THE ROLE OF COMPLEMENT AND NEUTROPHILS AT THE CERVICO-VAGINAL INTERFACE IN MICROBIAL-DRIVEN PRETERM BIRTH

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DECLARATION

Statement of originality

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

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ABSTRACT

Preterm birth (PTB) is a syndrome with many underlying aetiologies that occurs in 10% of pregnancies, and affects approximately 15 million newborns each year. There are clear associations between vaginal microbial composition and the risk of cervical shortening and preterm birth. *Lactobacillus crispatus* dominance is protective and associated with uncomplicated pregnancies, whereas *L. iners* and high diversity communities confer a higher risk. However, the mechanisms driving microbial-host immune interactions and PTB remain poorly understood.

This PhD has investigated how the innate and adaptive immune responses at the cervicovaginal interface contribute to microbial-driven cervical shortening and preterm birth. To do so, three specific aims were established: 1) to determine if the complement system modulates the risk of spontaneous preterm birth in women who have a high-risk microbial signature; 2) to determine if the immunophenotype of cervical neutrophils influences the local immune milieu in response to a high-risk microbial signature, cervical shortening and preterm labour; and 3) to determine if the recognition of vaginal microbiota by opsonins differs by microbial composition, and in cases of cervical shortening and preterm birth.

This thesis reports on an increase in cytokines and complement proteins in association with the local cervicovaginal microbial composition, cervical shortening and preterm birth. It was found that cervical neutrophils were more activated than peripheral neutrophils and that their immunophenotype correlated with vaginal microbial composition and increased concentrations of cytokines and complement proteins.

Cervicovaginal microbial composition differed in opsonisation status as determined by flow cytometry, with higher percentages of opsonisation in women with *L. crispatus* vaginal dominance. This was associated with lower concentrations of complement proteins and term birth, suggesting a potential role for opsonins in vaginal homeostasis during pregnancy.

This thesis highlights the role of the immune response in host-microbe interactions and pregnancy outcome. These results support the use for immunomodulators, complement therapeutics and live biotherapeutics targets for the prevention of inflammation and microbial driven PTB.

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ABBREVIATIONS

£	Pounds
ACTH	Adrenocorticotropin hormone
AF	Amniotic fluid
Ag	Antigen
ANOVA	Analysis of variance
APC	Allophycocyanin
ASV	Amplicon sequence variant
BCR	B cell receptor
BMI	Body Mass Index
bp	Base pair
BSA	Bovine serum albumin
BV	Bacterial Vaginosis
C5aR	C5a Receptor
Cat #	Catalogue number
CD	Cluster of differentiation
CL	Cervical length
COX	Cyclooxygenase
CRH	Corticotropin-releasing hormone
CRH-R	Corticotropin-releasing hormone receptor
C-section	Caesarean section
CST	Community State Type
C-STITCH	Cerclage Suture Type for an Insufficient Cervix and its effect on Health Outcomes
CV	Coefficient of variation
CVF	Cervicovaginal fluid
CWH	Chelsea and Westminster Hospital
Cx	Cervical
DAMPS	Damage-associated molecular patterns
DESI	Desorption Electrospray Ionization
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DNA	Deoxyribonucleic acid

dNK	Decidual NK
e.g.	Exempli gratia (“for example”)
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMMPRIN	Extracellular matrix metalloproteinase inducer
Fab	Fragment antigen-binding
Fc	Fragment crystallisable
FDA	Food and Drug Administration
fFN	Fetal fibronectin
FITC	Fluorescein isothiocyanate
FM	Fetal membranes
FSC	Forward scatter
GBS	Group B Streptococcus
GM-CSF	Granulocyte-macrophage colony- stimulating factor
HIV	Human immunodeficiency virus
HMP	Human Microbiome Project
Hx	History
IAI	Intraamniotic infection
IBD	Inflammatory Bowel Disease
IFN	Interferon
Ig	Immunoglobulin
iHMP	Integrative Human Microbiome Project
IL	Interleukin
iMFI	Integrated MFI
iNKT	Invariant NKT cells
IRDB	Institute of Reproductive and Developmental Biology
IQR	Interquartile range
LDGs	Low-density granulocytes
LiH	Lithium Heparin
LLETZ	Large Loop Excision of the Transformation Zone
LMS	Learning Management System
LPS	Lipopolysaccharide

MAC	Membrane Attack Complex
MASP	Mannose binding lectin-associated serine proteases
MBL	Mannose Binding Lectin
MFI	Median Fluorescence Intensity
MHC	Major histocompatibility complex
MIAC	Microbial invasion of the Amniotic Cavity
mL	Millilitres
MMPs	Matrix metalloproteinases
MOMS-PI	Multi-omic microbiome study: pregnancy initiative
MRC	Medical Research Council
mRNA	Messenger RNA
MS	Mass spectrometry
MTL	Mid-trimester loss
n/a	Not applicable
NET	Neutrophil extracellular trap
NF-κβ	Nuclear factor κβ
ng	Nanograms
NGS	Next Generation Sequencing
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NICU	Neonatal intensive care unit
NIHR	National Institute for Health and Care Research
NK	Natural Killer
NMR	Nuclear Magnetic Resonance
NOD	Nucleotide oligomerisation domain
OOR	Out of range
PAMPS	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PET	Preeclampsia
PG	Prostaglandin
PMN	Polymorphonuclear cells

PMT	Photomultiplier tubes
PPROM	Preterm premature rupture of membranes
PR	Progesterone receptors
PRR	Pattern recognition receptors
PT	Spontaneous preterm delivery
PTB	Preterm birth
PTL	Preterm labour
QCCH	Queen Charlotte's and Chelsea Hospital
RBC	Red blood cells
RCT	Randomised Control Trial
REC	Research Ethics Committee
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
ROS	Reactive Oxygen Species
SCBU	Special care baby unit
sIgA	Secretory IgA
SLE	Systemic lupus erythematosus
SMH	St Mary's Hospital
SObs	Species Observed
spp.	Species
sPTB	Spontaneous preterm birth
SSC	Side scatter
T regs	T regulatory
Th	T helper
TI	Term intervention delivery
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TU	Term uncomplicated delivery
UCLH	University College London Hospital
uNK	Uterine NK
Unk	Unknown
USS	Ultrasound

VMET 2	Vaginal Microbiome and Metabonome in Pregnancy
W	Weeks
WHO	World Health Organization

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1. INTRODUCTION

1.1. Preterm birth

1.1.1. Definition

Preterm birth occurs before completing 37 weeks of pregnancy (WHO, World Health Organisation). Preterm birth can be further subclassified into extremely preterm (<28^{+0} weeks of gestation), very preterm (between 28^{+0} and 31^{+6} weeks of gestation) and moderate or late preterm birth (between 32^{+0} -36^{+6} weeks of gestation) (Blencowe et al., 2013) (Figure 1.1A). Preterm birth affects as many as 15 million newborns worldwide and accounts for 11% of all live births, with rates varying between 5% to 18% depending on the country (Blencowe et al., 2013).

Preterm birth and its associated complications are the leading causes of mortality and morbidity among children under five years of age (WHO), with severity of morbidity dependent on gestational age (Mercer, 2003) (Figure 1.1.B, C). Survivors have a greater risk of long-term health conditions when the gestational age of birth is lower, including impairment in vision, hearing, lung and neurological development, and cardiovascular diseases. In addition, preterm birth can have significant economic and social impact (Blencowe et al., 2013).

A

PREGNANCY								
	Second trimester			Third trimester			Term	
Gestational age at birth	16	20	24	28	32	36	40	
	Extremely preterm $<28^{+0}$ weeks			**Very preterm** 28^{+0}- 31^{+6} weeks	**Moderate or late preterm** 32^{+0} - 36^{+6} weeks		**Term** 37^{+0} - 41^{+6} weeks	**Post term** ≥42 weeks
	PRETERM BIRTH							

B **C**

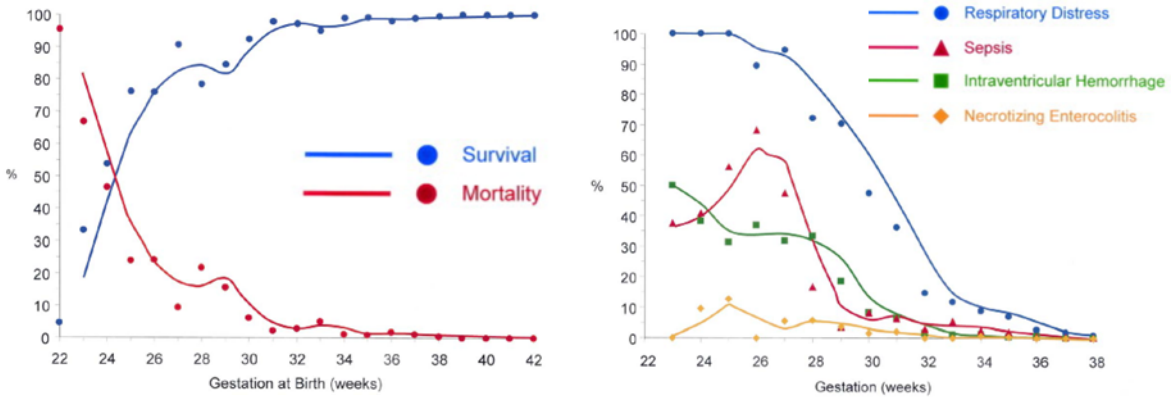


Figure 1.1. Definitions for preterm birth and related pregnancy outcomes. **A.** Preterm birth subclassifications. Adapted from Blencowe et al., 2013. **B**. Preterm birth infants have higher rates of neonatal mortality; **C.** and morbidity is inversely correlated to gestational age. Source: Mercer, 2003.

1.1.2. Clinical manifestation

Preterm birth can be subdivided into: 'indicated' or 'spontaneous'. The former is also known as 'iatrogenic' preterm birth and is defined as delivery initiated by the obstetric care provider (via labour induction or caesarean delivery) due to maternal or fetal indications (for example pre-eclampsia and fetal growth restriction). Around one third of preterm births are indicated/iatrogenic, while the rest are spontaneous. Spontaneous preterm birth includes women who present in labour with or without intact membranes, or who present initially with pre labour preterm premature rupture of the membranes (Goldenberg et al., 2008, 2000).

Preterm premature rupture of membranes (PPROM) is described as rupture of fetal membranes before 37^{+0} weeks of pregnancy prior to the onset of uterine contractions. PPROM is diagnosed via direct examination with a speculum to detect amniotic fluid in the vagina (NICE Guideline NG25, 2015). More than half of pregnant women experiencing PPROM will deliver within a week; with the rest of women

being at a higher risk of developing intrauterine infection (Mercer, 2003; Goldenberg et al., 2008). Preterm labour is described as regular contractions and the presence of cervical change. However, only a minority of women who present with preterm contractions will progress into labour and ultimately deliver. The process of spontaneous preterm birth requires contractions, cervical dilation and membrane rupture for preterm delivery to occur.

1.1.3. Causes of preterm birth

Preterm birth is a syndrome with multiple underlying aetiologies (Romero et al., 2014a; Gimeno-Molina et al., 2022a), including microbial-induced inflammation, chorion-decidual haemorrhage and vascular disease, decidual senescence, disruption of maternal-fetal tolerance, decline in progesterone action; and uterine overdistension (Figure 1.2).

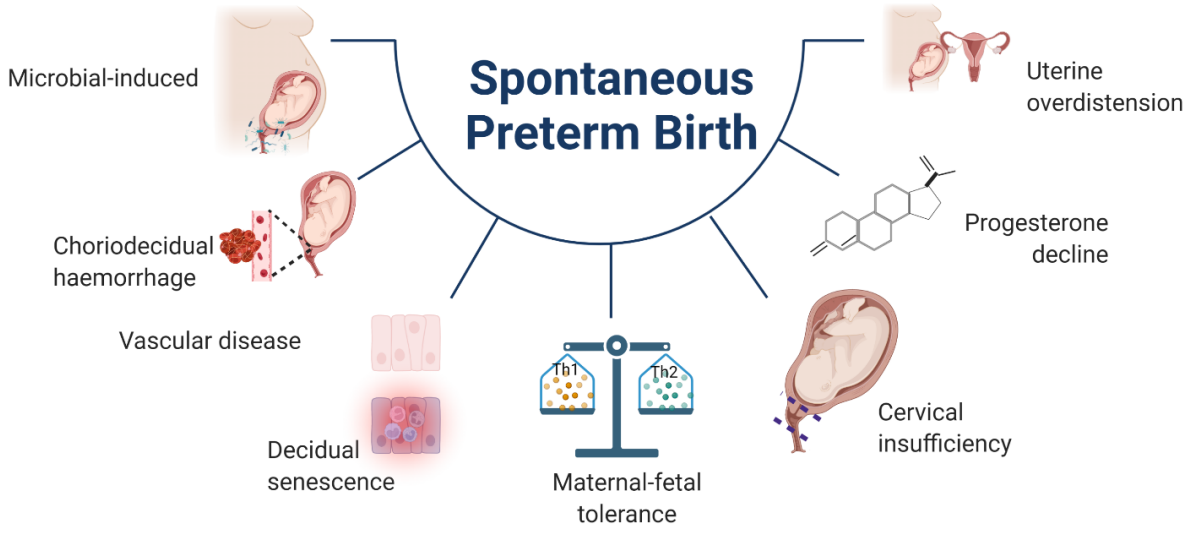


Figure 1.2. Causes of preterm birth. Proposed mechanisms implicated in spontaneous preterm birth (sPTB). Source: Gimeno-Molina et al., 2022a.

Preterm birth has also been associated with multiple risk factors, including maternal and socio-demographic factors (such as ethnicity, age, body mass index, economical/educational access, smoking status, among others) as well as past obstetric and gynaecological history such as previous PTB and cervical excisional treatment, and specific pregnancy related risk factors such as short interpregnancy interval, multiple pregnancy, and vaginal bleeding (Table 1.1) (Goldenberg et al., 2008; Shapiro-Mendoza and Lackritz, 2012; Koullali et al., 2016).

Defining the risk factors for PTB is useful for identifying women at risk early in pregnancy in order to provide the most appropriate preventative treatment as soon as possible. Additionally, it can contribute to better understanding of the mechanism of preterm birth. Furthermore, developing our understanding of the causes and mechanisms that lead to preterm birth can establish the basis for the development of new potential treatments.

There is a national and global drive to reduce the rates of preterm birth. The Department of Health have set an ambitious target to reduce preterm birth rates in the UK from 8% to 6% by 2025 (Safer Maternity Care, 2017). The WHO Sustainable Developmental Goal have set a target to end unnecessary neonatal and childhood deaths by 2030 and have acknowledged the essential need to reduce preterm birth to be able to achieve it (WHO SDG 3.2). We can only achieve these targets by increasing our mechanistic understanding of the causes and developing new and more targeted preventative strategies. This will improve individualised maternity care, as there is no “one size fits all” solution for preterm birth prevention.

