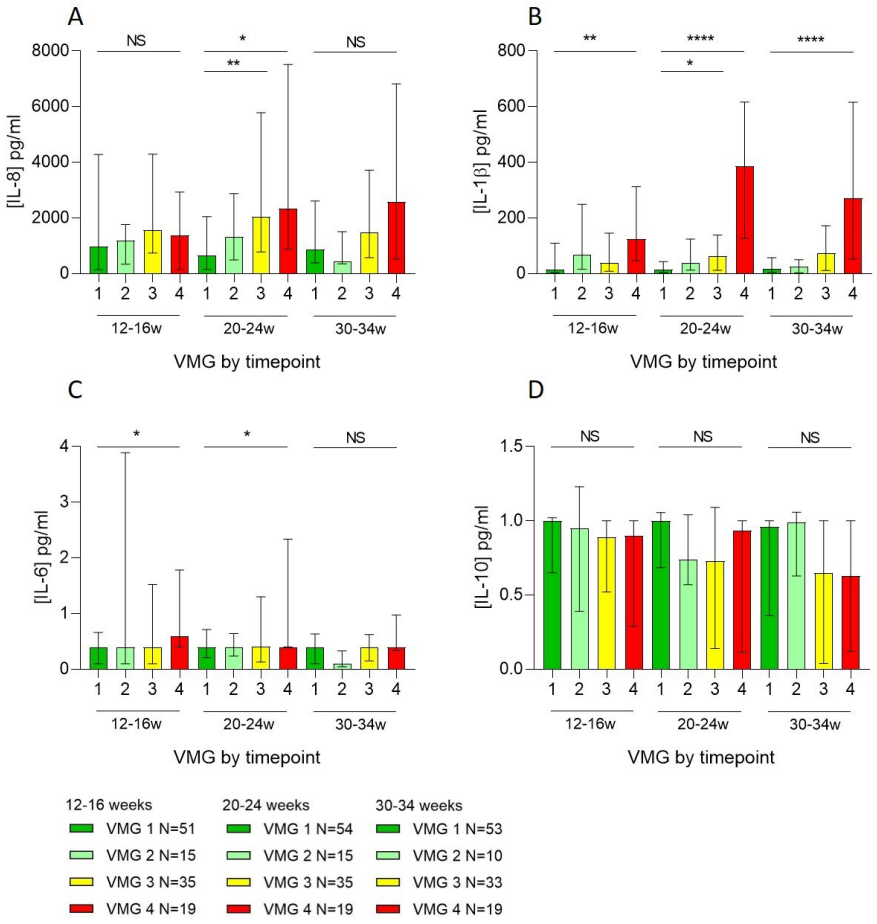


Figure 5.17 Cervico-vaginal cytokines according to vaginal microbial groups by gestation of sampling
IL-8 was significantly higher in VMG 4 compared to VMG 1, and significantly higher in VMG 3 compared to VMG 1 at 20-24 weeks (A). There were non-significant increases in VMG 4 and VMG 3 at 12-16 and 30-34 weeks. IL-1β was significantly higher in VMG 4 compared to VMG 1 at all three sampling timepoints. IL-1β was also significantly higher in VMG 3 compared to VMG I at 20-24 weeks (B). There was a non-significant increase in IL-6 in VMG IV compared to VMG I (C). There was a non-significant increase in IL-10 in VMG I compared to VMG IV (D). Statistical analysis was by the Kruskal-Wallis test with Dunn's multiple comparisons test. (* p<0.05, ** p<0.01, **** p<0.0001, NS = non-significant). VMG = vaginal microbial group. The results are expressed as median and interquartile range. At 12-16 weeks: VMG 1 N=51, VMG 2 N=15, VMG 3 N=35, VMG 4 N=19. At 20-24 weeks: VMG 1 N=54, VMG 2 N=15, VMG 3 N=35, VMG 4 N=19. At 30-34 weeks: VMG 1 N=53, VMG 2 N=10, VMG 3 N=33, VMG 4 N=19.

Concentrations of IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α and GM-CSF did not show any significant differences between VMG 1, 2, 3 and 4 across the three sampling timepoints, (p>0.05) Figure 5.18 A-G.

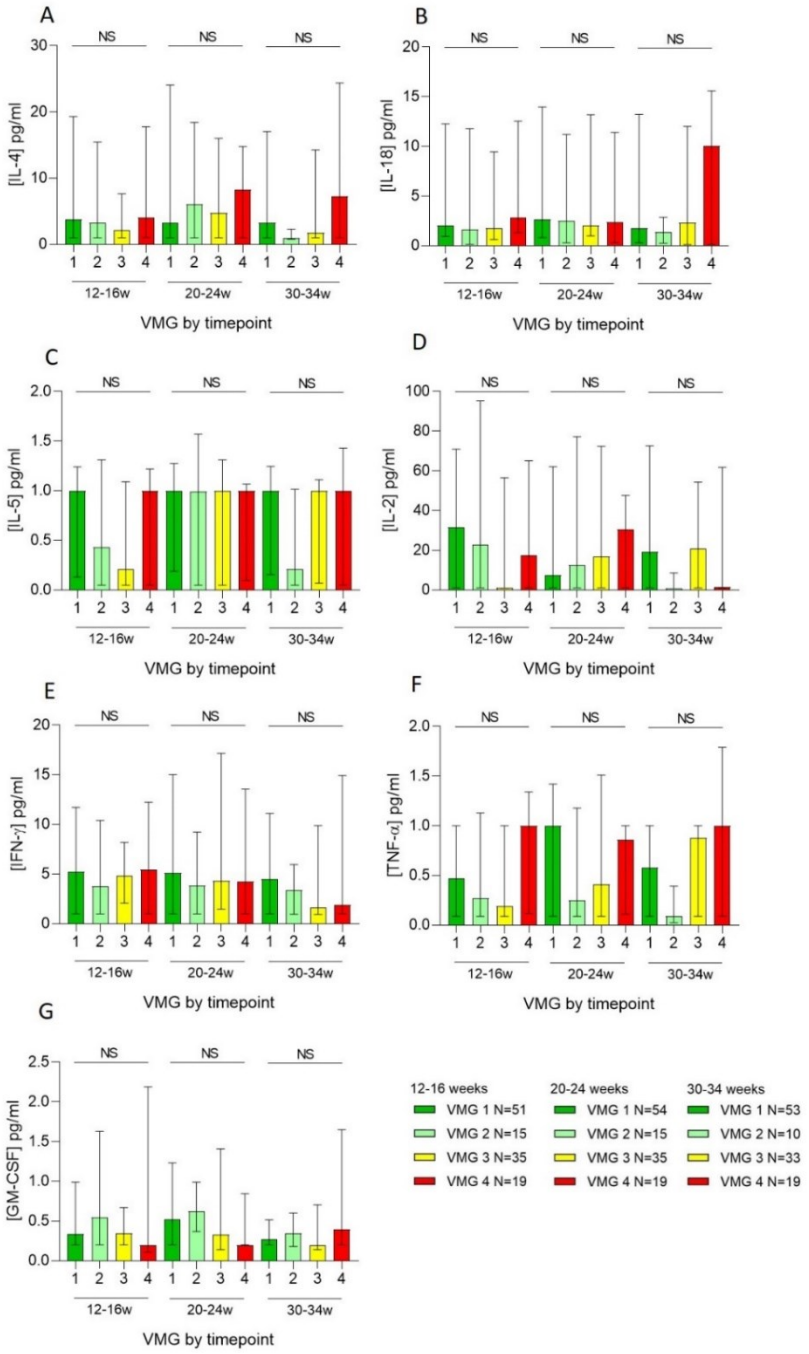


Figure 5.18 Cervico-vaginal cytokines according to vaginal microbial groups by gestation of sampling
There were no significant differences in IL-4, IL-18, IL-5, IL-2, IFN-γ, TNF-α or GM-CSF according to VMG 1, 2, 3 and 4, at any of the three sampling timepoints of 12-16, 20-24 and 30-34 weeks. Statistical analysis was by the Kruskal-Wallis with Dunn's multiple comparisons test, (NS = non-significant). VMG = vaginal microbial group. The results are expressed as median and interquartile range. At 12-16 weeks: VMG 1 N=51, VMG 2 N=15, VMG 3 N=35, VMG 4 N=19. At 20-24 weeks: VMG 1 N=54, VMG 2 N=15, VMG 3 N=35, VMG 4 N=19. At 30-34 weeks: VMG 1 N=53, VMG 2 N=10, VMG 3 N=33, VMG 4 N=19.

VMG 4 Diverse species was associated with significantly higher concentrations of C3b at all three sampling timepoints (p<0.0001, p<0.0001 and p<0.004) and C5 at the first two sampling timepoints (p=0.008 and p=0.02) compared to VMG 1 *L. crispatus*, Figure 5.19 A and D. VMG 2 *L. gasseri* was associated with significantly higher concentrations of C3b at the latter two sampling timepoints compared to VMG 1 *L. crispatus* (p=0.001 and p=0.007), Figure 5.19 C. C5a was significantly higher in VMG 2 *L. gasseri* compared to VMG 1 *L. crispatus* at the third sampling timepoint (p=0.007), but no other differences were observed at the other sampling timepoints. There were no differences in C5, C5a, MBL or C3b concentrations between VMG 3 *L. iners* and VMG 1 *L. crispatus*, (p>0.05) Figure 5.19 A-D.

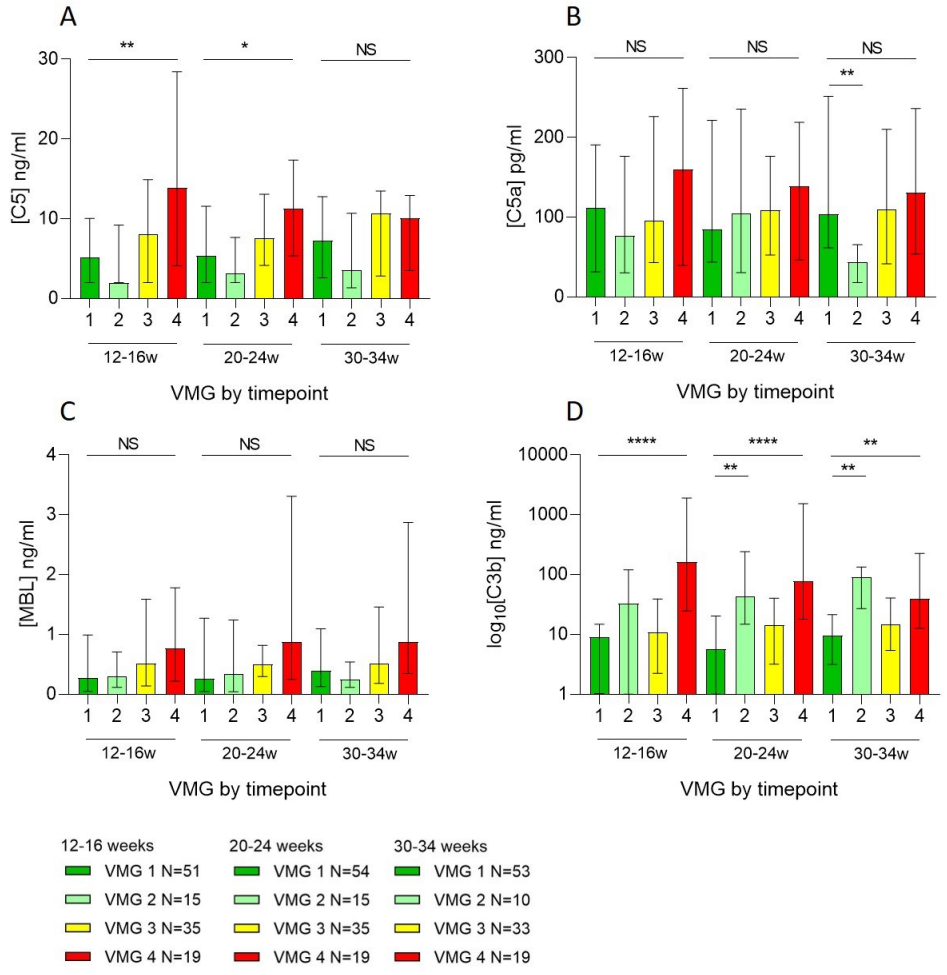


Figure 5.19 Cervico-vaginal complement according to vaginal microbial groups by gestation of sampling
C5 was significantly increased in VMG 5 compared to VMG 1 at 12-16 and 20-24 weeks gestation (A). C5a was significantly greater in VMG 2 compared to VMG 1 at 30-34 weeks gestation (B). There was a non-significant increase in MBL in VMG 4 compared to VMG 1, and VMG 3 compared to VMG 3 across all three timepoints (C). C3b was significantly increased in VMG 4 compared to VMG 1 at all three sampling timepoints. C3b was also significantly lower in VMG 1 compared to VMG 2 at 20-24 and 30-34 weeks gestation (D). Statistical analysis was by the Kruskal-Wallis with Dunn's multiple comparisons test. (* p<0.05, ** p<0.01, **** p<0.0001, NS = non-significant). VMG = vaginal microbial group. The results are expressed as median and interquartile range. At 12-16 weeks: VMG 1 N=51, VMG 2 N=15, VMG 3 N=35, VMG 4 N=19. At 20-24 weeks: VMG 1 N=54, VMG 2 N=15, VMG 3 N=35, VMG 4 N=19. At 30-34 weeks: VMG 1 N=53, VMG 2 N=10, VMG 3 N=33, VMG 4 N=19.

VMG 4 Diverse species was associated with significantly higher IgG2 at all three timepoints compared to VMG 1 *L. crispatus* (p=0.01, p=0.0002, p=0.001 respectively), Figure 5.20 B. VMG 4 Diverse species was also associated with significantly higher IgG3 at all three timepoints compared to VMG 1 *L. crispatus* (p=0.001, p<0.0001, p<0.001 respectively), Figure 5.20 C. VMG 4 Diverse species was associated with significantly higher IgG4 compared to VMG 1 *L. crispatus* at the latter two timepoints, (p<0.01 and p<0.05 respectively), Figure 5.20 D. VMG 4 Diverse species was associated with significantly higher IgM compared to VMG 1 *L. crispatus* at all three sampling timepoints, (p=0.0003, p=0.0002 and p=0.01), Figure 5.20 F.

In Figure 5.12 there was a significant adaptive immune response with significantly higher IgG2, IgG3, IgG3 and IgM in VMG 3 *L. iners* compared to VMG 1 *L. crispatus*, reflecting a true effect. However, there was a lack of statistical power to demonstrate this when the analysis was performed at each of the three sampling timepoints, (p>0.05) Figure 5.20.

Interestingly, VMG 1 *L. gasseri* was associated with significantly higher IgG2 compared to VMG 1 *L. crispatus* at all three timepoints of sampling, (p=0.001, p=0.007 and p=0.02 respectively), Figure 5.20 B. VMG 2 *L. gasseri* was associated with significantly higher IgG3 compared to VMG 1 *L. crispatus* again across all timepoints, (p=0.0005, p=0.0002 and p<0.0001 respectively), Figure 5.20 C.

In Figures 5.17-5.20, VMG IV Diverse species resulted in activation of the innate and adaptive immune responses and the bridging complement response. VMG 3 *L. iners* did not appear to significantly activate complement, therefore is likely to stimulate cytokine production independently of the complement cascade, perhaps via adhesion to epithelial cells rather than innate immune cells . VMG 2 *L. gasseri* was associated with activation of the adaptive immune response and the complement system, without downstream effects on cytokine production.

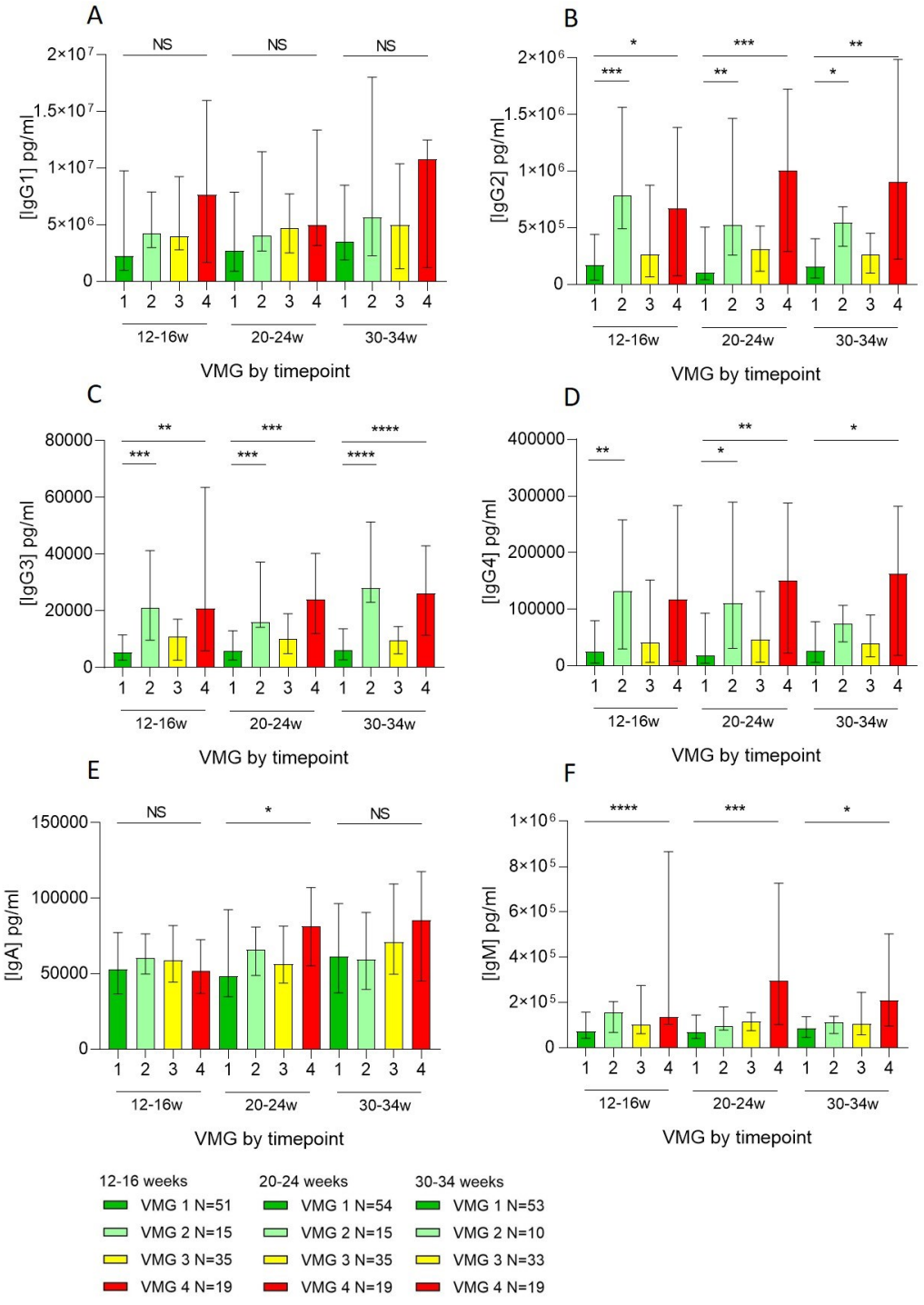


Figure 5.20 Cervico-vaginal immunoglobulins according to vaginal microbial groups by gestation of sampling
There was a non-significant increase in IgG1 in VMG 2, 3 and 4 compared to VMG 1 (A). IgG2 and IgG3 were significantly increased in VMG 4 compared to VMG 1 at all three sampling timepoints, and also significantly increased in VMG 2 compared to VMG 1 (B and C). IgG4 was significantly higher in VMG 4 compared to VMG 1 at 20-24 and 30-34 weeks, and significantly higher in VMG 2 compared to VMG 1 at 12-16 and 20-24 weeks (D). IgA was significantly higher in VMG 4 compared to VMG 1 at 20-24 weeks (E). IgM was significantly higher in VMG 4 compared to VMG 1 at all three sampling timepoints (F). Statistical analysis was by the Kruskal-Wallis test with Dunn's multiple comparisons test. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). VMG = vaginal microbial group. The results are expressed as median and interquartile range. At 12-16 weeks: VMG 1 N=51, VMG 2 N=15, VMG 3 N=35, VMG 4 N=19. At 20-24 weeks: VMG 1 N=54, VMG 2 N=15, VMG 3 N=35, VMG 4 N=19. At 30-34 weeks: VMG 1 N=53, VMG 2 N=10, VMG 3 N=33, VMG 4 N=19.

5.5.4 Vaginal microbial composition, diversity and richness according to risk factor for preterm delivery

In section 4.5.5 of Chapter 4, local immune profiles according to the risk factor for preterm delivery were explored. Study participants were divided into two main groups according to the reason why they were referred to the preterm birth prevention clinics. The first group consisted of women who had excisional cervical treatment but no other risk factors. The second group consisted of women who had previously had a mid-trimester loss and/or a preterm birth. As described in the previous chapter, there was a pro-inflammatory signature in women with previous MTL and/or PTB, compared to women with previous excisional cervical treatment. To see if this could be microbial driven, the vaginal microbial composition, diversity and richness were compared between 40 women with previous excisional cervical treatment and 56 women with previous MTL/PTB, at the 12-16 week sampling timepoint, before any cervical shortening and before the insertion of a cervical cerclage where applicable.

The microbial composition according to *Lactobacillus* dominance and depletion was similar in the group with previous cervical treatment and previous MTL and/or PTB, Figure 5.21 A. Comparing microbial composition according to the 4 most common vaginal microbial groups VMG 1-4 showed a higher proportion of women with VMG 1 *L. crispatus* in the previous cervical treatment group, and a higher proportion of women with VMG 3 *L. iners* in the previous MTL and/or PTB group, however this did not reach statistical significance Figure 5.21 B. The species diversity as measured by the inverse Simpson index was significantly higher in the previous MTL and/or PTB group ($p<0.05$), Figure 5.21 C, yet there was no difference in species richness ($p=0.14$) Figure 5.21 D.

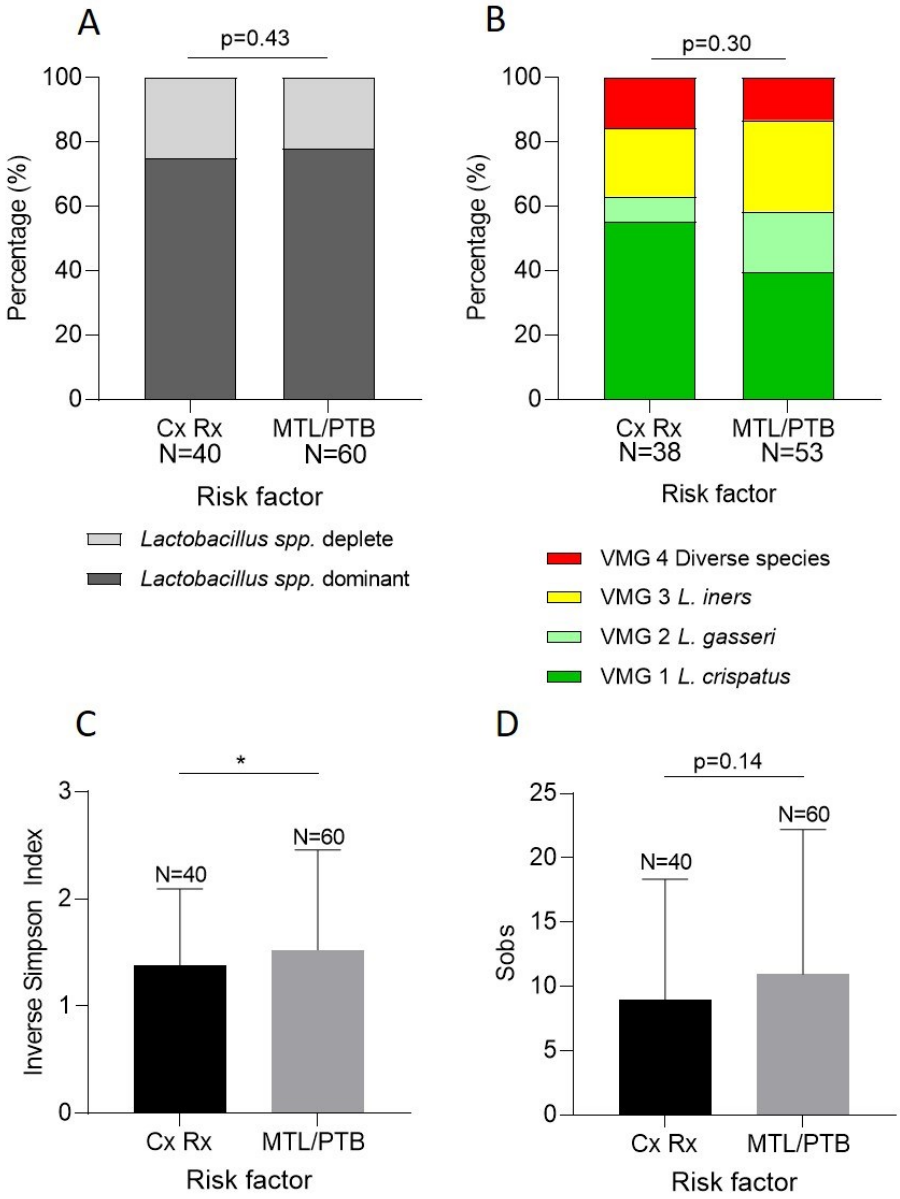


Figure 5.21 Vaginal microbial composition according to the risk factor for preterm birth
In samples collected at the 12-16 week timepoint, there was no difference in the percentage of *Lactobacillus* dominant or *Lactobacillus* deplete samples according to the risk factor of previous excisional cervical treatment (N=40) or previous MTL and/or PTB (N=60) (A). There was a greater proportion of women with VMG 1 *L. crispatus* in the group with previous excisional cervical treatment (N=38), and a greater proportion of women with VMG 3 *L. iners* in the group with previous mid-trimester miscarriage +/or preterm birth (N=53), however this did not reach statistical significance (B). The alpha diversity was significantly higher in the group with previous mid-trimester miscarriage +/or preterm birth (C). There was no significant increase in richness, as defined by the species observed, in the group with previous mid-trimester miscarriage +/or preterm birth (D). Statistical analysis was by Fisher's exact test for proportional data and Mann Whitney test for comparison between two groups. The results are expressed as mean and standard deviation (C and D). Cx Rx (N=40) and MTL/PTB (N=60), (C and D).
Abbreviations: Cx Rx = cervical treatment, MTL = mid trimester loss, PTB = preterm birth. *L.* deplete = *Lactobacillus* deplete, *L.* dominant = *Lactobacillus* dominant. Sobs = species observed.

Cytokines, complement and immunoglobulins in cervico-vaginal fluid were compared in *Lactobacillus* dominant and deplete samples in women grouped according to previous cervical treatment and women with previous MTL and/or PTB. This was performed in order to determine if the dysbiosis in women with previous MTL and/or PTB was associated with local inflammation.

Microbial driven inflammation in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples could be demonstrated by increased IL-1β in all women ($p<0.05$), Figure 5.22 B, and a significant increase in IL-6 in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples in women with previous MTL and/or PTB, ($p=0.01$), Figure 5.22 D. Women with previous MTL and/or PTB with *Lactobacillus* dominance also had significantly higher concentrations of pro-inflammatory IL-8, compared to women with previous excisional cervical treatment, ($p=0.006$), Figure 5.22 A. IL-10 concentrations were similar in *Lactobacillus* deplete and *Lactobacillus* dominant samples, or according to the risk factor for preterm delivery, Figure 5.22 C.

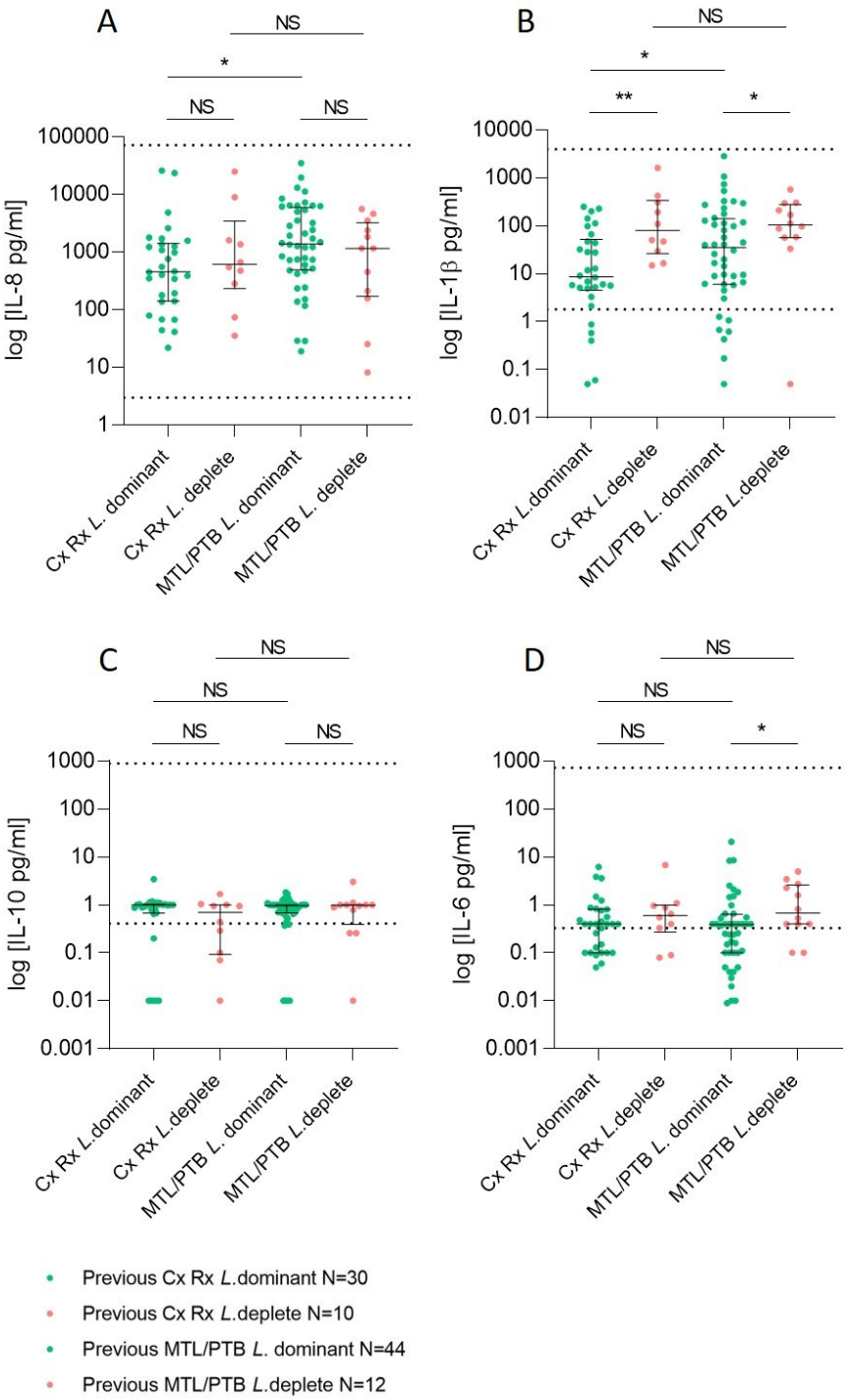


Figure 5.22 Vaginal microbial composition and the local cytokines according to the risk factor for preterm birth
At the 12-16 week timepoint, IL-1β was significantly higher in *Lactobacillus* depletion irrespective of the risk factor for preterm birth (B). IL-6 was significantly higher in *Lactobacillus* depletion in women with previous MTL/PTB (D). IL-8 and IL-1β were significantly higher in previous MTB/PTB compared to previous cervical treatment in the *Lactobacillus* dominant group (A and B). However there was no significant difference in IL-8, IL-1β, IL-6 or IL-10 in *Lactobacillus* depletion according to the risk factor for PTB (A-D). Statistical analysis was by Mann Whitney U test for comparison between two groups, *p<0.05, **p<0.01, N = non-significant. Abbreviations: Cx Rx = cervical treatment, MTL = mid trimester loss, PTB = preterm birth. *L.* deplete = *Lactobacillus* deplete, *L.* dominant = *Lactobacillus* dominant. The results are expressed as median and interquartile range. Previous Cx Rx *L.* dominant N=30, previous Cx Rx *L.* deplete N=10, previous MTL/PTB *L.* dominant N=44, previous MTL/PTB *L.* deplete N=12.

Similarly, for cervico-vaginal fluid complement, microbial driven inflammation could be demonstrated for C5, with increased concentrations demonstrated in *Lactobacillus* deplete compared to *Lactobacillus* dominance in the women with previous MTL and/or PTB, (p=0.05), Figure 5.23 A. C3b concentrations were significantly higher in *Lactobacillus* deplete compared to *Lactobacillus* dominance in all women (p=0.02 if previous MTL and/or PTB and p<0.0001 if previous cervical treatment), Figure 5.23 D. At the first sampling timepoint of 12^{+0} - 16^{+6} weeks concentrations of C5, C5a, MBL or C3b were similar in women with either risk factor for preterm delivery, for both *Lactobacillus* dominant and *Lactobacillus* deplete samples, (p>0.05) Figure 5.23 A-D.

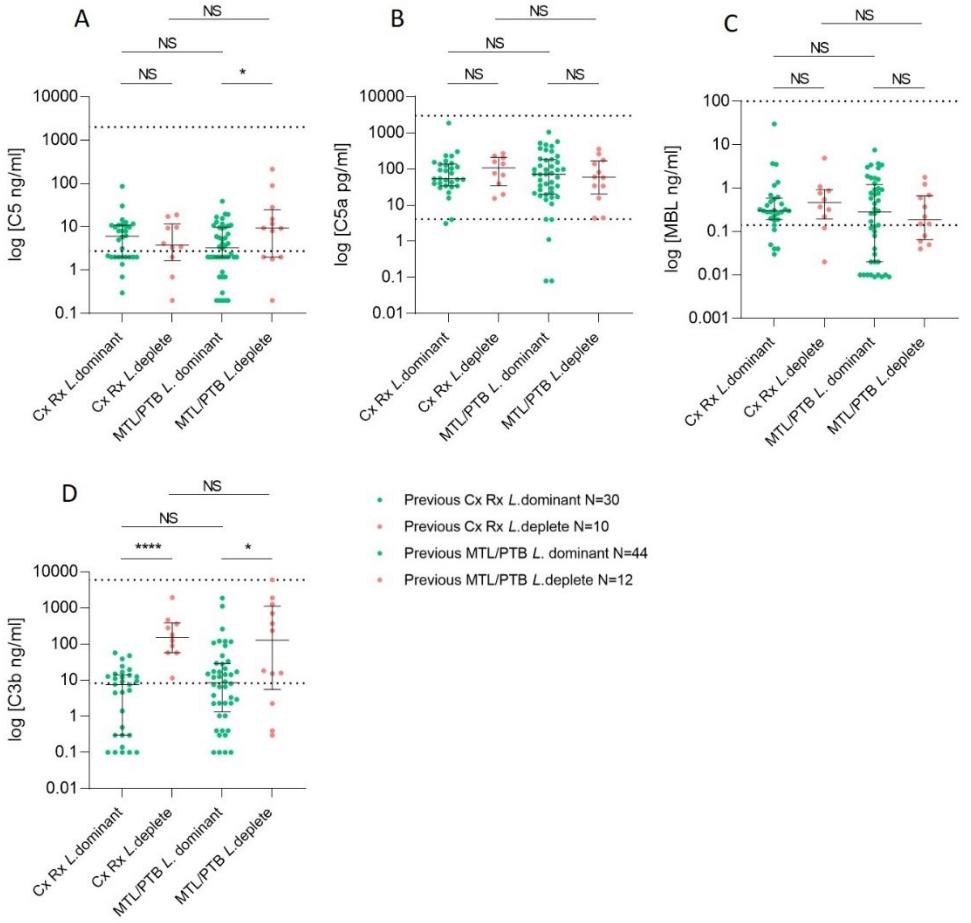


Figure 5.23 Vaginal microbial composition and the local complement according to the risk factor for preterm birth

At the 12-16 week timepoint, C5 and C3b were significantly higher in *Lactobacillus* deplete samples compared to *Lactobacillus* dominant in women with a risk for of previous PTB/MTL, (A and D). C3b was also significantly higher in *Lactobacillus* deplete samples in women with a risk factor of previous cervical treatment (D). However there was no significant difference in C5, C5a, MBL or C3b in *Lactobacillus* depletion according to the risk factor for PTB (A-D).

Statistical analysis was by Mann Whitney U test for comparison between two groups, *p<0.05, ****p<0.0001, NS = non-significant. Abbreviations: Cx Rx = cervical treatment, MTL = mid trimester loss, PTB = preterm birth. *L.* deplete = *Lactobacillus* deplete, *L.* dominant = *Lactobacillus* dominant. The results are expressed as median and interquartile range. Previous Cx Rx *L.* dominant N=30, previous Cx Rx *L.* deplete N=10, previous MTL/PTB *L.* dominant N=44, previous MTL/PTB *L.* deplete N=12.

In samples collected at 12^{+0} -16^{+6} weeks, IgG1 concentrations were no different in women with *Lactobacillus* depletion compared to *Lactobacillus* dominance, ($p>0.05$) Figure 5.24 A. However, IgG2 and IgG4 were significantly increased in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples in the group with previous cervical treatment ($p<0.00001$ and $p<0.0001$ respectively), but not in women with a history of previous MTL/PTB, ($p>0.05$) Figure 5.24 B and D. This is likely due to *L. iners* having a subtle effect on IgG2 and IgG4. IgG3 and IgM were significantly increased in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples in all women ($p<0.05$), Figure 5.24 C and F. IgA concentrations were similar in *Lactobacillus* deplete and *dominant* samples in women with either risk factor for preterm delivery, ($p>0.05$) Figure 5.24 E.

IgG4 showed a differential local immune response in women with *Lactobacillus* dominance, with significantly increased concentrations in women with previous MTL and/or PTB compared to women with previous cervical treatment ($p=0.04$), Figure 5.24 D. This pattern was also observed in IgG2, although the difference was not statistically significant, ($p=0.09$) Figure 5.24 B.

To summarise, women with previous MTL and/or PTB were been found to have dysregulated innate and adaptive immune responses, compared to women with previous excisional cervical treatment in early pregnancy. There is a synergistic effect with the vaginal microbial composition, and IL-8, IL-1β and IgG4 have been found to be present in higher concentrations in the presence of *Lactobacillus* dominance in women with previous MTL and/or PTB compared to previous excisional cervical treatment.