Table 1.1. Risk factors for preterm birth. Reproduced from Gimeno-Molina et al., 2022a.

	Factor		Associated risk	Reference
Maternal characteristics	Ethnicity		African and Afro-Caribbean are at higher risk than Caucasian	(Schaaf et al., 2013; Opondo et al., 2020)
	Sociodemographic factors		Socioeconomic status, maternal age, educational attainment, marital status, access to health care services	(Manuck, 2017; Sparks, 2009)
	Nutritional status		Low BMI and overweight and obese women	(Masho et al., 2013; Shaw et al., 2014)
	Lifestyle	Behavioural	Depression, stress, etc. Although psychosocial traits are interrelated to other parameters	(Grigoriadis et al., 2018; Grote et al., 2010)
		Smoking	Correlated to the number of cigarettes smoked per day. Associated with a systemic inflammatory response.	(Moore et al., 2016; Soneji and Beltrán-Sánchez, 2019)
		Drugs, alcohol	Heavy alcohol consumption during pregnancy increases the risk of low birthweight and PTB.	(Blencowe et al., 2013; Patra et al., 2011)
	Medical history: periodontal disease		Haematogenous transmission of oral microbial pathogens	(Corbella et al., 2012; Offenbacher et al., 1996)
Cervical history	Cervical surgery		Cone biopsy Large loop excision of the transformation zone (LLETZ)	(Crane, 2003; Wittmaack et al., 2019)
	Cervical insufficiency		Cervical length: higher risk when mid-pregnancy short cervix	(Berghella et al., 2007; Iams et al., 1996)
Pregnancy history	Short interpregnancy interval		Less than 6 months	(Blencowe et al., 2013)
	Previous preterm birth or mid-trimester loss		~20% risk of recurrence	(Kamphuis et al., 2018; Phillips et al., 2017)
Pregnancy characteristics	Multiple gestation		PTB accounts for half of the complications observed in this obstetrical population	(Conde-Agudelo et al., 2010; Fuchs and Senat, 2016)
	In-vitro fertilization		Assisted reproductive technologies involved implantation of multiple embryos and advanced maternal age	(Cavoretto et al., 2018; Jančar et al., 2018; Muglia and Katz, 2010)
	Vaginal bleeding		Placental abruption, placenta preavia	(Fishman and Chasen, 2011)
	Bacterial vaginosis Asymptomatic bacteriuria		Overgrowth of atypical microorganisms in the vagina, with or without symptomatology	(Goldenberg et al., 2000; Shimaoka et al., 2019)

1.1.4. Social impact of preterm birth

Preterm birth has a financial impact on health care services, which is usually measured in terms of hospitalisation (total length of stay, interventions, feeding, and transfers) (Khan et al., 2015). Neonatal care is expensive, highly technical and labour intense. The average length of stay of hospitalisation until initial discharge is 2.4 days for term births; however, it ranges from 65 to 116 days when infants are born between 24 to 28 weeks of gestation (Hinchliffe et al., 2013). The initial cost of hospitalisation can reach up to £4528 for late preterm births, £11629 for moderate preterm births (Khan et al., 2015); and even as high as £317166 for extremely preterm deliveries, compared to term deliveries (~£1000) (Petrou et al., 2011). During the first two years of age, moderate and late preterm births are estimated to cost £12037 and £5823 per birth, respectively; in contrast to an average of £2056 for a term infant (Khan et al., 2015). The economic impact of health sequelae (cumulative hospital readmissions, service utilisation) also persists into late childhood (8th and 11th year after birth) (Achana et al 2022; Hua et al 2023). Preterm birth is not only costly to healthcare services, but also impacts on other sectors of the economy, including family and carers, and society in general (Petrou et al., 2019). It has been estimated that just in England and Wales, the total economic burden to the public sector is of £2.946 billion over the 18-year time period (Mangham et al., 2009). This financial cost estimation does not include long term derived impact, such as the use of health and social care services, education, occupational status, income, and receipt of social welfare benefits (Petrou et al., 2019). Additional aspects of life and society are also influenced by preterm birth such as non-financial intangible consequences of pain, fear, emotional and social isolation (Petrou et al., 2011) and family-care services costs (day care, community health social care services, medicines and drugs, home adaptation, special equipment, loss of parental productivity) (Khan et al., 2015).

1.1.5. Management of preterm birth

The management of preterm birth includes strategies to predict and identify women at high-risk of delivering preterm, as well as prevention and treatment (acute management).

1.1.5.1. Prediction

Cervical length monitoring

Cervical shortening is one of the major risk factors for sPTB (Figure 1.2., Table 1.1.). Women who experience cervical shortening in the second trimester are at higher risk of delivering preterm

(Hassan et al., 2000; Iams et al., 1996). To monitor the cervical length there are three methods available: transperineal, transabdominal or transvaginal ultrasound, the latter being more accurate in identifying a short cervix (Glover and Manuck, 2018; Hernandez-Andrade et al., 2012). In a transvaginal ultrasound scan the cervical length is measured calculating the distance between the internal and the external cervical os. The transvaginal cervical length cut off to determine a normal or a short cervical length is on the 10th centile (25mm): over 25mm is considered a "normal" cervical length, whereas lengths of ≤25mm are considered "short" (Owen et al., 2001). The risk of PTB increases when the cervical length is shorter (Grimes-Dennis and Berghella, 2007) and the earlier it is detected in pregnancy (Berghella et al., 2007).

There is some controversy regarding universal transvaginal cervical length monitoring, since there is not enough evidence of its utility in low-risk populations (asymptomatic women with no pre-defined risk factor for PTB) (Berghella and Saccone, 2019; Miller et al., 2015). In contrast, it is widely adopted as a screening tool in women who have risk factors for preterm birth.

Fetal fibronectin

Fetal fibronectin (fFN) is an extracellular matrix glycoprotein present on the maternal-fetal interface (Glover and Manuck, 2018; Son and Miller, 2017). During pregnancy, it can be detected in cervicovaginal fluid at low levels, but when the concentration is higher than 50ng/mL there is a higher risk of sPTB (Lockwood et al., 1991). The fFN test is available for patients between 22 and 34 weeks of gestation, as a qualitative test using 50ng/mL as a cut off threshold. This test is most useful for its high negative predictive value; a negative fFN test reassures that delivery will not occur within the following 14 days in 98% of cases. The positive predictive value is limited. Recently, it has been demonstrated that establishing different quantitative thresholds can help to better stratify the risk of sPTB, being the positive predictive value higher with greater fFN concentrations (e.g. positive predictive value increased from 20% to 47% by using 500mg/mL concentration instead of 50-200mg/mL) (Abbott et al., 2015).

Both current strategies to predict preterm labour (cervical length surveillance and fFN test) target events that precede labour in women at high-risk. However, these predictive tests are not useful for women considered to be at low risk of preterm birth. The majority of women who deliver preterm have no identifiable risk factors, therefore, a better understanding of the mechanisms leading to preterm birth are required to further develop new and effective predictive tools useful to all pregnant women.

1.1.5.2. Prevention

Cervical cerclage

The functions of the cervix during pregnancy are (1) to maintain a physical and immune barrier between the intrauterine cavity and the vagina; and (2) to shorten and dilate at the correct time, permitting labour and vaginal delivery (Yellon, 2019). In physiological conditions of pregnancy, the cervix remains long and closed until the end of pregnancy, when it progressively starts to shorten and soften to prepare for labour and delivery (Alfirevic et al., 2017).

The event of early cervical shortening in the absence of uterine contractions occurs in cases of cervical insufficiency. Cervical insufficiency has been defined as the inability of the cervix to retain a pregnancy to term (American College of Obstetrics and Gynecologists, 2004). Gynaecologic practices involving cervical treatment such as large loop excision of the transformation zone (LLETZ), cone biopsy or previous caesarean section can weaken the cervix for subsequent pregnancies (American College of Obstetrics and Gynecologists, 2004). Patients with cervical insufficiency typically present to hospital with vaginal pressure and protruding membranes which can result in late second trimester miscarriage or early preterm birth (Romero et al., 2006).

In order to prevent or delay preterm birth, cervical cerclage, a procedure that involves placing a suture (stitch) around the cervix, is performed to give structural support to the cervix and to maintain the endocervical mucus plug. The intervention was first introduced by Shirodkar (Shirodkar, 1955) and later by McDonald (McDonald, 1957). McDonald described the intervention as the ligation of the cervix during pregnancy in cases of dilation and bulging forewaters, for women with a previous history of one or more miscarriages (McDonald, 1957). This intervention aims to place the stitch at the level of the internal cervical os. Later, the suture is removed at 37 weeks of gestation or when in labour (Alfirevic et al., 2017; McDonald, 1957).

Cervical cerclage can be offered as a prophylactic measure during or before pregnancy. Indications to consider cervical cerclage include previous pregnancy history (referred to as elective cerclage) or when the cervix is found to be short on ultrasound or open (referred to ultrasound indicated or emergency cerclage, respectively) (Alfirevic et al., 2017; NICE Guideline NG25, 2015).

Cervical cerclage can reduce the risk of preterm birth by 30% in women with singleton gestation, short cervix, and previous history of spontaneous preterm birth (Berghella et al., 2011). However, cervical cerclage is not always a successful intervention. Sakai et al. showed that patients with a short cervix in the presence of cervical inflammation (defined as high

concentrations of IL-8 in cervical mucus), cervical cerclage is not only ineffective, but might induce exacerbated inflammation, increasing the risk of preterm birth (Sakai et al., 2006). Also, braided suture material used for cerclage has been shown to be associated with vaginal dysbiosis, increased cervicovaginal inflammation, and a higher rate of preterm birth compared to monofilament cerclage (Chan et al., 2022; Kindinger et al., 2016). Although recent data from the C-STICH (Cerclage Suture Type for an Insufficient Cervix and its effect on Health outcomes) randomised control trial (RCT) found no differences in the rates of pregnancy loss and the gestational age at delivery between women who received a monofilament or a braided cerclage suture (Hodgetts Morton et al., 2022). In women whereby a cerclage is unsuccessful, it is likely that pathologies other than mechanical cervical weakness are driving the underlying causes of preterm labour.

Progesterone

The other widely available therapy used to prevent preterm birth is progesterone (NICE Guideline NG25, 2015). Progesterone is implicated in contractility modulation, and promotes uterine quiescence (Sykes and Bennett, 2018; Talati et al., 2017). Although many studies have shown that progesterone can reduce the risk of preterm labour (da Fonseca et al., 2003; Hassan et al., 2011), there are also many studies which have shown it to be ineffective (Crowther et al., 2017; Norman et al., 2016; O'Brien et al., 2007). It is likely that the discrepancies between studies are due to the multifactorial aetiology of preterm birth and the heterogeneity of women recruited into these studies. Better patient stratification strategies would be required to identify which population/s would benefit the most of this particular intervention (Sykes and Bennett, 2018). Furthermore, dosage, type and delivery methods appear to show variation in efficacy for the prevention of preterm birth and its associated adverse outcomes. Evidence suggests that vaginal progesterone administration is associated with lower rates of sPTB, lower adverse reactions and lower rates of NICU (neonatal intensive care unit) admission, compared to the administration of intramuscular 17 α-hydroxyprogesterone caproate (250mg weekly) (G. Saccone et al., 2017).

Overall, there is evidence that progesterone and cervical cerclage are equally efficacious in the prevention of preterm birth in women with singleton gestation, previous sPTB and short cervix (Conde-Agudelo et al., 2018).

Cervical pessary

A cervical pessary is a circular plastic device placed in the vagina. The cervical pessary was initially used to treat pelvic floor disease. Cross indicated the use of this device to control incompetence of internal cervical os in patients with history of miscarriage by inserting the pessary around the cervix at the level of the internal os, until a week before term delivery (Cross, 1959). Since then, vaginal pessaries have modified towards a softer and conical silicone design to facilitate their insertion (Arabin and Alfirevic, 2013).

Arabin conducted one of the first pilot studies describing the benefits of the cervical pessary as a preventive treatment for prolonging pregnancy in patients at risk for sPTB (Arabin et al., 2003). The Arabin vaginal pessary is designed for pregnant women to direct the cervix more posteriority, changing the inclination of the cervical canal (Arabin et al., 2003). This positioning of the device provides an acute uterocervical angle that might prevent further opening of the internal os, as well as simultaneous protection of the cervical mucus plug. Some of the benefits that have been proposed for this intervention are that it is easy, well tolerated for the patient and low-cost. However, it can cause oedema and laceration of the cervix (Arabin and Alfirevic, 2013). Some studies have shown a reduction of preterm birth rates with cervical pessary intervention (Abdel-Aleem et al., 2013; Goya et al., 2012; Gabriele Saccone et al., 2017). For example, in a randomised trial that included 300 pregnant women, women who received cervical pessary had significant lower rates (7.3%) of early spontaneous preterm birth (<34 weeks of gestation) compared to women who did not use it (15.3%) (Gabriele Saccone et al., 2017). However, there are also studies showing it to be ineffective (Jin et al., 2017; Nicolaides et al., 2016; Norman et al., 2021). Again, such discrepancies could be due to the multifactorial nature of preterm birth, revealing the need for better targeted prevention strategies and trials that compare the different benefits of the current treatment strategies (Hezelgrave et al., 2016; Tsikouras et al., 2018).

Environmental and lifestyle modification

There are other recommended preventative strategies targeting modifiable factors related to lifestyle habits, such as body mass index (BMI), exercise practice and smoking cessation (Savitz et al., 2012). For instance, it has been shown that there is an increase in the mean infant birthweight and a reduction on the number of babies born with low birthweight when pregnant women stop smoking (Chamberlain et al., 2017; McCowan et al., 2009). Beneficial modifiable factors that can be implemented before and between pregnancies include birth spacing (inter-

pregnancy interval), care of nutritional status, smoking cessation and administration of folic acid (Dean et al., 2013; Iams et al., 2008).

1.1.5.3. Acute management

Tocolytics

Women who present to hospital with threatened preterm labour can be given tocolytics to inhibit uterine contractility and cervical dilation, with the aim of delaying the process of labour. The predominant gain in delaying labour is to be able to administer corticosteroids to the mother to help reduce morbidity and mortality of the newborn and magnesium sulphate to provide neuroprotection for the newborn (Haas et al., 2014; Requejo et al., 2013).