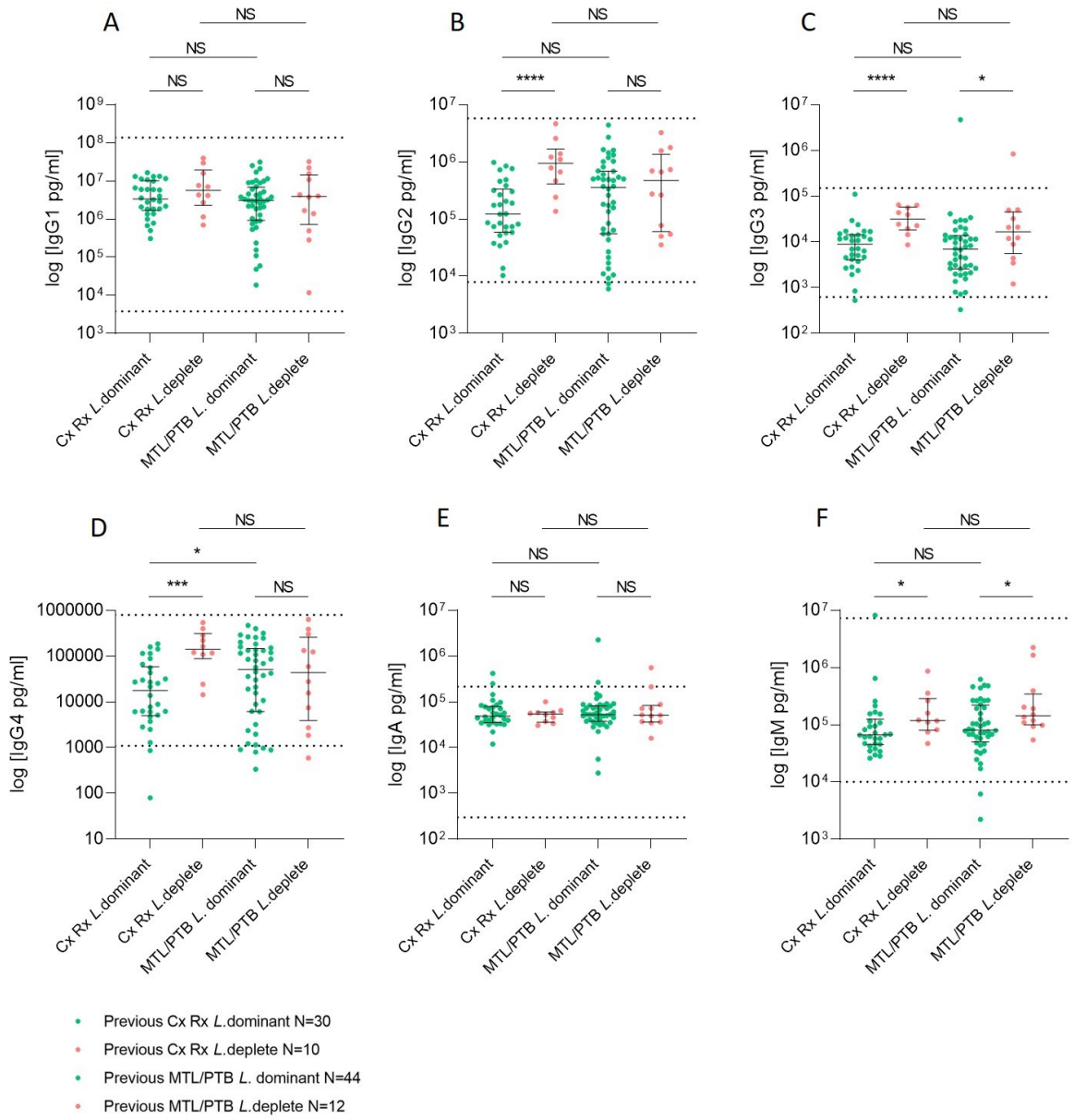


Figure 5.24 Vaginal microbial composition and IgG1-4 according to the risk factor for preterm birth
At the 12-16 week timepoint, IgG2, IgG3 and IgG4 were significantly higher in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples in women with previous cervical treatment (B-D). IgG3 was also significantly higher in *Lactobacillus* deplete in women with previous MTL/PTB. IgG4 was significantly higher in women with previous MTL/PTB compared to previous cervical treatment if they were *Lactobacillus* dominant (D). However, there was no significant difference in IgG1-4 in *Lactobacillus* depletion according to the risk factor for PTB (A-D). IgA concentrations were no different in *Lactobacillus* dominant or deplete samples (E). IgM was significantly higher in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples in both risk factors for preterm birth, previous cervical treatment and previous MTL/PTB (F). There was no significant difference in IgA or IgM concentrations in *Lactobacillus* deplete samples according to the risk factor for preterm birth. Statistical analysis was by Mann Whitney U test for comparison between two groups, *p<0.05, ***p<0.001, ****p<0.0001, NS = non-significant. Abbreviations: Cx Rx = cervical treatment, MTL = mid trimester loss, PTB = preterm birth. *L.* deplete = *Lactobacillus* deplete, *L.* dominant = *Lactobacillus* dominant. The results are expressed as median and interquartile range. Previous Cx Rx *L.* dominant N=30, previous Cx Rx *L.* deplete N=10, previous MTL/PTB *L.* dominant N=44, previous MTL/PTB *L.* deplete N=12.

5.5.5 Vaginal microbial composition and cervical shortening

Study participants had cervical length measurements performed at the same clinical visit when the cervico-vaginal fluid was sampled to determine the vaginal microbial composition. A cervical length of ≤25mm is widely used to identify a shortened cervix at transvaginal ultrasound before 24 weeks gestation and confers a high risk of preterm delivery [23].

At the first timepoint of sampling, 12^{+0} - 16^{+6} weeks, there were 15 women with a cervical length of ≤25mm. Their vaginal microbial composition was compared to 96 women who had a cervical length >25mm. None of the 96 women with a normal cervical length had a cervical cerclage in situ at the time of assessment. The gestation at delivery was significantly earlier in the group with cervical shortening ($p<0.01$), median gestation 38^{+1} weeks with cervical shortening, vs 38^{+6} weeks with normal cervical length. There was a higher proportion of preterm deliveries in the group with cervical shortening, however this did not reach statistical significance ($p<0.09$) (Table 5.3). There was a higher proportion of early preterm births <34 weeks (71%) compared to late preterm births >34 weeks (53%) in the group with cervical shortening, although this was not statistically significant ($p=0.37$).

At the second timepoint of sampling, 20^{+0} - 24^{+6} weeks, there were 18 women with a cervical length of ≤25mm. Their vaginal microbial composition was compared to 71 women who had a cervical length of >25mm. None of the 71 women with a normal cervical length had a cervical cerclage in situ at the time of assessment. The gestation at delivery was significantly earlier in the group with cervical shortening, median gestation 37^{+2} weeks with cervical shortening, vs 39^{+2} weeks with normal cervical length ($p<0.00001$) (Table 5.3).

Table 5.3 Clinical and demographical characteristics of study participants with cervical shortening and with normal cervical length, sampled at 12-16 and 20-24 weeks gestation

	12-16 weeks		
	Cervical shortening ≤25mm	Normal cervical length >25mm	p value
N (%)	15 (14%)	96 (86%)	
Gestation of sampling (weeks) median and IQ range	14^{+3} (13^{+4} - 15^{+0})	14^{+6} (13^{+4} - 15^{+3})	p=0.14
Gestation at delivery (weeks) median and IQ range	38^{+1} (31^{+0} - 39^{+0})	38^{+6} (36^{+5} - 40^{+1})	p<0.01 **
Early preterm </=33+6 weeks	6 (40%)	13 (13%)	Preterm vs Term p<0.09
Late preterm >/=34+0 weeks	1 (7%)	12 (13%)	
Term >37 weeks	8 (53%)	71 (74%)	
Cervical length mm (median and IQ range)	23 (19-25)	34 (31-39)	p<0.0001 ****
	20-24 weeks		
	Cervical shortening ≤25mm	Normal cervical length >25mm	p value
N (%)	18 (20%)	71 (80%)	
Gestation of sampling (weeks) median and IQ range	21^{+3} (20^{+3} - 22^{+1})	21^{+2} (20^{+2} - 22^{+5})	p=0.45
Gestation at delivery (weeks) median and IQ range	37^{+2} (30^{+2} - 38^{+3})	39^{+2} (37^{+3} - 40^{+3})	p<0.0001 ***
Early preterm </=33+6 weeks	5 (28%)	8 (11%)	Preterm vs Term p<0.11
Late preterm >/=34+0 weeks	2 (11%)	7 (10%)	
Term >37 weeks	11 (61%)	56 (79%)	
Cervical length mm (median and IQ range)	21 (19-25)	35 (32-37)	p<0.0001 ****

Data presented as median (interquartile range (IQ)) or number (%). P values: Mann Whitney U test for comparisons between two groups or Fisher's exact test for proportional data.

At 12^{+0} - 16^{+6} weeks and 20^{+0} - 24^{+6} weeks, there was no overall difference in the proportion of samples that were *Lactobacillus* dominant or *Lactobacillus* deplete according to cervical shortening ≤25mm, and normal cervical length >25mm, Figure 5.25 A and D. However, there was an over-representation of VMG 3 *L. iners* in the women with cervical shortening, whilst the most common VMG in women with a normal cervical length was *VMG 1 L. crispatus* at both sampling timepoints, Figure 5.25 B and E. Comparing the mean proportion of sequence reads with *L. iners* in cervico-vaginal swabs showed a significantly higher proportion in women with cervical length ≤25mm when sampling was performed at 12^{+0} - 16^{+6} weeks. The mean proportion of sequence reads with *L. iners* was 42% in those with cervical shortening and 21% in those with normal cervical length (p<0.05), Figure 5.25 C. At 20^{+0} - 24^{+6}, the mean proportion of sequence reads with *L. iners* was 34% in those with cervical shortening and 21% in those with normal cervical length (p=0.08).

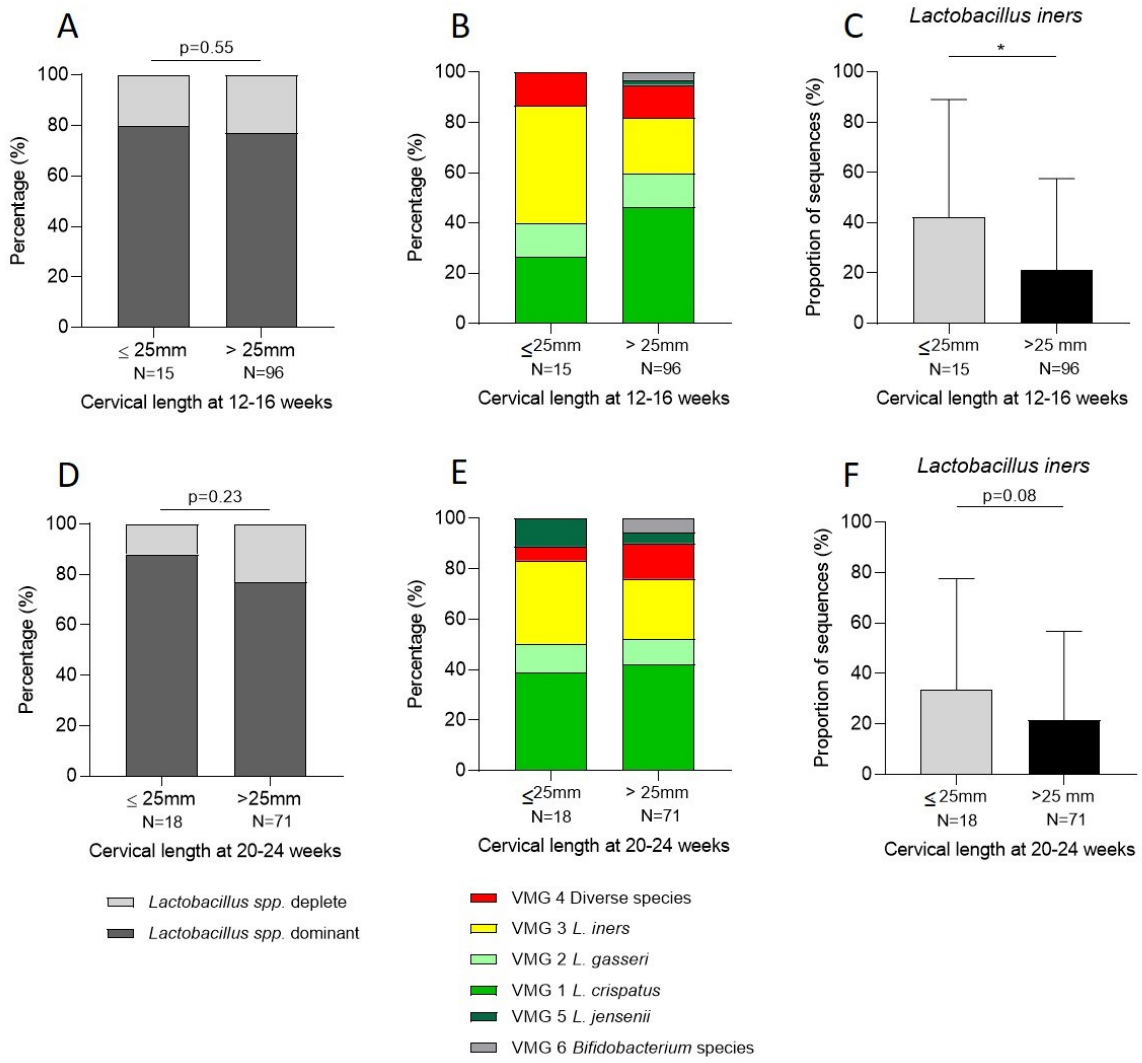


Figure 5.25 Vaginal microbial composition according to cervical length at 12-16 weeks and 20-24 weeks
Lactobacillus iners was associated with cervical shortening at 12-16 weeks gestation (A-C). There was a non-statistical increase in the proportion of *Lactobacillus iners* sequences with cervical shortening at 20-24 weeks (D-F). Statistical analyses were by Fisher's exact test and unpaired t-test, * p<0.05. The results are expressed as mean and standard deviation. At 12-16 weeks: ≤25mm N=15, >25mm N=96. At 20-24 weeks: ≤25mm N=18, >25mm N=71.

The local immune milieu in VMG 3 *L. iners* and VMG 1 *L. crispatus* samples in women with cervical shortening (≤25mm) and normal cervical length (>25mm) were compared independent of the sampling timepoint.

There was no difference in IL-8, IL-1β, IL-6 and IL-10 in women with VMG 1 *L. crispatus* with cervical shortening or normal cervical length, suggesting an alternative mechanism to microbial driven inflammation in cervical shortening in women with VMG 1 *L. crispatus*. In women with normal cervical length >25mm, there was significantly higher IL-8 in VMG 3 *L. iners* compared to VMG 1 *L. crispatus* (p=0.009), Figure 5.26 A. This possibly reflects a degree of regulated inflammation, without the collateral tissue injury seen in cervical remodelling and shortening. In women with cervical shortening, ≤25mm, there was also significantly higher IL-8 in VMG 3 *L. iners* compared to VMG 1 *L. crispatus* (p=0.004), Figure 5.26 A. In women with VMG 3 *L. iners*, IL-8, IL-6 and IL-1β were all significantly higher in women with cervical shortening, compared to women with normal cervical length, (p=0.009, p=0.04 and p=0.04 respectively), Figure 5.26 A – C. This may reflect a dysregulated inflammatory response leading to cervical remodelling and cervical shortening.

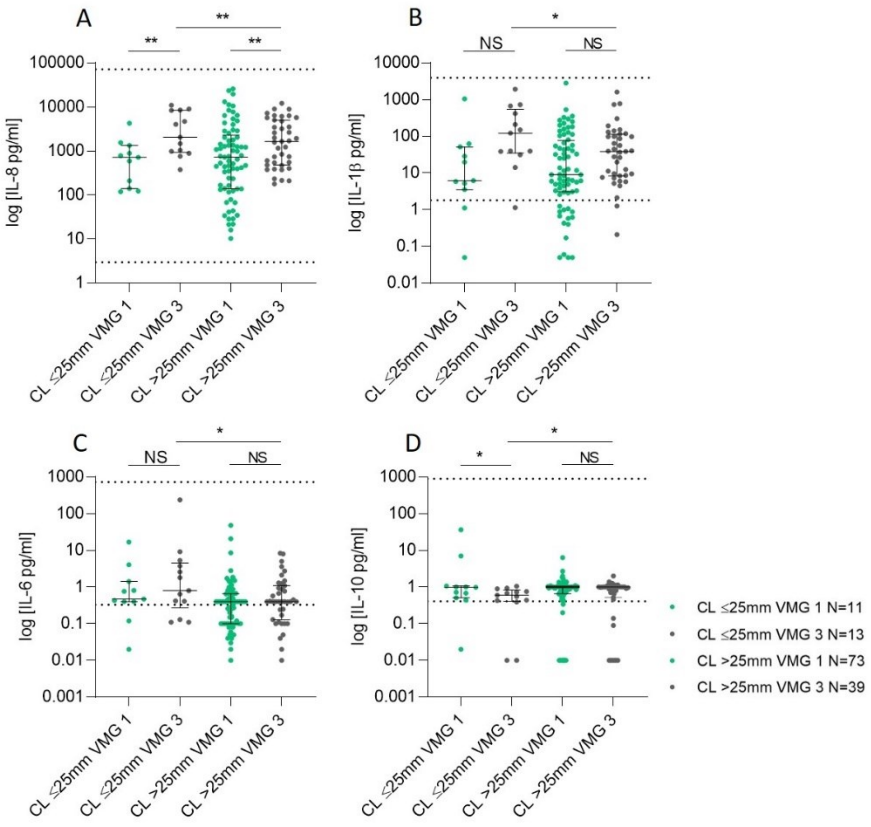


Figure 5.26 Cervico-vaginal fluid cytokines in VMG 1 *L. crispatus* and VMG 3 *L. iners* in women with and without cervical shortening

In women with and without cervical shortening, IL-8 concentrations were significantly higher in VMG 3 *L. iners* compared to VMG 1 *L. crispatus* (A). There was a non-significant trend for higher IL-1β in those with VMG 3 *L. iners* in both cervical shortening and normal cervical length (B). IL-6 concentrations were similar in VMG 3 *L. iners* and VMG 1 *L. crispatus*, irrespective of cervical length (C). IL-8, IL-1β and IL-6 concentrations were also significantly higher in VMG 3 *L. iners* in those with cervical shortening, compared to those with normal cervical length, whilst IL-10 was significantly lower. Statistical analysis was by Mann Whitney U test, *p<0.05, **p<0.01, NS = non-significant. CL=cervical length. The results are expressed as median and interquartile range. CL ≤25mm VMG 1 N=11, CL ≤25mm VMG 3 N=13, CL >25mm VMG 1 N=73, CL >25mm VMG 3 N=39.

There was no difference in C5, C5a, C3b and MBL in women with VMG 1 *L. crispatus* with cervical shortening or normal cervical length, suggesting that non-microbial driven, non-inflammation driven cervical shortening occurs in women with VMG 1 *L. crispatus*. In women with a normal cervical length, C5, C5a, C3b and MBL were similar in VMG 3 *L. iners* and VMG 1 *L. crispatus,* Figure 5.27 A – D. In women with cervical shortening, C3b was significantly higher in VMG 3 *L. iners* compared to VMG 1 *L. crispatus* (p=0.04), Figure 5.27 C. In women with VMG 3 *L. iners*, women with cervical shortening had significantly higher C5, C5a and C3b compared to women with normal cervical length (p=0.02, p=0.01 and p=0.004 respectively), Figure 5.26 A - C. This may also reflect a dysregulated inflammatory response leading to cervical remodelling and cervical shortening.

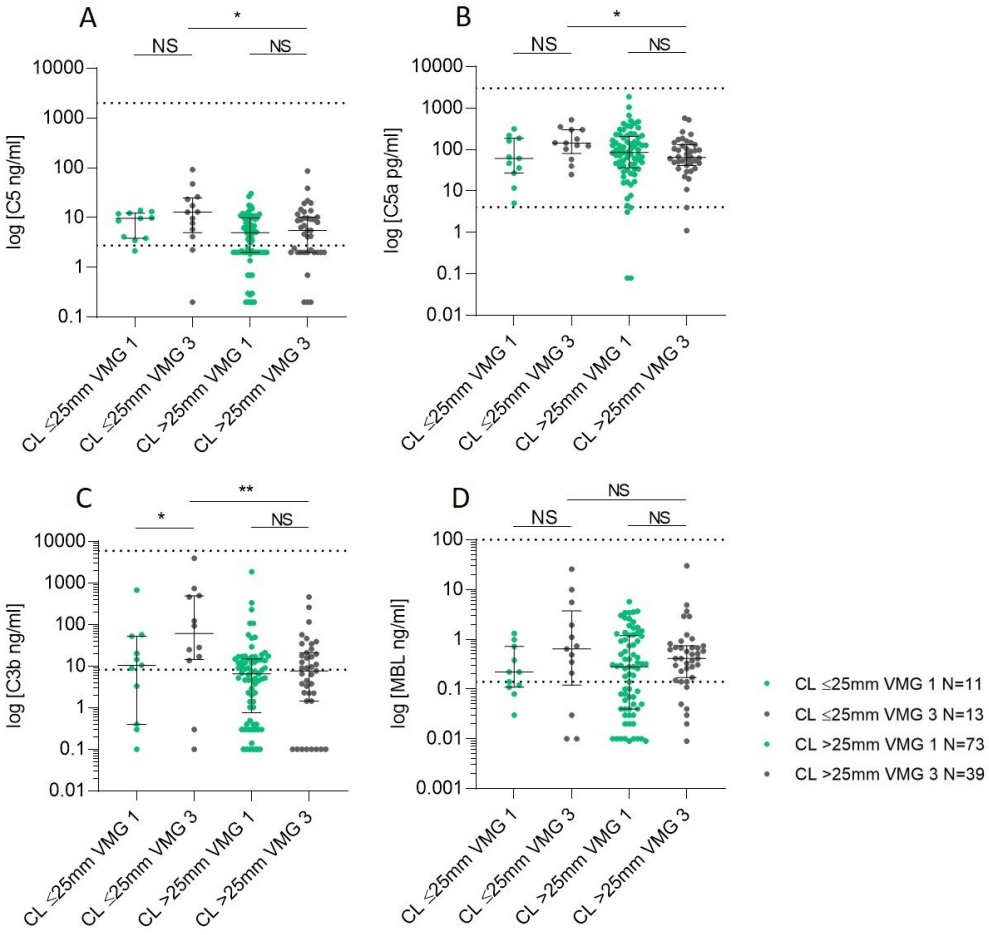


Figure 5.27 Cervico-vaginal fluid complement in VMG 1 *L. crispatus* and VMG 3 *L. iners* in women with and without cervical shortening

In women with and without cervical shortening concentrations of C5, C5a and MBL were similar in women with VMG 1 *L. crispatus* and VMG 3 *L. iners* (A, B and D). Only C3b concentrations were significantly higher in VMG 3 *L. iners* compared to VMG 1 *L. crispatus*, but only in the women with cervical shortening (C). In women with cervical shortening with VMG 3 *L. iners*, C5, C5a and C3b concentrations were significantly higher than women with normal cervical length with VMG 3 *L. iners* (A-C). Statistical analysis was by Mann Whitney U test, *p<0.05, **p<0.01, NS = non-significant. CL=cervical length. The results are expressed as median and interquartile range. CL ≤25mm VMG 1 N=11, CL ≤25mm VMG 3 N=13, CL >25mm VMG 1 N=73, CL >25mm VMG 3 N=39.

There was no difference in IgG1-4, IgA and IgM in women with VMG 1 *L. crispatus* with cervical shortening or normal cervical length, suggesting non-microbial driven inflammation is seen with cervical shortening in women with VMG 1 *L. crispatus*. In women with normal cervical length, IgG3, IgA and IgM were significantly higher in women with VMG 3 *L. iners* compared to VMG 1 *L. crispatus* (p=0.02, p=0.04 and p=0.01 respectively), Figure 5.28 C, E and F. This may reflect regulated inflammatory response which does not result in cervical remodelling and shortening. In women with cervical shortening, IgM was significantly higher in women with VMG 3 *L. iners* compared to VMG 1 *L. crispatus* (p=0.004), Figure 5.28 F. In women with VMG 3 *L. iners*, IgG2, IgG4 and IgM were significantly higher in women with cervical shortening, compared to normal cervical length, (p=0.04, p=0.05 and p=0.007 respectively), Figure 5.28 B, D and F. It is likely that IgG2, IgG4 and IgM binding to *L. iners* is driving the classical pathway of complement activation and inflammation.

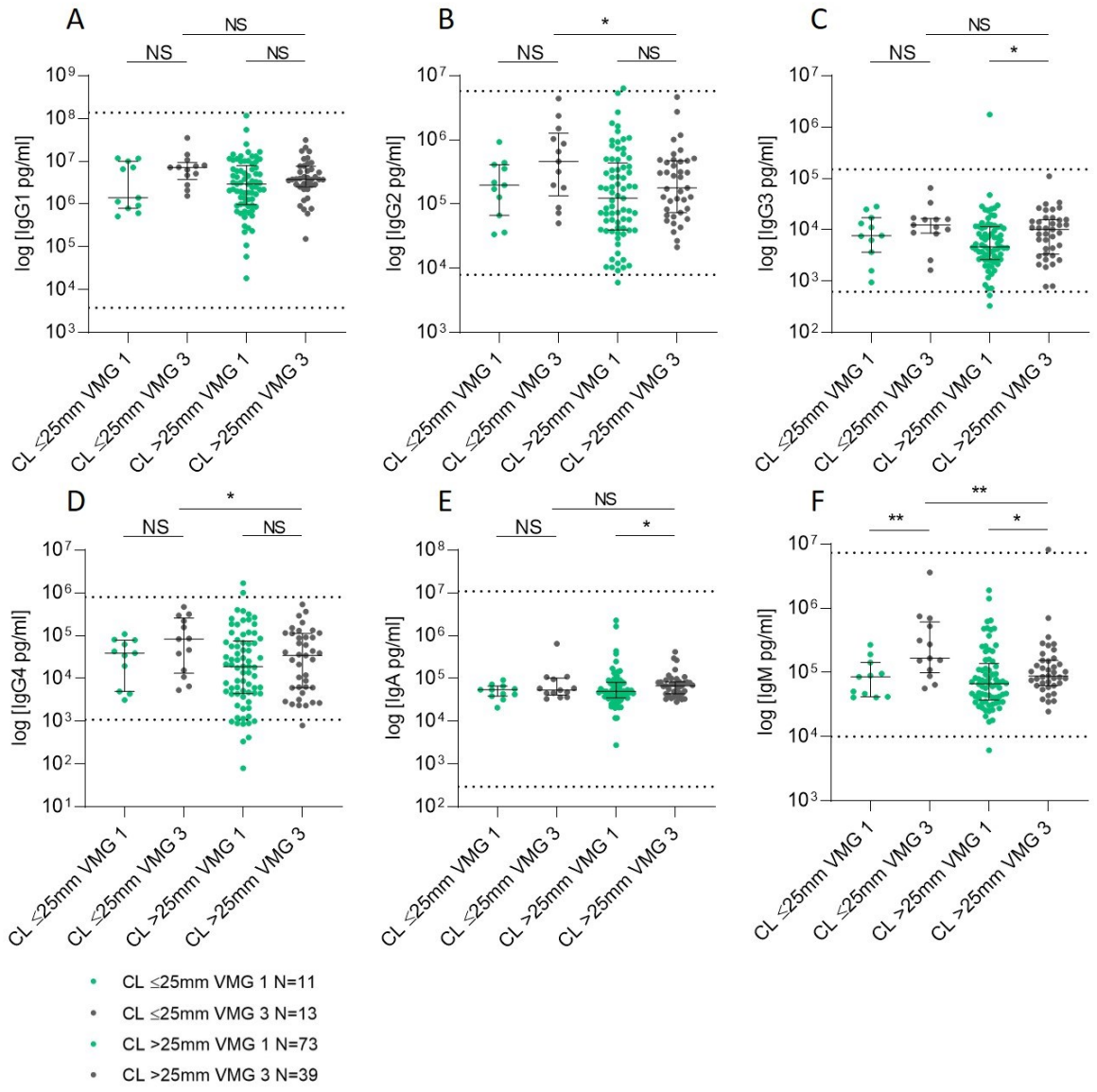


Figure 5.28 Cervico-vaginal fluid immunoglobulins in VMG 1 *L. crispatus* and VMG 3 *L. iners* in women with and without cervical shortening

In women with and without cervical shortening concentrations of IgG1, IgG2 and IgG4 were similar in women with VMG 1 *L. crispatus* and VMG 3 *L. iners* (A, B and D). However IgG3, IgA and IgM concentrations were significantly higher in VMG 3 *L. iners* compared to *L. crispatus*, but only in those with normal cervical length (C, E and F). Concentrations of IgG2, Ig4 and IgM were significantly higher in women with VMG 3 *L. iners* with cervical shortening, compared to women with VMG 3 *L. iners* with normal cervical length (B, D and F). Statistical analysis was by Mann Whitney U test, *p<0.05, **p<0.01, NS = non-significant. CL=cervical length. The results are expressed as median and interquartile range. CL ≤25mm VMG 1 N=11, CL ≤25mm VMG 3 N=13, CL >25mm VMG 1 N=73, CL >25mm VMG 3 N=39.

5.5.6 Vaginal microbial composition, diversity and richness pre and post cerclage

Of the 133 women in the study population, 59 (44%) had a cervical cerclage during their pregnancy. 19 (32%) were excluded from the following analysis because 17 did not have a vaginal swab collected prior to cervical cerclage insertion and 2 did not have a vaginal swab collected after cervical cerclage insertion.

There were 40 women who had vaginal swabs collected pre and post cervical cerclage allowing the comparison of vaginal microbial composition, diversity and richness before and after the clinical intervention. Clinical and demographical information for this cohort is provided in Chapter 4, Table 4.8.

There was a greater proportion of multiparous compared to nulliparous women (65% vs 35%). Sampling was performed on average 7 days prior to cervical cerclage and 50 days following cervical cerclage. The majority of women delivered at term gestation N=26 (65%), whilst the remaining 14 delivered preterm (35%). The number of history and ultrasound indicated cerclages were roughly similar, 19 and 21 respectively. 25 women received the monofilament Nylon cerclage, 14 received the braided Mersilene cerclage and there was one study participant where the cerclage material was not recorded. There was no difference in the cerclage material according to the indication for the cervical cerclage, (Fisher's test p=0.58).

The median gestation of delivery for women who had a Mersilene cerclage was 36^{+0} weeks, whilst it was 38^{+1} for women who had a Nylon cerclage. Of women who received Mersilene (N=14), the preterm birth rate was 50%, and of women who received Nylon (N=25), the preterm birth rate was 24%, which was statistically significant, (Fisher's exact test $p<0.05$).

There was a significant increase in the vaginal microbial diversity as measured by the inverse Simpson index following cervical cerclage, (median pre cerclage 1.03, vs median post cerclage 1.13), (p=0.02), but no significant difference in the species richness (p>0.05), Figure 5.29 A and B. The microbial composition according to the proportion of *Lactobacillus* dominant and *Lactobacillus* deplete samples and by vaginal microbial groups did not show any significant changes pre and post cervical cerclage, Figure 5.29 C and D.

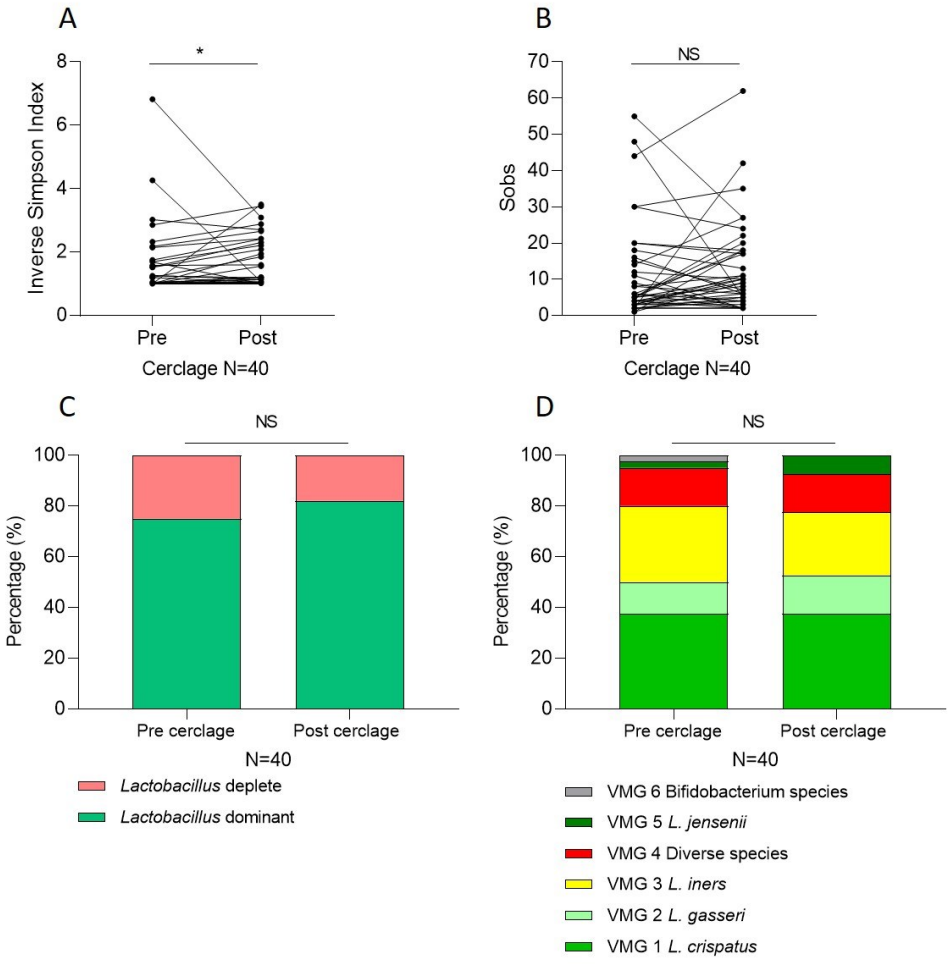


Figure 5.29 The vaginal microbial composition, diversity and richness pre and post cervical cerclage
The inverse Simpson index increased significantly after cervical cerclage (A). Overall, there was no significant change in species observed following cervical cerclage (B). The microbial composition by vaginal microbial groups was stable pre and post cervical cerclage (C and D). Statistical analysis was by Wilcoxon matched-pairs signed rank test comparing diversity and richness. Statistical analysis was by Fisher's exact test to compare microbial composition. N=40 matched samples pre and post cervical cerclage.

In this group of study participants who were sampled before and after cervical cerclage, the microbial composition before and after Mersilene and Nylon cerclage were compared. There was no difference in the alpha diversity and species richness according to the cerclage material. In section 4.5.7 in Chapter 4, there was an augmented local innate and adaptive immune response following Mersilene compared to Nylon cerclage. In women who had a Mersilene cerclage and who delivered preterm, there was further augmentation of the innate immune response, supporting a dysregulated amplification of complement activation and cytokine production. However, there was no significant change in microbial composition post Mersilene or Nylon cerclage, Figure 5.30. Notably, due to the study design with three sampling timepoints across pregnancy, the median number of days following cervical cerclage was 50 days.

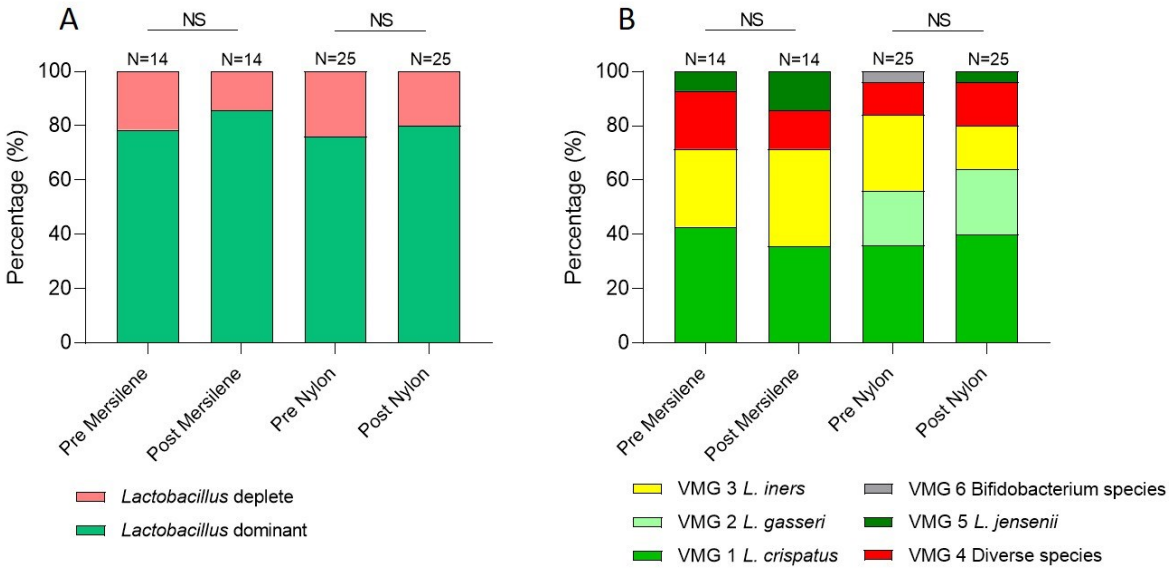


Figure 5.30 The vaginal microbial composition was largely stable pre and post Mersilene or Nylon cerclage
The overall vaginal microbial composition by *Lactobacillus* dominance and *Lactobacillus* depletion (A) and by vaginal microbial groups (B) was unchanged following Mersilene and Nylon cerclage (B) Statistical analysis was by Fisher's exact test. Mersilene cerclage N=14 and Nylon cerclage N=25.

5.5.7 Cervico-vaginal immune response and the vaginal microbial composition in women who deliver preterm and at term

The vaginal microbial composition was compared between women who had early (<34^{+0} weeks) and late preterm (34^{+1} - 36^{+6} weeks) deliveries with women who had uncomplicated term deliveries. At the 12^{+0} - 16^{+6} week timepoint of sampling, dominance of *Lactobacillus* species, (defined as >75% *Lactobacillus* species), occurred in similar proportions of women experiencing early preterm delivery (15/20, 75%), late preterm delivery (10/15, 67%) or uncomplicated term delivery (33/52, 75%). The same pattern was observed at the 20^{+0} - 24^{+6} and 30^{+0} - 34^{+6} week sampling timepoints, Figure 5.31 A-C.

By vaginal microbial groups, there a greater proportion of women with VMG 3 *L. iners* in the early preterm birth group, compared to the late preterm birth group, although this did not reach statistical significance. Additionally, the vaginal microbial composition was not significantly different in women who subsequently delivered at term Figure 5.31 D-F.