Guidelines recommend the tocolytic administration of an oxytocin receptor antagonist (Atosiban) or Calcium channel blocker (Nifedipine) (NICE Guideline NG25, 2015). There are other tocolytic agents such as betamimetics, prostaglandin inhibitors and nitrates that have been used as short-term tocolytics (Haas et al., 2014). However, they are not commonly used due to their side effect profiles. The key disadvantage of tocolytic agents is that they address the symptoms of preterm labour rather than the underlying causes (Romero et al., 2014a). It is possible that the reason tocolytics may not be effective in preventing preterm birth is that the biochemical process of labour is too advanced to reverse.

Magnesium sulphate

Management of women in preterm labour also includes the administration of magnesium sulphate for neuroprotection of the newborn (Requejo et al., 2013). One of the long-term impacts of preterm birth on survivors is neuro-developmental impairments (Blencowe et al., 2013). Pregnant women (between 24-30 weeks of gestation) who are in labour or planning to deliver within the next 24 hours are offered magnesium sulphate (NICE Guideline NG201, 2021). For more than 30 years a large number of studies have addressed the use magnesium sulphate (American College of Obstetricians and Gynecologists, 2010). Its mechanism of action is not fully understood but it has been suggested to act potentially via preventing neuronal cell apoptosis, prevention of inflammatory events or promotion of neurogenesis (Bachnas et al., 2019). There is plenty of evidence to support its use in women at risk of preterm birth (Doyle et al., 2009) that is considered to outweigh the potential adverse effects on the mother (nausea, headaches, vomiting).

Antibiotics

Since genital tract infection is an aetiological factor for preterm birth, it has been hypothesised that early intervention with antibiotic prophylaxis may reduce the risk of preterm birth. However, results of studies assessing this are inconclusive. Despite some evidence that in a subgroup of pregnant women with previous PTB and current BV (bacterial vaginosis) the rates of PTB were reduced by treatment with antibiotics (McDonald et al., 1997), in general, neither treatment of bacterial vaginosis nor asymptomatic bacteriuria during pregnancy correlate with a reduction of preterm birth rates (Nygren et al., 2008; Sangkomkamhang et al., 2015; Smaill and Vazquez, 2019). Discrepancies between studies could be explained by antibiotic heterogeneity, time of administration and the lack of specificity of antimicrobials. Therefore, currently there is no justification to give antibiotics to all pregnant women (Brocklehurst et al., 2013; Thinkhamrop et al., 2015), since there is not enough evidence showing a reduction on the risk of PPROM nor PTB. In addition, there is evidence that metronidazole use could be detrimental and lead to an increase in PTB rates (Shennan et al., 2006) and that erythromycin has long-term adverse neurodevelopmental effects in infants whose mothers were prescribed erythromycin whilst in threatened preterm labour with intact membranes (Kenyon et al., 2008).

1.1.5.4. Current perspective

The current available interventions are not effective for all cases, due to the complexity of the aetiology of preterm birth; and a single strategy cannot be used for all women to prevent the process of labour, due to the variable pathophysiologies (Requejo et al., 2013). Therefore, a better understanding of the mechanism and physiology and pathophysiology of cervical shortening and dilation in women who subsequently deliver preterm could allow us to improve the stratification of patients to better target patient specific therapies.

1.2. Physiology of term labour

The process of labour culminates with the delivery of the fetus and the placenta; this process requires the onset and maintenance of contractions, rupture of the fetal membranes and remodelling and dilatation of the cervix. The whole process is regulated by a combination of mechanical, inflammatory, and endocrine events (Golightly et al., 2011; Steer and Flint, 1999).

During labour, all the inhibitory effects present during pregnancy are eliminated. Pregnancy is characterised by the maintenance of cervical integrity and uterine quiescence (Myers, 2012;

Steer and Flint, 1999; Word et al., 2007), whereas labour is characterised by a softening of the cervix and the presence of frequent and regular uterine contractions (Steer and Flint, 1999). The whole process is stimulated by prostaglandins, cytokines and steroid modulator hormones (Challis et al., 2005; Steer and Flint, 1999). The process of labour can be divided into four phases (Figure 1.3.): Quiescence (Phase 0), Activation (Phase 1), Stimulation (Phase 2) and uterine involution (Phase 4) (Liao et al., 2005).

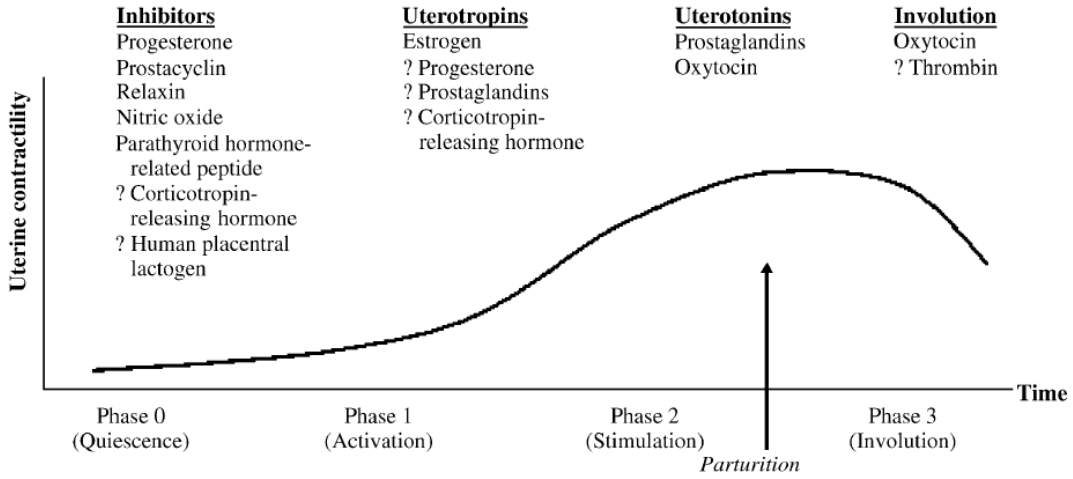


Figure 1.3. Phases of labour. The process of labour can be divided into four phases: Quiescence (Phase 0), Activation (Phase 1), Stimulation (Phase 2) and uterine involution (Phase 4). Source: Liao et al., 2005.

1.2.1. Uterine contractility

The process of labour requires the presence of frequent and regular uterine contractions. Two essential hormones involved in the transition from myometrial quiescence to contractility are progesterone and corticotropin-releasing hormone (CRH).

Progesterone maintains the uterus in a quiescent state, inhibiting contractility via several mechanisms (Sykes and Bennett, 2018; Talati et al., 2017). For example, progesterone promotes quiescence by modifying the expression of agonists and receptors involved in intracellular signalling pathways ,such as increased expression of $Ca2^{+}$ and K^{+} channels in myometrial cells to reduce myometrial contractions; and downregulating proteins involved in the cross-linking of actin-myosin (Soloff et al., 2011). Closer to labour, there is a reduction in the inhibitory effect of progesterone despite circulating levels of progesterone in maternal blood remaining high. This contrasts with the decreased progesterone levels observed just prior labour onset as seen in other viviparous species (e.g., rodents and sheep). Instead, it has been proposed that the decrease in progesterone action in human parturition is achieved via *functional* progesterone

withdrawal. Functional progesterone withdrawal can be a result of either progesterone receptors (PR) becoming inactive, changes in the transcriptional activity of PR isoforms or coactivators, or a combination of these activities (Mesiano, 2022). It has been hypothesised that progesterone withdrawal at the time of labour could be a response to inflammatory activation, involving the nuclear factor NF-κB (Mesiano, 2022).

CRH is produced by the placenta during pregnancy and acts on the fetal hypothalamic-pituitary adrenal axis, where it stimulates the production of adrenocorticotropin hormone (ACTH), which in turn stimulates the production of cortisol and dehydroepiandrosterone sulfate (DHEAS) (Figure 1.4.) (Challis and Hooper, 1989; Liao et al., 2005; Vrachnis et al., 2012). Consequently, DHEA-S promotes the synthesis of oestrogen; and cortisol itself stimulates the expression of surfactant protein A in fetal lungs. Increased levels of CRH induce infiltration of immune cells, that will further release cytokines (IL-1β, TNF-α), stimulating CRH and cortisol (Vrachnis et al., 2012), creating a positive feedback loop. Additionally, CRH can directly act on CRH receptors (CRH-R1 and CRH-R2) expressed on myometrial cells. Isoform CRH-R1 promotes relaxation of smooth muscles via inhibition of phosphorylation of contractile protein myosin light chains; whereas CRH-R2 can be stimulated by urocortins and will phosphorylate myosin light chain (Vrachnis et al., 2012).

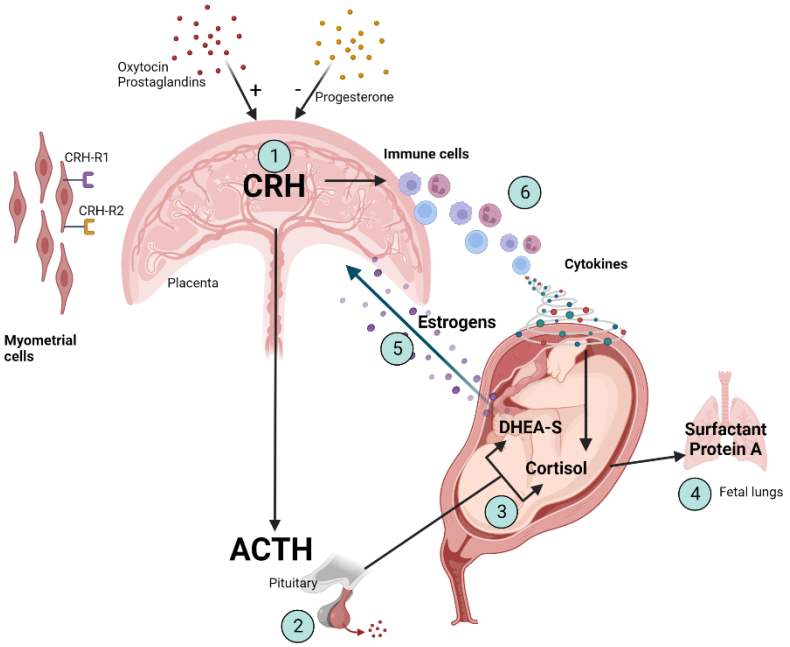


Figure 1.4. Placental CRH on fetal pituitary-adrenal axis. CRH: corticotropin releasing hormone. ACTH: adrenocorticotropin hormone. DHEA-S: dehydroepiandrosterone sulfate. CRH-R: corticotropin releasing hormone receptor. Created with BioRender.com.

Cortisol and DHEA-S are accountable for increasing oestrogen levels; this latter hormone is responsible for myometrial responsiveness via oxytocin receptors and prostaglandin receptors (Challis and Hooper, 1989; Golightly et al., 2011). The recruitment and communication of myometrial cells occurs through gap junctions, allowing the propagation of the action potential and the depolarisation move to and between adjacent myometrial cells, permitting an effective and coordinated electrical synchrony within myometrium (Liao et al., 2005; Steer and Flint, 1999).

1.2.2. Membrane activation

Rupture of fetal membranes is a controlled event which usually follows regular uterine contractions (Moore et al., 2016). This programmed weakening process requires extracellular matrix (ECM) remodelling and cellular apoptosis (Arikat et al., 2006; Kumar et al., 2006; Moore et al., 2006).

The physical properties of fetal membranes can be tested by assessing the tensile strength forces of each layer. Moore and colleagues showed that a specific zone in the membranes on the side overlying the cervix was weaker than the rest of the membranes and that had greater collagenase activity (El Khwad et al., 2005). This indicates that the biochemical properties of the membranes are not homogenous over the surface. When measuring force and displacement, they also showed different properties between the layers, with amnion having more tensile strength than the chorion (Moore et al., 2006). The sequence of events starts with choriodecidual rupture. The amnion and choriodecidual rupture occurs separately; after the choriodecidua shears apart, the amnion starts to herniate and distend; this non-elastic deformation culminates with amnion rupture (Arikat et al., 2006).

Membrane activation requires remodelling of the extracellular matrix (ECM). The ECM is an acellular structure that provides support (structural and biochemical) to the surrounding cells. The ECM can interact with cell surface receptors, and it is a source of growth factors, proteins and cytokines, that can directly impact cells. . Therefore, the ECM is considered a dynamic component whose content will determine the physical and biological properties of the tissue (Strauss, 2013). The main component of ECM is collagen. Fetal membranes are a viscoelastic avascular tissue composed of various layers with distinct collagen composition. Mainly, fibrillar collagen type I, II, III, and V are present, which are assembled into fibrils, and are responsible for the tensile strength (Strauss, 2013).

Stretching also affects the expression of ECM genes and matrix metalloproteinases (MMPs) (Strauss, 2013). MMPs are enzymes that hydrolyse and degrade collagen. The ECM is highly regulated by a balance between MMPs and TIMPs (tissue inhibitors of metalloproteinases) (Kumar et al., 2006; Moore et al., 2006). The weakest site of the membranes overlying the cervix showed an increased expression of MMP-9 and decreased TIMP-3 by western blot (El Khwad et al., 2005). Experiments of human fetal membranes cultured with proinflammatory cytokines (TNF-α, IL-1β) also revealed increased levels of MMP-9, and suppression of TIMP-3 (Kumar et al., 2006). Cytokines and prostaglandins are involved in the weakening process of the cervix weak zone (Moore et al., 2006). For example, TNF-α and IL-1β induce weakness in a dose- and time-dependent manner (Kumar et al., 2006). Proinflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) have been detected by immunohistochemistry in infiltrating leukocytes, decidual stromal cells, mesenchymal cells and extravillous trophoblast at the time of labour (Young et al., 2002).

1.2.3. Cervical ripening and dilation

During pregnancy, the cervix is closed and rigid. Its main functions are to provide a protective barrier and resisting the gravitational forces imposed by the expanding uterus. Prior to labour, the cervix experiences remarkable changes in consistency and length: mainly a progressive decrease in cervical rigidity, and dilatation (Sakamoto et al., 2005; Word et al., 2007). This process is known as cervical remodelling.

The process of cervical remodelling can be divided into several phases (Figure 1.5.). The first phase is known as softening: this is a slow phase in which collagen fibres are reorganized, and it is characterised by decline of the tensile strength and tissue compliance. Softening is followed by ripening, characterised by loss of integrity and collagen degradation. Finally, dilatation allows the passage of the fetus through the birth canal. The whole process culminates with a postpartum repair to recover cervical competence (Timmons et al., 2010; Word et al., 2007).

The cervix is also a dynamic structure constituted of changing connective tissue and collagenous structures that can be examined by histochemistry. Cervical tissue of non-pregnant women is characterised by a dense connective tissue and thick collagen; in contrast to cervical tissue examined intrapartum, where fibres are disturbed, spread and thinner (Junqueira et al., 1980). Deposits of depolymerised collagen can also be detected (Junqueira et al., 1980).

The human cervix is a fibrous organ with high connective tissue content. The ripening mechanism requires collagen remodelling, involving a decrease in the concentration of collagen,

an increase of collagenase activity and hyaluronan, and alteration of proteoglycan and water content, all contributing to collagen solubility and dispersion (Myers, 2012; Sakamoto et al., 2005; Sennström et al., 1997; Uldbjerg et al., 1983). Proteolytic enzymes such as MMPs are necessary to break down ECM components. Immunohistochemistry and quantification of mRNA of cervical biopsies has detected increased levels of MMP-2 and MMP-9 in cervical tissue from term parturition compared to non-pregnant tissues (Stygar et al., 2002).

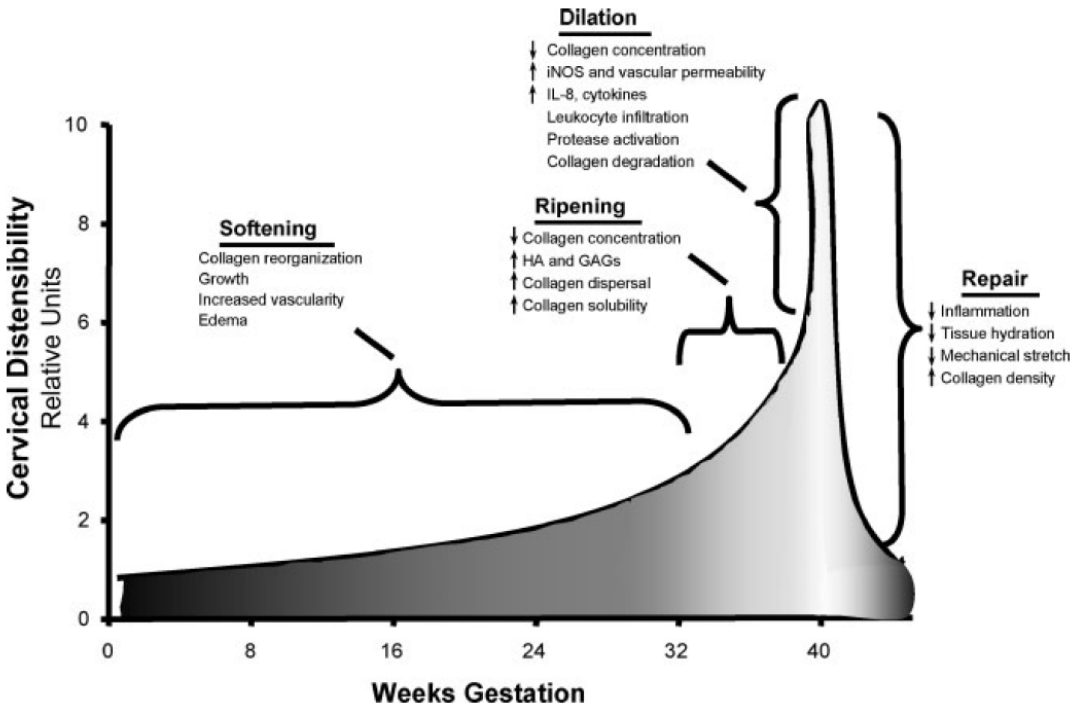


Figure 1.5. Phases of cervical remodelling. The phases of cervical remodelling are softening (slow and progressive reorganization of collagen fibres), ripening (rapid loss of cervical integrity), dilatation (further rapid reorganisation to let the baby pass through the birth canal) and repair (postpartum, to regain cervical competence). Source: (Word et al., 2007).

Cervical ripening is thought to be driven by an inflammatory response, evidenced by infiltration of leukocytes into the tissue. The attraction of leukocytes is stimulated by chemokines and cell adhesion molecules. Infiltrating leukocytes will further promote the expression of more cytokines in the cervix, stimulating tissue remodelling (Golightly et al., 2011). Leukocytes, mainly neutrophils and macrophages, have been detected in uterine cervical stroma (Bokström et al., 1997; Osman et al., 2003). IL-8 was first detected in the cervix from homogenised cervical tissues from non-pregnant and pregnant women by enzyme immunoassays, with a 6-fold increase in median IL-8 concentration observed in pregnant women not in labour and 11-fold increase in women after spontaneous term vaginal deliveries, compared to non-pregnant tissue (Sennström

et al., 1997). In addition, there is evidence of immunolocalisation of proinflammatory cytokines in cervical tissue and within the leukocytes themselves, including IL-1β, IL-6, IL-8 and TNF-α (Osman et al., 2003; Sakamoto et al., 2005; Sennström et al., 2000; Young et al., 2002), as well as increased cytokine concentration at the transcriptional level (Sennström et al., 2000).

1.3. Pathophysiology of preterm labour

There is an overlap of biochemical pathways between term and preterm labour (Figure 1.6.). Preterm labour also requires cervical ripening, uterine contractility, and rupture of membranes. The parturition pathway can be triggered with the premature activation of one or more of these components. The same pathways are activated, and proinflammatory cytokines, prostaglandins and infiltration leukocytes are localised in the maternal-fetal interface (Challis et al., 2009; Sykes et al., 2011). The inflammatory response during parturition is considered modest during term labour; but augmented during preterm labour, especially but not exclusively in the context of infection-driven aetiology (Challis et al., 2005).

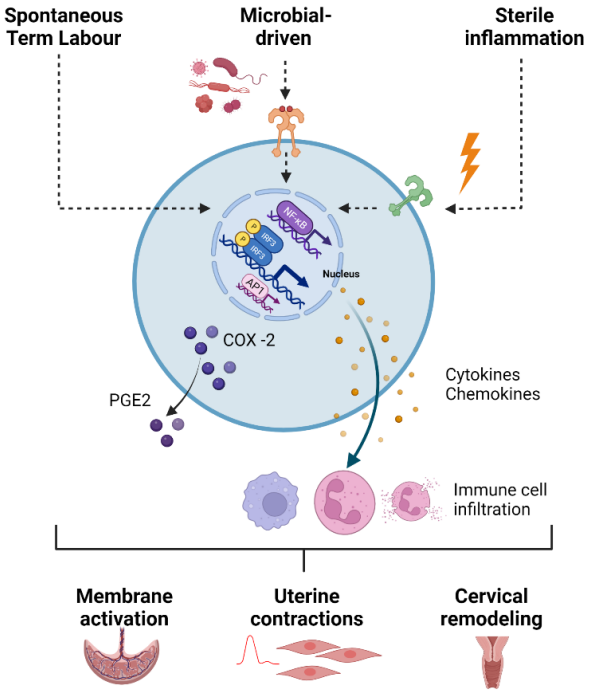


Figure 1.6. Potential infectious and non-infectious mechanisms leading to labour. Term labour is a proinflammatory process that requires the release of cytokines, chemokines, prostaglandins and immune cells infiltration. This proinflammatory event triggers membrane activation, uterine contractions and cervical remodelling, leading to labour. However, the same biochemical pathways can be activated when PRRs (pattern recognition receptors) recognise pathogenic molecules (microbial-driven), or endogenous signals in the absence of infection (sterile inflammation). COX: cyclooxygenase. PGE: prostaglandin. Created with Biorender.com

Preterm birth is a syndrome with many underlying causes (Figure 1.2.) (Gimeno-Molina et al., 2022a; Romero et al., 2014a), and there are several risk factors (Table 1.1.) implicated in the aetiology of preterm birth (Gilman-Sachs et al., 2018) as previously discussed. The most common causal aetiology of spontaneous preterm birth is inflammation and/or infection-driven.

1.3.1. Infection and inflammation

There are several ways in which microorganisms can gain access to the amniotic cavity (Figure 1.7): 1) ascending microorganisms from the vagina; 2) haematogenous spread; or 3) unintentionally, via invasive procedures (e.g., amniocentesis) (Goldenberg et al., 2008; Romero et al., 2007).

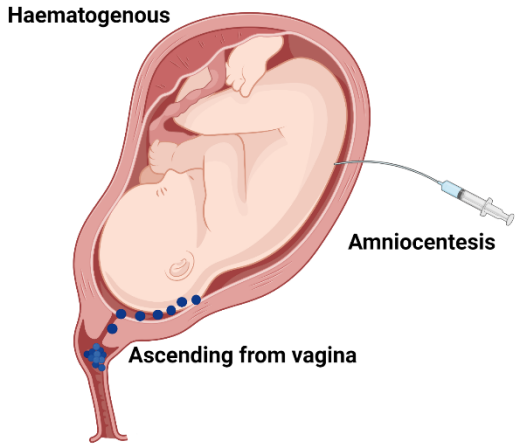


Figure 1.7. **Intrauterine microbial invasion.** Pathogenic microorganisms can access the amniotic cavity by ascending from the vagina, via haematogenous spread through the placenta or via invasive procedures such as amniocentesis. Reproduced from (Goldenberg et al., 2008). Created with Biorender.com.

Ascending infection from the vagina to the intrauterine cavity has long been implicated in the aetiology of preterm birth. Our group has previously described how vaginal dysbiosis (depletion of *Lactobacillus* species and increase of microbial diversity) increases the risk of premature birth (Brown et al., 2018; Chandiramani et al., 2014; Kindinger et al., 2017); among many others (Callahan et al., 2017; Elovitz et al., 2019; Fettweis et al., 2019; Stout et al., 2017; Tabatabaei et al., 2019). The role of the vaginal microbiome in preterm birth is further addressed in Section 4. Several animal models in mice (Gonzalez et al., 2011; Spencer et al., 2021; Suff et al., 2018) and primates (Coleman et al., 2021; Grigsby et al., 2010) have shown how vaginal (Gonzalez et al., 2011; Spencer et al., 2021; Suff et al., 2018) and intrauterine (Coleman et al., 2021; Grigsby et al., 2010) administration of bacteria or bacterial products can gain access to the choriodecidual space causing preterm birth. These studies implicate ascending vaginal infection with the

pathophysiology of choriodecidual inflammation, including the activation and recruitment of leukocytes, release of proinflammatory cytokines, prostaglandins, complement deposition and MMPs; and finally, cervical ripening and contractility.

The amniotic cavity is considered to be sterile, therefore the isolation of microorganisms from amniotic fluid is a pathological finding (Agrawal and Hirsch, 2012; Romero et al., 2007) and is often referred to as microbial invasion of the amniotic cavity (MIAC). Several microorganisms have been identified in the amniotic fluid (*Ureaplasma urealyticum*, *Bacteroides ureolytis*, *Sneathia sanguinegens*, *Ureaplasma peruum*, *Streptococcus agalactiae*, *Mycoplasma hominis*, *Gardnerella vaginalis*, *Enterococcus* spp., *Streptococcus* spp.) (Cappelletti et al., 2016); and the detection is commonly associated with elevated proinflammatory cytokine levels (Cappelletti et al., 2016; Gilman-Sachs et al., 2018). Moreover, intraamniotic infection and inflammation are common findings in pregnant women with cervical insufficiency (Lee et al., 2008) or PPROM (Romero et al., 1988) supporting the potential mechanism for ascending route of infection.

Intraamniotic infection is seen in association with histological chorioamnionitis (inflammation of the chorion and amnion). The hallmark of histological chorioamnionitis is neutrophil infiltration, whereby neutrophils migrate from the maternal blood into the fetal membranes along a chemotactic gradient. The prevalence of chorioamnionitis is higher when patients have MIAC (Romero et al., 2007); and it is also more prevalent in women with birth at earlier gestational ages (Russell, 1980).

1.3.2. Sterile inflammation

Preterm labour is normally triggered by inflammatory processes generated due to infection, however, it can also occur in the absence of microorganisms, known as sterile inflammation (Gilman-Sachs et al., 2018; Romero et al., 2014a). In these cases, intraamniotic inflammation can be due to other mechanisms such as necrosis or cellular stress that trigger activation of the innate immune system via the release of mediators and endogenous molecules known as alarmins.

Alarmins have been implicated in both healthy term labour and pregnancy complications such as sPTB. Examples of alarmins include IL-1α, high-mobility group box 1, cell free fetal DNA, S100 calcium binding protein B, heat shock proteins and uric acid (Nadeau-Vallée et al., 2016; Negishi et al., 2018; Romero et al., 2014d). For example, Romero et al. found that in cases of sterile intraamniotic inflammation (negative amniotic fluid culture, as well as no microbial footprint by

PCR, and IL-6 amniotic concentration ≥2.6ng/mL) the concentration of high-mobility group box 1 in amniotic fluid was higher in women with sterile intraamniotic inflammation compared to women without, as well as higher in women who delivered earlier after amniocentesis (Romero et al., 2014d).

1.4. Human vaginal microbiome

The next section is based on the publication: Preterm birth and the vaginal microbiomes. *Recent Advances in Obstetrics and Gynaecology* (2022). Belen Gimeno-Molina, Phillip R. Bennett, David A. MacIntyre & Lynne Sykes.

1.4.1. Microbiome: definition and research perspectives

The terms microbiome and microbiota tend to be used as interchangeable synonyms. However, the actual definitions reveal nuances between both terms. The term microbiota defines the entire collection of microorganisms that inhabit the human body; that includes not only bacteria but also, archaea, eukaryote (fungi, yeast, protozoa) and viruses (Methé et al., 2012). On the other hand, the term microbiome defines the microorganisms, together with their genomes and milieu conditions (Marchesi and Ravel, 2015).

Microbiota can be considered as an ecological system, as its composition is dynamic and complex. A healthy microbiota involves a diversity of species providing a benefit to the host development and health. The disruption of this homeostatic state will lead to dysbiosis, when there is a change in the composition of the resident commensal communities commonly found in healthy individuals. The impaired composition of the local community can be due to the loss of beneficial microbial organisms, the expansion of pathogenic microorganisms and/or loss of overall microbial diversity (Petersen and Round, 2014).

In the last 20 years, there have been significant advances in the understanding of the complexity and function of the human microbiota in health and disease. This has been facilitated by the development of culture-independent methodologies and international collaborative efforts such as the Human Microbiome Project (HMP, 2012). The HMP started in 2007 with the objective of characterising microbial communities from the vagina, skin and gut, to establish the “ideal” “healthy” composition of each different body site (Methé et al., 2012). However, soon it became apparent that the microbiome is complex and dynamic: communities can be perturbated by different conditions and disease states, metabolic functions, and host factors. Importantly, there

is no simplistic revelation of there being one specific taxon causally matching a disease phenotype. Therefore, this urged the second phase of the HMP, known as integrative Human Microbiome Project (Integrative HMP (iHMP) Research Network Consortium, 2019) that focused on the exploration of the host-microbiome interplay, and the biological properties and the relationships between immunity, metabolism, and molecular activity within the microbiome (Integrative HMP (iHMP) Research Network Consortium, 2019).