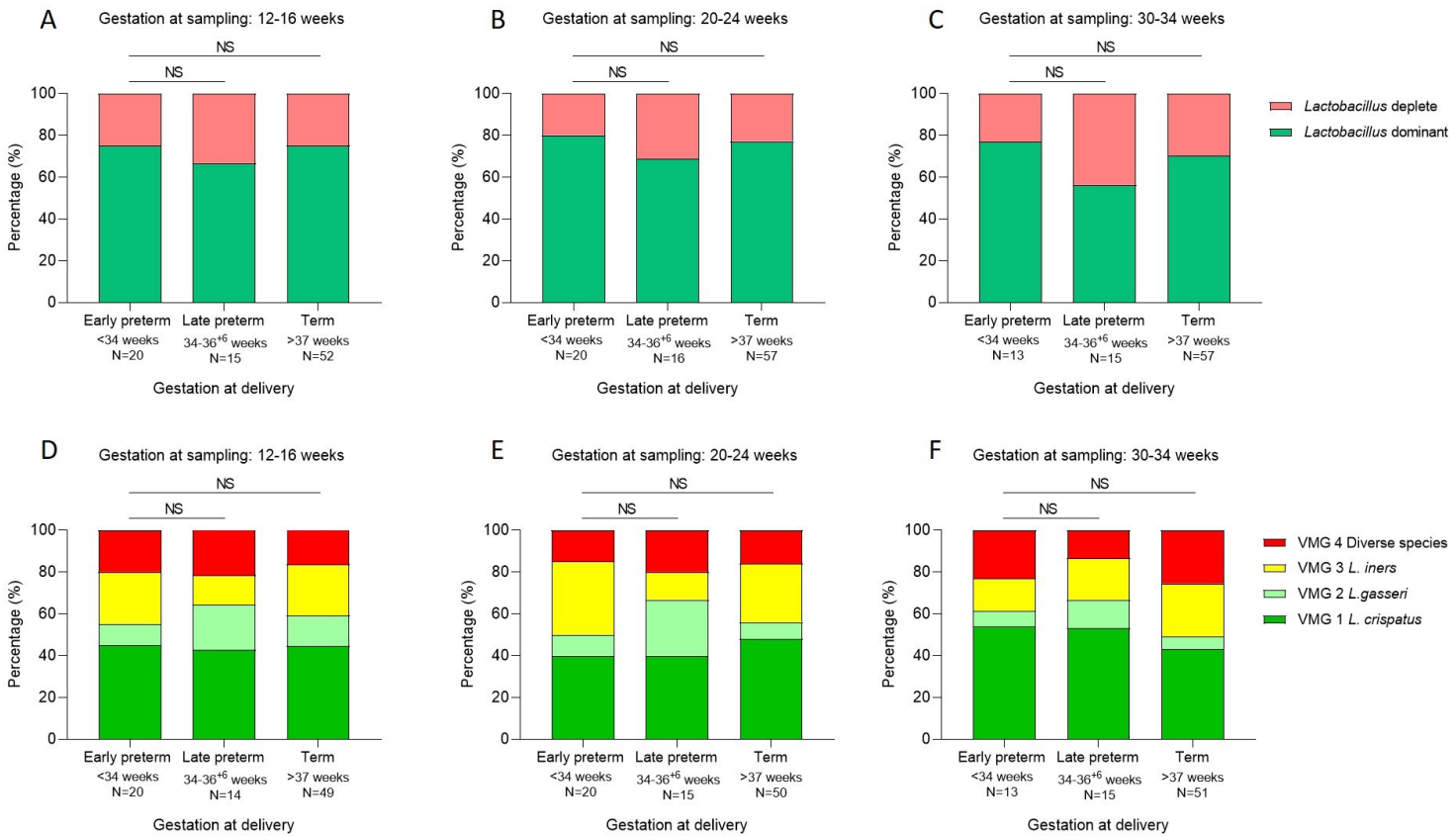


Figure 5.31 Vaginal microbial composition of early preterm, late preterm and term deliveries

Comparison of vaginal microbial composition by *Lactobacillus* dominance and *Lactobacillus* depletion (A-C) and vaginal microbial groups (VMG) (D-F) according to early preterm delivery (<34 weeks gestation), late preterm delivery (34-36^{+6} weeks gestation) and term delivery (>37 weeks gestation) did not show any significant differences, the three sampling time points 12-16, 20-24 and 30-34 weeks. Statistical analysis was by Fisher's exact test. According to *Lactobacillus* dominance and *Lactobacillus* depletion, at 12-16 weeks: early preterm birth N=20, late preterm birth N=15, term birth N=52, at 20-24 weeks: early preterm birth N=20, late preterm birth N=16, term birth N=57, and at 30-34 weeks: early preterm birth N=13, late preterm birth N=15, term birth N=57 (A-C). According to vaginal microbial group classification, at 12-16 weeks: early preterm birth N=20, late preterm birth N=14, term birth N=49, at 20-24 weeks: early preterm birth N=20, late preterm birth N=15, term birth N=50, and at 30-34 weeks: early preterm birth N=13, late preterm birth N=15, term birth N=51 (D-F).

So far, I have presented evidence to support microbial driven activation of the innate and adaptive immune response. However, we recognise that not all women who are *Lactobacillus* deplete, or have a predominance of VMG 3 *L. iners* and VMG 4 Diverse species, deliver preterm, and not all women who are classified as VMG 1 *L. crispatus* deliver at term. Elements of the local immune response to the microbial environment will be compared between women who deliver at preterm and those who deliver at term with the aim of increasing our understanding of the role of the immune response in microbial driven preterm birth.

Analysis of microbial-immune driven preterm birth at genera level

As demonstrated earlier, IL-8, IL-1β, IL-6, MBL, C3b, C5, IgM, and IgG1-4, expression were significantly increased in a *Lactobacillus* deplete environment. This next section explores if there are any distinguishing features in the immune response to *Lactobacillus* depletion in women who deliver at preterm and term gestations.

At the 12^{+0} - 16^{+6} week sampling timepoint, there was subtle increase in IL-8, IL-1β and IL-6 in *Lactobacillus* depletion compared to *Lactobacillus* dominance in both preterm and term deliveries, Figure 5.33 A, C, E. However, by the 20^{+0} - 24^{+6} week sampling point IL-8, IL-1β and IL-6 were significantly higher in *Lactobacillus* depletion compared to *Lactobacillus* dominance in women who delivered preterm (p=0.04, p=0.0002 and p=0.03 respectively), Figure 5.32 B, D and F. IL-1β was also significantly higher in *Lactobacillus* depletion compared to Lactobacillus dominance in term deliveries (p=0.004) at 20^{+0} - 24^{+6} weeks. Furthermore, median concentrations of IL-6 (p=0.02) and IL-1β (p=0.03), were higher in women who were *Lactobacillus* deplete and delivered preterm compared to women who were *Lactobacillus* deplete and delivered at term, supporting a dysregulated innate immune response in the preterm group, Figure 5.32 D, F. There were no significant differences in IL-8, IL-1β or IL-6 in *Lactobacillus* dominance in preterm compared to *Lactobacillus* dominance in term deliveries (p>0.05).

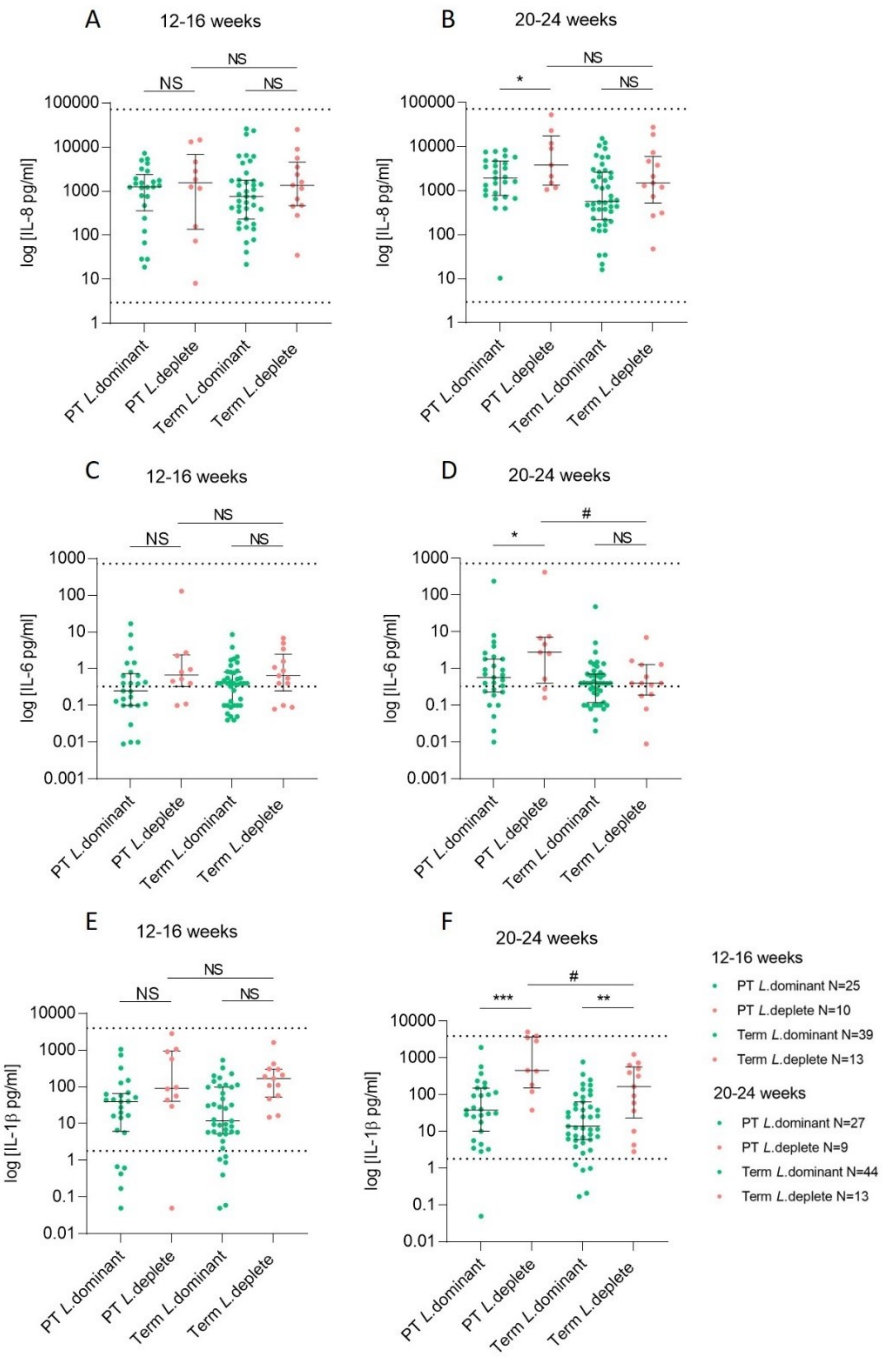


Figure 5.32 Cervico-vaginal fluid cytokines in relation to *Lactobacillus* dominance and depletion in preterm and term deliveries

IL-8, IL-1β and IL-6 concentrations were significantly higher in *Lactobacillus* deplete samples from women who deliver preterm, at 20-24 weeks of sampling (B, D, F). There was a non-significant increase at 12-16 weeks of sampling. (A, C, E). *Lactobacillus* depletion in women who had preterm deliveries was associated with significantly higher IL-6 and IL-1β compared to *Lactobacillus* depletion in women who had term deliveries (D and F). There was no significant difference in IL-8, IL-1β or IL-6 in *Lactobacillus* dominance in preterm compared to term deliveries. Statistical analysis comparing concentrations in *Lactobacillus* dominance and *Lactobacillus* depletion (*) and comparing concentrations in preterm and term *Lactobacillus* depletion (#) was by the Mann Whitney test. *p<0.05, # p<0.05, NS = non significant. The results are expressed as median and interquartile range. At 12-16 weeks, PT *L.* dominant N=25, PT *L.* deplete N=10, Term *L.* dominant N=39 and Term *L.* deplete N=13. At 20-24 weeks, PT *L.* dominant N=27, PT *L.* deplete N=9, Term *L.* dominant N=44 and Term *L.* deplete N=13.

A greater inflammatory response to *Lactobacillus* depletion could be seen at the later 20^{+0} - 24^{+6} week timepoint, rather than the 12^{+0} -16^{+6} week timepoint when assessing the complement analytes. At the 12^{+0} -16^{+6} week sampling timepoint, there was an increase in the median concentration of C5, C5a MBL and C3b in *Lactobacillus* depletion compared to *Lactobacillus* dominance in both preterm and term deliveries, although this did not reach statistical significance ($p>0.05$), Figure 5.33 A, C, E, G. However, at the 20^{+0} - 24^{+6} week timepoint, C5, MBL and C3b were significantly higher in *Lactobacillus* depletion compared to *Lactobacillus* dominance in the women who had preterm deliveries, ($p<0.0001$, $p=0.04$ and $p<0.0001$ respectively), Figure 5.33 B, F, H, but not in the term groups, apart from C3b ($p<0.0001$). Highest levels of C5, C5a, MBL and C3b were seen in women who delivered preterm and who were *Lactobacillus* deplete, Figure 5.33 B, D F, H. C5, MBL, and C3b concentrations where significantly higher, in women who were *Lactobacillus* deplete and delivered preterm compared to term ($p=0.002$, $p=0.02$ and $p=0.01$ respectively), supporting the concept that there is dysregulated complement activation in women who deliver preterm.

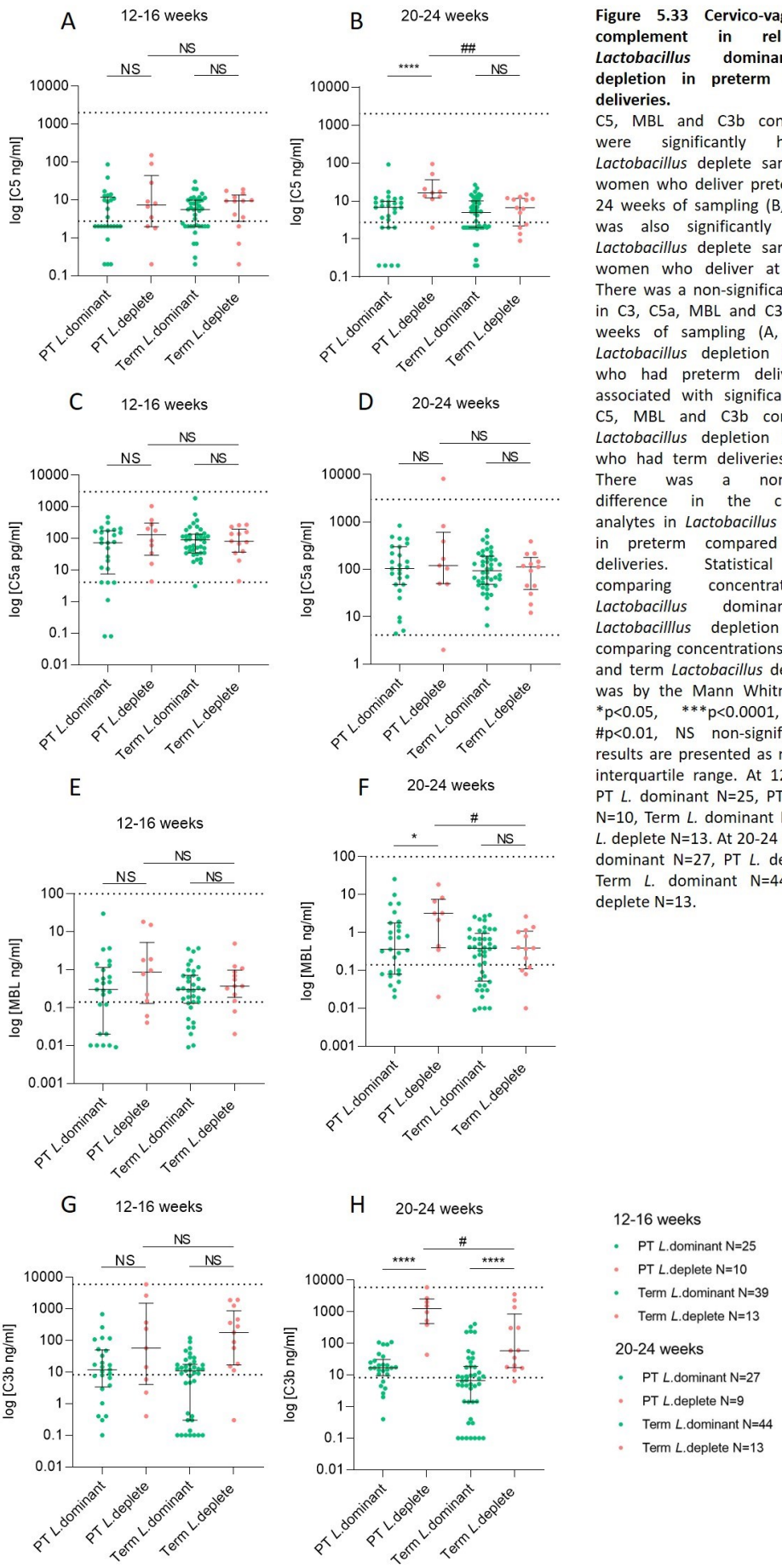


Figure 5.33 Cervico-vaginal fluid complement in relation to *Lactobacillus* dominance and depletion in preterm and term deliveries.

C5, MBL and C3b concentrations were significantly higher in *Lactobacillus* deplete samples from women who deliver preterm, at 20-24 weeks of sampling (B, F, H). C3b was also significantly higher in *Lactobacillus* deplete samples from women who deliver at term (H). There was a non-significant increase in C3, C5a, MBL and C3b at 12-16 weeks of sampling (A, C, E, G). *Lactobacillus* depletion in women who had preterm deliveries was associated with significantly higher C5, MBL and C3b compared to *Lactobacillus* depletion in women who had term deliveries (B, F, H). There was a non-significant difference in the complement analytes in *Lactobacillus* dominance in preterm compared to term deliveries. Statistical analysis comparing concentrations in *Lactobacillus* dominance and *Lactobacilllus* depletion (*) and comparing concentrations in preterm and term *Lactobacillus* depletion (#) was by the Mann Whitney U test. *p<0.05, ***p<0.0001, #p>0.05, #p<0.01, NS non-significant. The results are presented as median and interquartile range. At 12-16 weeks PT *L.* dominant N=25, PT *L.* deplete N=10, Term *L.* dominant N=39, Term *L.* deplete N=13. At 20-24 weeks PT *L.* dominant N=27, PT *L.* deplete N=9, Term *L.* dominant N=44, Term *L.* deplete N=13.

IgG2 and IgG3 concentrations were significantly higher in *Lactobacillus* depletion compared to *Lactobacillus* dominance at the later timepoint of 20^{+0} - 24^{+6} weeks in both women who had preterm and term deliveries ($p<0.05$), Figure 5.34 D and F.

There were no significant differences in IgG1 or IgG4 concentrations between women with *Lactobacillus* depletion and *Lactobacillus* dominance in women who had preterm or term deliveries at 12^{+0} -16^{+6} and 20^{+0} - 24^{+6} weeks, ($p>0.05$) Figure 5.34 A and B, G and H.

There were no significant differences in the concentrations of IgG1-4 in women who had *Lactobacillus* deplete samples between those who delivered preterm compared to at term, implying that the concentration of IgG subtypes do not independently alter outcome.

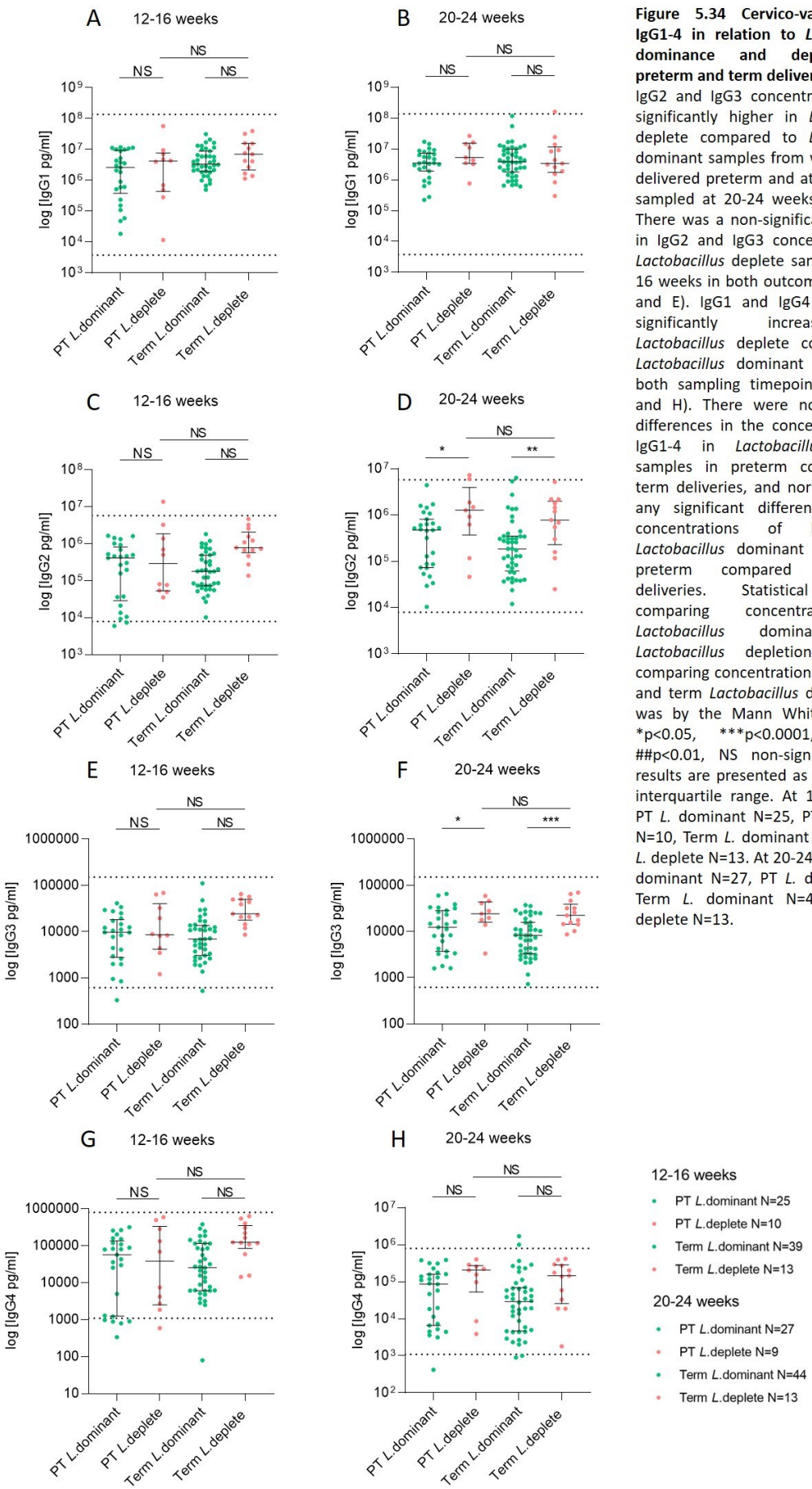


Figure 5.34 Cervico-vaginal fluid IgG1-4 in relation to *Lactobacillus* dominance and depletion in preterm and term deliveries.
IgG2 and IgG3 concentrations were significantly higher in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples from women who delivered preterm and at term when sampled at 20-24 weeks (D and F). There was a non-significant increase in IgG2 and IgG3 concentrations in *Lactobacillus* deplete samples at 12-16 weeks in both outcome groups (C and E). IgG1 and IgG4 were non-significantly increased in *Lactobacillus* deplete compared to *Lactobacillus* dominant samples at both sampling timepoints (A, B, G and H). There were no significant differences in the concentrations of IgG1-4 in *Lactobacillus* deplete samples in preterm compared to term deliveries, and nor were there any significant differences in the concentrations of IgG1-4 in *Lactobacillus* dominant samples in preterm compared to term deliveries. Statistical analysis comparing concentrations in *Lactobacillus* dominance and *Lactobacillus* depletion (*) and comparing concentrations in preterm and term *Lactobacillus* depletion (#) was by the Mann Whitney U test *p<0.05, ***p<0.0001, #p<0.05, ##p<0.01, NS non-significant. The results are presented as median and interquartile range. At 12-16 weeks PT *L.* dominant N=25, PT *L.* deplete N=10, Term *L.* dominant N=39, Term *L.* deplete N=13. At 20-24 weeks PT *L.* dominant N=27, PT *L.* deplete N=9, Term *L.* dominant N=44, Term *L.* deplete N=13.

IgA concentrations were similar in *Lactobacillus* dominant and *Lactobacillus* deplete samples when comparing women who delivered at preterm and term gestations. The concentrations of IgA were also similar when comparing *Lactobacillus* depletion in preterm compared to term deliveries, ($p>0.05$) Figure 5.35 A and B.

In contrast, IgM concentrations were significantly higher in *Lactobacillus* depletion compared to *Lactobacillus* dominance in women who had preterm and term deliveries when sampling occurred at 12^{+0} - 16^{+6} ($p<0.05$), Figure 5.35 C, but only in the women who had preterm deliveries when sampling occurred at 20^{+0} - 24^{+6} weeks ($p<0.01$), Figure 5.35 D. Furthermore, the IgM response distinguished between *Lactobacillus* depletion in preterm and term deliveries, with significantly higher concentrations in *Lactobacillus* depletion in preterm compared to term deliveries, ($p<0.01$), Figure 5.35 D, supporting its role in determining outcome.

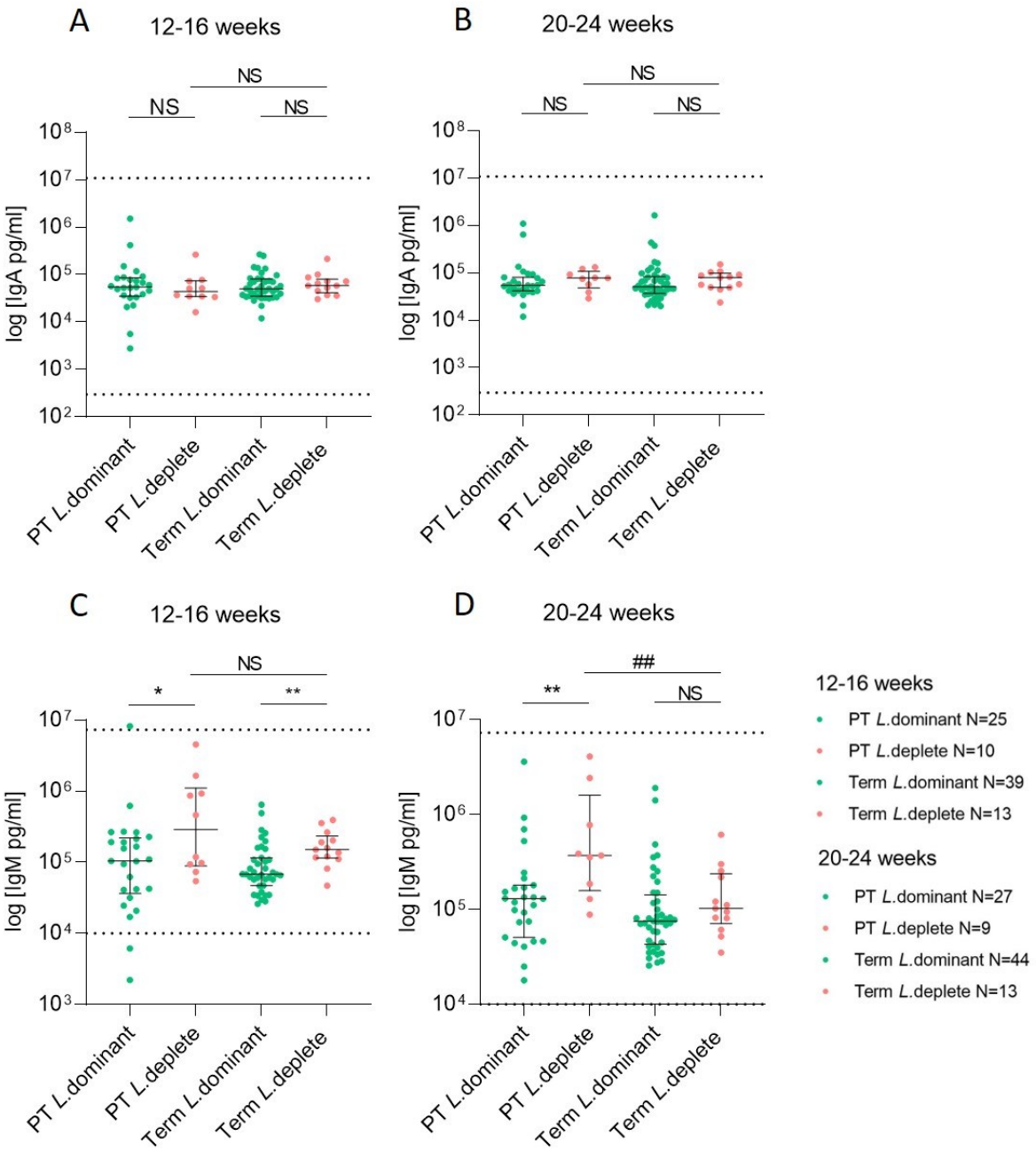


Figure 5.35 Cervico-vaginal fluid IgA and IgM in relation to *Lactobacillus* dominance and depletion in preterm and term deliveries

IgA concentrations were similar in *Lactobacillus* dominance and depletion at 12-6 and 20-24 weeks in preterm and term deliveries (A and B). IgM concentrations were significantly higher in *Lactobacillus* depletion compared to *Lactobacillus* dominance in women who had preterm and term deliveries at both sampling timepoints (C and D). At 20-24 weeks, IgM was also significantly higher in *Lactobacillus* deplete samples in women who had preterm compared to term deliveries (D). There was no difference in IgA or IgM when comparing their concentrations in *Lactobacillus* dominant samples in women who had preterm and term deliveries. Statistical analysis comparing concentrations in *Lactobacillus* dominance and *Lactobacillus* depletion (*) and comparing concentrations in preterm and term *Lactobacillus* depletion (#) was by the Mann Whitney test. *p<0.05, # p<0.05, NS = non significant. The results are presented as median and interquartile range. At 12-16 weeks, PT *L.* dominant N=25, PT *L.* deplete N=10, Term *L.* dominant N=39 and Term *L.* deplete N=13. At 20-24 weeks, PT *L.* dominant N=27, PT *L.* deplete N=9, Term *L.* dominant N=44 and Term *L.* deplete N=13.

Analysis of microbial-immune driven preterm birth at species level

IL-6, IL-1β, C5, C3b, MBL and IgM have been shown to be present in higher concentrations in *Lactobacillus* depletion in women who deliver preterm compared to women who deliver at term gestations. It is plausible that IgM and MBL play a role in microbe recognition, leading to activation of the complement cascade via the classical and lectin pathways, which ultimately lead to C5 and C3b production, immune cell recruitment and thus leading to dysregulated cytokine production. Therefore analyses at the species level was performed in order to distinguish if the immune response differed between women who deliver preterm and at term.

Concentrations of key inflammatory cytokines IL-8, IL-1β and IL-6 were compared in women who had preterm and term deliveries according to the three major vaginal microbial groups in this study population, VMG 1 *L. crispatus*, VMG 3 *L. iners* and VMG 4 Diverse species.

The inflammatory response as measured by IL-8, IL-1β and IL-6 to VMG 1 *L. crispatus* in women who had preterm and term deliveries were similar at both sampling timepoints, 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks, Figure 5.36 A-F, possibly reflecting preterm birth secondary to non-microbial/non-immune mediated pathology.

At 12^{+0} - 16^{+6} weeks there were significantly higher concentrations of IL-8 and IL-1β in women with preterm deliveries with VMG 3 *L. iners* compared to women with term deliveries with VMG 3 *L. iners* (p=0.04 and p=0.05), Figure 5.36 A and E. At 20^{+0} - 24^{+6} weeks, there were significantly higher concentrations of IL-1β and IL-6 in women with preterm deliveries with VMG 3 *L. iners* compared to women with term deliveries with VMG 3 *L. iners* (p=0.01 and p=0.008 respectively), Figure 5.36 D and F.

At the 20^{+0} - 24^{+6} weeks, IL-8, IL-1β and IL-6 were significantly higher in women with preterm deliveries with VMG 4 Diverse species compared to women with term deliveries with VMG 4 Diverse species (p=0.05, p=0.05 and p=0.01 respectively), Figure 5.36 B, D and F.

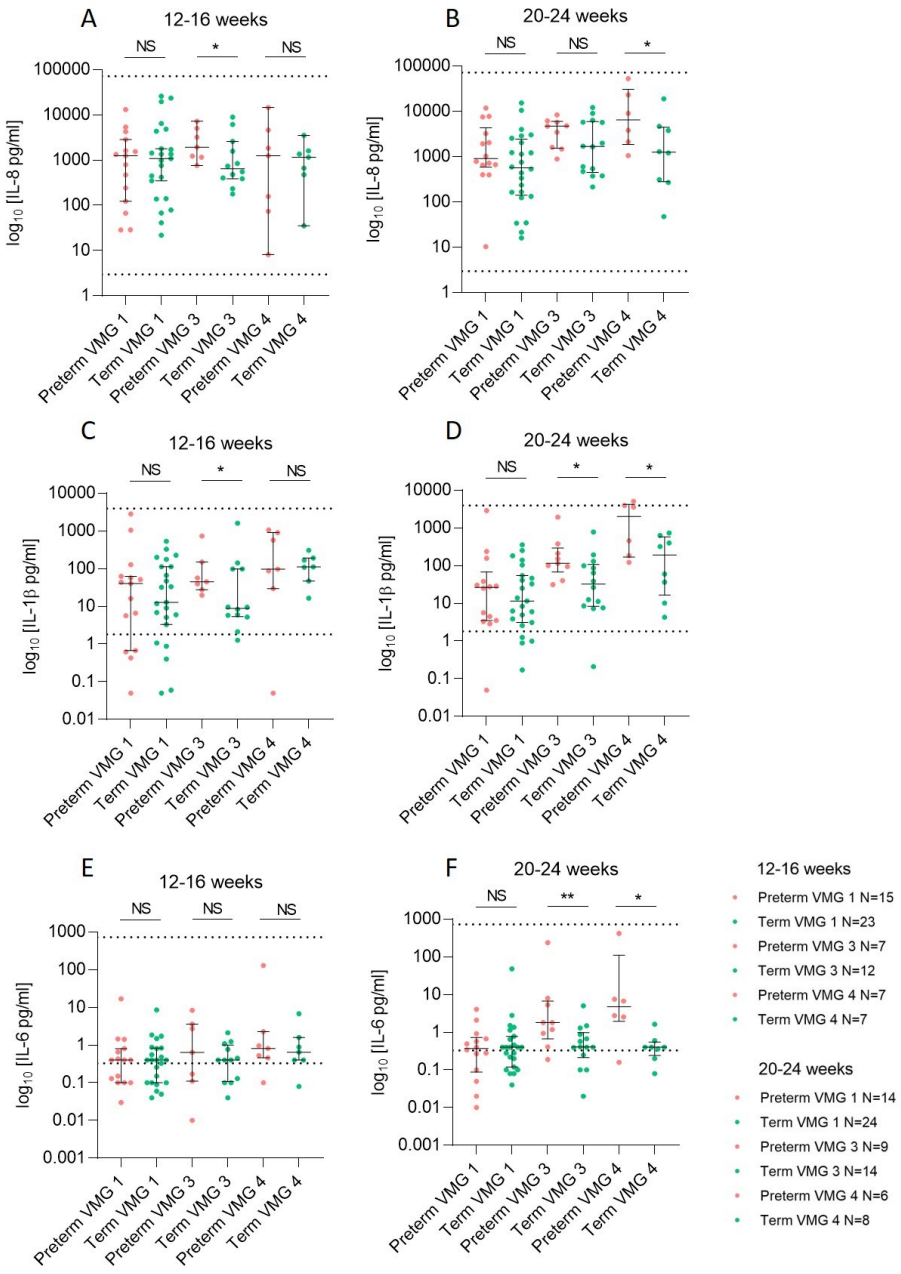


Figure 5.36 Cervico-vaginal fluid cytokines in relation to vaginal microbial groups 1, 3 and 4 in preterm and term deliveries

At the 12-16 week timepoint for VMG 3 *L. iners,* IL-8 and IL-1β were significantly higher in the preterm compared to term group ($p<0.05$) (A and E). At the 20-24 week timepoint for VMG 3 *L. iners,* IL-1β and IL-6 were significantly higher in the preterm compared to the term group ($p<0.05$ and $p<0.01$ respectively) (D and F). At the 20-24 week timepoint for VMG 4 Diverse species, IL-8, IL-1β and IL-6 were significantly higher in the preterm compared to the term group ($p<0.05$) (B, D and F), but no significant difference was observed at the 12-16 week timepoint (A, C and E). There were no differences in the expression of IL-8, IL-1β and IL-6 for VMG 1 *L. crispatus* according to preterm and term delivery at either 12-16 weeks or 20-24 weeks (A-F). Statistical analysis comparing concentrations in VMG 1 *L. crispatus*, VMG 3 *L. iners* and VMG 4 Diverse species in preterm and term deliveries was by the Mann Whitney U test. *$p<0.05$, **$p<0.01$, NS non-significant. The results are presented as median and interquartile range. At 12-16 weeks, Preterm VMG 1 N=15, Term VMG 1 N=23, Preterm VMG 3 N=7, Term VMG 3 N=12, Preterm VMG 4 N=7 and Term VMG 4 N=7. At 20-24 weeks, Preterm VMG 1 N=14, Term VMG 1 N=24, Preterm VMG 3 N=9, Term VMG 3 N=14, Preterm VMG 4 N=6 and Term VMG 4 N=8.

The complement response as measured by C5, C5a, MBL and C3b to VMG 1 *L. crispatus* in women who had preterm and term deliveries were similar at both sampling timepoints, 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks, Figure 5.37 A-H.

At 20^{+0} - 24^{+6} weeks there were significantly higher concentrations of MBL and C3b in women with preterm deliveries with VMG 3 *L. iners* compared to women with term deliveries with VMG 3 *L. iners* ($p=0.05$), Figure 5.37 G and H, with no significant differences for C5 or C5a ($p>0.05$), Figure 5.37 E and F.

At 20^{+0} - 24^{+6} weeks, there were significantly higher concentrations of C5, MBL and C3b in women with preterm deliveries with VMG 4 Diverse species compared to women with term deliveries with VMG 4 Diverse species ($p=0.0007$, $p=0.03$ and $p=0.004$ respectively), Figure 5.38 E, G and H, with no significant difference for C5a ($p>0.05$), Figure 5.37 F.

There were no differences in the expression of C5, C5a, MBL or C3b for VMG 3 *L. iners* or VMG 4 Diverse species according to preterm and term delivery at the first sampling timepoint, 12^{+0} - 16^{+6} weeks, ($p>0.05$) Figure 5.37 A-D.