Traditionally, microbiota analysis has relied on culture-dependent methodologies to isolate and identify microorganisms implicated in infectious diseases. However, these methods are time-consuming, and not all bacterial species can be cultivated as it is a challenge to identify the specific medium and conditions that are required for each species (Sommer, 2015). Culture-independent characterisation of microbial communities (Figure 1.8) allows a greater knowledge of the diversity of the human microbiome.

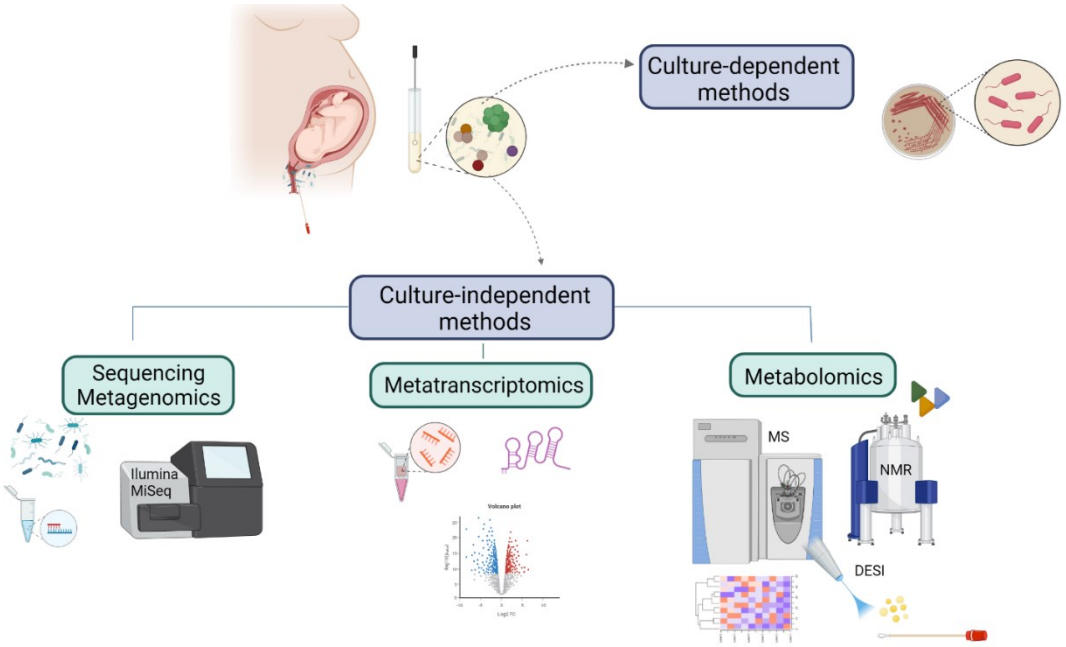


Figure 1.8. Methodologies for the detection of the vaginal microbiome. Traditionally, the study of vaginal microbiome relies on culture-dependent methods. Recent advances have been made in developing culture-independent methodologies for a more comprehensive understanding of the complexities of the human microbiome. Sequencing of the 16S rRNA gene is the most common molecular technique to assess the vaginal microbiome, assigning a taxonomy to the sequences observed. Other methods include metatranscriptomics, based on RNA approaches to understand activated pathways. Metaproteomics and metabolomics rely on technologies such as mass spectrometry (MS), nuclear magnetic resonance (NMR) or Desorption electrospray ionization (DESI) to understand which proteins and metabolites reflect microbial activity. Image created with BioRender.com. From (Gimeno-Molina et al., 2022a).

In the genomics field, the use of high-throughput sequencing and NGS (next generation sequencing) techniques have made it possible to identify bacteria by their genetic signature, in more economic and rapid ways (Barb et al., 2016; Van Der Pol et al., 2019). Although metagenomic sequencing is becoming more accessible, a huge amount of microbiome research relies on 16S rRNA gene sequencing. The bacterial 16S rRNA gene (Figure 1.9. A) contains nine hypervariable regions (V1-9) that demonstrate considerable sequence diversity among different bacteria. Universal primers target the conserved regions flanking the hypervariable regions; allowing amplification and further sequencing to finally assign a taxonomy (Chakravorty et al., 2007).

It is important to consider that no single hypervariable region can differentiate among all bacteria (Chakravorty et al., 2007) (Figure 1.9). Several studies have aimed to determine which hypervariable regions provide the best species-level diagnosis of pathogenic bacterial in general (Chakravorty et al., 2007; Marchesi et al., 1998) and in the vaginal environment (Van Der Pol et al., 2019). In general, the V4 region appears to display consistent results. But perhaps a multiple region approach and/or focus on the type of bacteria that the researcher is hoping to identify should be taken into account when selecting the primers (Barb et al., 2016). For example, since *Lactobacillus* is the dominant genus in vaginal microbiome, it is important to further classify this genus down to species level to reflect the detailed microbial profile (Ma et al., 2014). The V1-V3 regions robustly classifies the most common *Lactobacillus* spp. (Figure 1.9).

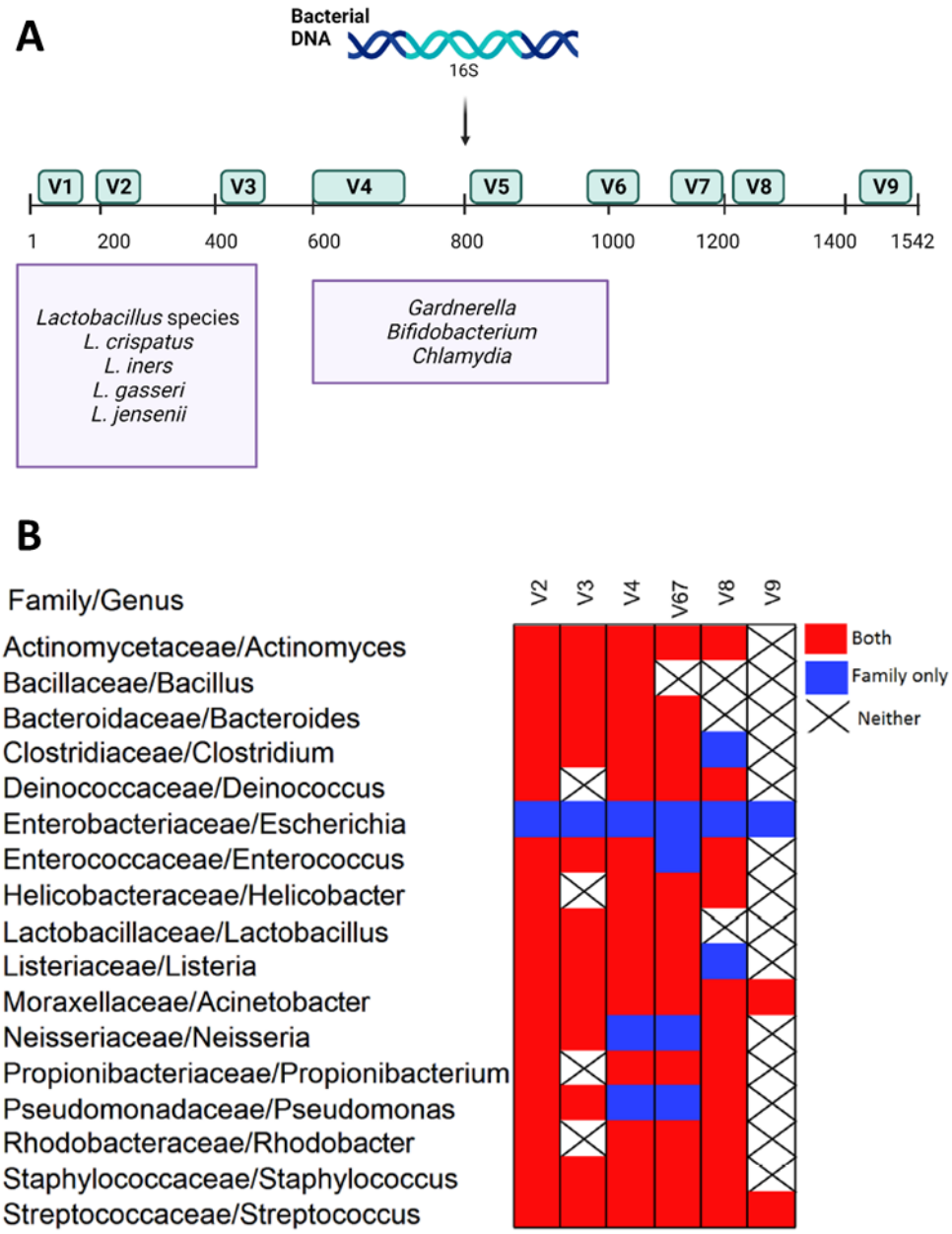


Figure 1.9. Bacterial 16S rRNA gene. **A)** Schematic representation of *16S rRNA* gene. This gene has 1542 bp (base-pair) and nine hypervariable regions can be differentiated: each one is flanked by conserved stretches, enabling targets for universal primers. Adapted from (Gimeno-Molina et al., 2022a). Created with Biorender.com. **B)** Incidence of bacteria at family and genus level, according to variable regions. Sourced from (Barb et al., 2016).

1.4.2. Vaginal microbiome and reproductive health

The establishment of the local microbiota starts as early as birth, following the rupture of fetal membranes, the passage through the birth canal, and the close contact with the maternal vaginal ecosystem (Dominguez-Bello et al., 2010). The mode of delivery can shape the initial structure of the microbial colonisation in newborns; the gut microbiota of vaginally delivered newborns resembles the vaginal microbiota of their mothers (with *Lactobacillus*, *Prevotella*, *Atopobium*, *Sneathia*); whereas the gut microbiota of babies born via caesarean is more similar to their mother's skin (*Staphylococcus*) (Dominguez-Bello et al., 2010).

The colonisation of the newborn vagina is characterised by the presence of residual maternal oestrogen, a thick epithelium and glycogen as a carbon source. This environment is colonised by lactic acid-producing bacteria. After the first month of life, the levels of oestrogen and glycogen decrease; the epithelium is less stratified, and the pH is neutral or alkaline as the presence of lactic acid producing bacteria is reduced (Farage and Maibach, 2006). During childhood and pre-puberty (until menarche), the vaginal microbiome is highly diverse (Hill et al., 1995). At the onset of menstruation, the vaginal mucosa responds to ovarian steroid hormone cycling, the epithelium gets thicker and stratified (Farage and Maibach, 2006) and the microbiota starts to become dominated by lactobacilli (Hickey et al., 2015). In this context it is evident that the establishment of the microbiota occurs in response to circulating hormone levels. Rising oestrogen levels promotes glycogen deposition and growth of vaginal epithelial cells, that release lactic acid into the microenvironment, provoking a pH decrease ideal for the proliferation of acidophilic bacteria such as *Lactobacillus* (Linhares et al., 2011). The cell-free glycogen serves as a carbohydrate substrate for the growth of *Lactobacillus* spp. in the genital tract, and it has been established that there is a negative correlation between glycogen levels and vaginal pH and progesterone (Mirmonsef et al., 2016).

Ravel was the first to characterise the vaginal microbial composition of reproductive-age women using sequencing of bacterial 16S rRNA genes. (Ravel et al., 2011). Using hierarchichal clustering of relative abundance data, five major cervicovaginal community state types (CSTs) were identified. Four of these were dominated by a single *Lactobacillus* species: CST I: *L. crispatus*; CST II: *L. gasseri*; CST III: *L. iners*; and CST V: *L. jensenii*. In contrast, CST IV was characterised by *Lactobacillus* species depletion and enrichment of anaerobic bacteria (*Prevotella*, *Dialister*, *Atopobium*, *Gardnerella*, *Megasphaera*, *Peptonipilus*, *Sneathia*, *Aerococcus*, *Mobilincus*) (Ravel et al., 2011).

Although the vaginal microbiome is relatively stable, there can be deviations in the composition over short periods of time, such as during the menstrual cycle (Gajer et al., 2012); or as a result of sexual habits and hygiene practices (Gajer et al., 2012; Noyes et al., 2018; Vodstrcil et al., 2017). Variations in the microbial composition and pH levels can also be detected between different ethnic groups (Anahtar et al., 2015; Ravel et al., 2011). Therefore, the establishment of a normal/natural composition of the vaginal microbiome can be difficult, as the vaginal microbiome of reproductive-age women can be heterogeneous and fluctuate over time as well as within and between individuals (Witkin and Ledger, 2012).

In general, the vaginal microbiota is dominated by *Lactobacillus*. The *Lactobacillus* genus has been associated with promoting vaginal health, due to the production of lactic acid and the maintenance of a low pH, establishing a protective barrier that avoids and inhibits the growth of endogenous pathogenic bacteria (Ravel et al., 2011; Witkin and Ledger, 2012). Moreover, *Lactobacillus* spp. are able to produce hydrogen peroxide and bacteriocins (such as acidolin, lactacin B, lactocin 160) that also inhibit the growth of microorganisms like *Gardnerella* spp. (Aroutcheva et al., 2001; Petricevic et al., 2014).

1.4.3. Vaginal microbiota during pregnancy

The composition and the stability of the vaginal microbiota differs between pregnant and non-pregnant women (Romero et al., 2014c). During pregnancy, there is a significant decrease in overall diversity and increased stability in the vaginal microbiota; mainly with enrichment of *Lactobacillus* species (Aagaard et al., 2012; DiGiulio et al., 2015; Romero et al., 2014c), reflecting the potential importance and the protective role of this species. Fettweis noted that women who began pregnancy with high diversity communities lean towards a more homogeneous lactobacilli-dominated microbiome, which is already established by the second trimester (Fettweis et al., 2019).There is a shift of the vaginal microbiota at delivery, characterised by a reduction in *Lactobacillus* spp. and increased alpha diversity (DiGiulio et al., 2015) and these changes persist postpartum (MacIntyre et al., 2015).

1.4.4. Vaginal microbiota in women who deliver preterm

In the last decade, there has been growing interest among research groups to establish associations between vaginal microbial composition and pregnancy outcome (Table 1.2). In general, the dominance of *Lactobacillus* species is associated with uncomplicated term

deliveries, especially *L. crispatus* dominance (CST I); whereas vaginal dysbiosis and heterogeneous and anaerobic communities deplete in *Lactobacillus* increase the risk of preterm birth (Brown et al., 2018), which is further noted when this diverse and less stable vaginal microbiome dominates in early pregnancy (Gupta et al., 2020). There is also evidence that dominance of *L. iners* (CST III) can be less protective and considered as a transition state between more diverse communities (Petricevic et al., 2014), as well as being associated with PTB (Kindinger et al., 2017).

When comparing studies and interpreting results of vaginal microbial composition, it is likely that there are confounding factors affecting their results, such as sample size, demographics, and sampling techniques (Gupta et al., 2020). It is likely that ethnicity influences both microbial composition and host response, which will impact on pregnancy outcome. One study (Romero et al., 2014b) did not find differences in the local vaginal microbial composition between women who had preterm (n=18/72) and term deliveries (n=54/72), in a predominantly African American population. This finding was consistent with a later study by Nelson, also conducted in African American women (Nelson, 2016). In contrast, studies of predominantly white populations, showed that multiple anaerobic bacteria (typically CST IV) are associated with sPTB (Table 1.2). DiGiulio et al. reported that *Gardnerella* and *Ureaplasma* can help to stratify preterm birth risk. *Gardnerella*, *Ureaplasma*, *Prevotella* and *Mycoplasma* are the most common reported taxa associated with sPTB (DiGiulio et al., 2015). Some of these taxa, along with others such as *Prevotella*, *Sneathia*, TM7-H1 (Fettweis et al., 2019), *Mobilincus curtsii/mulieris*, *Atopobium*, and *Megasphera* have been reported in women of African American ancestry too (Elovitz et al., 2019), adding to the complexity of the effect of population specific preterm birth risk. Regardless of these differences, recent metanalyses have confirmed the relationship between high vaginal bacterial diversity and increased preterm risk (Gudnadottir et al., 2022; Kosti et al., 2020).

As part of the iHMP, the MOMS-PI (Multi-Omic Microbiome Study: Pregnancy Initiative) has focused on establishing microbiome associations with preterm birth. They have collected multiple samples from mums (vaginal, oral, rectum, nasal, blood, urine) and their babies (cord blood, meconium, stool); and are aiming to combine 16S rRNA taxonomic, metagenomic, metatranscriptomic, cytokine profiling and lipidomics analysis for a comprehensive and mechanistic and integrative process of PTB (Integrative HMP (iHMP) Research Network Consortium, 2019). Results so far are consistent with the current literature: pregnancy is associated with a transition towards, and domination of *Lactobacillus* spp.; and if the vaginal microbiome is dominated by *L. crispatus* it is less likely to experience a preterm delivery (Fettweis et al., 2019).

PPROM, that precedes 30% of sPTBs, also has some specific vaginal microbiome aetiological aspects. At the time of rupture there is a misrepresentation of lactobacilli species, and a high diversity composition (Baldwin et al., 2015). Our group previously described the vaginal microbiome leading to PPROM and found that by mid-gestation, women who ultimately delivered at term were dominated with *Lactobacillus* spp. and had low richness and diversity; whereas at the same gestational age, those who experienced PPROM displayed higher diversity, low *Lactobacillus* spp. dominance, and enrichment in taxa like *Bacteroides*, *Fusobacteria* and *Clostridiales* (Brown et al., 2018). Another risk factor for PTB is cervical shortening (≤25mm), that has been associated with *L. iners* dominance (Chan et al., 2022; Kindinger et al., 2017) as early as 16 weeks of gestation (Kindinger et al., 2017)

However, to date, no single taxon *per se* has been shown to be a single causative microbe (Chu et al., 2018; Gupta et al., 2020), with many studies reporting on association rather than mechanism. Recently, there has been a growing interest in the identification of microbial signatures during pregnancy including some associations to immune factors as modulators for preterm birth (Chan et al., 2022; Elovitz et al., 2019; Florova et al., 2021). However, the mechanisms of the local cervicovaginal immune profiles during pregnancy are still not clearly understood.

Table 1.2. Relation between gestational vaginal microbiome and spontaneous preterm birth.

	STUDY	Ethnicity	CONTROL n	PTB n	Sampling	Background risk	Diversity/ Dysbiosis/BV	L. crispatus (or L. Dominance	Associations PTB	Methodology
2014	(Hyman et al., 2014)	White	66	17	Cross sectional	Mixed	Increased Risk		Preterm outcome could not be predicted based on CST	Chain -Terminator (Sanger) sequencing of 16S rRNA
2014	(Romero et al., 2014b)	88% African - American	72	18	Longitudinal	Not reported	No		No specific bacteria was identified	V1-V3 region 454 pyrosequencing (Roche) Defined as <34weeks
2014	(Petricevic et al., 2014)	Not reported	98	13	Cross sectional	Low	Not assessed	Decreased risk	*L. iners* (transition, lack of protection)	DGGE (denaturing gradient gel electrophoresis) for major *Lactobacillus* spp (cannot detect other phatobionts)
2015	(DiGiulio et al., 2015)	White (60%)	34	15	Longitudinal	Low	Increased risk		*Gardnerella* *Ureaplasma*	V3-V5 region by 454 pyrosequencing V4 by Illumina HiSeq2500
2016	(Nelson, 2016)	African American	27	13	Cross sectional	Low	No			V4 region (Illumina Miseq)
2017	(Kindinger et al., 2017)	Caucasian (65%)	127	34	Cross sectional	High	No	Decreased risk	*L. iners*	V1-V3 (Illumina MiSeq)
2017	(Stout et al., 2017)	African-American (69%)	53	24	Longitudinal	Mixed	PTB characterised with instability richness and diversity		No association Differences occur in the first trimester	V1-V3 for *Lactobacillus* discrimination V3-V5 for *Gardnerella* discrimination Roche 454 platform
2017	(Callahan et al., 2017)	White (Caucasian and Asian)	30	9	Longitudinal	Low Caucasian USA	Increased in White		Low *L. crispatus* (both cohorts)	Midvaginal swabs V4 region Illumina Hiseq 2500

	STUDY	Ethnicity	CONTROL n	PTB n	Sampling	Background risk	Diversity/ Dysbiosis/BV	L. crispatus (or L. Dominance	Associations PTB	Methodology
		Black (African-American)	55	41	Longitudinal	High African-American Previous PTB	No		*G. vaginalis* (Sequence variant 2) *Prevotella* Low *L. jensenii, L gasseri* in AA	
2018	(Freitas et al., 2018)	Majority white (50%)	170	46	Cross sectional	Low, mixed	Increased Risk		Mollicutes (*Mycoplasma*) and *Prevotella* spp. Higher richness rather than specific CST	*Cpn60* universal target
2018	(Brown et al., 2018)		102	20 PPROM	Longitudinal	High	Increased risk			V1-V2 region (Illumina MiSeq) Erythromycin treatment
2019	(Tabatabaei et al., 2019)	Mainly white European	356	17 early 77 late	First trimester	Low	Increased (<34w) CST IV risk of early PTB *G. vaginalis, A. vaginae Veillonellaceae*	Decreased risk	*L. gasseri/jonsonii, L. crispatus, L. acidophilus, L. iners, B. longum* and *B. breve* decreased risk	V4 region (Illumina) Early < 34w Late: 34-36w
2019	(R. G. Brown et al., 2019)	Mainly Caucasian (54%)	36	38 high 22 low PPROM	Longitudinal	Mixed	Increased Risk	Decreased risk	*Prevotella, Peptoniphilus, Dialister, Streptococcus, Catonella, Parvimonas, Anaerococcus* discriminatory PPROM	V1-V2 region (Illumina MiSeq)

	STUDY	Ethnicity	CONTROL n	PTB n	Sampling	Background risk	Diversity/ Dysbiosis/BV	L. crispatus (or L. Dominance	Associations PTB	Methodology
2019	(Elovitz et al., 2019)	Mixed, mostly AA	432	107	Longitudinal	Mixed	Increased in White	Decreased risk	*Mobiluncus curtsii/mulieris* *Sneathia Sanguinegens* *Atopobium spp* *Megasphera* *L. iners* in non-AA	V3-V4 regions Illumina HiSeq 2500 Association B defensin2
2019	(R. Brown et al., 2019)	Black (45%) risk population. Control 50% Caucasian	20	30	Longitudinal	Very high	Increased Risk	Decreased risk	Non-Lactobacillus = cervical dilation. *G. vaginalis* over-represented in women with symptoms. *G. vaginalis* prior cerclage related with poor outcomes	V1-V2 (Illumina MiSeq)
2019	(Fettweis et al., 2019)	African American	90	45	Longitudinal	Low	Increased risk	Decreased risk	Low *L. crispatus* High BVAB1 *Sneathia amnii* TM7-H1 *Prevotella Meghasphaera* Overall diversity was increased	V1-v3 region Ilumina Miseq Cytokines negative correlated to *L. crispatus* Few higher in PTB

1.5. Immunology of pregnancy, term and preterm labour

The immunological changes that occur during pregnancy are highly regulated, yet dynamic and complex. In the most simplistic terms, it has long been accepted that there is a degree of immune suppression to tolerate the semi-allogenic fetus whilst maintaining the ability to mount immune responses to be able to protect the mother and the baby from infection (Aghaeepour et al., 2017; Veenstra van Nieuwenhoven et al., 2003).

The concept of the fetus as an allograft arose with Medawar's work. He proposed that successful semi-allogenic fetus survival was due to maternal immune suppression, since there was no rejection of the fetus despite it being a product of two histoincompatible individuals. In addition, it was proposed that the fetus is antigenically immature, and that there is complete anatomical separation from the mother *in utero* (Medawar, 1953, 1948). Wegman and colleagues were the first to hypothesise that there was a bias towards Th2 responses (Wegmann et al., 1993), with evidence of Th2 cytokines (GM-CSF, IL-3) promoting growth and differentiation of trophoblast and intrauterine growth in pregnant mice. In contrast, adverse effects were seen with IL-2, IFN-γ and TNF-α (Th1 cytokines) in pregnant mice. The importance of the Th1/Th2 altered balance in pregnancy was reinforced by the effect of pregnancy on Th1/Th2 driven autoimmune diseases. For example, the Th1 driven condition rheumatoid arthritis, tends to improve during pregnancy, and flares post-partum (Adams Waldorf and Nelson, 2008; Barrett et al., 1999).

However, considering pregnancy as a state of immune suppression is too simplistic. Rather, the immunological state of pregnancy represents highly regulated yet dynamic immune homeostasis. Immune adaptation begins in the early stages of pregnancy. At implantation, there are already immune cells, both innate and adaptive, that apart of being implicated in tolerance and protection, are active participants of key processes such as trophoblast invasion, spiral artery transformation and decidual differentiation(Mor et al., 2017). It is broadly accepted that the first trimester is a proinflammatory state that facilitates implantation. During the second trimester there is a shift towards an anti-inflammatory Th2 response. And finally, towards the end of pregnancy, the immune response shifts back to a proinflammatory Th1 response leading to labour (uterine contractility, membrane activation, cervical ripening, and dilation (Figure 1.10.).

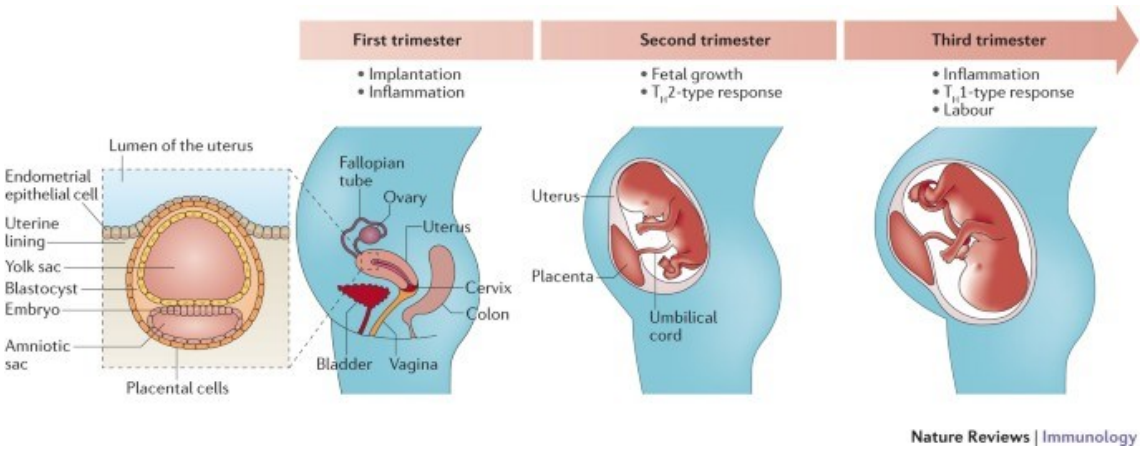


Figure 1.10. Immunological stages of pregnancy. Source: (Mor et al., 2017).

Recently, the existence of an "Immune clock" of pregnancy has been suggested, with chronological and timed immune response adaptations characterised using mass cytometry-based analysis of maternal peripheral blood collected from term pregnancies and mathematical modelling (cell signalling based Elastic NET) (Aghaeepour et al., 2017). In this study, changes in the immune response were linked with gestational age, suggesting that deviation from the "normal" pathway could be potentially linked to pathological events and pregnancy complications (Aghaeepour et al., 2017).

During pregnancy, both the adaptive and innate immune system (Table 1.3., Figure 1.11) participate in regulating the maternal immune response to ensure healthy outcomes for both mother and baby. Multiple reviews exist in the literature describing the role of immune cells and mediators in pregnancy and labour (Bonney, 2016; Cappelletti et al., 2016; Gomez-Lopez et al., 2014; Negishi et al., 2018; Sykes et al., 2012) with specific interest in certain cell types such as T cells (Kieffer et al., 2019; Krop et al., 2020; Miller et al., 2020), neutrophils (Bert et al., 2021; Gimeno-Molina et al., 2022b; Tong and Abrahams, 2020) and macrophages (Mor and Abrahams, 2003). The main limitations of studies to date are the focus on the peripheral immune response, and the paucity of studies exploring the role of the immune response to the vaginal microbiome at the cervicovaginal interface.

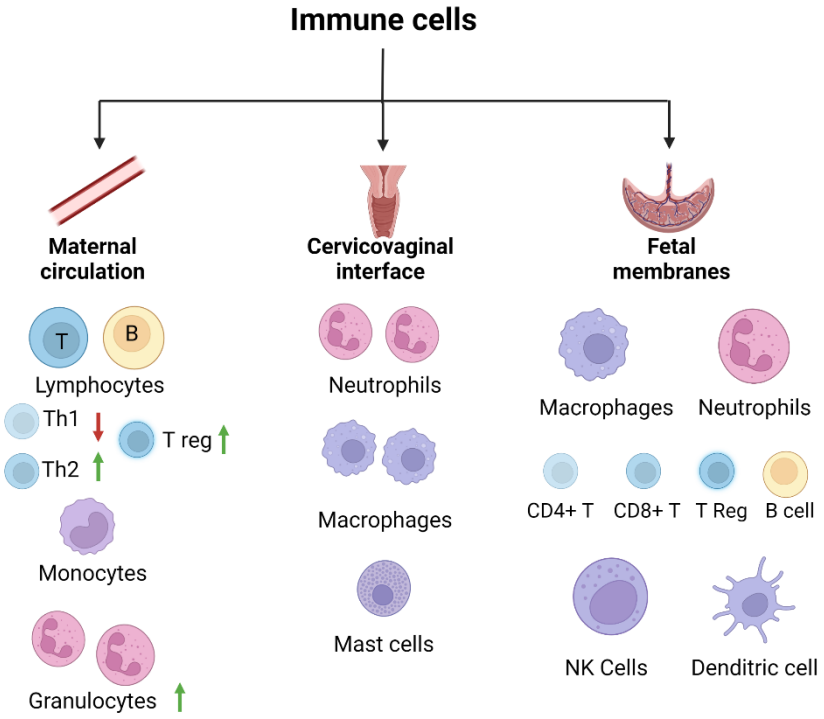


Figure 1.11. Immune cells in pregnancy. Innate and adaptive immune cells are present in the peripheral maternal circulation, cervicovaginal interface (cervix, cervical mucus plug) and maternal fetal interface (decidua, myometrium). Image created with Biorender.com.