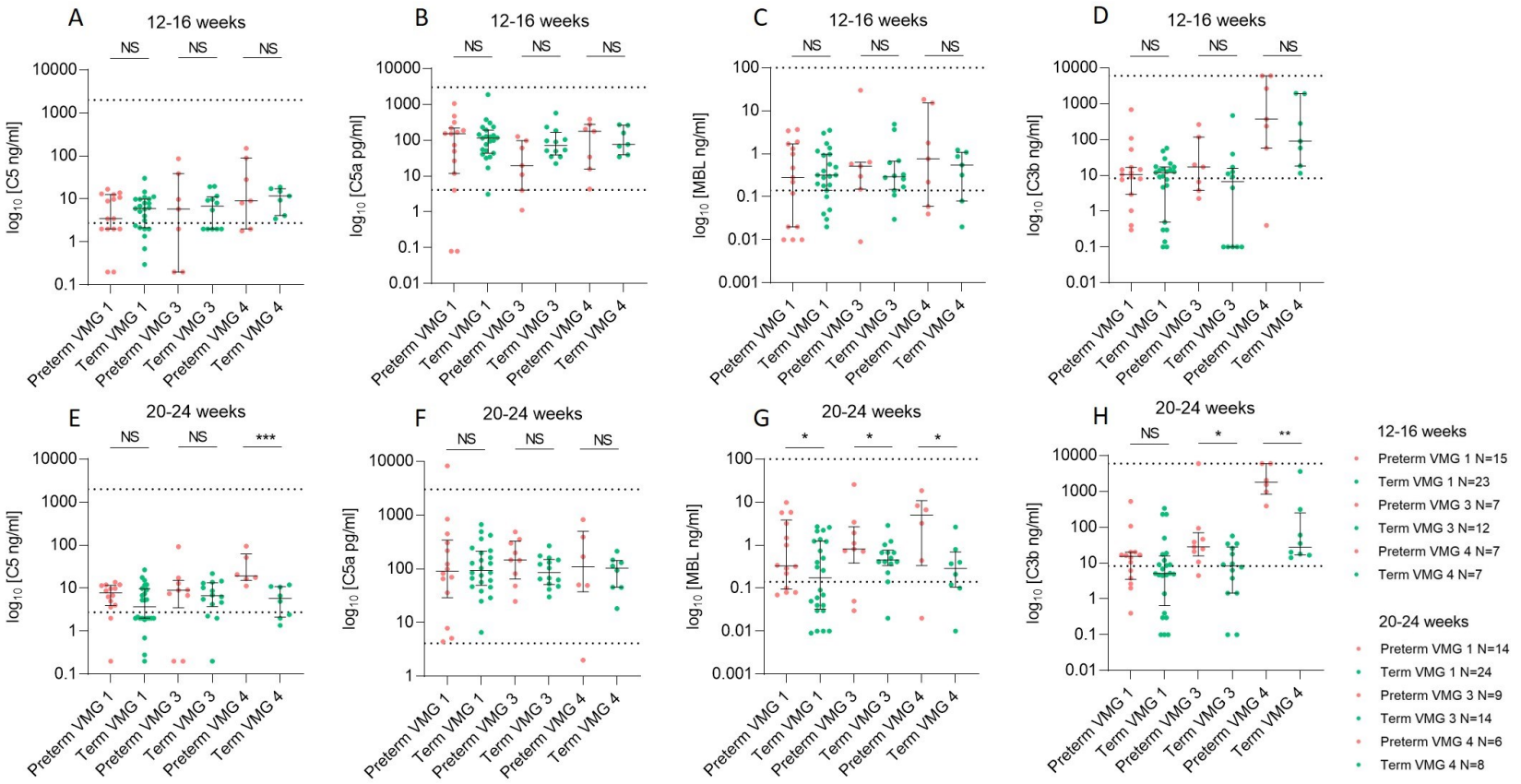


Figure 5.37 Cervico-vaginal fluid complement in relation to vaginal microbial groups 1, 3 and 4 in preterm and term deliveries

At the 20-24 week timepoint for VMG 3 L. iners, MBL and C3b were significantly higher in the preterm compared to the term group ($p<0.05$), (G and H), with no significant difference in C5 or C5a. At the 20-24 week timepoint for VMG 4 Diverse species, C5, MBL and C3b were significantly higher in the preterm compared to the term group ($p<0.001$, $p<0.05$ and $p<0.01$ respectively), (E, G and H), with no significant difference in C5a. There were no differences in the expression of C5, C5a, MBL or C3b for VMG 1 *L. crispatus,* VMG 3 *L. iners or* VMG 4 Diverse species according to preterm and term delivery at 12-16 weeks (A-D). Statistical analysis comparing concentrations in VMG 1 *L. crispatus*, VMG 3 *L. iners* and VMG 4 Diverse species in preterm and term deliveries was by the Mann Whitney U test. *p<0.05, **p<0.01, ***p<0.001, NS non-significant. The results are presented as median and interquartile range. At 12-16 weeks, Preterm VMG 1 N=15, Term VMG 1 N=23, Preterm VMG 3 N=7, Term VMG 3 N=12, Preterm VMG 4 N=7 and Term VMG 4 N=7. At 20-24 weeks, Preterm VMG 1 N=14, Term VMG 1 N=24, Preterm VMG 3 N=9, Term VMG 3 N=14, Preterm VMG 4 N=6 and Term VMG 4 N=8.

IgG1-4 concentrations in VMG 1 *L. crispatus* in women who had preterm and term deliveries were similar at both sampling timepoints, 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks, Figure 5.38 A-H.

There were no differences in the concentrations of IgG1-4 for VMG 3 *L. iners* according to preterm and term delivery at the first sampling timepoint, 12^{+0} - 16^{+6} weeks, however by 20^{+0} - 24^{+6} weeks there were significantly higher concentrations of IgG2, IgG3 and IgG4 in women with preterm deliveries with VMG 3 *L. iners* compared to women with term deliveries with VMG 3 *L. iners* (p=0.02, p=0.03 and p=0.03 respectively), Figure 5.38 F-H.

At both 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks, there were no significant differences in the concentrations of IgG1-4 in women with preterm deliveries with VMG 4 Diverse species compared to women with term deliveries with VMG 4 Diverse species, (p>0.05) Figure 5.38 A-H. Median concentrations of IgG2 and IgG4 were higher in those with VMG 4 Diverse species, but they did not discriminate between preterm and term delivery, Figure 3.38 F and H.

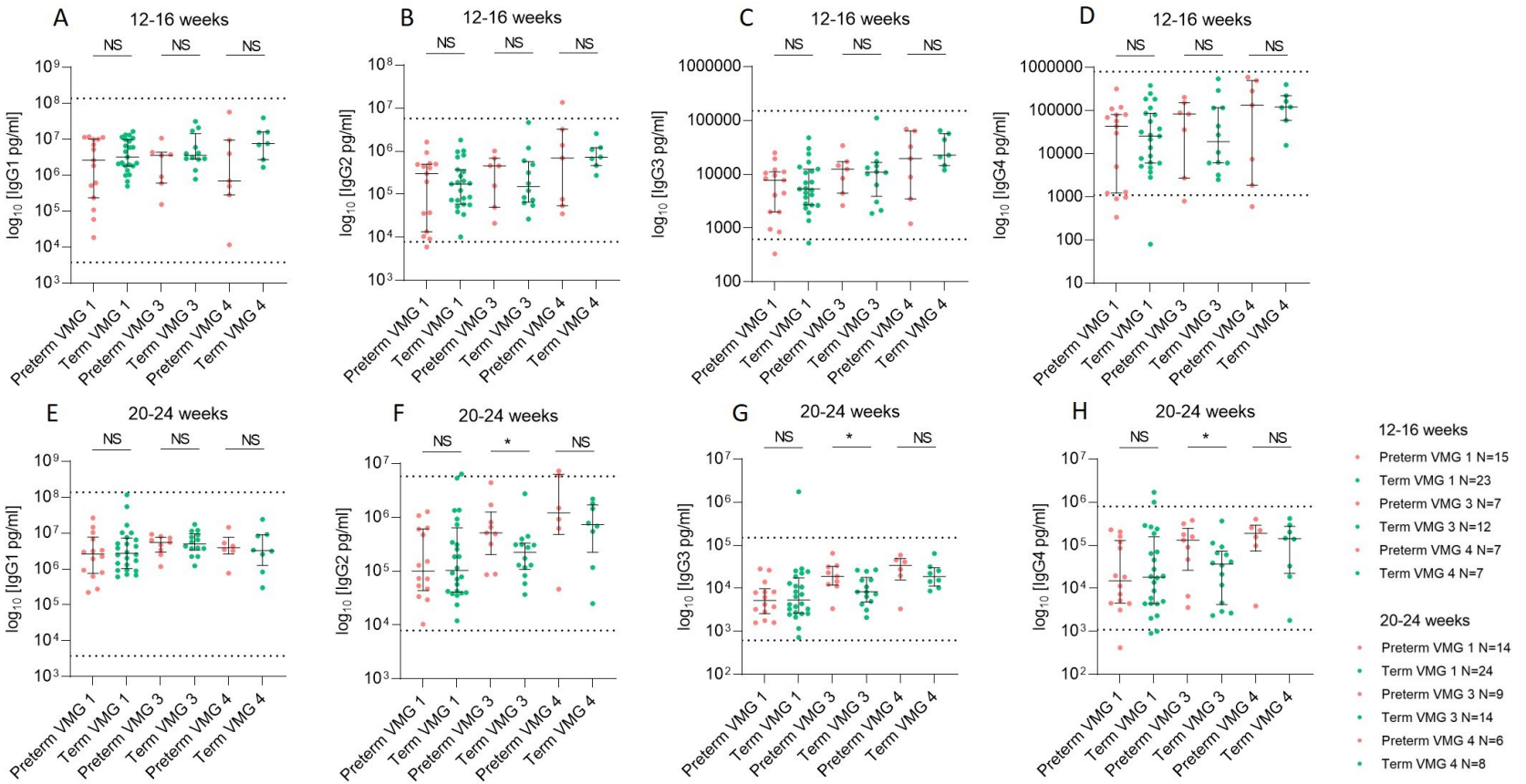


Figure 5.38 Cervico-vaginal fluid IgG1-4 in relation to vaginal microbial groups 1, 3 and 4 in preterm and term deliveries

At the 20-24 week timepoint for VMG 3 *L. iners*, IgG2, IgG3 and IgG4 were significantly higher in the preterm compared to the term group ($p<0.05$), (F and H), with no differences at the 12-16 week timepoint. There were no differences for IgG1-4 for VMG 4 Diverse species according to preterm and term delivery at the 12-16 or 20-24 week timepoints. There were no differences for IgG1-4 for VMG 1 *L. crispatus* according to preterm and term delivery at the 12-16 or 20-24 week timepoints (A-H).

Statistical analysis comparing concentrations in VMG 1 *L. crispatus*, VMG 3 *L. iners* and VMG 4 Diverse species in preterm and term deliveries was by the Mann Whitney U test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, NS non-significant. The results are presented as median and interquartile range. At 12-16 weeks, Preterm VMG 1 N=15, Term VMG 1 N=23, Preterm VMG 3 N=7, Term VMG 3 N=12, Preterm VMG 4 N=7 and Term VMG 4 N=7. At 20-24 weeks, Preterm VMG 1 N=14, Term VMG 1 N=24, Preterm VMG 3 N=9, Term VMG 3 N=14, Preterm VMG 4 N=6 and Term VMG 4 N=8.

IgA and IgM concentrations in VMG 1 *L. crispatus* in women who had preterm and term deliveries were similar at both sampling timepoints, 12^{+0} - 16^{+6} and 20^{+0} - 24^{+6} weeks, Figure 5.39 A-H.

At 20^{+0} - 24^{+6} weeks there were significantly higher concentrations of IgM in women with preterm deliveries with VMG 3 *L. iners* compared to women with term deliveries with VMG 3 *L. iners* (p=0.005), Figure 5.39 D, with a non-significant increase observed at 12^{+0} - 16^{+6} weeks, (p=0.48) Figure 5.39 B. IgA concentrations were similar in women with preterm deliveries with VMG 3 *L. iners* compared to women with term deliveries with VMG 3 *L. iners* at both sampling timepoints, Figure 5.39 A and C.

At 20^{+0} - 24^{+6} weeks there were significantly higher concentrations of IgM in women with preterm deliveries with VMG 4 Diverse species compared to women with term deliveries with VMG 4 Diverse species (p=0.0007), Figure 5.39 D, with a non-significant increase observed at 12^{+0} - 16^{+6} weeks (p=0.16), Figure 5.39 B. IgA concentrations were similar in women with preterm deliveries with VMG 4 Diverse species compared to women with term deliveries with VMG 4 Diverse species at both sampling timepoints, (p>0.05) Figure 5.39 A and C.

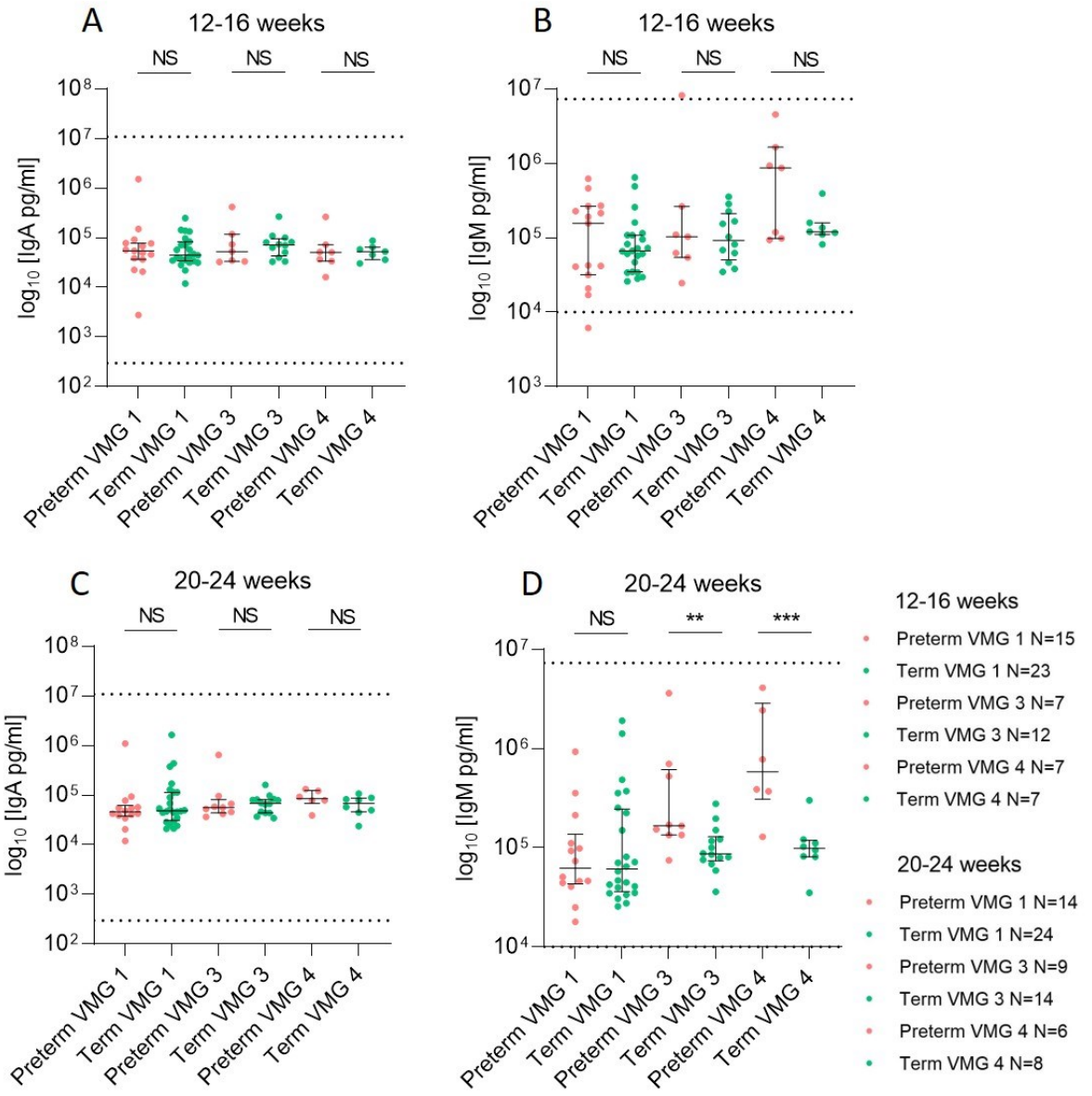


Figure 5.39 Cervico-vaginal fluid IgA and IgM in relation to vaginal microbial groups 1, 3 and 4 in preterm and term deliveries

At the 12-16 and 20-24 week timepoint IgA concentrations were similar for VMG 1 *L. crispatus*, VMG 3 *L. iners* and VMG 4 Diverse species for preterm and term deliveries (A and C). At the 20-24 week timepoint IgM expression was significantly higher for VMG 3 *L. iners* and VMG 4 Diverse species in the women who delivered preterm compared to at term, ($p<0.01$ and ($p<0.001$ respectively), (D), with no difference at the 12-16 week timepoint (B). IgA and IgM concentrations were no different in preterm and term deliveries for VMG I *L. crispatus* at either timepoints (A-D).

Statistical analysis comparing concentrations in VMG 1 *L. crispatus*, VMG 3 *L. iners* and VMG 4 Diverse species in preterm and term deliveries was by the Mann Whitney U test. **$p<0.01$, ***$p<0.001$, NS non-significant. The results are presented as median and interquartile range. At 12-16 weeks, Preterm VMG 1 N=15, Term VMG 1 N=23, Preterm VMG 3 N=7, Term VMG 3 N=12, Preterm VMG 4 N=7 and Term VMG 4 N=7. At 20-24 weeks, Preterm VMG 1 N=14, Term VMG 1 N=24, Preterm VMG 3 N=9, Term VMG 3 N=14, Preterm VMG 4 N=6 and Term VMG 4 N=8.

The previous section demonstrated that in women who have a dysbiotic vaginal microbiota, expression of cervico-vaginal fluid IL-1β, IL-8, IL-6, C3b, MBL, IgG and IgM are significantly increased in women who deliver preterm compared to women who deliver at term.

Microbial-immune interactions with activation of the classical pathway of the complement cascade via IgM and IgG, or via MBL and the mannose-binding lectin pathway of the complement cascade, results in the cleavage of C3 to C3a and C3b subunits. C3b opsonises pathogens targeting them for immune clearance by phagocytosis. C3a and C5a enhance phagocyte recruitment. The final lytic pathway of the complement cascade involves activation of C5, and C5b combines with C6 C7, C8 and C9 to form the membrane attack complex, inducing bacterial cell lysis. IL-8 and IL-1β are produced by neutrophils and macrophages with phagocytic activity. Together with MMPs and prostaglandins, they are known to be key inflammatory mediators of cervical remodelling, membrane rupture and uterine contractions, key processes in labour.

In women with *Lactobacillus* depletion who delivered preterm, IgM correlated positively with C3b, C5, C5a, IL-8 and IL-1β, Figure 5.40, supporting IgM mediated activation of the classical complement pathway leading to cytokine production. Similarly, MBL correlated positively with C3b, C5, C5a, IL-8 and IL-1β, Figure 5.41, supporting activation of the lectin complement pathway leading to cytokine production. Furthermore, the chemoattractant C5a correlated positively with downstream inflammatory cytokines IL-8 and IL-1β, whilst C3b correlated positively with IL-1β, Figure 5.42. This demonstrates that in some women, dysregulated activation of complement pathways and subsequent cytokine production as a result of a dysbiotic vaginal microbial environment may be a mechanism for infection/inflammation mediated preterm delivery.

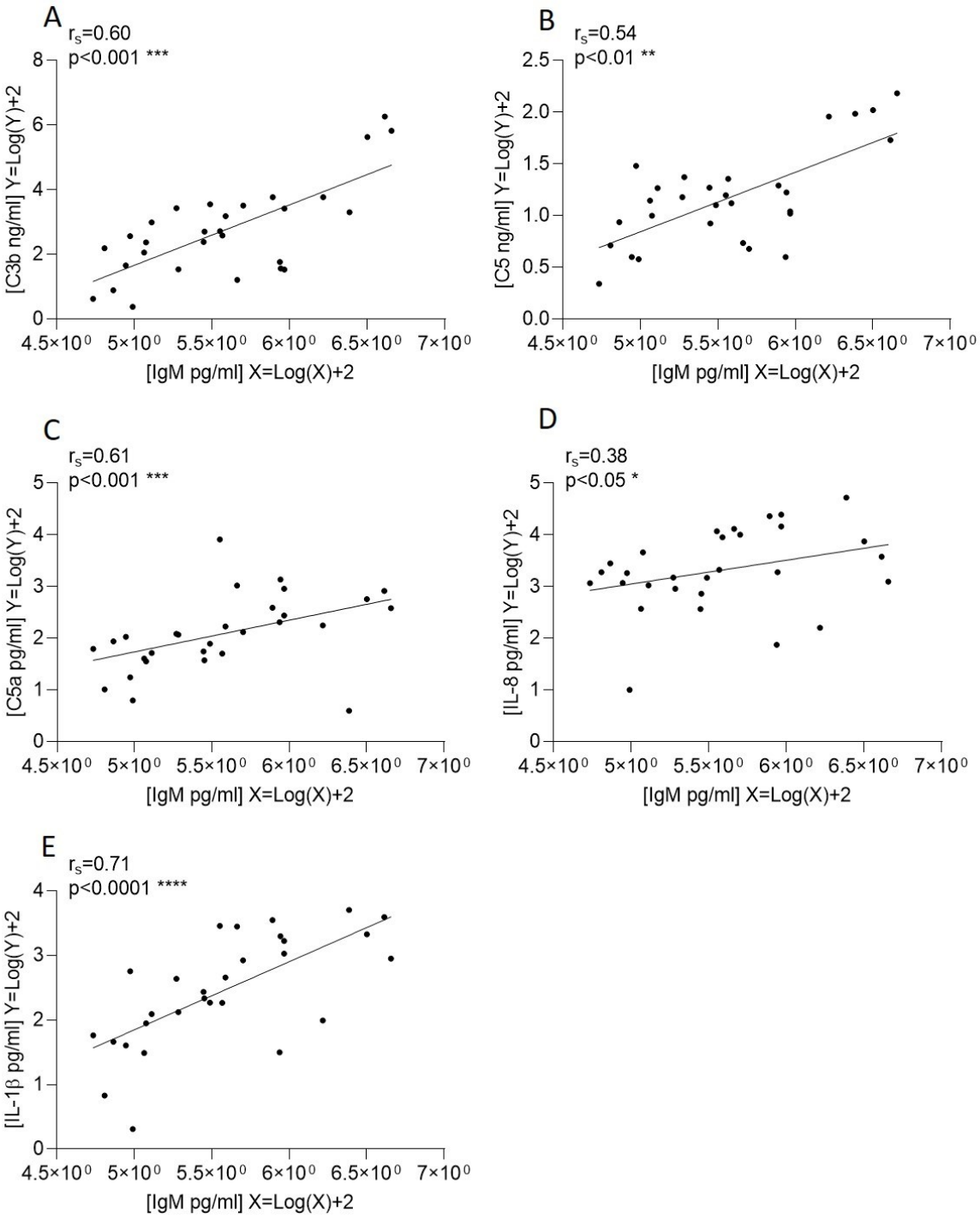


Figure 5.40 Activation of the classical complement pathway in women with preterm deliveries with *Lactobacillus* depletion

IgM correlated positively with C3b, C5, C5a, IL-8 and IL-1β. Log transformation of non-parametric data, Y=Log(Y)+2 and X=Log(X)+2. Statistical analysis was by Spearman's correlation, *p<0.05, **p<0.01, ***p<0.001. N=29 *Lactobacillus* deplete samples. N=29.

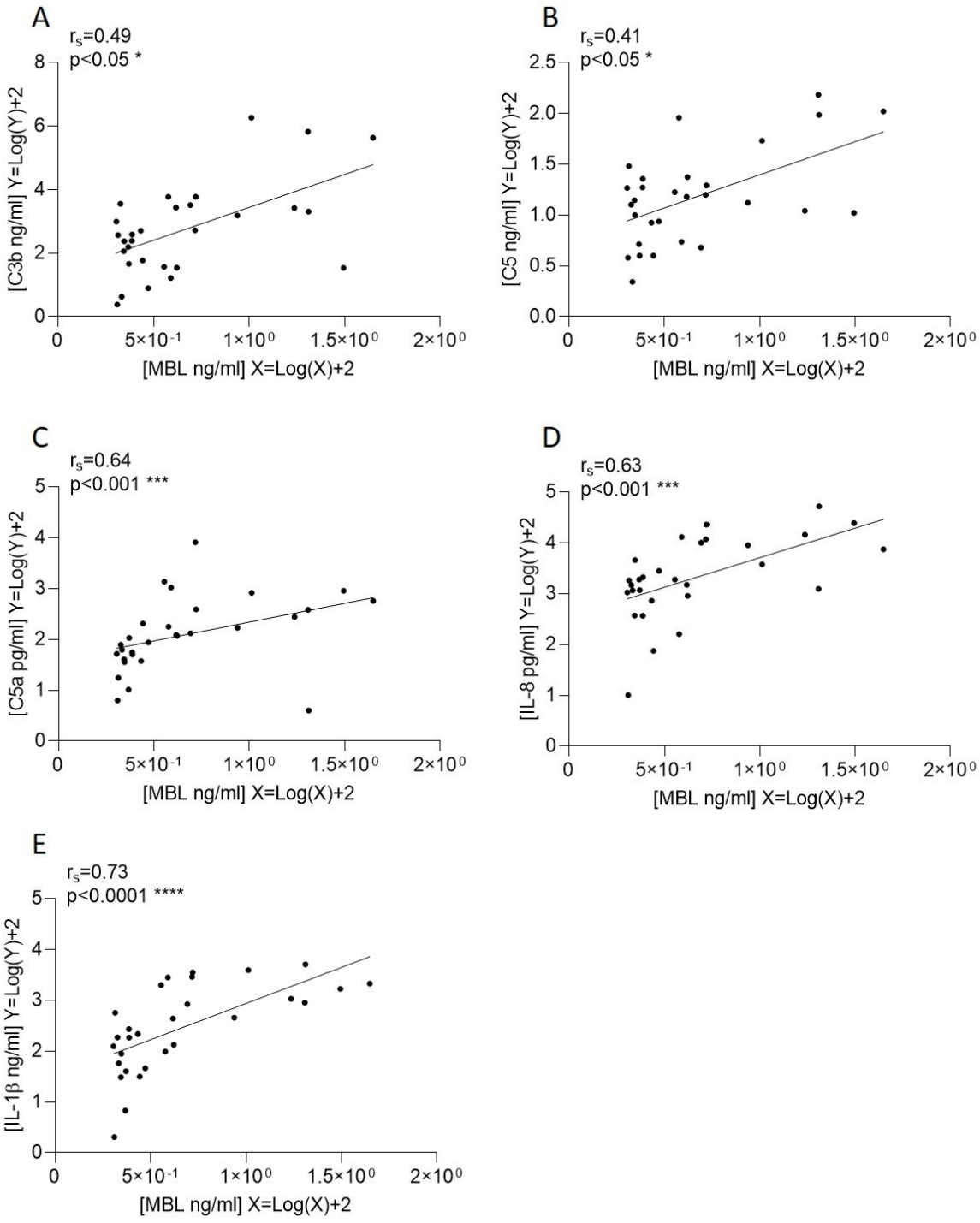


Figure 5.41 Correlation of MBL with the complement cascade and the innate immune cytokines in women with preterm deliveries with *Lactobacillus* depletion

MBL correlated positively with C3b, C5, C5a, IL-8 and IL-1β. Log transformation of non-parametric data, Y=Log(Y)+2 and X=Log(X)+2. Statistical analysis was by Spearman's correlation, *p<0.05, **p<0.01, ***p<0.001. N=29 *Lactobacillus* deplete samples. N=29.

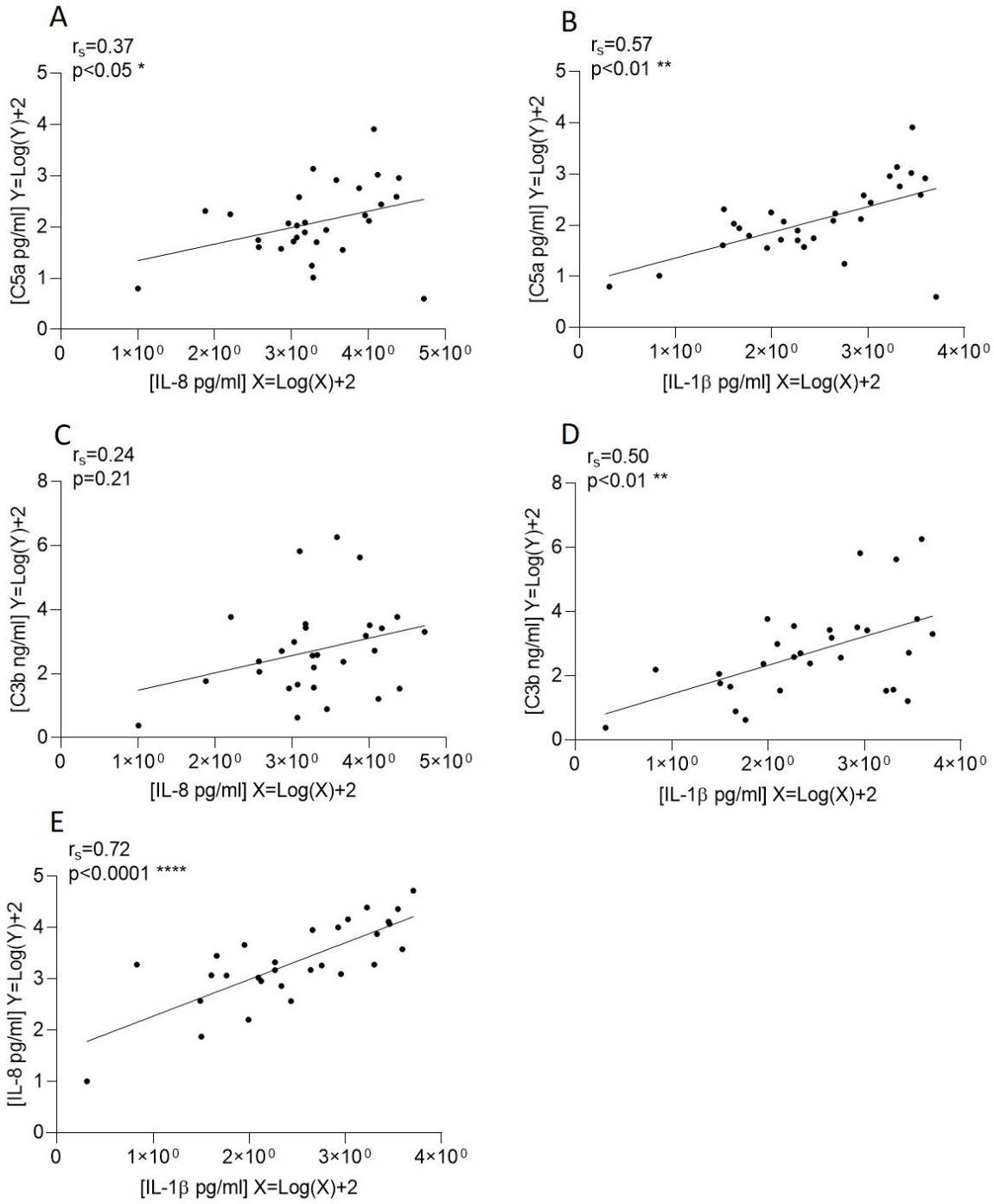


Figure 5.42 Correlation of chemoattractants C5a and C3b with IL-8 and IL-1β in women with preterm deliveries with *Lactobacillus* depletion

C5a correlated positively with IL-8 and IL-1β, and C3b correlated positively with IL-1β. IL-8 and IL-1β also correlated positively with each other. Log transformation of non-parametric data, Y=Log(Y)+2 and X=Log(X)+2. Statistical analysis was by Spearman's correlation, *p<0.05, **p<0.01, ***p<0.001. N=29 *Lactobacillus* deplete samples. N=29.

5.5.8 Correlation between the vaginal microbial composition and the peripheral immune response

The host microbial-local immune interactions have been shown to be complex during pregnancy. In order to study if there is a relationship between the peripheral immune response and the vaginal microbial composition, 78 study participants were identified who had data for their PBMC subsets and their effector markers and vaginal microbial composition and 31 study participants were identified who had data for their plasma cytokines and complement and vaginal microbial composition. This allowed a cross-sectional analysis of the peripheral immune response to *Lactobacillus* dominant or *Lactobacillus* deplete vaginal microbial environment. Analysis was performed individually at the three sampling timepoints, 12-16 weeks, 20-24 weeks and 30-34 weeks.

The proportions of peripheral mononuclear cells subsets and their markers of cell activity were compared in matched *L.* dominant and *L.* deplete vaginal microbial samples across pregnancy. There were no differences in the proportions of the PBMC subsets according to *Lactobacillus* dominant or *Lactobacillus* deplete vaginal microbial environments, lymphocytes, monocytes, CD4+ T cells, CD8+ T cells, Treg cells, CD4+ NK T cells, CD8+ NK T cells, B cells, NK cells, γδ T cells and monocytes (or subsets classical, intermediate and non-classical), ($p>0.05$) Table 5.4. There were also no differences in the cell effector markers as measured by the median fluorescence intensity of CD4+ CD25, CD4+ CD38, CD4+ HLA-DR, CD4+ PD-1, CD8+ CD25, CD8+ CD38, CD8+ HLA-DR, CD8+ PD-1, Treg CD25, Treg HLA-DR, monocytes HLA-DR, B cells IgD and NK CD38, ($p>0.05$) Table 5.4. The vaginal microbial composition did not have a significant influence on the PBMC subsets or their markers of cell activity.

The concentrations of plasma IL-18, IFN-γ, IL-2, IL-4, IL-8, TNF-α, GM-CSF, IL-5, IL-1β, IL-6, C5, C5a, or MBL were similar in *Lactobacillus* dominant and *Lactobacillus* deplete microbial environments (Table 5.5). C3b did appear to demonstrate an increased response to a *L.* deplete vaginal microbial environment at 12^{+0} - 16^{+6} weeks, however there was no significant difference by mid and late pregnancy.

Table 5.4 Peripheral blood mononuclear cell proportions and cell effector status in matched *Lactobacillus* dominant and *Lactobacillus* deplete vaginal microbial environments

	12-16 weeks			20-24 weeks			30-34 weeks		
Mean percentage % (standard deviation)	*L.* dominant N=41	*L.* deplete N=18	p-value	*L.* dominant N=49	*L.* deplete N=20	p-value	*L.* dominant N=57	*L.* deplete N=19	p-value
Lymphocytes	90.5 (6.5)	90.7 (5.3)	0.40	91.6 (6.4)	92.4 (5.5)	0.59	91.3 (5.7)	89.4 (7.8)	0.56
CD3+ lymphocytes	77.1 (6.6)	80.2 (7.6)	0.11	77.6 (5.7)	76.8 (7.7)	0.28	76.5 (7.5)	75.6 (8.3)	0.64
CD4+ T cells	64.9 (7.4)	63.7 (6.9)	0.28	65.5 (7.2)	63.4 (7.5)	0.27	65.8 (8.2)	63.8 (6.8)	0.35
CD8+ T cells	30.7 (6.6)	31.8 (6.5)	0.28	30.3 (6.6)	31.9 (6.8)	0.37	30.0 (7.4)	31.7 (6.4)	0.40
Treg	6.9 (2.1)	8.0 (2.7)	0.06	7.2 (2.7)	7.8 (3.5)	0.94	7.6 (2.9)	8.2 (2.7)	0.30
CD4+ NK T cells	0.7 (1.1)	0.4 (0.3)	0.25	0.6 (1.4)	0.7 (1.1)	0.36	0.6 (1.2)	0.5 (0.6)	0.84
CD8+ NK T cells	9.8 (7.3)	7.1 (5.7)	0.08	9.0 (6.7)	8.5 (7.9)	0.96	7.5 (6.1)	9.0 (6.9)	0.45
B cells	47.3 (12.6)	42.7 (16.4)	0.12	47.5 (15.1)	48.8 (13.5)	0.74	46.9 (14.2)	48.32 (16.4)	0.71
NK cells	26.5 (10.9)	27.4 (14.3)	0.40	23.9 (13.5)	23.6 (11.6)	0.92	20.1 (11.7)	20.7 (11.1)	0.85
γδ T cells	2.4 (2.2)	2.8 (2.9)	0.43	2.4 (2.1)	2.1 (1.4)	0.99	2.3 (2.0)	2.4 (2.4)	0.89
Monocytes	9.5 (6.5)	9.3 (5.3)	0.40	8.4 (6.5)	7.2 (5.7)	0.40	8.7 (5.7)	10.3 (8.1)	0.72
Classical monocytes	83.7 (4.6)	83.3 (3.2)	0.35	83 (4.3)	84.7 (5.2)	0.16	83.2 (3.9)	83.9 (3.9)	0.49
Intermediate monocytes	1.8 (1.7)	0.7 (5.5)	0.11	1.3 (1.4)	1.3 (1.2)	0.72	1.6 (2.0)	0.8 (0.7)	0.20
Non-classical monocytes	6.6 (4.1)	5.1 (4.5)	0.07	5.4 (3.5)	5.2 (3.3)	0.94	6.0 (4.2)	5.3 (4.6)	0.30
Mean of median fluorescence intensity (standard deviation)									
CD4+ CD25	2248 (741)	2315 (811)	0.38	2268 (691)	2227 (1075)	0.26	2319 (736)	2240 (800)	0.73
CD4+ CD38	8574 (5721)	10203 (3825)	0.14	9826 (5652)	1117 (4055)	0.36	10350 (5795)	10437 (3236)	0.95
CD4+ HLA-DR	8489 (1776)	8710 (2145)	0.68	9107 (2736)	8036 (2222)	0.54	9428 (2688)	9280 (2143)	0.83
CD4+ PD-1	1786 (382)	1847 (292)	0.54	1783 (384)	1731 (345)	0.60	1761 (354)	1712 (322)	0.60
CD8+ CD25	1344 (568)	1570 (597)	0.09	1357 (502)	1234 (668)	0.41	1412 (574)	1496 (664)	0.60
CD8+ CD38	5244 (2775)	6805 (3206)	0.06	6055 (2466)	8153 (2589)	0.07	7454 (2893)	8562 (3580)	0.18
CD8+ HLA-DR	10507 (3248)	11530 (4810)	0.28	10887 (3795)	10108 (2115)	0.71	10070 (3559)	10634 (2165)	0.07
CD8+ PD-1	1874 (419)	1916 (445)	0.73	1871 (449)	1680 (338)	0.09	1828 (451)	1754 (400)	0.53
Treg CD25	11427 (2744)	11379 (2672)	0.48	10658 (2434)	10694 (2704)	0.96	10758 (2637)	10517 (2504)	0.73
Treg HLA-DR	5510 (1273)	5723 (903)	0.52	5267 (1234)	5526 (1551)	0.88	5319 (1336)	5611 (1376)	0.42
Monocytes HLA-DR	66181 (18871)	69967 (18871)	0.24	64125 (22891)	69479 (21313)	0.37	60485 (21139)	68314 (23396)	0.18
B cells IgD	50178 (22473)	47398 (21367)	0.33	51789 (24381)	47906 (20449)	0.53	54363 (20855)	53363 (23641)	0.86
NK CD38	95815 (44069)	91394 (28796)	0.70	107468 (4611)	116160 (41137)	0.47	130917 (44943)	131051 (39588)	0.99

Statistical analysis was by unpaired t-test or Mann Whitney test. *L.* dominant = *Lactobacillus* dominant. *L.* deplete = *Lactobacillus* deplete.