Table 1.3. The immune system of the female reproductive tract. Adapted from (Hickey et al., 2011)

Cell/Mediator	ROLE	FUNCTION	DISTRIBUTION
Natural Killer (NK) cells	Innate	Cytotoxic activity Proinflammatory cytokines	10% systemic 70% endometrial
Macrophages	Innate	Phagocytosis Antigen presentation	Cervicovaginal interface Endometrial stroma Myometrial connective tissue
Neutrophils	Innate	Tissue remodelling (by MMPs) Phagocytosis Reactive oxygen species (ROS) production Degranulation Clear infection NETosis	Upper reproductive tract
Dendritic cells	Innate	Antigen presentation	Endometrium, vaginal epithelium
Complement proteins	Innate	Opsonization Lysis Synthesis anaphylotoxins Leukocyte recruitment	Endometrium Fetal membranes Cervicovaginal fluid
Secretions	Immune mediators	Antimicrobials Cytokines and chemokines Gradient recruitment	Systemically and infiltration into tissues
CD4+ T cell	Adaptive	Th1-Th2 paradigm	Upper reproductive tract
CD8+ T cell	Adaptive	TH1 cell-mediated apoptosis	Upper reproductive tract
B cells	Adaptive	Humoral response (antibody production) Maturation IgA and IgG secreting plasma cells	Upper reproductive tract

1.5.1. Innate immune system

The innate immune system is the first line of defence. The responses are not specific, and they rely on cells and on a group of proteins that become rapidly activated. The innate activation is based on the recognition of molecular signatures by pattern recognition receptors (PRRs). PRRs can recognise pathogen-associated molecular patterns (PAMPs) from foreign and microbial origins. However, PRRs can also sense endogenous signals via DAMPs (damage-associated molecular patterns). PRRs include TLRs (Toll-like receptors), C-type lectins, and NOD-like receptors. The activation of PRRs triggers intranuclear signals that converge on the activation of transcriptional factor NF-κB, further initiating a proinflammatory cascade of cytokines, chemokines and prostaglandins that participate in several processes of labour (Agrawal and Hirsch, 2012; Challis et al., 2009; Romero et al., 2007).

The innate immune response comprises different cell types and circulating soluble proteins that mediate and enhance the innate immune response, as well as acting as a bridge with the adaptive immune response.

1.5.1.1. Cellular response

Natural killer (NK) are cytotoxic innate lymphocytes. They have two distinct phenotypes. NK $CD56^{dim}CD16^{+}$ are typically found in the periphery and are characterised by high cytotoxic and lytic activity. Whereas in tissue (uterine, uNK; or in the placenta, dNK) they express distinct surface markers ($CD56^{high}CD16^{-}$) and are involved in cytokine secretion (Negishi et al., 2018), vascular remodelling and trophoblast invasion (Moffett-King, 2002). In the uterus NK cells play an important role during implantation but their levels decrease from mid-pregnancy and are absent in labour (Moffett-King, 2002). In peripheral blood, NK cells levels also decrease as pregnancy progresses, but a higher CD69 expression (marker associated with cytotoxicity and cell lysis) was observed in the third trimester of pregnancy (Mosimann et al., 2013).

NKT cells are a different subset described with both NK and T cell markers that act as a bridge between the innate and the adaptive immune system. Dysregulation of the Type I invariant NKT cells (iNKT) has been associated with adverse pregnancy outcomes such as pregnancy loss (Boyson 2006) and preeclampsia (Miko et al., 2008). This latter study also found a higher expression of CD69 in women with preeclampsia, together with higher expression of perforin and IFN-γ (Miko et al., 2008).

Monocytes are circulating white blood cells, accounting for 5-10% of the circulating PBMC (peripheral blood mononuclear cells). Monocytes mature in the circulation and eventually migrate into tissue, becoming macrophages. There are 3 subsets of monocytes: most of the population (approximately 90%) are described as classical monocytes ($CD14^{high}$, $CD16^{-}$) and the rest (~10%) are described as intermediate monocytes ($CD14^{high}$, $CD16^{+}$) or non-classical monocytes ($CD14^{low}$, $CD16^{high}$) (Wong et al., 2011). Compared to the non-pregnant state, classical monocytes decrease in pregnancy (Melgert et al., 2012). During pregnancy, levels of monocytes are stable, but at the end of pregnancy, they exhibit a higher expression of CD11a, CD54 and CD64 (Luppi et al., 2002). Monocytes from pregnant women that were LPS stimulated are also able to produce IL-1β, IL-6, IL-8 and TNF-α (Luppi et al., 2002). In cases of preterm delivery, a higher CD11b expression was observed in monocytes, compared to monocytes from term pregnancies (Gervasi et al., 2001). Monocytes from preterm labour samples also had a higher ratio of oxidative burst (from the basal intracellular ROS (reactive oxygen species) production) (Gervasi et al., 2001).

Macrophages have been reported to play an important role at different stages of pregnancy, including implantation, where they mediate maternal-fetal tolerance, and during to labour, where they are involved in cervical remodelling and membrane rupture (Abrahams et al., 2004; Gomez-Lopez et al., 2014). Macrophages can phenotypically be classified as M1 and M2, depending on the proinflammatory (secretion of IL-12, IL-1β, TNF-α and IL-6) or anti-inflammatory (secreting IL-10) effects, respectively (Bonney, 2016; Yao et al., 2019). Macrophages, together with neutrophils, have been detected in cervical stroma (Bokström et al., 1997; Osman et al., 2003), and fetal membranes (Hamilton et al., 2012). In fact, numbers of macrophages infiltrating in the decidua are higher when comparing tissue from labour to non-labour (Hamilton et al., 2012). Due to their ability to produce matrix metalloproteinases (Newby, 2016; Pan et al., 2022), prostaglandins (Norwitz et al., 1992) and collagenases (Dubicke et al., 2016) macrophages are considered to be one of the major contributors of labour, since these activities are closely interrelated with uterine contractility, membrane activation and cervical ripening and dilation in both physiological term labour and pathophysiological preterm labour. Recently, it has been described that during labour, both at term and preterm, there is a decidual M1 macrophage population, but that M2 macrophage population is reduced in cases of preterm (Xu et al., 2016).

Neutrophils are the first line of defence against pathogens due to the variety of effector functions that they can perform (Gimeno-Molina et al., 2022b; Rosales, 2018). Neutrophils are well known for their antimicrobial properties (Segal, 2005). However, their ability to degranulate

and secrete inflammatory mediators (Othman et al., 2022) has long implicated their potential for playing a role in the process of cervical ripening (Choi et al., 2018; Junqueira et al., 1980). It is well established that during pregnancy, granulocyte numbers increase in maternal peripheral blood (Aghaeepour et al., 2017; Luppi et al., 2002; Plum et al., 1978; Pramanik et al., 2007; von Dadelszen et al., 1999; Zhang et al., 2017). Von Dadelszen et al. suggested that delayed neutrophil apoptosis could be responsible for the neutrophilia observed during pregnancy and complications, as well as contributing to systemic inflammation (von Dadelszen et al., 1999). Not only is the peripheral neutrophil count higher during pregnancy, but they are more activated too. Evidence includes higher levels of reactive oxygen species (ROS), CD11b expression (von Dadelszen et al., 1999), phagocytic activity and detection of Leishman stain bands (Pramanik et al., 2007). The immunophenotype of peripheral granulocytes is altered in preterm birth, with higher expression of CD11b and CD66b; and higher ratio of oxidative burst (Gervasi et al., 2001), compared to peripheral granulocytes from healthy term pregnancies. Neutrophils have been detected in fetal membranes closer to labour (Osman et al., 2003). This is particularly relevant in cases of infection and inflammation, where neutrophil infiltration is the hallmark of chorioamnionitis (Kim et al., 2015). Recently, it has been shown that amniotic fluid neutrophils can phagocytose bacteria (Gomez-Lopez et al., 2017), proposing a mechanism of protection and microbial killing in cases of intra-amniotic infection, revealing the potential of the protective role of neutrophils in the intrauterine cavities.

Dendritic cells participate in antigen recognition and presentation to T cells. They are known to be a connection between innate and adaptive immunity, (Liu, 2001; Moser, 2003) by producing cytokines that drive the differentiation of naïve T cells into several subsets such as: T helper type 1 (Th1), type 2 (Th2), type 17 (Th17) and Treg (Eisenbarth, 2019). At the maternal fetal interface, they are mainly found in the decidua (Gardner and Moffett, 2003). Several subsets (tolerogenic and inflammatory) have been described and are key for the maternal-fetal immune tolerance during pregnancy, implantation and parturition (Wei et al., 2021). They can interact with T cells, natural killer cells and macrophages. Dysregulation can lead to an imbalance activation triggering preterm birth, as reported in an LPS-PTB mouse model (Bizargity et al., 2009).

1.5.1.2. Soluble proteins

This section will firstly review the role of the complement system. These group of proteins can be activated directly by pathogens or indirectly by pathogens recognised by antibodies, leading to a cascade of enzymatic reactions that can trigger various effector functions. Secondly and lastly, the role of cytokines in pregnancy will be evaluated. Cytokines and chemokines are

secreted proteins as a result of immune activation that can also determine and control the nature of immune responses and immune cell trafficking.

Complement proteins

The complement system is named after the role it plays to "complement" the adaptive immune response. The complement system acts to bridge the innate and the adaptive immune response and consists of more than 35 proteins. The key function of the complement system is to facilitate the clearance of pathogens, immune complexes and cell debris (Dunn et al., 2017; Sjöberg et al., 2009). The complement system involves three highly regulated pathways: the classical pathway, the lectin pathway and the alternative pathway (Figure 1.12). The classical pathway is triggered via immune complexes binding C1q. The lectin pathway is initiated by the recognition of polysaccharides expressed on bacterial and pathogenic surfaces, by Mannose-Binding Lectin (MBL) or ficolins. Finally, the alternative pathway can be both initiated as an autoactivation of unstable protein C3; or as a direct effect of the amplification loop from the other two pathways generating C3b. The three pathways converge on the activation of C3 and C5 convertases, that cleave each respective factor (C3 or C5, respectively). The proteolytic cleavage results in the release of proinflammatory anaphylatoxins (C3a, C5a), opsonisation of the target (e.g., pathogen or cell debris) by C3b; and the formation of the Membrane Attack Complex (MAC) (Dunn et al., 2017; Sjöberg et al., 2009)

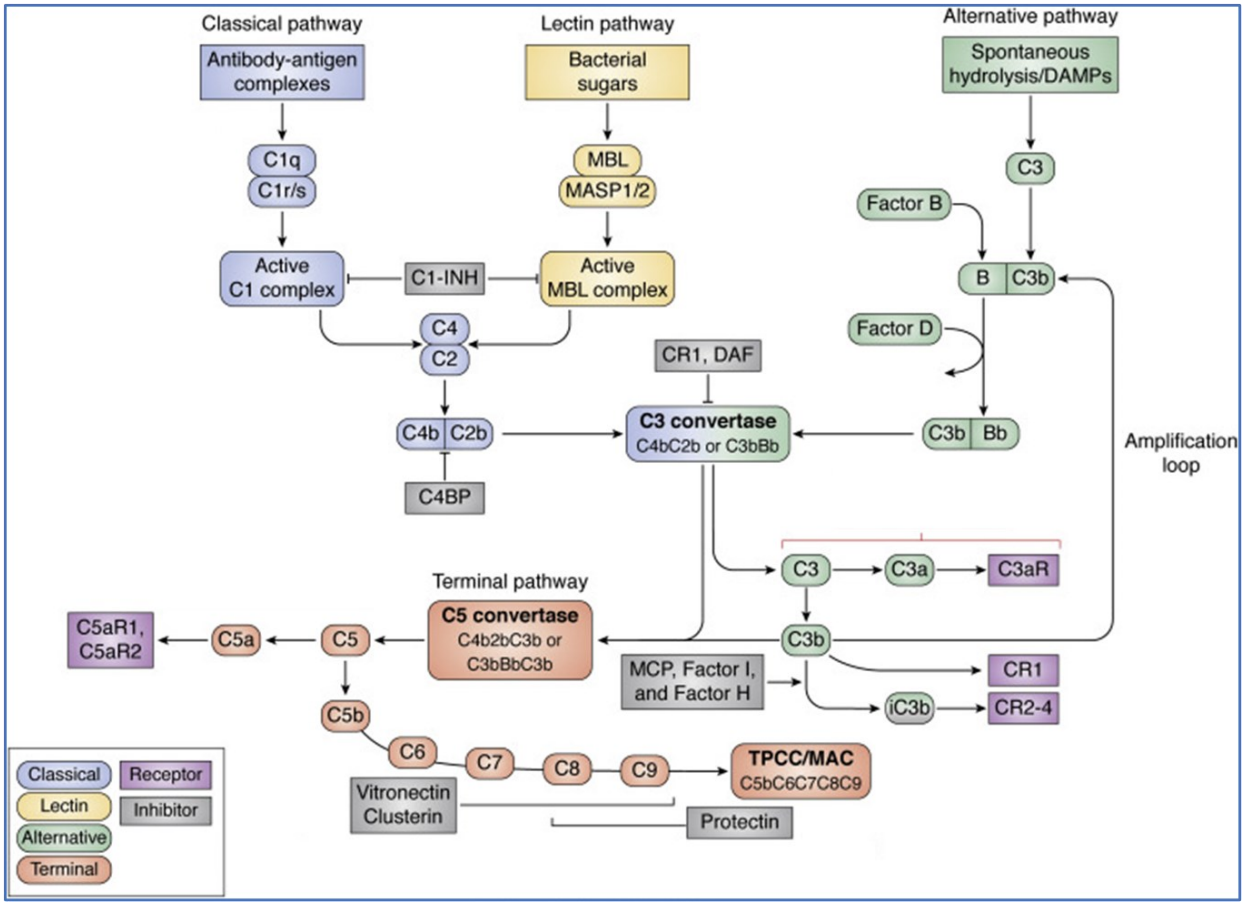


Figure 1.12. The complement system. The complement system is part of the innate immune system and can be activated via 3 different pathways. The classical pathway (blue) is initiated via recognition of immune complexes by C1q. The lectin pathway (yellow) is initiated by recognition of bacterial saccharides by MBL or ficolins. The alternative pathway (green) can be autoactivated by C3 instability or as a result of the amplification loop after C3 cleavage. The three pathways converge on the activation of C3 convertase (blue/green) and C5 convertase (orange). The proteolytic cleavage of C3 and C5 will result in the release of anaphylatoxins C3a and C5a; opsonization by C3b and formation of the Membrane Attack Complex (MAC). Complement inhibitors are indicated in grey; and complement receptors in purple. Reproduced and adapted from (Warwick et al., 2021).