Table 5.5 Plasma cytokines and complement concentrations in matched *Lactobacillus* dominant and *Lactobacillus* deplete vaginal microbial environments

	12-16 weeks			20-24 weeks			30-34 weeks		
Plasma analytes Mean concentration (standard deviation)	*L.* dominant N=20	*L.* deplete N=9	p-value	*L.* dominant N=22	*L.* deplete N=10	p-value	*L.* dominant N=23	*L.* deplete N=16	p-value
IL-18 pg/ml	79.6 (54.4)	86.5 (61.3)	0.75	101 (58.4)	115 (74.5)	0.80	113.8 (74.8)	142.5 (105.1)	0.61
IFN-γ pg/ml	67.4 (95)	163 (295)	0.63	62 (89)	134 (178)	0.29	74.6 (109)	140.1 (143.1)	0.28
IL-2 pg/ml	51.9 (102)	5.9 (0.08)	0.23	35.2 (83.7)	32.1 (62.6)	0.44	42.0 (87.9)	26.1 (51.8)	0.57
IL-4 pg/ml	15.1 (25.6)	14.9 (28.1)	0.30	13.8 (24.3)	17.8 (27.3)	0.57	17.0 (28.3)	15.6 (24.2)	0.82
IL-8 pg/ml	4.9 (11.8)	2.7 (6.1)	0.48	3.0 (6.7)	6.9 (15.9)	0.91	5.0 (11.7)	2.8 (5.3)	0.94
TNF-α pg/ml	3.6 (9.0)	2.2 (6.1)	0.35	2.6 (6.5)	4.6 (10.3)	0.58	3.9 (9.0)	2.4 (5.5)	0.77
GM-CSF pg/ml	3.6 (8.7)	2.9 (7.0)	0.53	2.2 (6.7)	4.7 (9.6)	0.94	3.4 (8.7)	2.8 (5.8)	0.85
IL-5 pg/ml	1.2 (1.9)	0.77 (0.77)	0.52	0.92 (0.89)	1.5 (2.6)	0.75	1.2 (1.8)	0.50 (0.004)	0.44
IL-1β pg/ml	1.7 (3.0)	1.3 (0.9)	0.99	2.5 (4.9)	1.1 (0.3)	0.99	1.7 (3.5)	1 (1.1)	0.99
IL-6 pg/ml	0.26 (0.7)	0.3 (0.5)	0.47	0.3 (0.7)	0.3 (0.30)	0.41	0.3 (0.9)	0.5 (0.8)	0.07
C5 ng/ml	13827 (3181)	13156 (6052)	0.87	13928 (3650)	16867 (3268)	0.15	14489 (5371)	13057 (2752)	0.74
C5a pg/ml	341 (553)	339 (458)	0.89	316 (579)	522 (412)	0.22	393 (501)	358 (424)	0.84
MBL ng/ml	5476 (4415)	5425 (4580)	0.86	5030 (4428)	6801 (4445)	0.31	5786 (6434)	7009 (5072)	0.36
C3b ng/ml	76393 (44479)	122618 (55287)	<0.05 *	77710 (42922)	114888 (68511)	0.09	82143 (51272)	117000 (78128)	0.11

Statistical analysis was by Mann Whitney test * p<0.05. *L.* dominant = *Lactobacillus* dominant. *L.* deplete = *Lactobacillus* deplete.

5.6 Discussion

Key findings

In this Chapter I have shown that vaginal dysbiosis is associated with increased activation of the local innate and adaptive immune response. *L. crispatus* is immunologically inert and it is likely that preterm birth seen with *L. crispatus* is neither microbial nor inflammation driven. The presence of *L. iners* is associated with an increase in cytokines and immunoglobulins. However, complement activation is only seen when *L. iners* is associated with cervical shortening, or with subsequent preterm delivery, which implies that the complement cascade plays a significant role in the pathophysiology of preterm birth. VMG 4 Diverse species increases cytokines, complement and immunoglobulins regardless of outcome, however the response is greater in those who deliver preterm. This raises the possibility that a degree of immune regulation occurs in women who deliver at term, and in contrast, dysregulation leads to preterm labour. Data presented supports a mechanism for microbial-immune driven preterm birth with VMG 3 *L. iners* and VMG 4 Diverse species, however it is not clear what determines a regulated response, and what factors lead to a dysregulated response.

Both VMG 3 *L. iners* and VMG 4 Diverse species were associated with higher IgM and MBL concentrations in women who delivered preterm compared to at term. Figure 5.43 and figure 5.44 summarise a proposed mechanistic pathway for microbial induced inflammation and preterm birth to VMG 3. L.iners and VMG 4 Diverse species. IgM is a potent trigger of the classical pathway, followed by IgG3 > IgG1 > IgG2. The classical pathway is activated by antibody-antigen complexes binding to C1. The mannose-binding lectin pathway is activated by the binding of MBL to mannose particles on the pathogen. They converge on to C3, which by C3 convertase divides into two components C3a and C3b. C3b is significantly higher in the presence of VMG 3 *L. iners* and VMG 4 Diverse species in women who deliver preterm compared to term. The final common pathway occurs when C5 is activated. The membrane attack complex then induces bacterial cell lysis. Complement fragments C3a and C5a are anaphylatoxins and chemoatttractants which promote further local inflammation and immune cell recruitment. Although C3a was not measured, we can hypothesise that it did increase given that there was a significant increase in C3b. The median concentration of C5a was higher in women who delivered preterm, although, narrowly missed out on statistical significance. It is plausible that this increase in C5a leads to neutrophil recruitment, explaining the increase in IL-8, lL-1β and IL-6. Amplification of this cascade is likely to ensue, as IL-8 has the ability to chemoattract more immune cells [361]. Furthermore, neutrophils may then contribute to an environment rich in prelabour mediators, as they release prostaglandins, and matrix metalloproteinases [326, 328]. Laudisi et al have

described that complement can induce the secretion of IL-1β via the nucleotide-binding oligomerisation domain and leucine-rich repeat-containing receptors, NLRP3 inflammasome [362]. IL-1β stimulates adhesion molecules, CD54 and vascular cell adhesion molecule 1 (VCAM-1) to recruit leukocytes to the sites of inflammation, and induces chemokines including MCP-1 [363].

Cervical remodelling involves several pro-inflammatory pathways. There is an influx of immune cells including neutrophils, macrophages and T cells into the extracellular matrix of the cervix with a marked increase in local pro-inflammatory cytokines such as IL-8, IL-1β, IL-6 and TNF-α [221, 327, 364]. Also contributing to cervical remodelling are MMPs and prostaglandins. The proposed mechanism for dysregulated complement activation in response to a dysbiotic vaginal microbiome for infection mediated preterm birth therefore incorporates elements of the pro-inflammatory pathways known to be activated during cervical remodelling.

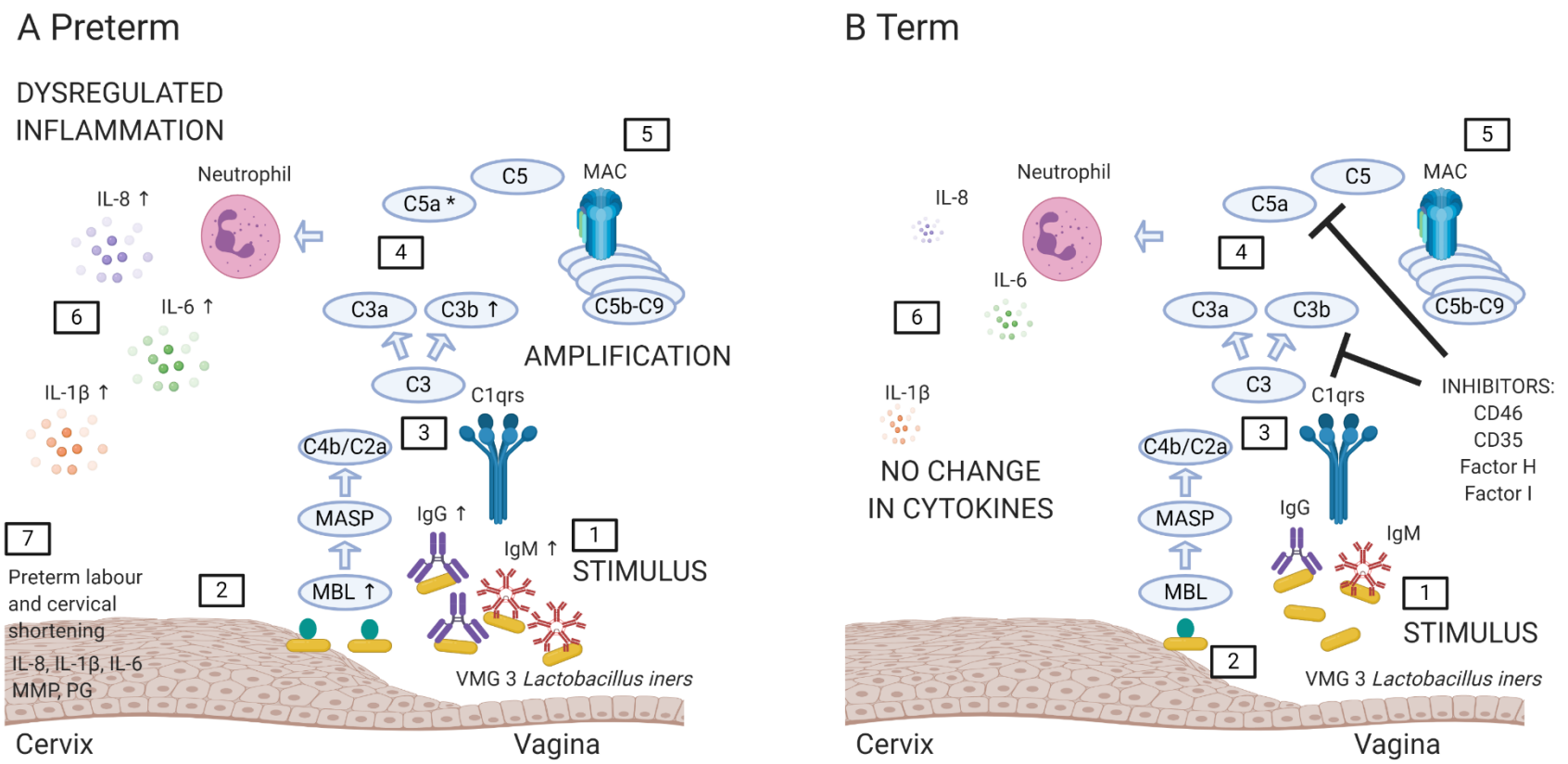


Figure 5.43 Dysregulated local complement activation to VMG 3 *Lactobacillus iners* occurs in cervical shortening and preterm birth

In women who deliver preterm (A), VMG 3 *L. iners* can activate the classical complement pathway via antibody-antigen complexes (1), or by the mannose binding lectin pathway (2). C3 convertase hydrolyses C3 to C3a and C3b, with amplification of the complement cascade (3). C3a and C5a enhance phagocyte recruitment (4). C5a was non-significantly increased in women who delivered preterm with VMG 3 *L. iners* (C5a*). C3b attaches to the surface of bacteria, thereby opsonising pathogens. The common final lytic pathway occurs when C5 is activated. C6, C7, C8 and C9 combine with C5b and the resulting membrane attack complex (MAC) induces bacterial cell lysis (5). IL-8 and IL-1β are secreted for phagocytes and IL-8 recruits further neutrophils (6). IL-8, IL-1β, IL-6, matrix metalloproteinases (MMPs) and prostaglandins (PGs) are key effectors involved in preterm labour and cervical shortening (7). In women who deliver at term (B), VMG 3 *L. iners* is recognised by IgM and IgG (1), and by MBL (2), but concentrations were significantly lower compared to the women who deliver preterm. C3 convertase hydrolyses C3 to C3a and C3b, but this step of the complement cascade is likely to be regulated by complement inhibitors, possibly involving, CD46, CD35, Factor H and Factor I (4 and 5), with no change in the concentrations of local cytokines IL8, IL-6 and IL-1β (6).

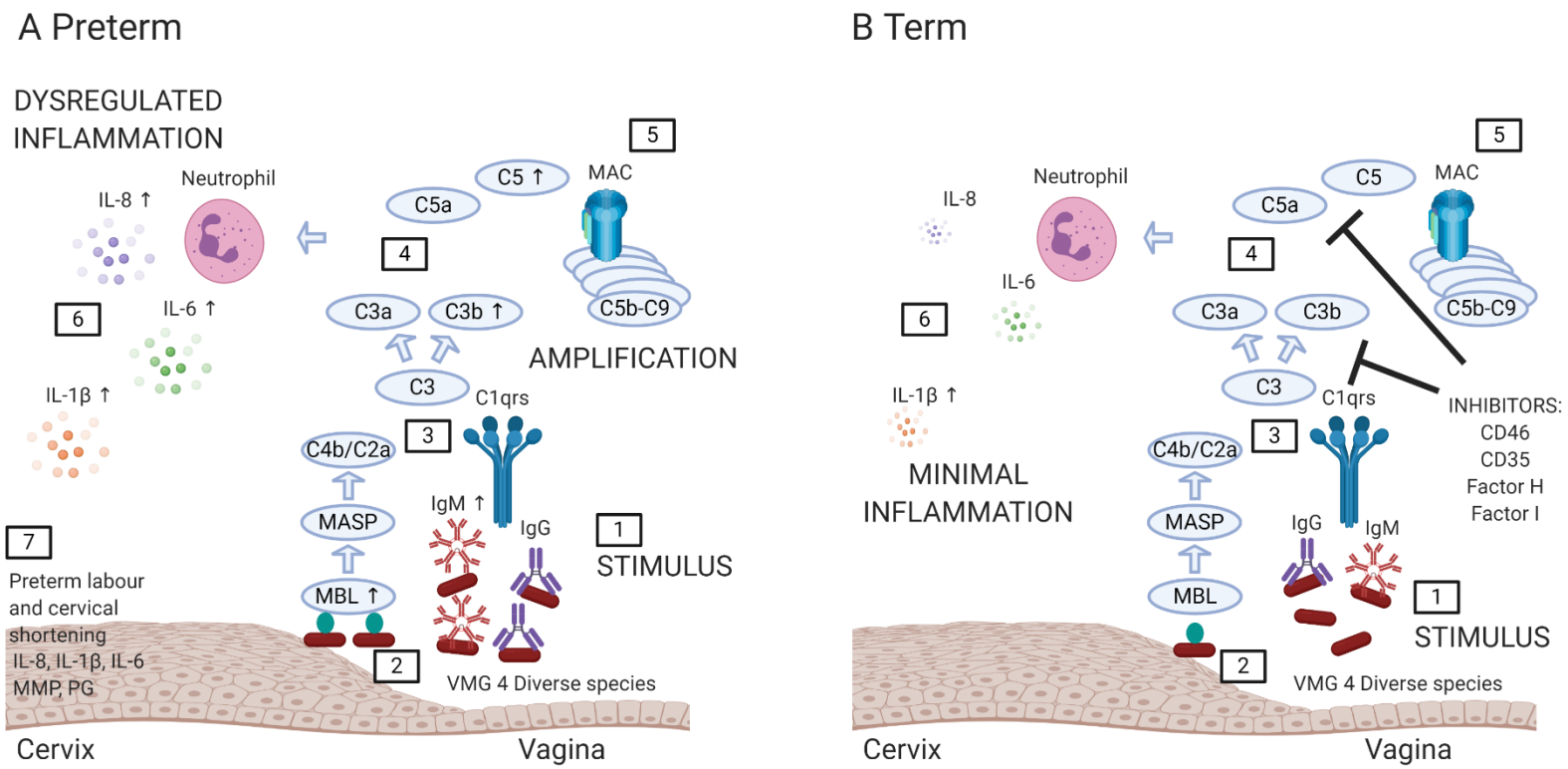


Figure 5.44 Dysregulated local complement activation to VMG 4 Diverse species occurs in cervical shortening and preterm birth

In women who deliver preterm (A), bacteria classified as VMG 4 Diverse species can activate the classical complement pathway via antibody-antigen complexes (1), or by the mannose binding lectin pathway (2). C3 convertase hydrolyses C3 to C3a and C3b, with amplification of the complement cascade (3). C3a and C5a enhance phagocyte recruitment (4). C3b attaches to the surface of bacteria, thereby opsonising pathogens. The common final lytic pathway occurs when C5 is activated. C6, C7, C8 and C9 combine with C5b and the resulting membrane attack complex (MAC) induces bacterial cell lysis (5). IL-8 and IL-1β are secreted for phagocytes and IL-8 recruits further neutrophils (6). IL-8, IL-1β, IL-6, matrix metalloproteinases (MMPs) and prostaglandins (PGs) are key effectors involved in cervical shortening and preterm labour (7).

In women who deliver at term (B) , bacteria classified as VMG 4 Diverse species were recognised by IgG, IgM (1) and MBL (2), but local concentrations may have not been sufficiently high enough for the antibodies to neutralise or opsonise the microbes. There was regulated complement activation (3-5), C3 convertase hydrolyses C3 to C3a and C3b, but this step of the complement cascade is likely to be regulated by complement inhibitors, possibly involving, CD46, CD35, Factor H and Factor I (4 and 5). There was minimal downstream inflammation with no change in IL-8 and IL-6, and a minimal increase in IL-1β , likely representing a residual inflammatory response (6).

The cervico-vaginal immune response and the vaginal microbial composition

Associations between the vaginal microbial composition and cervico-vaginal immune milieu were explored in this Chapter. Vaginal dysbiosis characterised by *Lactobacillus* species depletion has been shown in multiple studies to be associated with spontaneous preterm labour and PPROM, whilst *Lactobacillus crispatus* confers a degree of protection [147, 150, 155-158, 161, 365]. Some groups have shown that a mechanism of action by which this may occur is by an exaggerated local immune response associated with vaginal dysbiosis, however only cytokines which are downstream effectors of inflammation have been explored so far.

Kindinger et al have previously demonstrated that vaginal dysbiosis is associated with a local inflammation with increased expression of pro-inflammatory mediators in cervico-vaginal fluid: ICAM-1, IL-1β, IL-6, MMP-1, MCP-1, TNF-α, GM-CSF and IFN-γ [49]. In a South African population, Manhanzva et al also detected higher concentrations of local pro-inflammatory cytokines in *Lactobacillus* deplete individuals [366]. These cytokines were IL-8, IL-6, IL-1α, IL-1β, MIP-1α/β. In this chapter, the findings of increased pro-inflammatory IL-8, IL-1β and IL-6 in cervico-vaginal fluid in *Lactobacillus* depletion compared to *Lactobacillus* dominance are therefore consistent with this.

This Chapter explored the immune response from the point at which microbes are recognised by immunoglobulins and complement analytes, as well as by downstream cytokines. At genera level, I was able to demonstrate a significant increase in local complement activation involving the classical pathway with increased concentrations of IgG1-3 and IgM in *Lactobacillus* depletion compared to *Lactobacillus* dominance, and also the lectin pathway with increased concentrations of MBL. Converging the two pathways, C3b was also significantly higher in *Lactobacillus* depletion, and then following on from this also C5. C3b opsonises bacteria, and C3a and C5a are chemoattractants for phagocytes. As a consequence, inflammatory mediators IL-8, IL-6 and IL-1β were also significantly increased in *Lactobacillus* depletion compared to *Lactobacillus* dominance.

Further analysis performed at species level revealed additional differences in the local immune response to the vaginal microbiota. The majority of samples classified as VMG 3 *L. iners*, were categorised as *Lactobacillus* species dominant. Therefore, it was pertinent to report that when analysis was performed at species level cervico-vaginal fluid concentrations of IL-8 and IL-1β were also significantly higher in VMG 3 *L. iners* compared to VMG 1 *L. crispatus*, thus adding weight to the argument that *Lactobacillus iners* has more pathogenic than commensal characteristics [367]. Compared to other *Lactobacillus* species*, Lactobacillus iners* has a much smaller genome which is

typical of bacteria that usually exists in a symbiotic state with other bacteria. The genome of *Lactobacillus iners* encodes inerolysin, a pore forming toxin which is related to the vaginal pathobiont *Gardnerella vaginalis* [368].

Samples classified as VMG 4 Diverse species had significantly higher concentrations of IL-8, IL-1β, IL-6, C5, MBL, C3b, IgG1-4 and IgM compared to VMG 1 *L. crispatus*. However, whilst VMG 3 *L. iners* was associated with significantly higher concentrations of IL-8, IL-1β, IgG2-4 and IgM compared to VMG 1 *L. crispatus*, there were no differences when comparing the complement analytes. Therefore *Lactobacillus iners* may represent an intermediate state of dysbiosis. Supporting the notion that *Lactobacillus crispatus* is protective, cervico-vaginal fluid concentrations of anti-inflammatory IL-10 were significantly higher in VMG 1 *L. crispatus* compared to both VMG 3 *L. iners* and VMG 4 Diverse species.

Another notable difference in the local immune response to bacterial species was an increase in C3b, IgG2-4 and IgM in VMG 2 *L. gasseri* compared to VMG 1 *L. crispatus*. Alpha diversity and species richness were significantly higher in VMG 2 *L. gasseri* samples compared to VMG 1 *L. crispatus*. The immune modulatory activity of *L. crispatus* is believed to be exerted by lactic acid, and the balance between the L- and D- isomers. *L. crispatus* and *L. gasseri* are phylogenetically similar, but there may still be subtle differences in the amount of lactic acid isomers produced which may explain the different local immune responses. Lactic acid maintains a pH below 4.5 in the female genital tract projecting against the colonisation of pathogenic bacteria [369]. Verstraelen et al also describe that *L. crispatus* promotes are more stable vaginal microbial composition compared to *L. gasseri* and *L. iners*[370], whilst the latter two confer less resistance to colonisation by pathobionts.

The vaginal microbial composition and risk factors for preterm birth

In Chapter 4, I explored the hypothesis that women with a previous history of MTL and/or PTB were more likely to have inflammation driven preterm birth whilst the women with a previous history of excisional cervical treatment were more likely to have mechanical cervical insufficiency as the aetiology for preterm birth. The immune profiles for these two groups of women did show subtle differences and in this Chapter I was able to demonstrate that there were differences in the vaginal microbial composition between the two groups of women consistent with microbial driven inflammation priming the immune response in early pregnancy in women with a previous history of MTL and/or PTB. There may already be a degree of microbial-driven inflammation and cervical remodelling in those with a previous history of MTL and/or PTB. There are important implications for

the clinical translation of these findings. If vaginally administered probiotics can be shown to colonise the vagina they are likely to benefit those women with microbial-driven inflammation rather than those with primary mechanical cervical insufficiency. Additionally, cervical cerclage may have worse outcomes for those with an existing primed pro-inflammatory immune response.

The vaginal microbial composition in cervical shortening

The impact of cervical shortening on the vaginal microbial composition was explored in this Chapter. A cervical length less than 25mm in the second trimester is a recognised risk factor for preterm birth and is a clinically detectable manifestation of premature cervical remodelling. Our group have previously explored the interaction between cervical shortening and the vaginal microbial composition. There was a significant association between *Lactobacillus iners* and cervical shortening at 16 weeks gestation [158]. In comparison, a recent study by Gerson et al. reported women with CST IV were twice as likely to have cervical shortening compared to women with CST I, II and III [371].

In this Chapter, consistent with the former study, I present that *Lactobacillus iners* was significantly more common in women with cervical shortening than women with normal cervical length when sampled at 12-16 weeks gestation. In women with a dominant *Lactobacillus iners* vaginal microbiota, cervical shortening was found to be associated with a strong local inflammatory immune response.

Gerson et al describe a few explanations for the differences in the studies. The gestation of sampling in Gerson's et al's study was four weeks later than mine and Kindinger et al's. There was a much greater proportion of study participants from African ancestry in Gerson's et al's study and it is recognised that CST IV is more common in women of black ethnicity. *L. iners* has been associated with microbial imbalance and increased susceptibility to bacterial vaginosis [372, 373]. The concept of shifting between CST during pregnancy has been described with *L. iners* conferring an increased likelihood of shifting to CST IV [370]. Gerson et al describe a possible explanation that some women in their study who were CST IV may have had a vaginal microbial composition dominated by *Lactobacillus iners* earlier in their pregnancy.

In women who had cervical shortening, those with *L. iners* had significantly higher concentrations of IL-8, C3b, and IgM, and lower concentrations of IL-10 compared to those with *L. crispatus*. Increased local inflammation in women with normal cervical length with VMG 3 *L. iners* compared to VMG 1 *L. crispatus* may reflect regulated inflammation that does not result in cervical remodelling and shortening. However, the increased local inflammatory response in women with VMG 3 *L. iners* with

cervical shortening compared to normal cervical length reflects a dysregulated immune response that does result in collateral injury to the cervix and shortening. The difference to changes in local complement and immunoglobulins in cervical shortening are novel and offer a link between microbial recognition and activation of downstream inflammatory pathways known to be implicated in cervical shortening. It is important to note that cervical shortening can occur independently of vaginal dysbiosis, especially in those with primary mechanical cervical insufficiency, and not all women with *L. iners* have cervical shortening, therefore local inflammation that occurs with cervical shortening may only be microbial driven in some.

The vaginal microbial composition following cervical cerclage

Insertion of a cervical cerclage has been shown to induce local inflammatory processes, therefore this Chapter explored if there was an aspect of microbial induced inflammation. Although there was an overall increase in the alpha diversity post cervical cerclage, this was not related to the cerclage material. This study also did not demonstrate a significant change in vaginal microbial composition post cervical cerclage.

Our research group have previously published on the impact of the a braided Mersilene cerclage compared to a monofilament Nylon cerclage on the vaginal microbiota. Kindinger et al reported a shift towards vaginal microbial dysbiosis with a reduction of *Lactobacillus* spp. with the braided Mersilene suture material with a local inflammatory response, whilst the monofilament Nylon cerclage had little impact on the vaginal microbiota or the local immune milieu [(49)].

The study design was different from the one presented in this Chapter. In Kindinger et al's study, forty-nine study participants with cervical shortening were randomised to either Mersilene or Nylon cerclage material and the same Obstetrician performed the surgical procedure using the same technique. Study participants were sampled before the procedure, and longitudinally at 4, 8, 12 and 16 weeks post cerclage insertion. The greatest shift in dysbiosis was detected at the 4 week timepoint post cerclage insertion. Kindinger et al's study was designed to specifically interrogate vaginal microbial changes according to the type of cervical cerclage material.

The results in this thesis Chapter are not directly comparable for a number of reasons. This was a multi-centre study with variation in the surgeon and therefore the technique of cerclage insertion. Whether to use additional surgical knots to bury the cerclage material and to prescribe antibiotics in the post-operative period was at the surgeon's discretion. Study participants were sampled at

different timepoints post cerclage insertion, and the median number of days at which sampling occurred post cerclage was 50 days. It is possible that the impact of a change in microbial composition may no longer be detectable when there is such a significant length of time until sampling occurred. However, what is clear is that there is an augmented lasting local immune response to a braided Mersilene cervical cerclage, and further augmentation occurred in those who delivered preterm. It is also plausible that the non-specific innate immune response seen with Mersilene cerclage was due to the material used, rather than the vaginal microbial composition. Additionally, in this Chapter, Mersilene was associated with more preterm births. Important to also note in the Kindinger et al study were the results for a retrospective multi-centre cohort of 678 women who received a cervical cerclage and braided cerclage was associated with a significant increase in preterm birth and intrauterine death. In other clinical settings braided Mersilene mesh or cerclage has also been associated with poorer clinical outcomes [374, 375].

Microbial-immune interactions in preterm birth

Some groups have also begun to explore how vaginal dysbiosis alters some aspects of the immune response and subsequently influences pregnancy outcome. Notably, Fettweis et al reported on longitudinal analyses of vaginal microbial composition, cytokine, metagenomic and metatranscriptomic profiles of 45 preterm and 90 term controls [156]. They found that bacteria taxa found in higher abundances in women with preterm compared to term delivery included *Sneathia* species, *Prevotella* species, BVAB1 which positively correlated with pro-inflammatory immune mediators in cervico-vaginal fluid including interferon-γ-induced protein (IP)-10/chemokine ligand (CXCL)10, a strong pro-inflammatory chemokine [376]. In a study with over 100 cases of spontaneous PTB, Elovitz et al detected significantly lower concentrations of anti-microbial β-defensin-2 in vaginal microbial communities dominated by *Lactobacillus iners* and anaerobic species in women who delivered preterm [246]. Gomez-Lopez's study of 18 preterm birth cases reported a negative correlation between CXCL10 and dysbiotic vaginal microbial communities [377].

Study participants in Fettweis, Elotvitz and Gomez-Lopez's studies were mostly of African ancestry. It is recognised that there are racial differences in vaginal microbial composition with a higher prevalence of *Lactobacillus iners* and bacterial species seen in bacterial vaginosis in women of black and Hispanic origin compared to Caucasian and Asian women [145, 151, 360]. Therefore, large population studies with study participants from a variety of ethnic backgrounds are required to acquire more generalisable conclusions.

In this chapter I have described that the mucosal innate and adaptive immune response is similar in women with VMG 1 *L. crispatus* in preterm and term deliveries. Those women who delivered preterm and yet had a vaginal microbial composition of VMG 1 *L. crispatus* were likely to have a non-microbial driven aetiology. Women with VMG 3 *L. iners* who deliver preterm have a dysregulated local immune response compared to women who deliver at term with significantly higher concentrations of IL-1β, IL-6, MBL, C3b, IgG3, IgG4 and IgM. Women with VMG 4 Diverse species who deliver preterm have a dysregulated immune response compared to women who deliver at term with significantly higher concentrations of IL-8, IL-1β, IL-6, C5, C3b, MBL, and IgM in cervico-vaginal fluid. The difference in immune response to VMG 3 *L. iners* and VMG 4 Diverse species may represent slightly different mechanisms of action, with greater activation of complement in the latter. This could mean that complement inhibition is better targeted for those with VMG 4 Diverse species, rather than VMG 3 *L. iners*. Additionally, cytokine and immunoglobulin analytes were also higher in VMG 4 compared to VMG 3 *L. iners*, which implies a greater degree of immune dysregulation.

A dysbiotic vaginal microbiota and activated immune response did not differentiate between all term and preterm deliveries. It is possible that some women will be able to mount a regulated immune response to a microbial insult resulting in a term delivery, whilst other women will have a dysregulated microbial driven immune result that culminates in preterm birth.

There are other aspects of microbial-immune interactions that are beyond the scope of this Chapter. As well as ethnic variations in the vaginal microbial composition, there are highly likely to be ethnic variations in mucosal immunity to be considered and the hormonal influence on microbial-immune interactions [(378)]. Susceptibility to infection and inflammatory conditions varies across the world. Specific to pregnancy and the innate immune response, Nguyen et al report different ethnic dependent polymorphisms in genes encoding IL-1ra IL-4 and MBL [(379)].

A key influencer of microbial-immune interactions in the female reproductive tract is oestrogen. It is well recognised that high concentrations of oestrogen promotes the accumulation of glycogen in vaginal epithelial cells, which is then metabolised by amylase to maltose, maltotriose and α-dextrines, which in turn are metabolised to lactic acid by *Lactobacillus* spp. High oestrogen environments favours the proliferation of *Lactobacilli* to the detriment of other pathobionts. Although pregnancy is a hyperoestrogenic state and circulating oestrogens increases with gestational age [(380)], it is possible that individual variations can influence the vaginal microbiome composition, and this is an area that has not been explored in this Chapter.

Oestrogens also have an important role in parturition: increasing the number of prostaglandin and oxytocin receptors in the myometrium, upregulating enzymes required for muscle contraction, e.g. myosin light chain kinase and calmodulin [381], facilitating coordinated uterine contractions by increasing gap junction formation in the myometrium [382], and mediating the activity of collagenases and elastase involved in cervical ripening [383]. Sex hormones interact with genetic and environmental factors to determine key aspects of immunity. TLR expression varies according to gender, with TLR2 and TLR4 more highly expressed in males, and TLR3, 7 and 9 more highly expressed in females [384]. With regards to the immune cell populations, women have lower NK cells and memory Treg cells compared to men [385]. Women generate greater antibody responses, maintaining high immune reactivity following viral infections and have higher antibody levels following vaccination compared to men [386, 387]. There is evidence that sex hormones can impact the microbial composition and the host immune response via secondary metabolites binding to oestrogen receptors and peroxisome proliferator-activated receptors [388]. For these reasons, there may be a degree of heterogeneity in the influences of sex hormones on microbial-immune interactions in a pregnant population.

Study strengths and limitations

This large, longitudinal study with a highly phenotyped population allowed a broad interrogation of microbial-host immune relationships. Microbial interactions at the level of microbial recognition by the local immune system with complement and immunoglobulins, and downstream effector cytokines, as well as the interaction between the vaginal microbial environment and the peripheral immune response were explored. The results presented offer a mechanism for microbial driven PTB in the form of local microbial recognition, complement activation, local innate immune cell recruitment and dysregulated cytokine production; all of which converge to result in cervical remodelling, fetal membrane activation and uterine contractions. The findings presented will contribute to developing more targeted, individualised care to the women who visit the preterm birth prevention clinics and to those who may be classified as low risk at booking.

The number of study participants experiencing PTB in this study cohort is a key limiting factor. A greater number would have allowed further phenotyping to differentiate between those with and without PPROM, those who delivered preterm with and without cervical cerclage and/or progesterone, and allow comparisons of the role of microbial driven inflammation in early and late preterm births. Additionally, measuring the concentrations of additional analytes in cervico-vaginal fluid including C3a, and inhibitory Factors H and I, may strengthen the proposed mechanism of dysregulated complement activation in microbial driven PTB.

CHAPTER 6: SUMMARY DISCUSSION

6.1 Summary discussion, future perspectives and translation into clinical practice

Preterm birth is a worldwide health burden and remains the leading cause of neonatal morbidity and mortality [389]. The incidence continues to rise and is estimated to be approximately 11% of all live births worldwide [390]. The challenge to reduce its prevalence remains due to its complex pathophysiology as a result of multiple aetiologies. Without improving our knowledge on the aetiology and pathophysiology of preterm labour, prediction and prevention remain a challenge.

In healthy pregnancy, the maternal immune system undergoes highly regulated temporal adaptions according to the different physiological stages of pregnancy whilst still being alert to respond appropriately to pathogens [175]. A dysregulated immune response has been implicated in the pathogenesis of preterm birth [391]. A stable low diversity, *Lactobacillus* dominant vaginal microbiota is typical of healthy term pregnancy, whilst a dysbiotic vaginal microbiota which is *Lactobacillus* deplete is more common in women who deliver preterm [145, 147, 150]. However not all women with a dysbiotic vaginal microbial composition deliver preterm, therefore the interactions between the vaginal microbiota and an individual's host immune response has major implications for the pregnancy outcome. These concepts have underpinned the investigations in this thesis to explore the maternal immune responses in pregnancy and preterm birth and their interactions with the vaginal microbiota.

In Chapter 3, I explored the trajectory of the peripheral immune response in pregnancy through the assessment of peripheral blood mononuclear cells, plasma cytokines and plasma complement. Temporal adaptions to the adaptive immune response were demonstrated with an increase in CD4+ T cells and their activity measured by CD25 MFI and a gestational increase in CD4+ CD38 MFI and CD8+ CD38 MFI. Concerning the innate immune response, NK cell activity as measured by CD38 MFI also increased with advancing gestation. These findings are consistent with the hypothesis of extravasation of immune cells from the periphery to gestation tissues as labour nears as CD38 potentiates the ability of lymphocytes to adhere to endothelial cells [217]. These temporal changes support the theory of an immune clock in pregnancy, a chronology of well-timed immune adaptations. A key paper to make advancements with this theory was that by Aghaeepour et al who reported progressive increase in peripheral IL-2 production and activation of STAT1 in response to IFN-α stimulation in NK and dendritic cells, and an increase in STAT5a signally in CD4+ T cells [175].

In this thesis, there were no significant differentiating features in the peripheral immune response between women who had preterm and term deliveries. Furthermore, there were no immune signatures related to the risk factor for preterm birth, cervical shortening, or the insertion of a cervical cerclage. Therefore, immune targets for prediction and/or prevention of preterm labour are unlikely to be found in the periphery.

In contrast, data presented in Chapter 4 revealed that a proportion of women who delivered preterm had evidence of local immune activation, compared to women who delivered at term. Women who were referred to the preterm birth prevention clinic because of a previous MTL and/or PTB were shown to have a subtle primed local immune response as early as 12-16 weeks, compared to women who had excisional cervical treatment. In Chapter 5, analysis of the vaginal microbial profiles of the two groups of women showed an increase in alpha diversity in the previous MTL and/or PTB group which supports a microbial driven increase in local inflammation. It is plausible that these early differences may reflect the two main pathophysiological mechanisms for early preterm labour: microbial driven inflammation/infection and mechanical cervical insufficiency. The impact of improving our understanding of different mechanisms for preterm birth in women depending on their underlying risk factor, can lead to earlier patient stratification and more targeted therapeutic strategies.

Cervical shortening is a clinical manifestation that can be readily recognised sonographically in women who may be on a trajectory towards preterm birth. There was an innate and adaptive local immune response in the presence of cervical shortening. However, limited by numbers, it was not possible to confidently identify if local inflammation precedes cervical shortening, or is an effect of shortening. An important finding in the context of cervical shortening was that *Lactobacillus iners* was more commonly associated with cervical shortening compared to *Lactobacillus crispatus*, supporting previous findings by our research group [158]. In addition, in this study, *Lactobacillus iners* was associated with greater local inflammation compared to *Lactobacillus crispatus* with increased concentrations of innate immune mediators IL-1β and IL-6, and activators of the complement pathway IgM and IgG. IL-1β, IL-6, IL-8 and TNF-α promote angiogenesis, vasodilation and increase vascular permeability encouraging further recruitment of more neutrophils and macrophages. MMPs and prostaglandins from neutrophils solubilise collagen fibres of the cervix leading to cervical remodelling, thus linking *Lactobacillus iners* with cervical shortening.

There was also a marked difference in local inflammation between a dysbiotic vaginal microbiota - VMG 4 or CST IV (*Lactobacillus* depletion) compared to *Lactobacillus crispatus*. Demonstrated in Chapter 5 was an increase in mediators that facilitate pathogen recognition, IgM, IgG and MBL which resulted in an increase in local C3b and C5a. With an associated increase in IL-8, IL-1β and IL-6, there was potentially an increase in local phagocytic activity, and a corresponding increase in MMPs and prostaglandins from neutrophils. Activation of the NF-κB transcription factor upregulates genes encoding IL-8, IL-1β and IL-6 increasing COX-2, prostaglandin production and MMPs which are known to play a key role in cervical remodelling, membrane activation and uterine contractions, thus linking microbial-driven inflammation with preterm birth. Complement inhibitors are already used in certain conditions, for example Eculizumab, a recombinant monoclonal antibody which inhibits complement activation of C5 in the treatment of paroxysmal nocturnal haemoglobinuria [(392)]. The IL-1 receptor antagonist, Anakinra is used in the management of idiopathic recurrent pericarditis [(393)]. Immunomodulators targeting complement inhibition and IL-1 receptor antagonists may be therapeutic targets to explore for infection mediated preterm birth.

There were also more subtle but nevertheless important differences in local inflammation between *Lactobacillus gasseri* and *Lactobacillus crispatus*, with higher local concentrations of C3b, Ig2-4 and IgM in *Lactobacillus gasseri* compared to *Lactobacillus crispatus*. These points are clinically important from a pharmacological point of view in the selection of which strains of *Lactobacillus species* are used in the development of vaginally administered probiotics. Recent randomised controlled trials of vaginally administered *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Lactobacillus crispatus* have been shown to reduce recurrence of bacterial vaginosis [(394, 395)]. Our group are studying the ability of a *Lactobacillus crispatus* pessary to colonise the vagina and its influence on the local immune response in pregnancy.

There is some controversy regarding the optimal type of suture material to perform a cervical cerclage. In Chapter 4, I presented evidence to support the activation of a strong innate and adaptive immune response following braided Mersilene cerclage insertion whilst monofilament Nylon in contrast was immunologically inert. Additionally, in this study population, the incidence of preterm birth was significantly higher following Mersilene versus Nylon cerclage. Our group have previously shown that Mersilene also induces a shift towards vaginal dysbiosis [(49)] and in Chapter 5, I have demonstrated that increased bacterial diversity especially in VMG 4 resulted in a marked local pro-inflammatory cytokine, complement and immunoglobulin response. These findings support the use of Nylon over Mersilene cerclage in order to prevent iatrogenic microbial-inflammation driven preterm birth.

Microbial-driven inflammation leads to activation of the innate and adaptive immune response with the complement cascade bridging the two. An augmented immune response is seen in women who deliver preterm suggesting a degree of dysregulation as opposed to a regulated response in women who deliver at term. What determines a differential immune response requires further exploration.

Future work

Future work will focus on how and why individuals have different immune responses to the different vaginal microbial communities. Beginning with components that activate the complement cascade, from this study high CVF concentrations of IgM, MBL and C3b were seen in women who were *Lactobacillus* deplete and who delivered preterm. In collaboration with a group in Valencia who use bacterial fluorescence cell sorting of IgA opsonised and IgA non-opsonised stool microbiota [(396)], we have optimised this technique to determine the percentage and median fluorescent intensity of IgA and IgG opsonised and non-opsonised vaginal microbiota. Work is currently underway in our research group to optimise assessing immune recognition of vaginal microbiota by IgM, MBL and C3b. This work together with 16S rRNA sequencing will increase our understanding of immune recognition of vaginal microbiota at the genera and species level and determine if this has any effect on local inflammation and pregnancy outcome.

For the complement cascade itself, future work would be directed at interrogating the ability to inhibit the different activation pathways. C5a and C3a are key chemoattractants and targeting them is likely to significantly impact on the activity of local neutrophils and macrophages. Effector molecules in cervico-vaginal fluid and their relationship with the vaginal microbiota were comprehensively studied in this thesis. IL-8, IL-6 and IL-1β were consistently increased in response to VMG 3 *Lactobacillus iners*, VMG 4 Diverse species, preterm delivery and cervical shortening. It is not yet known if the source of these cytokines are predominantly from cervical/vaginal epithelial cells, or via neutrophils that would plausibly be attracted via C5a/C3a as a result of activation of the complement cascade. It is possible that neutrophil recognition of pathogens, aided by opsonisation with C3b, leads to neutrophil degranulation and dysregulated production of MMPs [(328)], prostaglandins [(361, 397)] and pro-inflammatory cytokines [(221, 326, 327, 398)]. As a result, this leads to cervical remodeling, fetal membrane rupture and uterine contractility, and ultimately preterm birth [(364)]. With my expertise in the use of the Cytek™ Aurora, I have supported the research group in developing a technique to assess the activation status of cervical vaginal neutrophils to establish their role in microbial-inflammation driven preterm birth.

Another area of interest in the of study microbial-host interactions is the analysis of N- and O-glycosylation of local immune cells through mass spectrometry techniques [399]. Glycan-glycan interactions between those coating the surface of immune cells and those on activated endothelium is important for cell migration. Aberrant neutrophil glycan profiles have been found to correlate with some disease states [400], therefore mass spectrometry glycomic profiling would allow the structural characterisation of neutrophils during pregnancy and preterm birth. In collaboration with the Glycobiology and Glycosciences laboratory at Imperial College, future work aims to determine if there are differences in both the bacterial and immune cell-glycan profiles that determine the risk of preterm birth.

6.2 Final conclusions

- There is a peripheral immune signature consistent with an immune clock of healthy pregnancy.
- There is no peripheral immune signature associated with preterm birth, therefore we are unlikely to find a peripheral biomarker for prediction or therapeutic intervention.
- Cervical shortening and preterm labour is associated with early activation of the local innate and adaptive immune response.
- *Lactobacillus crispatus* does not activate pro-inflammatory cytokines, complement or immunoglobulins and therefore holds promise as a probiotic to prevent preterm birth.
- *Lactobacillus iners* and *Lactobacillus* depletion leads to activation of the innate and adaptive immune response and complement dysregulation occurs in women who deliver preterm and this could represent a group whereby complement inhibitors as pessaries could be effective.
- Nylon is immunologically inert, whereas Mersilene leads to an augmented innate immune response and complement activation and is associated with higher rates of preterm birth so should not be used.

The work presented in this thesis significantly enhances our understanding of the peripheral and local immune response in healthy pregnancy and in women who deliver preterm. The data presented provides novel insight into microbial-host immune interactions during healthy pregnancy and in pregnancies complicated by cervical shortening and preterm labour. This is the first study to describe a mechanistic pathway for microbial-inflammation driven preterm birth and involves a complex interplay between the innate and adaptive immune response, bridged by the complement cascade. The impact of such mechanistic insight is the ability to a) develop new therapies such as probiotics and immune modulators and b) improve patient stratification in order to prevent preterm labour and adverse neonatal outcomes.

CHAPTER 7: REFERENCES

1. WHO. Preterm Birth Factsheet 2018 https://www.who.int/news-room/fact-sheets/detail/preterm-birth.
2. Liu L, Oza S, Hogan D, Chu Y, Perin J, Zhu J, et al. Global, regional, and national causes of under-5 mortality in 2000-15: an updated systematic analysis with implications for the Sustainable Development Goals. Lancet (London, England). 2016;388(10063):3027-35.
3. Purisch SE, Gyamfi-Bannerman C. Epidemiology of preterm birth. Semin Perinatol. 2017;41(7):387-91.
4. Lawn JE, Kinney MV, Belizan JM, Mason EM, McDougall L, Larson J, et al. Born too soon: accelerating actions for prevention and care of 15 million newborns born too soon. Reproductive health. 2013;10 Suppl 1:S6.
5. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. Lancet. 2008;371(9606):75-84.
6. Cavazos-Rehg PA, Krauss MJ, Spitznagel EL, Bommarito K, Madden T, Olsen MA, et al. Maternal age and risk of labor and delivery complications. Maternal and child health journal. 2015;19(6):1202-11.
7. Baer RJ, Yang J, Berghella V, Chambers CD, Coker TR, Kuppermann M, et al. Risk of preterm birth by maternal age at first and second pregnancy and race/ethnicity. Journal of perinatal medicine. 2018;46(5):539-46.
8. Smith GC, Pell JP, Dobbie R. Interpregnancy interval and risk of preterm birth and neonatal death: retrospective cohort study. BMJ (Clinical research ed). 2003;327(7410):313.
9. DeFranco EA, Stamilio DM, Boslaugh SE, Gross GA, Muglia LJ. A short interpregnancy interval is a risk factor for preterm birth and its recurrence. American journal of obstetrics and gynecology. 2007;197(3):264.e1-6.
10. Muglia LJ, Katz M. The enigma of spontaneous preterm birth. The New England journal of medicine. 2010;362(6):529-35.
11. Bloom SL, Yost NP, McIntire DD, Leveno KJ. Recurrence of preterm birth in singleton and twin pregnancies. Obstet Gynecol. 2001;98(3):379-85.
12. Esplin MS, O'Brien E, Fraser A, Kerber RA, Clark E, Simonsen SE, et al. Estimating recurrence of spontaneous preterm delivery. Obstet Gynecol. 2008;112(3):516-23.
13. Poon LC, Savvas M, Zamblera D, Skyfta E, Nicolaides KH. Large loop excision of transformation zone and cervical length in the prediction of spontaneous preterm delivery. Bjog. 2012;119(6):692-8.
14. Edlow AG, Srinivas SK, Elovitz MA. Second-trimester loss and subsequent pregnancy outcomes: What is the real risk? Am J Obstet Gynecol. 2007;197(6):581.e1-6.
15. Patel RR, Steer P, Doyle P, Little MP, Elliott P. Does gestation vary by ethnic group? A London-based study of over 122,000 pregnancies with spontaneous onset of labour. International journal of epidemiology. 2004;33(1):107-13.
16. Hua M, Odibo AO, Longman RE, Macones GA, Roehl KA, Cahill AG. Congenital uterine anomalies and adverse pregnancy outcomes. American journal of obstetrics and gynecology. 2011;205(6):558.e1-5.
17. Zeitlin J, Saurel-Cubizolles MJ, De Mouzon J, Rivera L, Ancel PY, Blondel B, et al. Fetal sex and preterm birth: are males at greater risk? Human reproduction (Oxford, England). 2002;17(10):2762-8.
18. Kistka ZA, Palomar L, Lee KA, Boslaugh SE, Wangler MF, Cole FS, et al. Racial disparity in the frequency of recurrence of preterm birth. Am J Obstet Gynecol. 2007;196(2):131.e1-6.
19. Macones GA, Parry S, Elkousy M, Clothier B, Ural SH, Strauss JF, 3rd. A polymorphism in the promoter region of TNF and bacterial vaginosis: preliminary evidence of gene-environment interaction in the etiology of spontaneous preterm birth. Am J Obstet Gynecol. 2004;190(6):1504-8; discussion 3A.
20. Wang H, Parry S, Macones G, Sammel MD, Kuivaniemi H, Tromp G, et al. A functional SNP in the promoter of the SERPINH1 gene increases risk of preterm premature rupture of membranes in African Americans. Proc Natl Acad Sci U S A. 2006;103(36):13463-7.
21. England N. Saving Babies Lives Care Bundle Version 2 2019 [updated March 2019. 2:[Available from: https://www.england.nhs.uk/wp-content/uploads/2019/07/saving-babies-lives-care-bundle-version-two-v5.pdf.
22. Medley N, Vogel JP, Care A, Alfirevic Z. Interventions during pregnancy to prevent preterm birth: an overview of Cochrane systematic reviews. Cochrane Database Syst Rev. 2018;11(11):Cd012505.
23. Iams JD, Goldenberg RL, Meis PJ, Mercer BM, Moawad A, Das A, et al. The length of the cervix and the risk of spontaneous premature delivery. National Institute of Child Health and Human Development Maternal Fetal Medicine Unit Network. N Engl J Med. 1996;334(9):567-72.
24. Crane JM, Hutchens D. Transvaginal sonographic measurement of cervical length to predict preterm birth in asymptomatic women at increased risk: a systematic review. Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2008;31(5):579-87.
25. NICE. Preterm Labour and Birth NICE Guideline NG25 2019 [updated 2nd August 2019. Available from: https://www.nice.org.uk/guidance/ng25.
26. Heath VC, Southall TR, Souka AP, Elisseou A, Nicolaides KH. Cervical length at 23 weeks of gestation: prediction of spontaneous preterm delivery. Ultrasound Obstet Gynecol. 1998;12(5):312-7.

27. Hassan SS, Romero R, Berry SM, Dang K, Blackwell SC, Treadwell MC, et al. Patients with an ultrasonographic cervical length < or =15 mm have nearly a 50% risk of early spontaneous preterm delivery. Am J Obstet Gynecol. 2000;182(6):1458-67.
28. Taipale P, Hiilesmaa V. Sonographic measurement of uterine cervix at 18-22 weeks' gestation and the risk of preterm delivery. Obstet Gynecol. 1998;92(6):902-7.
29. Esplin MS, Elovitz MA, Iams JD, Parker CB, Wapner RJ, Grobman WA, et al. Predictive Accuracy of Serial Transvaginal Cervical Lengths and Quantitative Vaginal Fetal Fibronectin Levels for Spontaneous Preterm Birth Among Nulliparous Women. Jama. 2017;317(10):1047-56.
30. Peaceman AM, Andrews WW, Thorp JM, Cliver SP, Lukes A, Iams JD, et al. Fetal fibronectin as a predictor of preterm birth in patients with symptoms: a multicenter trial. American journal of obstetrics and gynecology. 1997;177(1):13-8.
31. Abbott DS, Hezelgrave NL, Seed PT, Norman JE, David AL, Bennett PR, et al. Quantitative fetal fibronectin to predict preterm birth in asymptomatic women at high risk. Obstet Gynecol. 2015;125(5):1168-76.
32. Watson HA, Carter J, Seed PT, Tribe RM, Shennan AH. The QUiPP App: a safe alternative to a treat-all strategy for threatened preterm labor. Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2017;50(3):342-6.
33. Pirjani R, Moini A, Almasi-Hashiani A, Farid Mojtahedi M, Vesali S, Hosseini L, et al. Placental alpha microglobulin-1 (PartoSure) test for the prediction of preterm birth: a systematic review and meta-analysis. J Matern Fetal Neonatal Med. 2019:1-13.
34. Akercan F, Kazandi M, Sendag F, Cirpan T, Mgoyi L, Terek MC, et al. Value of cervical phosphorylated insulinlike growth factor binding protein-1 in the prediction of preterm labor. J Reprod Med. 2004;49(5):368-72.
35. Allport VC, Pieber D, Slater DM, Newton R, White JO, Bennett PR. Human labour is associated with nuclear factor-kappaB activity which mediates cyclo-oxygenase-2 expression and is involved with the 'functional progesterone withdrawal'. Molecular human reproduction. 2001;7(6):581-6.
36. Loudon JA, Elliott CL, Hills F, Bennett PR. Progesterone represses interleukin-8 and cyclo-oxygenase-2 in human lower segment fibroblast cells and amnion epithelial cells. Biology of reproduction. 2003;69(1):331-7.
37. Ruddock NK, Shi SQ, Jain S, Moore G, Hankins GD, Romero R, et al. Progesterone, but not 17-alpha-hydroxyprogesterone caproate, inhibits human myometrial contractions. American journal of obstetrics and gynecology. 2008;199(4):391.e1-7.
38. Oh SY, Kim CJ, Park I, Romero R, Sohn YK, Moon KC, et al. Progesterone receptor isoform (A/B) ratio of human fetal membranes increases during term parturition. Am J Obstet Gynecol. 2005;193(3 Pt 2):1156-60.
39. Stjernholm-Vladic Y, Wang H, Stygar D, Ekman G, Sahlin L. Differential regulation of the progesterone receptor A and B in the human uterine cervix at parturition. Gynecol Endocrinol. 2004;18(1):41-6.
40. Kirby MA, Heuerman AC, Custer M, Dobyns AE, Strilaeff R, Stutz KN, et al. Progesterone Receptor-Mediated Actions Regulate Remodeling of the Cervix in Preparation for Preterm Parturition. Reproductive sciences (Thousand Oaks, Calif). 2016;23(11):1473-83.
41. Csapo A. Progesterone block. The American journal of anatomy. 1956;98(2):273-91.
42. Conde-Agudelo A, Romero R, Nicolaides K, Chaiworapongsa T, O'Brien JM, Cetingoz E, et al. Vaginal progesterone vs. cervical cerclage for the prevention of preterm birth in women with a sonographic short cervix, previous preterm birth, and singleton gestation: a systematic review and indirect comparison metaanalysis. American journal of obstetrics and gynecology. 2013;208(1):42.e1-.e18.
43. Martinez de Tejada B, Karolinski A, Ocampo MC, Laterra C, Hosli I, Fernandez D, et al. Prevention of preterm delivery with vaginal progesterone in women with preterm labour (4P): randomised double-blind placebo-controlled trial. Bjog. 2015;122(1):80-91.
44. Sykes L, Bennett PR. Efficacy of progesterone for prevention of preterm birth. Best Pract Res Clin Obstet Gynaecol. 2018;52:126-36.
45. (FDA) UFaDA. Hydroxyprogesterone caproate (17P), U.S. Food and drug administration approved drug products 2018 [Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/021945s012lbl.pdf.
46. Berghella V, Rafael TJ, Szychowski JM, Rust OA, Owen J. Cerclage for short cervix on ultrasonography in women with singleton gestations and previous preterm birth: a meta-analysis. Obstet Gynecol. 2011;117(3):663-71.
47. Berghella V, Ciardulli A, Rust OA, To M, Otsuki K, Althuisius S, et al. Cerclage for sonographic short cervix in singleton gestations without prior spontaneous preterm birth: systematic review and meta-analysis of randomized controlled trials using individual patient-level data. Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2017;50(5):569-77.
48. Harger JH. Comparison of success and morbidity in cervical cerclage procedures. Obstet Gynecol. 1980;56(5):543-8.
49. Kindinger LM, MacIntyre DA, Lee YS, Marchesi JR, Smith A, McDonald JA, et al. Relationship between vaginal microbial dysbiosis, inflammation, and pregnancy outcomes in cervical cerclage. Sci Transl Med. 2016;8(350):350ra102.

50. Israfil-Bayli F, Toozs-Hobson P, Lees C, Slack M, Daniels J, Vince A, et al. Cervical cerclage and type of suture material: a survey of UK consultants' practice. J Matern Fetal Neonatal Med. 2014;27(15):1584-8.
51. (BCTU) BCTU. Cerclage Suture Type for an Insufficient Cervix and its effect on Health (C-STITCH) 2000 [Available from: https://www.birmingham.ac.uk/research/bctu/trials/womens/C-Stich/index.aspx.
52. Arabin B, Alfirevic Z. Cervical pessaries for prevention of spontaneous preterm birth: past, present and future. Ultrasound Obstet Gynecol. 2013;42(4):390-9.
53. Nicolaides KH, Syngelaki A, Poon LC, Picciarelli G, Tul N, Zamprakou A, et al. A Randomized Trial of a Cervical Pessary to Prevent Preterm Singleton Birth. The New England journal of medicine. 2016;374(11):1044-52.
54. Jin XH, Li D, Huang LL. Cervical Pessary for Prevention of Preterm Birth: A Meta-Analysis. Scientific reports. 2017;7:42560.
55. Care A, Muller-Myhsok B, Olearo E, Todros T, Caradeux J, Goya M, et al. Should phenotype of previous preterm birth influence management of women with short cervix in subsequent pregnancy? Comparison of vaginal progesterone and Arabin pessary. Ultrasound Obstet Gynecol. 2019;53(4):529-34.
56. Shor S, Zimerman A, Maymon R, Kovo M, Wolf M, Wiener I, et al. Combined therapy with vaginal progesterone, Arabin cervical pessary and cervical cerclage to prevent preterm delivery in high-risk women. J Matern Fetal Neonatal Med. 2019:1-5.
57. Norman JE, Norrie J, Maclennan G, Cooper D, Whyte S, Cunningham Burley S, et al. Open randomised trial of the (Arabin) pessary to prevent preterm birth in twin pregnancy with health economics and acceptability: STOPPIT-2-a study protocol. BMJ Open. 2018;8(12):e026430.
58. Le J, Briggs GG, McKeown A, Bustillo G. Urinary tract infections during pregnancy. The Annals of pharmacotherapy. 2004;38(10):1692-701.
59. Romero R, Oyarzun E, Mazor M, Sirtori M, Hobbins JC, Bracken M. Meta-analysis of the relationship between asymptomatic bacteriuria and preterm delivery/low birth weight. Obstet Gynecol. 1989;73(4):576-82.
60. Hauth JC, Goldenberg RL, Andrews WW, DuBard MB, Copper RL. Reduced incidence of preterm delivery with metronidazole and erythromycin in women with bacterial vaginosis. The New England journal of medicine. 1995;333(26):1732-6.
61. Thinkhamrop J, Hofmeyr GJ, Adetoro O, Lumbiganon P, Ota E. Antibiotic prophylaxis during the second and third trimester to reduce adverse pregnancy outcomes and morbidity. The Cochrane database of systematic reviews. 2015(6):Cd002250.
62. Simcox R, Sin WT, Seed PT, Briley A, Shennan AH. Prophylactic antibiotics for the prevention of preterm birth in women at risk: a meta-analysis. The Australian & New Zealand journal of obstetrics & gynaecology. 2007;47(5):368-77.
63. Kenyon SL, Taylor DJ, Tarnow-Mordi W. Broad-spectrum antibiotics for preterm, prelabour rupture of fetal membranes: the ORACLE I randomised trial. ORACLE Collaborative Group. Lancet. 2001;357(9261):979-88.
64. Shennan A, Crawshaw S, Briley A, Hawken J, Seed P, Jones G, et al. A randomised controlled trial of metronidazole for the prevention of preterm birth in women positive for cervicovaginal fetal fibronectin: the PREMET Study. Bjog. 2006;113(1):65-74.
65. Martin JN, Jr., D'Alton M, Jacobsson B, Norman JE. In Pursuit of Progress Toward Effective Preterm Birth Reduction. Obstet Gynecol. 2017;129(4):715-9.
66. Roberts D, Brown J, Medley N, Dalziel SR. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. The Cochrane database of systematic reviews. 2017;3:Cd004454.
67. Organisation W-WH. WHO recommendation on antenatal corticosteroid therapy for women at risk of preterm birth from 24 weeks to 34 weeks of gestation 2015 [updated 2015. Available from: https://extranet.who.int/rhl/topics/preconception-pregnancy-childbirth-and-postpartum-care/pregnancy-complications/preterm-birth/who-recommendation-antenatal-corticosteroid-therapy-women-risk-preterm-birth-24-weeks-34-weeks.
68. Gyamfi-Bannerman C, Thom EA, Blackwell SC, Tita AT, Reddy UM, Saade GR, et al. Antenatal Betamethasone for Women at Risk for Late Preterm Delivery. The New England journal of medicine. 2016;374(14):1311-20.
69. Haas DM, Caldwell DM, Kirkpatrick P, McIntosh JJ, Welton NJ. Tocolytic therapy for preterm delivery: systematic review and network meta-analysis. BMJ (Clinical research ed). 2012;345:e6226.
70. Hammers AL, Sanchez-Ramos L, Kaunitz AM. Antenatal exposure to indomethacin increases the risk of severe intraventricular hemorrhage, necrotizing enterocolitis, and periventricular leukomalacia: a systematic review with metaanalysis. American journal of obstetrics and gynecology. 2015;212(4):505.e1-13.
71. Mackeen AD, Seibel-Seamon J, Muhammad J, Baxter JK, Berghella V. Tocolytics for preterm premature rupture of membranes. The Cochrane database of systematic reviews. 2014(2):Cd007062.
72. Doyle LW, Crowther CA, Middleton P, Marret S. Antenatal magnesium sulfate and neurologic outcome in preterm infants: a systematic review. Obstet Gynecol. 2009;113(6):1327-33.
73. Doyle LW, Crowther CA, Middleton P, Marret S, Rouse D. Magnesium sulphate for women at risk of preterm birth for neuroprotection of the fetus. The Cochrane database of systematic reviews. 2009(1):Cd004661.

74. Chollat C, Sentilhes L, Marret S. Fetal Neuroprotection by Magnesium Sulfate: From Translational Research to Clinical Application. Frontiers in neurology. 2018;9:247.
75. Burhouse A, Lea C, Ray S, Bailey H, Davies R, Harding H, et al. Preventing cerebral palsy in preterm labour: a multiorganisational quality improvement approach to the adoption and spread of magnesium sulphate for neuroprotection. BMJ Open Qual. 2017;6(2):e000189.
76. Flenady V, Hawley G, Stock OM, Kenyon S, Badawi N. Prophylactic antibiotics for inhibiting preterm labour with intact membranes. The Cochrane database of systematic reviews. 2013(12):Cd000246.
77. Thomson AJ. Care of Women Presenting with Suspected Preterm Prelabour Rupture of Membranes from 24(+0) Weeks of Gestation: Green-top Guideline No. 73. Bjog. 2019;126(9):e152-e66.
78. Brown RG, Marchesi JR, Lee YS, Smith A, Lehne B, Kindinger LM, et al. Vaginal dysbiosis increases risk of preterm fetal membrane rupture, neonatal sepsis and is exacerbated by erythromycin. BMC Med. 2018;16(1):9.
79. Prevention of Early-onset Neonatal Group B Streptococcal Disease: Green-top Guideline No. 36. Bjog. 2017;124(12):e280-e305.
80. Yellon SM. Immunobiology of Cervix Ripening. Front Immunol. 2019;10:3156.
81. Word RA, Li XH, Hnat M, Carrick K. Dynamics of cervical remodeling during pregnancy and parturition: mechanisms and current concepts. Semin Reprod Med. 2007;25(1):69-79.
82. Nold C, Anton L, Brown A, Elovitz M. Inflammation promotes a cytokine response and disrupts the cervical epithelial barrier: a possible mechanism of premature cervical remodeling and preterm birth. Am J Obstet Gynecol. 2012;206(3):208.e1-7.
83. Bokström H, Brännström M, Alexandersson M, Norström A. Leukocyte subpopulations in the human uterine cervical stroma at early and term pregnancy. Hum Reprod. 1997;12(3):586-90.
84. Word RA, Tang DC, Kamm KE. Activation properties of myosin light chain kinase during contraction/relaxation cycles of tonic and phasic smooth muscles. J Biol Chem. 1994;269(34):21596-602.
85. Sanborn BM, Ku CY, Shlykov S, Babich L. Molecular signaling through G-protein-coupled receptors and the control of intracellular calcium in myometrium. J Soc Gynecol Investig. 2005;12(7):479-87.
86. Woodcock NA, Taylor CW, Thornton S. Effect of an oxytocin receptor antagonist and rho kinase inhibitor on the [Ca++]i sensitivity of human myometrium. Am J Obstet Gynecol. 2004;190(1):222-8.
87. Woodcock NA, Taylor CW, Thornton S. Prostaglandin F2alpha increases the sensitivity of the contractile proteins to Ca2+ in human myometrium. Am J Obstet Gynecol. 2006;195(5):1404-6.
88. Kim SH, MacIntyre DA, Firmino Da Silva M, Blanks AM, Lee YS, Thornton S, et al. Oxytocin activates NF-kappaB-mediated inflammatory pathways in human gestational tissues. Molecular and cellular endocrinology. 2015;403:64-77.
89. Mesiano S, Chan EC, Fitter JT, Kwek K, Yeo G, Smith R. Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor A expression in the myometrium. The Journal of clinical endocrinology and metabolism. 2002;87(6):2924-30.
90. Tan H, Yi L, Rote NS, Hurd WW, Mesiano S. Progesterone receptor-A and -B have opposite effects on proinflammatory gene expression in human myometrial cells: implications for progesterone actions in human pregnancy and parturition. The Journal of clinical endocrinology and metabolism. 2012;97(5):E719-30.
91. Grammatopoulos DK, Hillhouse EW. Role of corticotropin-releasing hormone in onset of labour. Lancet (London, England). 1999;354(9189):1546-9.
92. Li W, Challis JR. Corticotropin-releasing hormone and urocortin induce secretion of matrix metalloproteinase-9 (MMP-9) without change in tissue inhibitors of MMP-1 by cultured cells from human placenta and fetal membranes. The Journal of clinical endocrinology and metabolism. 2005;90(12):6569-74.
93. Bryant-Greenwood GD. The extracellular matrix of the human fetal membranes: structure and function. Placenta. 1998;19(1):1-11.
94. Strauss JF, 3rd. Extracellular matrix dynamics and fetal membrane rupture. Reproductive sciences (Thousand Oaks, Calif). 2013;20(2):140-53.
95. Malak TM, Bell SC. Structural characteristics of term human fetal membranes: a novel zone of extreme morphological alteration within the rupture site. British journal of obstetrics and gynaecology. 1994;101(5):375-86.
96. Goldenberg RL, Andrews WW, Hauth JC. Choriodecidual infection and preterm birth. Nutrition reviews. 2002;60(5 Pt 2):S19-25.
97. Daalderop LA, Wieland BV, Tomsin K, Reyes L, Kramer BW, Vanterpool SF, et al. Periodontal Disease and Pregnancy Outcomes: Overview of Systematic Reviews. JDR Clin Trans Res. 2018;3(1):10-27.
98. Bard E, Riethmuller D, Biichlé S, Meillet D, Prétet JL, Mougin C, et al. Validation of a high sensitive immunoenzymatic assay to establish the origin of immunoglobulins in female genital secretions. J Immunoassay Immunochem. 2002;23(2):145-62.
99. Valenti P, Rosa L, Capobianco D, Lepanto MS, Schiavi E, Cutone A, et al. Role of Lactobacilli and Lactoferrin in the Mucosal Cervicovaginal Defense. Front Immunol. 2018;9:376.
100. Cole AM. Innate host defense of human vaginal and cervical mucosae. Curr Top Microbiol Immunol. 2006;306:199-230.

101. Ming L, Xiaoling P, Yan L, Lili W, Qi W, Xiyong Y, et al. Purification of antimicrobial factors from human cervical mucus. Hum Reprod. 2007;22(7):1810-5.
102. Stock SJ, Duthie L, Tremaine T, Calder AA, Kelly RW, Riley SC. Elafin (SKALP/Trappin-2/proteinase inhibitor-3) is produced by the cervix in pregnancy and cervicovaginal levels are diminished in bacterial vaginosis. Reprod Sci. 2009;16(12):1125-34.
103. Linhares IM, Summers PR, Larsen B, Giraldo PC, Witkin SS. Contemporary perspectives on vaginal pH and lactobacilli. Am J Obstet Gynecol. 2011;204(2):120.e1-5.
104. Mitchell C, Marrazzo J. Bacterial vaginosis and the cervicovaginal immune response. Am J Reprod Immunol. 2014;71(6):555-63.
105. Hart KM, Murphy AJ, Barrett KT, Wira CR, Guyre PM, Pioli PA. Functional expression of pattern recognition receptors in tissues of the human female reproductive tract. J Reprod Immunol. 2009;80(1-2):33-40.
106. Kawai T, Akira S. Signaling to NF-kappaB by Toll-like receptors. Trends Mol Med. 2007;13(11):460-9.
107. Khanjani S, Terzidou V, Johnson MR, Bennett PR. NFκB and AP-1 drive human myometrial IL8 expression. Mediators Inflamm. 2012;2012:504952.
108. Wylie KM, Wylie TN, Cahill AG, Macones GA, Tuuli MG, Stout MJ. The vaginal eukaryotic DNA virome and preterm birth. Am J Obstet Gynecol. 2018;219(2):189.e1-.e12.
109. Ma X, Sun D, Li C, Ying J, Yan Y. Chronic hepatitis B virus infection and preterm labor(birth) in pregnant women-an updated systematic review and meta-analysis. J Med Virol. 2018;90(1):93-100.
110. Wedi CO, Kirtley S, Hopewell S, Corrigan R, Kennedy SH, Hemelaar J. Perinatal outcomes associated with maternal HIV infection: a systematic review and meta-analysis. Lancet HIV. 2016;3(1):e33-48.
111. Cardenas I, Mor G, Aldo P, Lang SM, Stabach P, Sharp A, et al. Placental viral infection sensitizes to endotoxin-induced pre-term labor: a double hit hypothesis. Am J Reprod Immunol. 2011;65(2):110-7.
112. Rasheed ZBM, Lee YS, Kim SH, Rai RK, Ruano CSM, Anucha E, et al. Differential Response of Gestational Tissues to TLR3 Viral Priming Prior to Exposure to Bacterial TLR2 and TLR2/6 Agonists. Frontiers in Immunology. 2020;11(1899).
113. Ou CW, Orsino A, Lye SJ. Expression of connexin-43 and connexin-26 in the rat myometrium during pregnancy and labor is differentially regulated by mechanical and hormonal signals. Endocrinology. 1997;138(12):5398-407.
114. Adams Waldorf KM, Singh N, Mohan AR, Young RC, Ngo L, Das A, et al. Uterine overdistention induces preterm labor mediated by inflammation: observations in pregnant women and nonhuman primates. American journal of obstetrics and gynecology. 2015;213(6):830.e1-.e19.
115. Watson HA, Carter J, David AL, Seed PT, Shennan AH. Full dilation cesarean section: a risk factor for recurrent second-trimester loss and preterm birth. Acta Obstet Gynecol Scand. 2017;96(9):1100-5.
116. Levine LD, Sammel MD, Hirshberg A, Elovitz MA, Srinivas SK. Does stage of labor at time of cesarean delivery affect risk of subsequent preterm birth? Am J Obstet Gynecol. 2015;212(3):360.e1-7.
117. Noehr B, Jensen A, Frederiksen K, Tabor A, Kjaer SK. Depth of cervical cone removed by loop electrosurgical excision procedure and subsequent risk of spontaneous preterm delivery. Obstet Gynecol. 2009;114(6):1232-8.
118. Khalid S, Dimitriou E, Conroy R, Paraskevaidis E, Kyrgiou M, Harrity C, et al. The thickness and volume of LLETZ specimens can predict the relative risk of pregnancy-related morbidity. Bjog. 2012;119(6):685-91.
119. Volozonoka L, Rots D, Kempa I, Kornete A, Rezeberga D, Gailite L, et al. Genetic landscape of preterm birth due to cervical insufficiency: Comprehensive gene analysis and patient next-generation sequencing data interpretation. PLoS One. 2020;15(3):e0230771.
120. McLean M, Bisits A, Davies J, Woods R, Lowry P, Smith R. A placental clock controlling the length of human pregnancy. Nat Med. 1995;1(5):460-3.
121. Ruiz RJ, Gennaro S, O'Connor C, Dwivedi A, Gibeau A, Keshinover T, et al. CRH as a Predictor of Preterm Birth in Minority Women. Biol Res Nurs. 2016;18(3):316-21.
122. Williams MA, Mittendorf R, Lieberman E, Monson RR. Adverse infant outcomes associated with first-trimester vaginal bleeding. Obstet Gynecol. 1991;78(1):14-8.
123. Al-Memar M, Vaulet T, Fourie H, Bobdiwala S, Farren J, Saso S, et al. First-trimester intrauterine hematoma and pregnancy complications. Ultrasound Obstet Gynecol. 2020;55(4):536-45.
124. Koifman A, Levy A, Zaulan Y, Harlev A, Mazor M, Wiznitzer A, et al. The clinical significance of bleeding during the second trimester of pregnancy. Arch Gynecol Obstet. 2008;278(1):47-51.
125. Harger JH, Hsing AW, Tuomala RE, Gibbs RS, Mead PB, Eschenbach DA, et al. Risk factors for preterm premature rupture of fetal membranes: a multicenter case-control study. Am J Obstet Gynecol. 1990;163(1 Pt 1):130-7.
126. Elovitz MA, Ascher-Landsberg J, Saunders T, Phillippe M. The mechanisms underlying the stimulatory effects of thrombin on myometrial smooth muscle. Am J Obstet Gynecol. 2000;183(3):674-81.
127. Grand RJ, Turnell AS, Grabham PW. Cellular consequences of thrombin-receptor activation. Biochem J. 1996;313 (Pt 2)(Pt 2):353-68.

128. Kim YM, Bujold E, Chaiworapongsa T, Gomez R, Yoon BH, Thaler HT, et al. Failure of physiologic transformation of the spiral arteries in patients with preterm labor and intact membranes. Am J Obstet Gynecol. 2003;189(4):1063-9.
129. Kim YM, Chaiworapongsa T, Gomez R, Bujold E, Yoon BH, Rotmensch S, et al. Failure of physiologic transformation of the spiral arteries in the placental bed in preterm premature rupture of membranes. Am J Obstet Gynecol. 2002;187(5):1137-42.
130. Strauss JF, 3rd, Romero R, Gomez-Lopez N, Haymond-Thornburg H, Modi BP, Teves ME, et al. Spontaneous preterm birth: advances toward the discovery of genetic predisposition. Am J Obstet Gynecol. 2018;218(3):294-314.e2.
131. Zhang G, Feenstra B, Bacelis J, Liu X, Muglia LM, Juodakis J, et al. Genetic Associations with Gestational Duration and Spontaneous Preterm Birth. N Engl J Med. 2017;377(12):1156-67.
132. Capece A, Vasieva O, Meher S, Alfirevic Z, Alfirevic A. Pathway analysis of genetic factors associated with spontaneous preterm birth and pre-labor preterm rupture of membranes. PLoS One. 2014;9(9):e108578.
133. Modi BP, Teves ME, Pearson LN, Parikh HI, Haymond-Thornburg H, Tucker JL, et al. Mutations in fetal genes involved in innate immunity and host defense against microbes increase risk of preterm premature rupture of membranes (PPROM). Mol Genet Genomic Med. 2017;5(6):720-9.
134. Roberts AK, Monzon-Bordonaba F, Van Deerlin PG, Holder J, Macones GA, Morgan MA, et al. Association of polymorphism within the promoter of the tumor necrosis factor alpha gene with increased risk of preterm premature rupture of the fetal membranes. Am J Obstet Gynecol. 1999;180(5):1297-302.
135. Marchesi JR, Ravel J. The vocabulary of microbiome research: a proposal. Microbiome. 2015;3:31.
136. Cummings LA, Kurosawa K, Hoogestraat DR, SenGupta DJ, Candra F, Doyle M, et al. Clinical Next Generation Sequencing Outperforms Standard Microbiological Culture for Characterizing Polymicrobial Samples. Clinical chemistry. 2016;62(11):1465-73.
137. Chakravorty S, Helb D, Burday M, Connell N, Alland D. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. Journal of microbiological methods. 2007;69(2):330-9.
138. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature. 2007;449(7164):804-10.
139. The Integrative Human Microbiome Project. Nature. 2019;569(7758):641-8.
140. Petrova MI, Lievens E, Malik S, Imholz N, Lebeer S. Lactobacillus species as biomarkers and agents that can promote various aspects of vaginal health. Front Physiol. 2015;6:81.
141. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, et al. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A. 2011;108 Suppl 1:4680-7.
142. Gajer P, Brotman RM, Bai G, Sakamoto J, Schütte UM, Zhong X, et al. Temporal dynamics of the human vaginal microbiota. Sci Transl Med. 2012;4(132):132ra52.
143. Chaban B, Links MG, Jayaprakash TP, Wagner EC, Bourque DK, Lohn Z, et al. Characterization of the vaginal microbiota of healthy Canadian women through the menstrual cycle. Microbiome. 2014;2:23.
144. Roy EJ, Mackay R. The concentration of oestrogens in blood during pregnancy. The Journal of obstetrics and gynaecology of the British Empire. 1962;69:13-7.
145. MacIntyre DA, Chandiramani M, Lee YS, Kindinger L, Smith A, Angelopoulos N, et al. The vaginal microbiome during pregnancy and the postpartum period in a European population. Sci Rep. 2015;5:8988.
146. Aagaard K, Riehle K, Ma J, Segata N, Mistretta TA, Coarfa C, et al. A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy. PLoS One. 2012;7(6):e36466.
147. DiGiulio DB, Callahan BJ, McMurdie PJ, Costello EK, Lyell DJ, Robaczewska A, et al. Temporal and spatial variation of the human microbiota during pregnancy. Proc Natl Acad Sci U S A. 2015;112(35):11060-5.
148. Brotman RM, He X, Gajer P, Fadrosh D, Sharma E, Mongodin EF, et al. Association between cigarette smoking and the vaginal microbiota: a pilot study. BMC infectious diseases. 2014;14:471.
149. Aslan E, Bechelaghem N. To 'douche' or not to 'douche': hygiene habits may have detrimental effects on vaginal microbiota. Journal of obstetrics and gynaecology : the journal of the Institute of Obstetrics and Gynaecology. 2018;38(5):678-81.
150. Romero R, Hassan SS, Gajer P, Tarca AL, Fadrosh DW, Bieda J, et al. The vaginal microbiota of pregnant women who subsequently have spontaneous preterm labor and delivery and those with a normal delivery at term. Microbiome. 2014;2:18.
151. Zhou X, Brown CJ, Abdo Z, Davis CC, Hansmann MA, Joyce P, et al. Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. Isme j. 2007;1(2):121-33.
152. Benirschke K. Routes and types of infection in the fetus and the newborn. AMA J Dis Child. 1960;99:714-21.
153. Jones HE, Harris KA, Azizia M, Bank L, Carpenter B, Hartley JC, et al. Differing prevalence and diversity of bacterial species in fetal membranes from very preterm and term labor. PLoS One. 2009;4(12):e8205.
154. Romero R, Sirtori M, Oyarzun E, Avila C, Mazor M, Callahan R, et al. Infection and labor. V. Prevalence, microbiology, and clinical significance of intraamniotic infection in women with preterm labor and intact membranes. Am J Obstet Gynecol. 1989;161(3):817-24.

155. Callahan BJ, DiGiulio DB, Goltsman DSA, Sun CL, Costello EK, Jeganathan P, et al. Replication and refinement of a vaginal microbial signature of preterm birth in two racially distinct cohorts of US women. Proc Natl Acad Sci U S A. 2017;114(37):9966-71.
156. Fettweis JM, Serrano MG, Brooks JP, Edwards DJ, Girerd PH, Parikh HI, et al. The vaginal microbiome and preterm birth. Nat Med. 2019;25(6):1012-21.
157. Petricevic L, Domig KJ, Nierscher FJ, Sandhofer MJ, Fidesser M, Krondorfer I, et al. Characterisation of the vaginal Lactobacillus microbiota associated with preterm delivery. Sci Rep. 2014;4:5136.
158. Kindinger LM, Bennett PR, Lee YS, Marchesi JR, Smith A, Cacciatore S, et al. The interaction between vaginal microbiota, cervical length, and vaginal progesterone treatment for preterm birth risk. Microbiome. 2017;5(1):6.
159. Tabatabaei N, Eren AM, Barreiro LB, Yotova V, Dumaine A, Allard C, et al. Vaginal microbiome in early pregnancy and subsequent risk of spontaneous preterm birth: a case-control study. Bjog. 2019;126(3):349-58.
160. Parry S, Strauss JF, 3rd. Premature rupture of the fetal membranes. N Engl J Med. 1998;338(10):663-70.
161. Brown RG, Al-Memar M, Marchesi JR, Lee YS, Smith A, Chan D, et al. Establishment of vaginal microbiota composition in early pregnancy and its association with subsequent preterm prelabor rupture of the fetal membranes. Transl Res. 2019;207:30-43.
162. Mitra A, MacIntyre DA, Lee YS, Smith A, Marchesi JR, Lehne B, et al. Cervical intraepithelial neoplasia disease progression is associated with increased vaginal microbiome diversity. Sci Rep. 2015;5:16865.
163. Mitra A, MacIntyre DA, Ntritsos G, Smith A, Tsilidis KK, Marchesi JR, et al. The vaginal microbiota associates with the regression of untreated cervical intraepithelial neoplasia 2 lesions. Nat Commun. 2020;11(1):1999.
164. Wiik J, Sengpiel V, Kyrgiou M, Nilsson S, Mitra A, Tanbo T, et al. Cervical microbiota in women with cervical intra-epithelial neoplasia, prior to and after local excisional treatment, a Norwegian cohort study. BMC Womens Health. 2019;19(1):30.
165. Witkin SS, Mendes-Soares H, Linhares IM, Jayaram A, Ledger WJ, Forney LJ. Influence of vaginal bacteria and D- and L-lactic acid isomers on vaginal extracellular matrix metalloproteinase inducer: implications for protection against upper genital tract infections. mBio. 2013;4(4).
166. Amabebe E, Anumba DOC. The Vaginal Microenvironment: The Physiologic Role of Lactobacilli. Front Med (Lausanne). 2018;5:181.
167. Voskuhl R, Momtazee C. Pregnancy: Effect on Multiple Sclerosis, Treatment Considerations, and Breastfeeding. Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics. 2017;14(4):974-84.
168. Barrett JH, Brennan P, Fiddler M, Silman AJ. Does rheumatoid arthritis remit during pregnancy and relapse postpartum? Results from a nationwide study in the United Kingdom performed prospectively from late pregnancy. Arthritis and rheumatism. 1999;42(6):1219-27.
169. Sykes L, MacIntyre DA, Yap XJ, Teoh TG, Bennett PR. The Th1:th2 dichotomy of pregnancy and preterm labour. Mediators Inflamm. 2012;2012:967629.
170. Siegel I, Gleicher N. Changes in peripheral mononuclear cells in pregnancy. Am J Reprod Immunol. 1981;1(3):154-5.
171. Siegel I, Gleicher N. Peripheral white blood cell alterations in early labor. Diagn Gynecol Obstet. 1981;3(2):123-6.
172. Riella LV, Paterson AM, Sharpe AH, Chandraker A. Role of the PD-1 pathway in the immune response. Am J Transplant. 2012;12(10):2575-87.
173. Meggyes M, Miko E, Szigeti B, Farkas N, Szereday L. The importance of the PD-1/PD-L1 pathway at the maternal-fetal interface. BMC Pregnancy Childbirth. 2019;19(1):74.
174. Mor G, Aldo P, Alvero AB. The unique immunological and microbial aspects of pregnancy. Nat Rev Immunol. 2017;17(8):469-82.
175. Aghaeepour N, Ganio EA, McIlwain D, Tsai AS, Tingle M, Van Gassen S, et al. An immune clock of human pregnancy. Sci Immunol. 2017;2(15).
176. Apps R, Kotliarov Y, Cheung F, Han KL, Chen J, Biancotto A, et al. Multimodal immune phenotyping of maternal peripheral blood in normal human pregnancy. JCI Insight. 2020;5(7).
177. Moura E, Mattar R, de Souza E, Torloni MR, Gonçalves-Primo A, Daher S. Inflammatory cytokine gene polymorphisms and spontaneous preterm birth. J Reprod Immunol. 2009;80(1-2):115-21.
178. Bashirova AA, Martin MP, McVicar DW, Carrington M. The killer immunoglobulin-like receptor gene cluster: tuning the genome for defense. Annu Rev Genomics Hum Genet. 2006;7:277-300.
179. Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood. 2010;115(11):2167-76.
180. Wei SQ, Fraser W, Luo ZC. Inflammatory cytokines and spontaneous preterm birth in asymptomatic women: a systematic review. Obstet Gynecol. 2010;116(2 Pt 1):393-401.
181. Tang MX, Hu XH, Liu ZZ, Kwak-Kim J, Liao AH. What are the roles of macrophages and monocytes in human pregnancy? Journal of reproductive immunology. 2015;112:73-80.

182. Kim J, Ko Y, Kwon K, Koo S, Rhee Y, Kang B, et al. Analysis of monocyte subsets and toll-like receptor 4 expression in peripheral blood monocytes of women in preterm labor. Journal of reproductive immunology. 2012;94(2):190-5.
183. Pawelczyk E, Nowicki BJ, Izban MG, Pratap S, Sashti NA, Sanderson M, et al. Spontaneous preterm labor is associated with an increase in the proinflammatory signal transducer TLR4 receptor on maternal blood monocytes. BMC pregnancy and childbirth. 2010;10:66.
184. Bakker R, Pierce S, Myers D. The role of prostaglandins E1 and E2, dinoprostone, and misoprostol in cervical ripening and the induction of labor: a mechanistic approach. Arch Gynecol Obstet. 2017;296(2):167-79.
185. Bemark M, Holmqvist J, Abrahamsson J, Mellgren K. Translational Mini-Review Series on B cell subsets in disease. Reconstitution after haematopoietic stem cell transplantation - revelation of B cell developmental pathways and lineage phenotypes. Clinical and experimental immunology. 2012;167(1):15-25.
186. Petroff MG. Review: Fetal antigens--identity, origins, and influences on the maternal immune system. Placenta. 2011;32 Suppl 2(Suppl 2):S176-81.
187. Bonney EA. Immune Regulation in Pregnancy: A Matter of Perspective? Obstet Gynecol Clin North Am. 2016;43(4):679-98.
188. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. Front Immunol. 2014;5:520.
189. Vazquez MI, Catalan-Dibene J, Zlotnik A. B cells responses and cytokine production are regulated by their immune microenvironment. Cytokine. 2015;74(2):318-26.
190. Michimata T, Tsuda H, Sakai M, Fujimura M, Nagata K, Nakamura M, et al. Accumulation of CRTH2-positive T-helper 2 and T-cytotoxic 2 cells at implantation sites of human decidua in a prostaglandin D(2)-mediated manner. Mol Hum Reprod. 2002;8(2):181-7.
191. Tsuda H, Michimata T, Sakai M, Nagata K, Nakamura M, Saito S. A novel surface molecule of Th2- and Tc2-type cells, CRTH2 expression on human peripheral and decidual CD4+ and CD8+ T cells during the early stage of pregnancy. Clin Exp Immunol. 2001;123(1):105-11.
192. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol Today. 1993;14(7):353-6.
193. Raghupathy R. Th1-type immunity is incompatible with successful pregnancy. Immunol Today. 1997;18(10):478-82.
194. Robertson SA, Guerin LR, Bromfield JJ, Branson KM, Ahlström AC, Care AS. Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. Biol Reprod. 2009;80(5):1036-45.
195. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. Nat Immunol. 2004;5(3):266-71.
196. Rowe JH, Ertelt JM, Xin L, Way SS. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. Nature. 2012;490(7418):102-6.
197. Peck A, Mellins ED. Plasticity of T-cell phenotype and function: the T helper type 17 example. Immunology. 2010;129(2):147-53.
198. Richani K, Soto E, Romero R, Espinoza J, Chaiworapongsa T, Nien JK, et al. Normal pregnancy is characterized by systemic activation of the complement system. J Matern Fetal Neonatal Med. 2005;17(4):239-45.
199. Mathern DR, Heeger PS. Molecules Great and Small: The Complement System. Clin J Am Soc Nephrol. 2015;10(9):1636-50.
200. Makhseed M, Raghupathy R, El-Shazly S, Azizieh F, Al-Harmi JA, Al-Azemi MM. Pro-inflammatory maternal cytokine profile in preterm delivery. Am J Reprod Immunol. 2003;49(5):308-18.
201. Gargano JW, Holzman C, Senagore P, Thorsen P, Skogstrand K, Hougaard DM, et al. Mid-pregnancy circulating cytokine levels, histologic chorioamnionitis and spontaneous preterm birth. J Reprod Immunol. 2008;79(1):100-10.
202. Xiong H, Zhou C, Qi G. Proportional changes of CD4+CD25+Foxp3+ regulatory T cells in maternal peripheral blood during pregnancy and labor at term and preterm. Clin Invest Med. 2010;33(6):E422.
203. Koucký M, Malíčková K, Cindrová-Davies T, Germanová A, Pařízek A, Kalousová M, et al. Low levels of circulating T-regulatory lymphocytes and short cervical length are associated with preterm labor. J Reprod Immunol. 2014;106:110-7.
204. Ito M, Nakashima A, Hidaka T, Okabe M, Bac ND, Ina S, et al. A role for IL-17 in induction of an inflammation at the fetomaternal interface in preterm labour. J Reprod Immunol. 2010;84(1):75-85.
205. Lorenz E, Mira JP, Frees KL, Schwartz DA. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. Arch Intern Med. 2002;162(9):1028-32.
206. Krediet TG, Wiertsema SP, Vossers MJ, Hoeks SB, Fleer A, Ruven HJ, et al. Toll-like receptor 2 polymorphism is associated with preterm birth. Pediatr Res. 2007;62(4):474-6.
207. Lynch AM, Gibbs RS, Murphy JR, Byers T, Neville MC, Giclas PC, et al. Complement activation fragment Bb in early pregnancy and spontaneous preterm birth. Am J Obstet Gynecol. 2008;199(4):354.e1-8.

208. Lynch AM, Gibbs RS, Murphy JR, Giclas PC, Salmon JE, Holers VM. Early elevations of the complement activation fragment C3a and adverse pregnancy outcomes. Obstet Gynecol. 2011;117(1):75-83.
209. Gonzalez JM, Franzke CW, Yang F, Romero R, Girardi G. Complement activation triggers metalloproteinases release inducing cervical remodeling and preterm birth in mice. Am J Pathol. 2011;179(2):838-49.
210. Gonzalez JM, Pedroni SM, Girardi G. Statins prevent cervical remodeling, myometrial contractions and preterm labor through a mechanism that involves hemoxygenase-1 and complement inhibition. Mol Hum Reprod. 2014;20(6):579-89.
211. Lappas M, Woodruff TM, Taylor SM, Permezel M. Complement C5A regulates prolabor mediators in human placenta. Biol Reprod. 2012;86(6):190.
212. Ruocco MG, Chaouat G, Florez L, Bensussan A, Klatzmann D. Regulatory T-cells in pregnancy: historical perspective, state of the art, and burning questions. Front Immunol. 2014;5:389.
213. Born WK, Reardon CL, O'Brien RL. The function of gammadelta T cells in innate immunity. Curr Opin Immunol. 2006;18(1):31-8.
214. Morita CT, Mariuzza RA, Brenner MB. Antigen recognition by human gamma delta T cells: pattern recognition by the adaptive immune system. Springer Semin Immunopathol. 2000;22(3):191-217.
215. Bai L, Deng S, Reboulet R, Mathew R, Teyton L, Savage PB, et al. Natural killer T (NKT)-B-cell interactions promote prolonged antibody responses and long-term memory to pneumococcal capsular polysaccharides. Proc Natl Acad Sci U S A. 2013;110(40):16097-102.
216. Létourneau S, van Leeuwen EM, Krieg C, Martin C, Pantaleo G, Sprent J, et al. IL-2/anti-IL-2 antibody complexes show strong biological activity by avoiding interaction with IL-2 receptor alpha subunit CD25. Proc Natl Acad Sci U S A. 2010;107(5):2171-6.
217. Dianzani U, Funaro A, DiFranco D, Garbarino G, Bragardo M, Redoglia V, et al. Interaction between endothelium and CD4+CD45RA+ lymphocytes. Role of the human CD38 molecule. J Immunol. 1994;153(3):952-9.
218. Thibult ML, Mamessier E, Gertner-Dardenne J, Pastor S, Just-Landi S, Xerri L, et al. PD-1 is a novel regulator of human B-cell activation. Int Immunol. 2013;25(2):129-37.
219. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. Immunol Rev. 2010;236:219-42.
220. Geisberger R, Lamers M, Achatz G. The riddle of the dual expression of IgM and IgD. Immunology. 2006;118(4):429-37.
221. Osman I, Young A, Ledingham MA, Thomson AJ, Jordan F, Greer IA, et al. Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. Mol Hum Reprod. 2003;9(1):41-5.
222. Guzeloglu-Kayisli O, Kayisli UA, Semerci N, Basar M, Buchwalder LF, Buhimschi CS, et al. Mechanisms of chorioamnionitis-associated preterm birth: interleukin-1beta inhibits progesterone receptor expression in decidual cells. The Journal of pathology. 2015;237(4):423-34.
223. Bae J, Park D, Lee YS, Jeoung D. Interleukin-2 promotes angiogenesis by activation of Akt and increase of ROS. Journal of microbiology and biotechnology. 2008;18(2):377-82.
224. Chatterjee P, Chiasson VL, Bounds KR, Mitchell BM. Regulation of the Anti-Inflammatory Cytokines Interleukin-4 and Interleukin-10 during Pregnancy. Frontiers in immunology. 2014;5:253.
225. Ekman-Ordeberg G, Dubicke A. Preterm Cervical Ripening in humans. Facts, views & vision in ObGyn. 2012;4(4):245-53.
226. Sakai M, Ishiyama A, Tabata M, Sasaki Y, Yoneda S, Shiozaki A, et al. Relationship between cervical mucus interleukin-8 concentrations and vaginal bacteria in pregnancy. American journal of reproductive immunology (New York, NY : 1989). 2004;52(2):106-12.
227. Rizzo G, Capponi A, Vlachopoulou A, Angelini E, Grassi C, Romanini C. The diagnostic value of interleukin-8 and fetal fibronectin concentrations in cervical secretions in patients with preterm labor and intact membranes. Journal of perinatal medicine. 1997;25(6):461-8.
228. Sakai M, Sasaki Y, Yoneda S, Kasahara T, Arai T, Okada M, et al. Elevated interleukin-8 in cervical mucus as an indicator for treatment to prevent premature birth and preterm, pre-labor rupture of membranes: a prospective study. American journal of reproductive immunology (New York, NY : 1989). 2004;51(3):220-5.
229. Dubicke A, Fransson E, Centini G, Andersson E, Bystrom B, Malmstrom A, et al. Pro-inflammatory and anti-inflammatory cytokines in human preterm and term cervical ripening. Journal of reproductive immunology. 2010;84(2):176-85.
230. Gomez-Lopez N, StLouis D, Lehr MA, Sanchez-Rodriguez EN, Arenas-Hernandez M. Immune cells in term and preterm labor. Cellular & molecular immunology. 2014;11(6):571-81.
231. Eubank TD, Roberts R, Galloway M, Wang Y, Cohn DE, Marsh CB. GM-CSF induces expression of soluble VEGF receptor-1 from human monocytes and inhibits angiogenesis in mice. Immunity. 2004;21(6):831-42.
232. Chandiramani M, Seed PT, Orsi NM, Ekbote UV, Bennett PR, Shennan AH, et al. Limited relationship between cervico-vaginal fluid cytokine profiles and cervical shortening in women at high risk of spontaneous preterm birth. PLoS One. 2012;7(12):e52412.

233. Fortunato SJ, Menon R, Lombardi SJ. Role of tumor necrosis factor-alpha in the premature rupture of membranes and preterm labor pathways. American journal of obstetrics and gynecology. 2002;187(5):1159-62.
234. Bradley JR. TNF-mediated inflammatory disease. J Pathol. 2008;214(2):149-60.
235. Turner MW. Mannose-binding lectin (MBL) in health and disease. Immunobiology. 1998;199(2):327-39.
236. Bodamer OA, Mitterer G, Maurer W, Pollak A, Mueller MW, Schmidt WM. Evidence for an association between mannose-binding lectin 2 (MBL2) gene polymorphisms and pre-term birth. Genet Med. 2006;8(8):518-24.
237. Pellis V, De Seta F, Crovella S, Bossi F, Bulla R, Guaschino S, et al. Mannose binding lectin and C3 act as recognition molecules for infectious agents in the vagina. Clin Exp Immunol. 2005;139(1):120-6.
238. Kutteh WH, Moldoveanu Z, Mestecky J. Mucosal immunity in the female reproductive tract: correlation of immunoglobulins, cytokines, and reproductive hormones in human cervical mucus around the time of ovulation. AIDS Res Hum Retroviruses. 1998;14 Suppl 1:S51-5.
239. Masson L, Passmore JA, Liebenberg LJ, Werner L, Baxter C, Arnold KB, et al. Genital inflammation and the risk of HIV acquisition in women. Clin Infect Dis. 2015;61(2):260-9.
240. Nazli A, Chan O, Dobson-Belaire WN, Ouellet M, Tremblay MJ, Gray-Owen SD, et al. Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. PLoS Pathog. 2010;6(4):e1000852.
241. Arnold KB, Burgener A, Birse K, Romas L, Dunphy LJ, Shahabi K, et al. Increased levels of inflammatory cytokines in the female reproductive tract are associated with altered expression of proteases, mucosal barrier proteins, and an influx of HIV-susceptible target cells. Mucosal Immunol. 2016;9(1):194-205.
242. Atashili J, Poole C, Ndumbe PM, Adimora AA, Smith JS. Bacterial vaginosis and HIV acquisition: a meta-analysis of published studies. Aids. 2008;22(12):1493-501.
243. Gosmann C, Anahtar MN, Handley SA, Farcasanu M, Abu-Ali G, Bowman BA, et al. Lactobacillus-Deficient Cervicovaginal Bacterial Communities Are Associated with Increased HIV Acquisition in Young South African Women. Immunity. 2017;46(1):29-37.
244. Borgdorff H, Gautam R, Armstrong SD, Xia D, Ndayisaba GF, van Teijlingen NH, et al. Cervicovaginal microbiome dysbiosis is associated with proteome changes related to alterations of the cervicovaginal mucosal barrier. Mucosal Immunol. 2016;9(3):621-33.
245. Aldunate M, Srbinovski D, Hearps AC, Latham CF, Ramsland PA, Gugasyan R, et al. Antimicrobial and immune modulatory effects of lactic acid and short chain fatty acids produced by vaginal microbiota associated with eubiosis and bacterial vaginosis. Front Physiol. 2015;6:164.
246. Elovitz MA, Gajer P, Riis V, Brown AG, Humphrys MS, Holm JB, et al. Cervicovaginal microbiota and local immune response modulate the risk of spontaneous preterm delivery. Nat Commun. 2019;10(1):1305.
247. Yarbrough VL, Winkle S, Herbst-Kralovetz MM. Antimicrobial peptides in the female reproductive tract: a critical component of the mucosal immune barrier with physiological and clinical implications. Hum Reprod Update. 2015;21(3):353-77.
248. Witkin SS, Moron AF, Ridenhour BJ, Minis E, Hatanaka A, Sarmento SGP, et al. Vaginal Biomarkers That Predict Cervical Length and Dominant Bacteria in the Vaginal Microbiomes of Pregnant Women. mBio. 2019;10(5).
249. Bianchi-Jassir F, Seale AC, Kohli-Lynch M, Lawn JE, Baker CJ, Bartlett L, et al. Preterm Birth Associated With Group B Streptococcus Maternal Colonization Worldwide: Systematic Review and Meta-analyses. Clin Infect Dis. 2017;65(suppl_2):S133-s42.
250. Boldenow E, Gendrin C, Ngo L, Bierle C, Vornhagen J, Coleman M, et al. Group B Streptococcus circumvents neutrophils and neutrophil extracellular traps during amniotic cavity invasion and preterm labor. Sci Immunol. 2016;1(4).
251. Vornhagen J, Quach P, Boldenow E, Merillat S, Whidbey C, Ngo LY, et al. Bacterial Hyaluronidase Promotes Ascending GBS Infection and Preterm Birth. mBio. 2016;7(3).
252. Hillier SL, Ferrieri P, Edwards MS, Ewell M, Ferris D, Fine P, et al. A Phase 2, Randomized, Control Trial of Group B Streptococcus (GBS) Type III Capsular Polysaccharide-tetanus Toxoid (GBS III-TT) Vaccine to Prevent Vaginal Colonization With GBS III. Clin Infect Dis. 2019;68(12):2079-86.
253. Nolan JP, Condello D. Spectral flow cytometry. Curr Protoc Cytom. 2013;Chapter 1:Unit1.27.
254. BioLegend. Spectral Cytometry 2020 [Available from: https://www.biolegend.com/en-us/spectral-cytometry.
255. Elshal MF, McCoy JP. Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA. Methods (San Diego, Calif). 2006;38(4):317-23.
256. Systems RD. Luminex Assay Principle 2020 [Available from: https://www.rndsystems.com/resources/technical/luminex-assay-principle.
257. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol. 2013;79(17):5112-20.
258. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73(16):5261-7.
259. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26(19):2460-1.

260. Parks DH, Beiko RG. Identifying biologically relevant differences between metagenomic communities. Bioinformatics. 2010;26(6):715-21.
261. Zhang J, Shynlova O, Sabra S, Bang A, Briollais L, Lye SJ. Immunophenotyping and activation status of maternal peripheral blood leukocytes during pregnancy and labour, both term and preterm. J Cell Mol Med. 2017;21(10):2386-402.
262. Reichhardt MP, Lundin K, Lokki AI, Recher G, Vuoristo S, Katayama S, et al. Complement in Human Pre-implantation Embryos: Attack and Defense. Front Immunol. 2019;10:2234.
263. Calleja-Agius J, Jauniaux E, Muttukrishna S. Inflammatory cytokines in maternal circulation and placenta of chromosomally abnormal first trimester miscarriages. Clin Dev Immunol. 2012;2012:175041.
264. Romero R, Dey SK, Fisher SJ. Preterm labor: one syndrome, many causes. Science. 2014;345(6198):760-5.
265. Qiu L, Pan M, Zhang R, Ren K. Maternal peripheral blood platelet-to-white blood cell ratio and platelet count as potential diagnostic markers of histological chorioamnionitis-related spontaneous preterm birth. J Clin Lab Anal. 2019;33(4):e22840.
266. Cook J, Bennett PR, Kim SH, Teoh TG, Sykes L, Kindinger LM, et al. First Trimester Circulating MicroRNA Biomarkers Predictive of Subsequent Preterm Delivery and Cervical Shortening. Sci Rep. 2019;9(1):5861.
267. Menon R, Debnath C, Lai A, Guanzon D, Bhatnagar S, Kshetrapal PK, et al. Circulating Exosomal miRNA Profile During Term and Preterm Birth Pregnancies: A Longitudinal Study. Endocrinology. 2019;160(2):249-75.
268. Ferrer-Font L, Pellefigues C, Mayer JU, Small SJ, Jaimes MC, Price KM. Panel Design and Optimization for High-Dimensional Immunophenotyping Assays Using Spectral Flow Cytometry. Curr Protoc Cytom. 2020;92(1):e70.
269. Mallone R, Funaro A, Zubiaur M, Baj G, Ausiello CM, Tacchetti C, et al. Signaling through CD38 induces NK cell activation. Int Immunol. 2001;13(4):397-409.
270. Mikhaylova VA, Onokhina YC, Ovchinnikova OM, Maksimova IM, Arzhanova ON, Sokolov DI, et al. Phenotypical characteristics of peripheral blood monocytes in normal pregnancy and gestosis. Bull Exp Biol Med. 2013;154(4):471-5.
271. Naccasha N, Gervasi MT, Chaiworapongsa T, Berman S, Yoon BH, Maymon E, et al. Phenotypic and metabolic characteristics of monocytes and granulocytes in normal pregnancy and maternal infection. Am J Obstet Gynecol. 2001;185(5):1118-23.
272. Kraus TA, Engel SM, Sperling RS, Kellerman L, Lo Y, Wallenstein S, et al. Characterizing the pregnancy immune phenotype: results of the viral immunity and pregnancy (VIP) study. J Clin Immunol. 2012;32(2):300-11.
273. Luppi P, Haluszczak C, Betters D, Richard CA, Trucco M, DeLoia JA. Monocytes are progressively activated in the circulation of pregnant women. J Leukoc Biol. 2002;72(5):874-84.
274. Svensson J, Jenmalm MC, Matussek A, Geffers R, Berg G, Ernerudh J. Macrophages at the fetal-maternal interface express markers of alternative activation and are induced by M-CSF and IL-10. J Immunol. 2011;187(7):3671-82.
275. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. Nat Med. 2006;12(9):1065-74.
276. Le Gars M, Seiler C, Kay AW, Bayless NL, Starosvetsky E, Moore L, et al. Pregnancy-Induced Alterations in NK Cell Phenotype and Function. Front Immunol. 2019;10:2469.
277. Mosimann B, Wagner M, Shehata H, Poon LC, Ford B, Nicolaides KH, et al. Natural killer cells and their activation status in normal pregnancy. Int J Reprod Med. 2013;2013:906813.
278. Medina KL, Smithson G, Kincade PW. Suppression of B lymphopoiesis during normal pregnancy. J Exp Med. 1993;178(5):1507-15.
279. Grimaldi CM, Cleary J, Dagtas AS, Moussai D, Diamond B. Estrogen alters thresholds for B cell apoptosis and activation. J Clin Invest. 2002;109(12):1625-33.
280. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. The Journal of experimental medicine. 2000;192(7):1027-34.
281. Good-Jacobson KL, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. Nature immunology. 2010;11(6):535-42.
282. Khan AR, Hams E, Floudas A, Sparwasser T, Weaver CT, Fallon PG. PD-L1hi B cells are critical regulators of humoral immunity. Nature communications. 2015;6:5997.
283. Hughes GC, Clark EA, Wong AH. The intracellular progesterone receptor regulates CD4+ T cells and T cell-dependent antibody responses. J Leukoc Biol. 2013;93(3):369-75.
284. Robinson DP, Klein SL. Pregnancy and pregnancy-associated hormones alter immune responses and disease pathogenesis. Horm Behav. 2012;62(3):263-71.
285. Shah NM, Herasimtschuk AA, Boasso A, Benlahrech A, Fuchs D, Imami N, et al. Changes in T Cell and Dendritic Cell Phenotype from Mid to Late Pregnancy Are Indicative of a Shift from Immune Tolerance to Immune Activation. Front Immunol. 2017;8:1138.

286. Somerset DA, Zheng Y, Kilby MD, Sansom DM, Drayson MT. Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset. Immunology. 2004;112(1):38-43.
287. Santner-Nanan B, Peek MJ, Khanam R, Richarts L, Zhu E, Fazekas de St Groth B, et al. Systemic increase in the ratio between Foxp3+ and IL-17-producing CD4+ T cells in healthy pregnancy but not in preeclampsia. J Immunol. 2009;183(11):7023-30.
288. Shima T, Sasaki Y, Itoh M, Nakashima A, Ishii N, Sugamura K, et al. Regulatory T cells are necessary for implantation and maintenance of early pregnancy but not late pregnancy in allogeneic mice. J Reprod Immunol. 2010;85(2):121-9.
289. Teles A, Thuere C, Wafula PO, El-Mousleh T, Zenclussen ML, Zenclussen AC. Origin of Foxp3(+) cells during pregnancy. Am J Clin Exp Immunol. 2013;2(3):222-33.
290. Mjösberg J, Svensson J, Johansson E, Hellström L, Casas R, Jenmalm MC, et al. Systemic reduction of functionally suppressive CD4dimCD25highFoxp3+ Tregs in human second trimester pregnancy is induced by progesterone and 17beta-estradiol. J Immunol. 2009;183(1):759-69.
291. Tilburgs T, Roelen DL, van der Mast BJ, de Groot-Swings GM, Kleijburg C, Scherjon SA, et al. Evidence for a selective migration of fetus-specific CD4+CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy. J Immunol. 2008;180(8):5737-45.
292. Kisielewicz A, Schaier M, Schmitt E, Hug F, Haensch GM, Meuer S, et al. A distinct subset of HLA-DR+-regulatory T cells is involved in the induction of preterm labor during pregnancy and in the induction of organ rejection after transplantation. Clin Immunol. 2010;137(2):209-20.
293. Psarra K, Kapsimali V, Tarassi K, Dendrinos S, Athanasiadis T, Botsis D, et al. TCRgammadelta + T lymphocytes in unexplained recurrent spontaneous abortions. Am J Reprod Immunol. 2001;45(1):6-11.
294. Li L, Tu J, Jiang Y, Zhou J, Schust DJ. Regulatory T cells decrease invariant natural killer T cell-mediated pregnancy loss in mice. Mucosal Immunol. 2017;10(3):613-23.
295. Boyson JE, Nagarkatti N, Nizam L, Exley MA, Strominger JL. Gestation stage-dependent mechanisms of invariant natural killer T cell-mediated pregnancy loss. Proc Natl Acad Sci U S A. 2006;103(12):4580-5.
296. Slaats J, Ten Oever J, van de Veerdonk FL, Netea MG. IL-1β/IL-6/CRP and IL-18/ferritin: Distinct Inflammatory Programs in Infections. PLoS Pathog. 2016;12(12):e1005973.
297. Ida A, Tsuji Y, Muranaka J, Kanazawa R, Nakata Y, Adachi S, et al. IL-18 in pregnancy; the elevation of IL-18 in maternal peripheral blood during labour and complicated pregnancies. J Reprod Immunol. 2000;47(1):65-74.
298. Petrovsky N, McNair P, Harrison LC. Diurnal rhythms of pro-inflammatory cytokines: regulation by plasma cortisol and therapeutic implications. Cytokine. 1998;10(4):307-12.
299. Suzuki K, Nakaji S, Yamada M, Totsuka M, Sato K, Sugawara K. Systemic inflammatory response to exhaustive exercise. Cytokine kinetics. Exerc Immunol Rev. 2002;8:6-48.
300. de Jager W, Bourcier K, Rijkers GT, Prakken BJ, Seyfert-Margolis V. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. BMC Immunol. 2009;10:52.
301. Holtan SG, Chen Y, Kaimal R, Creedon DJ, Enninga EA, Nevala WK, et al. Growth modeling of the maternal cytokine milieu throughout normal pregnancy: macrophage-derived chemokine decreases as inflammation/counterregulation increases. J Immunol Res. 2015;2015:952571.
302. Curry AE, Vogel I, Skogstrand K, Drews C, Schendel DE, Flanders WD, et al. Maternal plasma cytokines in early- and mid-gestation of normal human pregnancy and their association with maternal factors. J Reprod Immunol. 2008;77(2):152-60.
303. Kraus TA, Sperling RS, Engel SM, Lo Y, Kellerman L, Singh T, et al. Peripheral blood cytokine profiling during pregnancy and post-partum periods. Am J Reprod Immunol. 2010;64(6):411-26.
304. Denney JM, Nelson EL, Wadhwa PD, Waters TP, Mathew L, Chung EK, et al. Longitudinal modulation of immune system cytokine profile during pregnancy. Cytokine. 2011;53(2):170-7.
305. Arababadi MK, Aminzadeh F, Hassanshahi G, Khorramdelazad H, Norouzi M, Zarandi ER, et al. Cytokines in Preterm Delivery. Laboratory Medicine. 2012;43(4):27-30.
306. Jarocki S, Redźko S, Przepieść J, Urban J. [Maternal serum Th1 and Th2 cytokines in preterm and term delivery]. Ginekol Pol. 2007;78(4):284-7.
307. Tency I. Inflammatory response in maternal serum during preterm labour. Facts Views Vis Obgyn. 2014;6(1):19-30.
308. Regal JF, Gilbert JS, Burwick RM. The complement system and adverse pregnancy outcomes. Mol Immunol. 2015;67(1):56-70.
309. Girardi G. Complement activation, a threat to pregnancy. Semin Immunopathol. 2018;40(1):103-11.
310. Agostinis C, Bulla R, Tripodo C, Gismondi A, Stabile H, Bossi F, et al. An alternative role of C1q in cell migration and tissue remodeling: contribution to trophoblast invasion and placental development. J Immunol. 2010;185(7):4420-9.
311. Roos A, Xu W, Castellano G, Nauta AJ, Garred P, Daha MR, et al. Mini-review: A pivotal role for innate immunity in the clearance of apoptotic cells. Eur J Immunol. 2004;34(4):921-9.

312. Derzsy Z, Prohászka Z, Rigó J, Jr., Füst G, Molvarec A. Activation of the complement system in normal pregnancy and preeclampsia. Mol Immunol. 2010;47(7-8):1500-6.
313. He YD, Xu BN, Song D, Wang YQ, Yu F, Chen Q, et al. Normal range of complement components during pregnancy: A prospective study. Am J Reprod Immunol. 2020;83(2):e13202.
314. Vaisbuch E, Romero R, Erez O, Mazaki-Tovi S, Kusanovic JP, Soto E, et al. Activation of the alternative pathway of complement is a feature of pre-term parturition but not of spontaneous labor at term. Am J Reprod Immunol. 2010;63(4):318-30.
315. Christiansen OB, Kilpatrick DC, Souter V, Varming K, Thiel S, Jensenius JC. Mannan-binding lectin deficiency is associated with unexplained recurrent miscarriage. Scand J Immunol. 1999;49(2):193-6.
316. Kruse C, Rosgaard A, Steffensen R, Varming K, Jensenius JC, Christiansen OB. Low serum level of mannan-binding lectin is a determinant for pregnancy outcome in women with recurrent spontaneous abortion. Am J Obstet Gynecol. 2002;187(5):1313-20.
317. Annells MF, Hart PH, Mullighan CG, Heatley SL, Robinson JS, Bardy P, et al. Interleukins-1, -4, -6, -10, tumor necrosis factor, transforming growth factor-beta, FAS, and mannose-binding protein C gene polymorphisms in Australian women: Risk of preterm birth. Am J Obstet Gynecol. 2004;191(6):2056-67.
318. Silva L, Javorski N, André Cavalcanti Brandão L, Lima MC, Crovella S, Eickmann SH. Influence of MBL2 and NOS3 polymorphisms on spontaneous preterm birth in North East Brazil: genetics and preterm birth. J Matern Fetal Neonatal Med. 2020;33(1):127-35.
319. Wang LK, Huang MC, Liu CC, Chen CP. Second-trimester plasma mannose-binding lectin levels and risk of preterm birth. J Matern Fetal Neonatal Med. 2017;30(6):678-83.
320. Frakking FN, Brouwer N, Zweers D, Merkus MP, Kuijpers TW, Offringa M, et al. High prevalence of mannose-binding lectin (MBL) deficiency in premature neonates. Clin Exp Immunol. 2006;145(1):5-12.
321. Segura-Cervantes E, Mancilla-Ramirez J, Zurita L, Paredes Y, Arredondo JL, Galindo-Sevilla N. Blood SC5b-9 complement levels increase at parturition during term and preterm labor. J Reprod Immunol. 2015;109:24-30.
322. Gonzalez JM, Romero R, Girardi G. Comparison of the mechanisms responsible for cervical remodeling in preterm and term labor. J Reprod Immunol. 2013;97(1):112-9.
323. Lu J, Clark AG. Impact of microRNA regulation on variation in human gene expression. Genome Res. 2012;22(7):1243-54.
324. Sakr M, Takino T, Sabit H, Nakada M, Li Z, Sato H. miR-150-5p and miR-133a suppress glioma cell proliferation and migration through targeting membrane-type-1 matrix metalloproteinase. Gene. 2016;587(2):155-62.
325. Ekman G, Malmström A, Uldbjerg N, Ulmsten U. Cervical collagen: an important regulator of cervical function in term labor. Obstet Gynecol. 1986;67(5):633-6.
326. Sennström MB, Ekman G, Westergren-Thorsson G, Malmström A, Byström B, Endrésen U, et al. Human cervical ripening, an inflammatory process mediated by cytokines. Mol Hum Reprod. 2000;6(4):375-81.
327. Young A, Thomson AJ, Ledingham M, Jordan F, Greer IA, Norman JE. Immunolocalization of proinflammatory cytokines in myometrium, cervix, and fetal membranes during human parturition at term. Biol Reprod. 2002;66(2):445-9.
328. Sennström MB, Brauner A, Byström B, Malmström A, Ekman G. Matrix metalloproteinase-8 correlates with the cervical ripening process in humans. Acta Obstet Gynecol Scand. 2003;82(10):904-11.
329. Stygar D, Wang H, Vladic YS, Ekman G, Eriksson H, Sahlin L. Increased level of matrix metalloproteinases 2 and 9 in the ripening process of the human cervix. Biol Reprod. 2002;67(3):889-94.
330. Akerud A, Dubicke A, Sennstrom M, Ekman-Ordeberg G, Malmstrom A. Differences in heparan sulfate production in cervical fibroblast cultures from women undergoing term and preterm delivery. Acta Obstet Gynecol Scand. 2008;87(11):1220-8.
331. Törnblom SA, Patel FA, Byström B, Giannoulias D, Malmström A, Sennström M, et al. 15-hydroxyprostaglandin dehydrogenase and cyclooxygenase 2 messenger ribonucleic acid expression and immunohistochemical localization in human cervical tissue during term and preterm labor. J Clin Endocrinol Metab. 2004;89(6):2909-15.
332. Thomson AJ, Lunan CB, Cameron AD, Cameron IT, Greer IA, Norman JE. Nitric oxide donors induce ripening of the human uterine cervix: a randomised controlled trial. Br J Obstet Gynaecol. 1997;104(9):1054-7.
333. Ledingham MA, Thomson AJ, Young A, Macara LM, Greer IA, Norman JE. Changes in the expression of nitric oxide synthase in the human uterine cervix during pregnancy and parturition. Mol Hum Reprod. 2000;6(11):1041-8.
334. Dubicke A, Akerud A, Sennstrom M, Hamad RR, Bystrom B, Malmstrom A, et al. Different secretion patterns of matrix metalloproteinases and IL-8 and effect of corticotropin-releasing hormone in preterm and term cervical fibroblasts. Mol Hum Reprod. 2008;14(11):641-7.
335. Fortunato SJ, Menon R, Lombardi SJ. The effect of transforming growth factor and interleukin-10 on interleukin-8 release by human amniochorion may regulate histologic chorioamnionitis. Am J Obstet Gynecol. 1998;179(3 Pt 1):794-9.

336. Sato TA, Keelan JA, Mitchell MD. Critical paracrine interactions between TNF-alpha and IL-10 regulate lipopolysaccharide-stimulated human choriodecidual cytokine and prostaglandin E2 production. J Immunol. 2003;170(1):158-66.
337. Fortunato SJ, Menon R, Lombardi SJ, LaFleur B. Interleukin-10 inhibition of gelatinases in fetal membranes: therapeutic implications in preterm premature rupture of membranes. Obstet Gynecol. 2001;98(2):284-8.
338. Brown NL, Alvi SA, Elder MG, Bennett PR, Sullivan MH. The regulation of prostaglandin output from term intact fetal membranes by anti-inflammatory cytokines. Immunology. 2000;99(1):124-33.
339. Israfil-Bayli F, Toozs-Hobson P, Lees C, Slack M, Ismail K. Cerclage outcome by the type of suture material (COTS): study protocol for a pilot and feasibility randomised controlled trial. Trials. 2014;15:415.
340. Riollet C, Rainard P, Poutrel B. Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with Escherichia coli and Staphylococcus aureus. Clin Diagn Lab Immunol. 2000;7(2):161-7.
341. Ahrenstedt O, Knutson L, Nilsson B, Nilsson-Ekdahl K, Odlind B, Hällgren R. Enhanced local production of complement components in the small intestines of patients with Crohn's disease. N Engl J Med. 1990;322(19):1345-9.
342. Ballow M, Donshik PC, Mendelson L. Complement proteins and C3 anaphylatoxin in the tears of patients with conjunctivitis. J Allergy Clin Immunol. 1985;76(3):473-6.
343. Passwell J, Schreiner GF, Nonaka M, Beuscher HU, Colten HR. Local extrahepatic expression of complement genes C3, factor B, C2, and C4 is increased in murine lupus nephritis. J Clin Invest. 1988;82(5):1676-84.
344. Hocini H, Bélec L, Iscaki S, Garin B, Pillot J, Becquart P, et al. High-level ability of secretory IgA to block HIV type 1 transcytosis: contrasting secretory IgA and IgG responses to glycoprotein 160. AIDS Res Hum Retroviruses. 1997;13(14):1179-85.
345. Devito C, Broliden K, Kaul R, Svensson L, Johansen K, Kiama P, et al. Mucosal and plasma IgA from HIV-1-exposed uninfected individuals inhibit HIV-1 transcytosis across human epithelial cells. J Immunol. 2000;165(9):5170-6.
346. Kutteh WH, Prince SJ, Hammond KR, Kutteh CC, Mestecky J. Variations in immunoglobulins and IgA subclasses of human uterine cervical secretions around the time of ovulation. Clin Exp Immunol. 1996;104(3):538-42.
347. Schaefer K, Brown N, Kaye PM, Lacey CJ. Cervico-vaginal immunoglobulin G levels increase post-ovulation independently of neutrophils. PLoS One. 2014;9(12):e114824.
348. Ashford K, Chavan NR, Wiggins AT, Sayre MM, McCubbin A, Critchfield AS, et al. Comparison of Serum and Cervical Cytokine Levels throughout Pregnancy between Preterm and Term Births. AJP Rep. 2018;8(2):e113-e20.
349. Amabebe E, Chapman DR, Stern VL, Stafford G, Anumba DOC. Mid-gestational changes in cervicovaginal fluid cytokine levels in asymptomatic pregnant women are predictive markers of inflammation-associated spontaneous preterm birth. J Reprod Immunol. 2018;126:1-10.
350. Jun JK, Yoon BH, Romero R, Kim M, Moon JB, Ki SH, et al. Interleukin 6 determinations in cervical fluid have diagnostic and prognostic value in preterm premature rupture of membranes. Am J Obstet Gynecol. 2000;183(4):868-73.
351. Park S, You YA, Yun H, Choi SJ, Hwang HS, Choi SK, et al. Cervicovaginal fluid cytokines as predictive markers of preterm birth in symptomatic women. Obstet Gynecol Sci. 2020;63(4):455-63.
352. Dunn AB, Dunlop AL, Hogue CJ, Miller A, Corwin EJ. The Microbiome and Complement Activation: A Mechanistic Model for Preterm Birth. Biol Res Nurs. 2017;19(3):295-307.
353. Schraufstatter IU, Khaldoyanidi SK, DiScipio RG. Complement activation in the context of stem cells and tissue repair. World J Stem Cells. 2015;7(8):1090-108.
354. Björk J, Hugli TE, Smedegård G. Microvascular effects of anaphylatoxins C3a and C5a. J Immunol. 1985;134(2):1115-9.
355. Drouin SM, Kildsgaard J, Haviland J, Zabner J, Jia HP, McCray PB, Jr., et al. Expression of the complement anaphylatoxin C3a and C5a receptors on bronchial epithelial and smooth muscle cells in models of sepsis and asthma. J Immunol. 2001;166(3):2025-32.
356. Schraufstatter IU, Trieu K, Sikora L, Sriramarao P, DiScipio R. Complement c3a and c5a induce different signal transduction cascades in endothelial cells. J Immunol. 2002;169(4):2102-10.
357. Kim YM, Park KH, Park H, Yoo HN, Kook SY, Jeon SJ. Complement C3a, But Not C5a, Levels in Amniotic Fluid Are Associated with Intra-amniotic Infection and/or Inflammation and Preterm Delivery in Women with Cervical Insufficiency or an Asymptomatic Short Cervix (≤ 25 mm). J Korean Med Sci. 2018;33(35):e220.
358. Monsanto SP, Daher S, Ono E, Pendeloski KPT, Trainá É, Mattar R, et al. Cervical cerclage placement decreases local levels of proinflammatory cytokines in patients with cervical insufficiency. Am J Obstet Gynecol. 2017;217(4):455.e1-.e8.
359. Mercer BM, Goldenberg RL, Moawad AH, Meis PJ, Iams JD, Das AF, et al. The preterm prediction study: effect of gestational age and cause of preterm birth on subsequent obstetric outcome. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. Am J Obstet Gynecol. 1999;181(5 Pt 1):1216-21.
360. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, et al. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A. 2011;108 Suppl 1(Suppl 1):4680-7.

361. Wright HL, Moots RJ, Bucknall RC, Edwards SW. Neutrophil function in inflammation and inflammatory diseases. Rheumatology (Oxford). 2010;49(9):1618-31.
362. Laudisi F, Spreafico R, Evrard M, Hughes TR, Mandriani B, Kandasamy M, et al. Cutting edge: the NLRP3 inflammasome links complement-mediated inflammation and IL-1β release. J Immunol. 2013;191(3):1006-10.
363. Weber A, Wasiliew P, Kracht M. Interleukin-1beta (IL-1beta) processing pathway. Sci Signal. 2010;3(105):cm2.
364. Dubicke A, Ekman-Ordeberg G, Mazurek P, Miller L, Yellon SM. Density of Stromal Cells and Macrophages Associated With Collagen Remodeling in the Human Cervix in Preterm and Term Birth. Reprod Sci. 2016;23(5):595-603.
365. Nelson DB, Hanlon A, Nachamkin I, Haggerty C, Mastrogiannis DS, Liu C, et al. Early pregnancy changes in bacterial vaginosis-associated bacteria and preterm delivery. Paediatr Perinat Epidemiol. 2014;28(2):88-96.
366. Manhanzva MT, Abrahams AG, Gamieldien H, Froissart R, Jaspan H, Jaumdally SZ, et al. Inflammatory and antimicrobial properties differ between vaginal Lactobacillus isolates from South African women with non-optimal versus optimal microbiota. Sci Rep. 2020;10(1):6196.
367. Petrova MI, Reid G, Vaneechoutte M, Lebeer S. Lactobacillus iners: Friend or Foe? Trends Microbiol. 2017;25(3):182-91.
368. Rampersaud R, Planet PJ, Randis TM, Kulkarni R, Aguilar JL, Lehrer RI, et al. Inerolysin, a cholesterol-dependent cytolysin produced by Lactobacillus iners. J Bacteriol. 2011;193(5):1034-41.
369. O'Hanlon DE, Moench TR, Cone RA. Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota. PLoS One. 2013;8(11):e80074.
370. Verstraelen H, Verhelst R, Claeys G, De Backer E, Temmerman M, Vaneechoutte M. Longitudinal analysis of the vaginal microflora in pregnancy suggests that L. crispatus promotes the stability of the normal vaginal microflora and that L. gasseri and/or L. iners are more conducive to the occurrence of abnormal vaginal microflora. BMC Microbiol. 2009;9:116.
371. Gerson KD, McCarthy C, Elovitz MA, Ravel J, Sammel MD, Burris HH. Cervicovaginal microbial communities deficient in Lactobacillus species are associated with second trimester short cervix. Am J Obstet Gynecol. 2020;222(5):491.e1-.e8.
372. Tamrakar R, Yamada T, Furuta I, Cho K, Morikawa M, Yamada H, et al. Association between Lactobacillus species and bacterial vaginosis-related bacteria, and bacterial vaginosis scores in pregnant Japanese women. BMC Infect Dis. 2007;7:128.
373. Africa CW, Nel J, Stemmet M. Anaerobes and bacterial vaginosis in pregnancy: virulence factors contributing to vaginal colonisation. Int J Environ Res Public Health. 2014;11(7):6979-7000.
374. Mehta P, Patel P, Olver JM. Functional results and complications of Mersilene mesh use for frontalis suspension ptosis surgery. Br J Ophthalmol. 2004;88(3):361-4.
375. Slack M, Sandhu JS, Staskin DR, Grant RC. In vivo comparison of suburethral sling materials. Int Urogynecol J Pelvic Floor Dysfunct. 2006;17(2):106-10.
376. Liu M, Guo S, Hibbert JM, Jain V, Singh N, Wilson NO, et al. CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. Cytokine Growth Factor Rev. 2011;22(3):121-30.
377. Florova V, Romero R, Tarca AL, Galaz J, Motomura K, Ahmad MM, et al. Vaginal host immune-microbiome interactions in a cohort of primarily African-American women who ultimately underwent spontaneous preterm birth or delivered at term. Cytokine. 2020;137:155316.
378. Sarkodie EK, Zhou S, Baidoo SA, Chu W. Influences of stress hormones on microbial infections. Microb Pathog. 2019;131:270-6.
379. Nguyen DP, Genc M, Vardhana S, Babula O, Onderdonk A, Witkin SS. Ethnic differences of polymorphisms in cytokine and innate immune system genes in pregnant women. Obstet Gynecol. 2004;104(2):293-300.
380. Weiss G. Endocrinology of parturition. J Clin Endocrinol Metab. 2000;85(12):4421-5.
381. Challis JRG, Matthews SG, Gibb W, Lye SJ. Endocrine and paracrine regulation of birth at term and preterm. Endocr Rev. 2000;21(5):514-50.
382. Kamel RM. The onset of human parturition. Arch Gynecol Obstet. 2010;281(6):975-82.
383. Andersson S, Minjarez D, Yost NP, Word RA. Estrogen and progesterone metabolism in the cervix during pregnancy and parturition. J Clin Endocrinol Metab. 2008;93(6):2366-74.
384. García-Gómez E, González-Pedrajo B, Camacho-Arroyo I. Role of sex steroid hormones in bacterial-host interactions. Biomed Res Int. 2013;2013:928290.
385. Lee BW, Yap HK, Chew FT, Quah TC, Prabhakaran K, Chan GS, et al. Age- and sex-related changes in lymphocyte subpopulations of healthy Asian subjects: from birth to adulthood. Cytometry. 1996;26(1):8-15.
386. Klein SL, Marriott I, Fish EN. Sex-based differences in immune function and responses to vaccination. Trans R Soc Trop Med Hyg. 2015;109(1):9-15.
387. Ruggieri A, Anticoli S, D'Ambrosio A, Giordani L, Viora M. The influence of sex and gender on immunity, infection and vaccination. Ann Ist Super Sanita. 2016;52(2):198-204.
388. Park HJ, Choi JM. Sex-specific regulation of immune responses by PPARs. Exp Mol Med. 2017;49(8):e364.

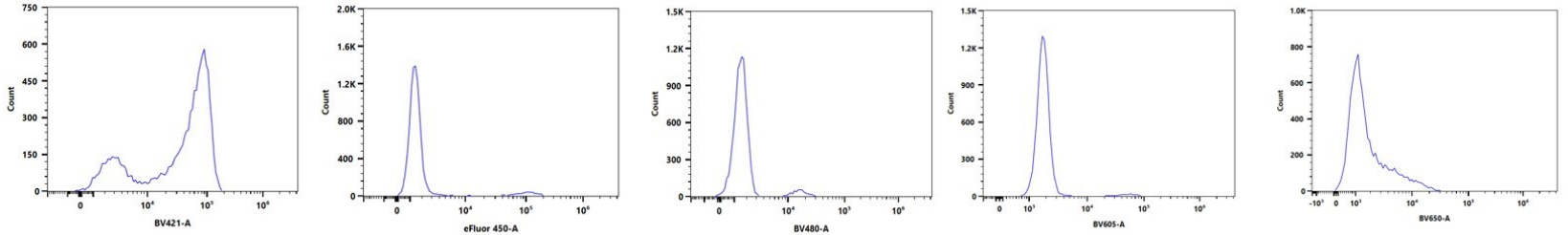
389. Liu L, Oza S, Hogan D, Perin J, Rudan I, Lawn JE, et al. Global, regional, and national causes of child mortality in 2000-13, with projections to inform post-2015 priorities: an updated systematic analysis. Lancet. 2015;385(9966):430-40.
390. Chawanpaiboon S, Vogel JP, Moller AB, Lumbiganon P, Petzold M, Hogan D, et al. Global, regional, and national estimates of levels of preterm birth in 2014: a systematic review and modelling analysis. Lancet Glob Health. 2019;7(1):e37-e46.
391. Green ES, Arck PC. Pathogenesis of preterm birth: bidirectional inflammation in mother and fetus. Semin Immunopathol. 2020;42(4):413-29.
392. Socié G, Caby-Tosi MP, Marantz JL, Cole A, Bedrosian CL, Gasteyger C, et al. Eculizumab in paroxysmal nocturnal haemoglobinuria and atypical haemolytic uraemic syndrome: 10-year pharmacovigilance analysis. Br J Haematol. 2019;185(2):297-310.
393. Baskar S, Klein AL, Zeft A. The Use of IL-1 Receptor Antagonist (Anakinra) in Idiopathic Recurrent Pericarditis: A Narrative Review. Cardiol Res Pract. 2016;2016:7840724.
394. Cohen CR, Wierzbicki MR, French AL, Morris S, Newmann S, Reno H, et al. Randomized Trial of Lactin-V to Prevent Recurrence of Bacterial Vaginosis. N Engl J Med. 2020;382(20):1906-15.
395. Russo R, Karadja E, De Seta F. Evidence-based mixture containing Lactobacillus strains and lactoferrin to prevent recurrent bacterial vaginosis: a double blind, placebo controlled, randomised clinical trial. Benef Microbes. 2019;10(1):19-26.
396. Dzidic M, Abrahamsson TR, Artacho A, Björkstén B, Collado MC, Mira A, et al. Aberrant IgA responses to the gut microbiota during infancy precede asthma and allergy development. J Allergy Clin Immunol. 2017;139(3):1017-25.e14.
397. Huang WC, Sala-Newby GB, Susana A, Johnson JL, Newby AC. Classical macrophage activation up-regulates several matrix metalloproteinases through mitogen activated protein kinases and nuclear factor-κB. PLoS One. 2012;7(8):e42507.
398. Pavlov O, Pavlova O, Ailamazyan E, Selkov S. Characterization of cytokine production by human term placenta macrophages in vitro. Am J Reprod Immunol. 2008;60(6):556-67.
399. Haslam SM, North SJ, Dell A. Mass spectrometric analysis of N- and O-glycosylation of tissues and cells. Curr Opin Struct Biol. 2006;16(5):584-91.
400. Letkemann R, Wittkowski H, Antonopoulos A, Podskabi T, Haslam SM, Föll D, et al. Partial correction of neutrophil dysfunction by oral galactose therapy in glycogen storage disease type Ib. Int Immunopharmacol. 2017;44:216-25.

CHAPTER 8: APPENDIX

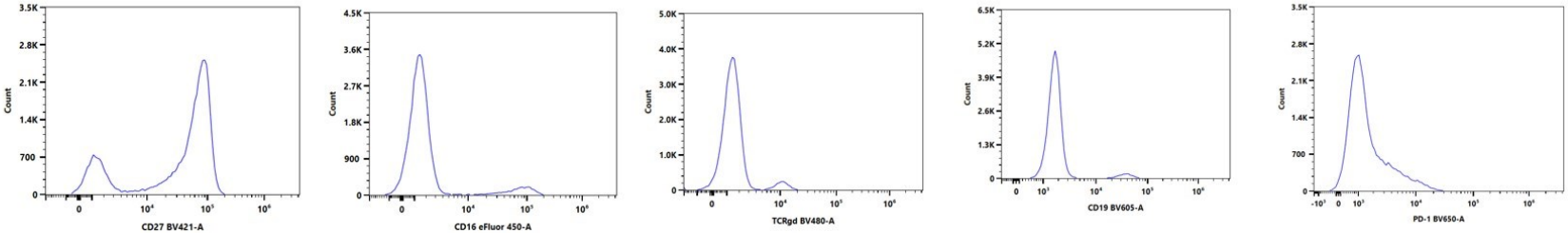
Appendix 1. Histograms of single stain versus multi-colour in flurochrome panel to stain PBMC

Good detection of each fluorochrome could be demonstrated if the histogram in the single stain control matched the histogram obtained in the multi-colour sample.

Single stained control

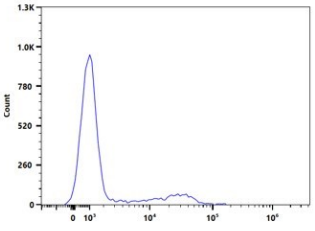


Multicolor sample

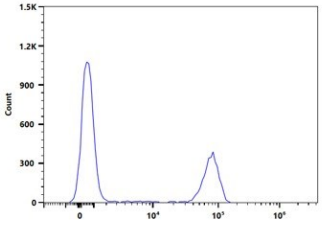


Appendix 1 continued

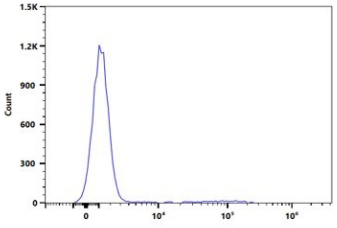
Single stained control



BV711



BV750



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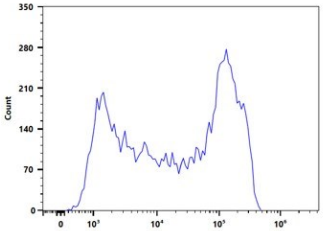
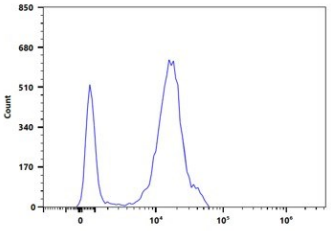
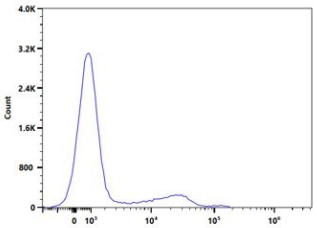


AF 488

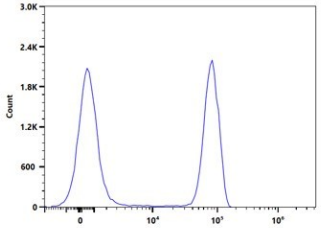


AF532

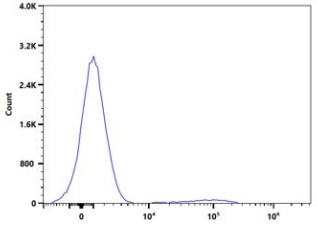
Multicolor sample



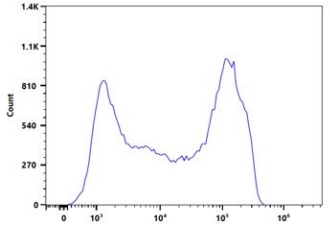
CD56 BV711



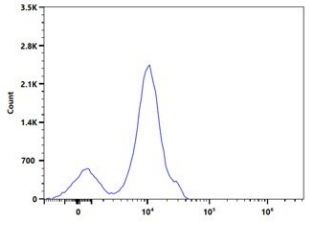
CD4 BV750



IgD BV785



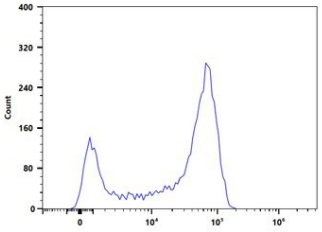
CD45RA AF 488



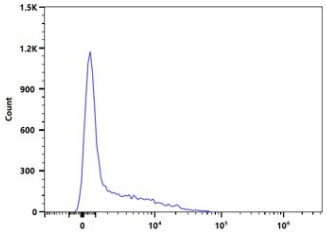
CD3 AF532

Appendix 1 continued

Single stained control



PerCP eFluor 710



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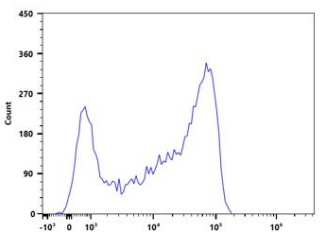
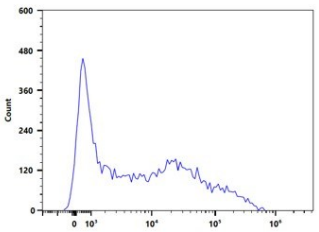
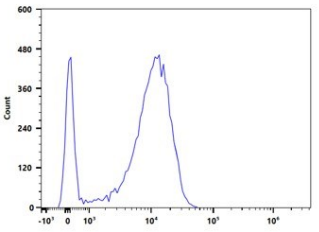


PE-Dazzle

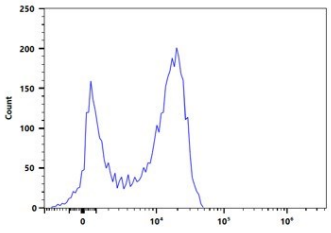


Pe-Cy7

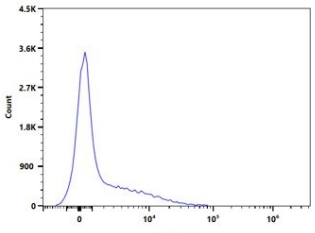


APC

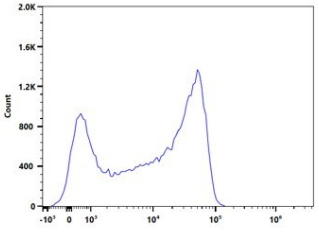
Multicolor sample



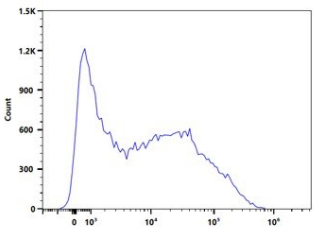
CD14 PerCP eFluor 710



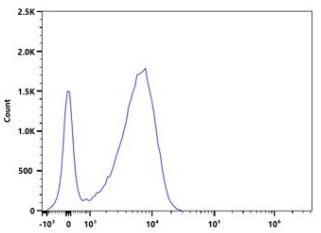
CD25 PE



CCR7 PE-Dazzle



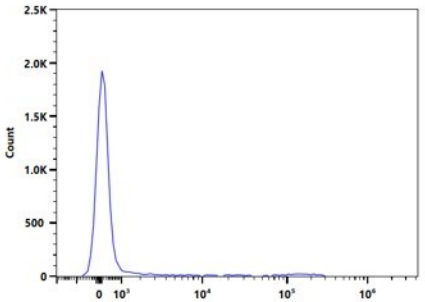
CD38 Pe-Cy7



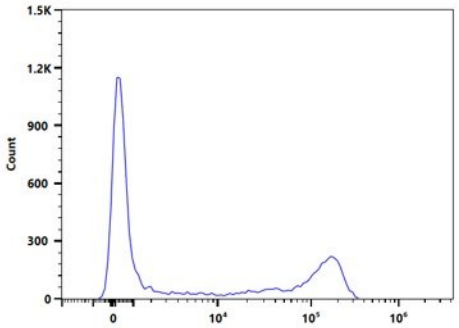
CD127 APC

Appendix 1 continued

Single stained control

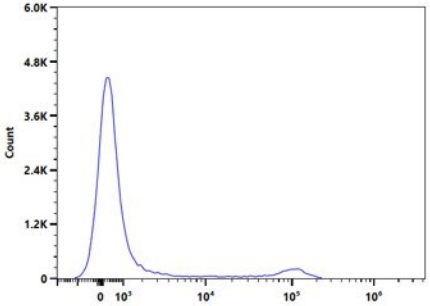


AF 700

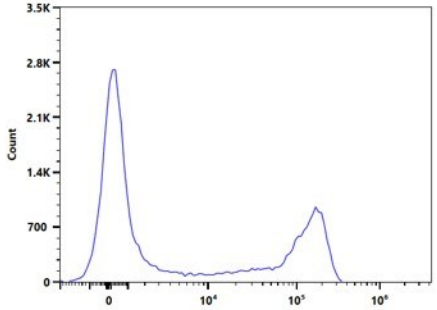


APC Fire 750

Multicolor sample



HLA-DR AF 700



CD8 APC Fire 750

Appendix 2. Proportions of PBMC across pregnancy

The proportions of PBMC subsets compared across pregnancy in the three pregnancy outcome groups, preterm (PT), term with no intervention (T), term with intervention (TI), N=78. The three timepoints were 12^{+0} - 16^{+6} (A), 20^{+0} - 24^{+6} (B) and 30^{+0} - 34^{+6} (C). The one-way ANOVA was performed with the uncorrected Dunnett's test and with the corrected Dunnett's multiple comparisons test for normally distributed data. The Kruskal-Wallis test was performed with the uncorrected Dunn's test and with the corrected Dunn's multiple comparisons test for non-parametric data.

	Uncorrected Dunnett's or Dunn's test						Corrected Dunnett's or Dunn's multiple comparisons test					
Outcome	PT	PT	T	T	TI	TI	PT	PT	T	T	TI	TI
Timepoint	A vs B	A vs C	A vs B	A vs C	A vs B	A vs C	A vs B	A vs C	A vs B	A vs C	A vs B	A vs C
PBMC												
Lymphocytes	0.09	0.61	0.48	0.66	0.90	0.51	0.18	0.99	0.98	0.99	0.99	0.99
CD3+ lymphocytes	0.57	0.77	0.58	0.46	0.38	0.13	0.79	0.94	0.80	0.67	0.58	0.22
B cells	0.51	0.99	0.46	0.57	0.34	0.32	0.73	0.99	0.67	0.79	0.52	0.50
NK cells	0.31	0.46	0.52	0.15	0.72	0.08	0.48	0.68	0.73	0.25	0.91	0.13
CD4+ T cells	0.97	0.91	0.28	0.16	0.35	0.58	0.99	0.99	0.44	0.27	0.45	0.64
CD8+ T cells	0.93	0.99	0.35	0.23	0.36	0.52	0.99	0.99	0.53	0.36	0.55	0.74
Treg	0.61	0.34	0.93	0.36	0.54	0.72	0.82	0.53	0.68	0.51	0.19	0.83
γδ T cells	0.33	0.12	0.29	0.23	0.58	0.52	0.67	0.25	0.58	0.46	0.99	0.99
CD4+ NK T	0.61	0.76	0.45	0.55	0.48	0.44	0.99	0.99	0.91	0.99	0.97	0.88
CD8+ NK T	0.98	0.85	0.93	0.93	0.83	0.33	0.99	0.99	0.99	0.99	0.99	0.66
Monocytes	0.09	0.60	0.37	0.66	0.90	0.51	0.18	0.99	0.75	0.99	0.99	0.99
Classical monocytes	0.66	0.91	0.84	0.87	0.68	0.43	0.87	0.99	0.97	0.98	0.88	0.64
Intermediate monocytes	0.97	0.29	0.93	0.58	0.44	0.98	0.99	0.58	0.99	0.99	0.88	0.99
Non-classical monocytes	0.65	0.22	0.66	0.89	0.95	0.86	0.99	0.44	0.99	0.99	0.99	0.99

Appendix 3. PBMC effector markers across pregnancy

The median fluorescence intensity of PBMC effector markers were compared across pregnancy in the three pregnancy outcome groups, preterm (PT), term with no intervention (T), term with intervention (TI), N=78. The three timepoints were 12^{+0} - 16^{+6} (A), 20^{+0} - 24^{+6} (B) and 30^{+0} - 34^{+6} (C). The one-way ANOVA was performed with the uncorrected Dunnett's test and with the corrected Dunnett's multiple comparisons test for normally distributed data. The Kruskal-Wallis test was performed with the uncorrected Dunn's test and with the corrected Dunn's multiple comparisons test for non-parametric data.

	Uncorrected Dunnett's or Dunn's test						Corrected Dunnett's or Dunn's multiple comparisons test					
Outcome	PT	PT	T	T	TI	TI	PT	PT	T	T	TI	TI
Timepoint	A vs B	A vs C	A vs B	A vs C	A vs B	A vs C	A vs B	A vs C	A vs B	A vs C	A vs B	A vs C
Cell effectors												
CD4+ CD25	0.68	0.49	0.89	0.44	0.76	0.48	0.88	0.71	0.99	0.65	0.93	0.70
CD4+ CD38	0.50	0.69	0.26	0.21	0.80	0.53	0.71	0.89	0.41	0.34	0.95	0.74
CD4+ HLA-DR	0.49	0.97	0.40	0.08	0.95	0.41	0.98	0.99	0.80	0.17	0.99	0.82
CD4+ PD-1	0.48	0.63	0.92	0.67	0.48	0.19	0.70	0.84	0.99	0.87	0.69	0.31
CD8+ CD25	0.53	0.95	0.97	p<0.05 *	0.35	0.71	0.75	0.99	0.99	p<0.05 *	0.53	0.90
CD8+ CD38	0.18	p<0.05 *	0.38	0.10	0.12	p<0.01 **	0.30	p<0.05 *	0.57	0.17	0.22	p<0.01 **
CD8+ HLA-DR	0.65	0.52	0.85	0.99	0.87	0.40	0.99	0.99	0.99	0.99	0.99	0.81
CD8+ PD-1	0.62	0.86	0.73	0.47	0.35	0.18	0.83	0.98	0.91	0.68	0.54	0.29
Treg CD25	0.28	0.25	0.25	0.41	0.46	0.38	0.45	0.40	0.40	0.61	0.67	0.58
Treg CD38	0.85	0.38	0.25	0.11	0.82	0.39	0.99	0.75	0.49	0.22	0.99	0.78
Treg HLA-DR	0.49	0.73	0.87	0.43	0.08	p<0.05 *	0.71	0.91	0.98	0.63	0.13	p<0.05 *
Monocyte HLA-DR	0.60	0.66	0.66	0.13	0.41	0.36	0.82	0.87	0.86	0.21	0.61	0.55
Monocytes PD-1	0.31	0.21	0.95	0.35	0.30	0.13	0.49	0.34	0.99	0.54	0.47	0.22
B cells IgD	0.59	0.87	0.64	0.21	0.75	0.63	0.81	0.98	0.84	0.34	0.92	0.84
B cells PD-1	0.65	0.07	0.65	0.90	0.48	0.19	0.86	0.12	0.86	0.99	0.69	0.31
NK cells CD38	0.47	p<0.01 **	0.08	p<0.0001 ***	0.31	p<0.05 *	0.69	p<0.05 *	0.14	p<0.05 *	0.48	0.09

Appendix 4. Cervico-vaginal fluid analytes across pregnancy

The concentrations of cervico-vaginal fluid cytokines, complement and immunoglobulins were compared across pregnancy in the three pregnancy outcome groups, preterm (PT), term with no intervention (T), term with intervention (TI), N=133. The three timepoints were 12^{+0} - 16^{+6} (A), 20^{+0} - 24^{+6} (B) and 30^{+0} - 34^{+6} (C). The Kruskal-Wallis test was performed with the uncorrected Dunn's test and with corrected Dunn's multiple comparisons test.

	Uncorrected Dunn's test						Corrected Dunn's multiple comparisons test					
Outcome	PT	PT	T	T	TI	TI	PT	PT	T	T	TI	TI
Timepoint	A vs B	A vs C	A vs B	A vs C	A vs B	A vs C	A vs B	A vs C	A vs B	A vs C	A vs B	A vs C
Cytokine												
IL-8	p<0.05 *	0.20	0.92	0.99	0.18	0.77	0.06	0.40	0.99	0.99	0.36	0.99
IL-6	0.08	0.77	0.95	0.65	0.22	0.27	0.18	0.99	0.99	0.99	0.43	0.55
IL-1β	0.20	0.23	0.96	0.89	0.31	0.26	0.41	0.47	0.99	0.99	0.62	0.47
IL-10	0.73	0.66	0.99	0.15	0.28	0.93	0.99	0.99	0.99	0.31	0.56	0.99
IL-4	0.32	0.96	0.40	0.55	0.57	0.23	0.63	0.99	0.79	0.99	0.99	0.47
IL-18	0.33	0.73	0.96	0.65	0.88	0.33	0.67	0.99	0.99	0.99	0.99	0.66
IL-5	0.23	0.29	0.45	0.56	0.95	0.75	0.47	0.58	0.89	0.99	0.99	0.99
IL-2	0.21	0.68	0.82	0.63	0.52	0.54	0.42	0.99	0.99	0.99	0.99	0.99
IFN-γ	0.30	0.42	0.67	0.77	0.05	p<0.01 **	0.60	0.84	0.99	0.99	0.11	p<0.01 **
TNF-α	0.36	0.71	0.82	0.78	0.99	0.99	0.72	0.99	0.99	0.99	0.99	0.99
GM-CSF	0.29	0.06	0.28	0.60	0.54	0.26	0.58	0.13	0.56	0.99	0.99	0.52
C5	0.21	0.21	0.95	0.53	0.54	0.26	0.41	0.43	0.99	0.99	0.99	0.99
C5a	0.17	0.51	0.50	0.29	0.63	0.78	0.34	0.99	0.99	0.57	0.99	0.99
MBL	0.22	0.17	0.86	0.43	0.62	0.75	0.44	0.34	0.99	0.86	0.99	0.99
C3b	0.17	0.28	0.93	0.75	0.94	0.82	0.35	0.57	0.99	0.99	0.99	0.99
IgG1	0.07	0.11	0.56	0.81	0.71	0.65	0.16	0.22	0.99	0.99	0.99	0.99
IgG2	0.19	0.24	0.76	0.68	0.58	0.61	0.38	0.49	0.99	0.99	0.99	0.99
IgG3	0.14	0.18	0.99	0.37	0.79	0.99	0.28	0.36	0.99	0.74	0.99	0.99
IgG4	0.13	0.17	0.71	0.72	0.65	0.60	0.27	0.35	0.99	0.99	0.99	0.99
IgA	0.27	0.15	0.38	0.27	0.93	p<0.05 *	0.53	0.29	0.75	0.53	0.99	p<0.05 *
IgM	0.40	0.26	0.75	0.77	0.36	0.27	0.81	0.51	0.99	0.99	0.72	0.53