Most of the studies addressing the role of the complement system in pregnancy have been focused on blood plasma. Richani et al. found that concentration of C3a, C4a and C5a were consistently higher during pregnancy compared to non-pregnant women (Richani et al., 2005). However, recently He et al. reported fluctuating levels of complement proteins in plasma samples collected across pregnancy. In this study, Factor B, C3, C3a and C4 were found to increase as gestational age advanced; other proteins (MBL, sC5b-9) remained stable or fluctuated (C5a, C3c) (He et al., 2020). There is a paucity of studies investigating the potential role of the complement system at the cervicovaginal interface. Livson found that there is more abundance of C3 in late pregnancy and labour than in non-pregnant samples; but C3 could potentially be less activated, since there were less fragments with reduced intensities detected in western blots (Livson et al., 2021). The same group also found that C4 activation in CVF correlated with certain bacterial species (*L. jensenii*, *G. vaginalis, Atopobium*) (Livson et al., 2022).

When assessing the role of complement in pregnancy specific complications, preeclampsia is the most studied condition (Denny et al., 2013; Lynch et al., 2008a, 2008b). But some interesting associations have been identified with preterm birth too. Plasma concentrations of fragment Bb (a marker for activation of the alternative pathway) were higher among patients that delivered preterm (Lynch et al., 2008a; Vaisbuch et al., 2010). C3a levels were also higher in peripheral blood from women who subsequently had pregnancy complications such as preterm birth, premature rupture of membranes or preeclampsia (Lynch et al., 2011). Recently, Kim et al. reported how elevated levels of C3a in amniotic fluid were associated with an increased risk of intra-amniotic infection and spontaneous preterm birth in women with cervical insufficiency or a short cervix (Kim et al., 2018). Several studies have additionally shown that increased levels of IL-6, MMP-9 and C3a and C4a in amniotic fluid were associated with both intra-amniotic infection and preterm parturition (Park et al., 2018; Soto et al., 2009). Less information is available regarding the cervicovaginal interface. Hong et al. studied CVF samples from PTL cases and found higher levels of C3a and C5a when comparing presence and absence of intraamniotic infection (Hong et al., 2020). In this study, no “controls” (term deliveries) were included, but levels of C3a and C5a stratified the subclassification of preterm birth, finding higher levels in women with an early preterm delivery than late preterm (Hong et al., 2020). Finally, to the best of our knowledge, only Chan (Chan et al., 2022) has begun to address the role of the complement system in association with vaginal microbial composition and pregnancy outcome. In this study, adverse vaginal microbial composition was associated with increased concentrations of MBL, C3b and C5, leading to an increased risk of sPTB. Furthermore, *L. iners* vaginal dominance

together with elevated C3b, C5 and C5a was associated with cervical shortening (Chan et al., 2022). Despite this work highlighting the role of the complement system in microbial-driven sPTB, only a few mediators (4 complement proteins) were evaluated, and a deeper assessment of the complement system is needed to understand the full extent in which the complement system is implicated. A summary of findings reporting on the role of the complement system in pregnancy and pregnancy complications is shown in Table 1.4

Table 1.4. The complement system in pregnancy and pregnancy complications

Study	Cohort/Cases	Sample type	Gestation	Findings
(He et al., 2020)	Normal pregnancy Non-pregnant	Maternal plasma Plasma	6-36w N/A	C1q and MBL stable across pregnancy C3, C4, C3a gradual increase Fluctuations in C3c, C5a and sC5b-9 C3a levels positive correlation to C3c C5a positive correlation to C3c C3c, C4 higher in pregnancy than non-pregnant
(Richani et al., 2005)	Normal pregnancy Non-pregnant	Maternal plasma Plasma	20-42w N/A	C3a, C4a, C5a higher in pregnant than non-pregnant No changes during pregnancy C3a levels positive correlation with other levels of complement
(Stabile et al., 1988)	Normal Pregnancy	Maternal plasma Fetal plasma Amniotic fluid	15-28w 15-28w 15-28w	Concentrations 10 times higher in maternal> fetal> amniotic fluid Positive correlation between maternal-fetal C3 and Factor B and I Positive correlation between maternal amniotic levels of Factor I Maternal levels not correlated with gestation Positive correlation in fetal levels C3, C4, Factor H with gestation
(Denny et al., 2013)	Normal pregnancy Gest. hypertension Preeclampsia	Maternal/fetal Plasma Maternal/fetal Plasma Maternal/fetal Plasma	36w 36w 34w	No difference in C3a levels C5a higher in pre-eclampsia both maternal and fetal plasma Positive correlation between maternal and fetal C5a levels
(Lynch et al., 2008b)	Pregnancy, PET	Maternal plasma	<20w	Bb levels significantly higher in those who developed pre-eclampsia
(Lynch et al., 2008a)	Pregnancy, PTB	Maternal plasma	<20w	Bb associated with early preterm birth (<34w) but not late PT Bb levels similar in late preterm and term deliveries
(Lynch et al., 2011)	Pregnancy Adverse outcomes	Maternal plasma	<20w	C3a predictive of adverse pregnancy outcomes C3in the upper quartile 3x more likely to have adverse outcome Including sPTB<37w and PPROM

Author	Cohort/Cases	Sample type	Gestation	Findings
(Lynch et al., 2016)	Preterm	Maternal plasma	10-15w	Factor B, Factor H and ficolin-3 highest risk ranking protein for PTB
	Term pregnancy	Maternal plasma	10-15w	MMP2 lower in PTB
(Ohmura et al., 2019)	Recurrent miscarriage	Plasma	N/A	Higher anti-C1q antibodies in RPL patients than controls
				Lower C3 in anti-C1q positive patients
				Mice model - high anti-C1q higher ratio of pregnancy loss and fetal growth restriction, placental tissue damage
				Mice model reverted with C5aR blockade
(Park et al., 2019)	Premature cervical dilation	Maternal plasma + amniotic fluid	17-29w	Higher C3a, C5a in women who delivered <32 compared to >32w
	Short cervix	Maternal plasma + amniotic fluid	17-29w	Only IL-6 predictive for intra-amniotic infection
				Plasma CRP correlated with IL-6, C3a, C5a
(Segura-Cervantes et al., 2015)	Pregnant	Maternal plasma	26-37w	SC5b-9 higher in labour, PTL and pprom than in non-labour
	Labour term	Maternal plasma +FM	39w	Deposition in FM no differences between term and preterm
	Labour preterm	Maternal plasma +FM	33w	No deposition detected from FM in PT who delivered by CS
	Labour PPROM	Maternal plasma +FM	33w	
(Vaisbuch et al., 2010)	Non-pregnant	Plasma	N/A	PTL leading to PTB higher Bb concentrations than term
	Normal Pregnancy	Maternal plasma	26-33w	No differences with or without IAI
	Term not in labour	Maternal plasma	38-39w	Spontaneous term labour not associated with change FbB; nor changes with pregnancy status or gestational age
	Spontaneous term labour	Maternal plasma	39-40w	
	PTL intact membrane delivered at term	Maternal plasma, AF	29-33w	Physiological activation of complement in maternal circulation
	PTL without IAI delivered preterm	Maternal plasma, AF	26-32w	PTL delivered at term, no difference to normal pregnant
	PTL with IAI delivered preterm	Maternal plasma, AF	24-32w	No difference with non-pregnant and normal pregnancy

Author	Cohort/Cases	Sample type	Gestation	Findings
(Soto et al., 2005)	Normal pregnancy	Maternal plasma	20-36w	C5a lower in term labour than not in labour
	Term not in labour	Maternal plasma	37-41w	C5a higher in preterm labour
	Term in labour	Maternal plasma	37-41w	IAI had higher C5a andC3a plasma than without IAI
	PTL delivered at term	Maternal plasma	20-33w	No change with advancing gestational age
	PTL without IAI PT	Maternal plasma	20-33w	
	PTL with IAI PT	Maternal plasma	20-33w	
(Derzsy et al., 2010)	Preeclampsia	Maternal plasma and serum	36-39w	Preeclampsia vs healthy pregnant higher higher serum CRP, plasma C4d, C3a, SC5b9 and lower C3
	Normal pregnancy	Maternal plasma and serum	36-37w	Decreased ratios denotes decompensation of pathway regulatory mechanisms - C1-inhibitor and C4b binding protein
	Non-pregnant	Plasma and serum	N/A	Acute phase protein CRP positive correlation with C3a
				Pre-eclampsia with IUGR higher plasma SC5b9
(Haeger et al., 1992)	Preeclampsia	Plasma	Unknown	Higher C5a, elastase and neopterin in preeclampsia than normal pregnancy
	Normal pregnancy	Plasma	Unknown	Activated neutrophils and macrophages
	Normal pregnancy who had C-section	Plasma	Unknown	*Ex vivo* incubation C5a+neutrophil dose-dependent release of PMN elastase
	Non-pregnant	Plasma	Unknown	Complement system may affect the function of neutrophils
(Kim et al., 2018)	Cervical insufficiency	Amniotic fluid	17-27w	C3a higher in IAI
	Short cervix	Amniotic fluid	17-27w	C3a associated with increased risk sPTB <32 weeks
(Soto et al., 2009)	Pregnancy	Amniotic fluid	14-18w	C5a higher in labour than pregnancy
	Term no labour/labour			MIAC higher C4a and C5a
	PTL delivered at term			
	PTL preterm without MIAC			
	PTL MIAC			
(Lappas et al., 2012)	Term delivery via elective caesarean section in the absence of labour	Placenta and fetal membrane - immunohistochemistry	Unknown	C5a stimulates production cytokines (IL6, IL8) COX2, PGE2, MMP9 and NFKB
				Responses reduced after treating with CD88 antagonist

Author	Cohort/Cases	Sample type	Gestation	Findings
(Chan et al., 2022)	Preterm birth	CVF	A: 12-16w	MBL higher in PTB B>A; CST IVB>CST I
	Term		B: 20-24w	MBL higher in deplete PT> deplete term; iv preterm>IV term
			C: 30-34 w	C3b higher in PT B>A
				C3b higher in CST IV> I; deplete preterm>term; III preterm> III term; IV preterm>term
				C5 CST IV> CST I; Deplete preterm> term; CST IV preterm>term
				C5a higher B>A in preterm but no CST differences
				Cervical shortening higher IgM, C5, C5a, IL-6, IL-10
(Hong et al., 2020)	PTL	Amniotic fluid	23-33w	C5a, IGFBP-2 significantly associated with IAI and SPTD
		CVF	23-33w	C3a IAI only
				CVF similar predictive value for IAI but higher value than AF for predicting SPTD
(Livson et al., 2022)	Late pregnancy	CVF	41-42w	*L.jensenii* negatively associated with C4 activation during pregnancy
	Active labour	CVF	38-42w	*Gardnerella* positively associated with C4 activation
				Factor B stronger in pregnancy than labour
				Labour might block complement (high levels Factor H)
(Livson et al., 2021)	Late pregnancy not in labour	CVF	41-42	Abundance of IgG (bands more intense in IgG than IgA; IgG cleavage; but IgA bands intact).
	Active labour	CVF	38-42	Higher abundance C3a in pregnancy/labour but less activated (more intensity of fragments in non-pregnant)
	Non-pregnant	CVF	N/A	Detection IgG-C3-complexes (western staining both antibodies)
				Constant C3 activation in this local environment

PET: preeclampsia. sPTB: spontaneous preterm birth. W: weeks. FM: fetal membranes. AF: amniotic fluid. CS: c-section. CVF: cervicovaginal fluid. N/A: not applicable

Cytokines

Cytokines are small proteins that can be produced by immune cells (and other cell types) and can modulate the balance between the humoral and cellular responses. IL-1β, IL-6 and IL-8 are proinflammatory cytokines whose role has been very well-established during pregnancy and labour. IL-1β has been suggested to play a role in implantation and placentation, mediating in the maternal-fetal immunotolerance interplay (Equils et al., 2020). IL-1β has also been implicated in mediating contractility (Vrachnis et al., 2012) and *ex vivo* experiments suggest a role for weakening fetal membranes (Kumar et al., 2006). IL-6 also participates in implantation and can stimulate trophoblast invasion (Equils et al., 2020). IL-8 (also known as CXCL8) is a proinflammatory chemokine whose main role described is the chemotaxis of neutrophils to sites of infection and inflammation (Hammond et al., 1995), but it can also play a role in monocyte-macrophage differentiation (Corre et al., 1999).

Cytokines have been very well studied in the periphery (K. Ashford et al., 2018; K. B. Ashford et al., 2018; Jarmund et al., 2021), amniotic fluid (Park et al., 2018), cervicovaginal fluid (Amabebe et al., 2018; K. B. Ashford et al., 2018; Chan et al., 2022; Kindinger et al., 2016) and in the maternal fetal interface (Osman et al., 2003; Sakamoto et al., 2005; Sennström et al., 2000, 1997; Young et al., 2002) during pregnancy, term and preterm labour. In the serum of term pregnancies, levels of proinflammatory cytokines (e.g., IL-1β, IL-8) are higher in the first trimester (Jarmund et al., 2021). During the second trimester levels generally remain stable (K. Ashford et al., 2018; Buxton et al., 2020) before increasing around the time of spontaneous term labour onset (Jarmund et al., 2021). Higher concentrations of IL-6 have been associated with increased risk of sPTB (Ferguson et al., 2014).

Cytokines can also be detected in amniotic fluid of normal pregnancies (Halgunset et al., 1994). However, increased levels of proinflammatory cytokines in the amniotic fluid are characteristic of intraamniotic infection and inflammation. Park et al. showed that detection of IL-6 in both maternal plasma and amniotic fluid can predict intra-amniotic infection in women with preterm labour (Park et al., 2018).

Cytokines have also been studied in cervicovaginal fluid (CVF). Overall, the concentration of cytokines is higher in CVF than in serum (K. Ashford et al., 2018; K. B. Ashford et al., 2018). In term pregnancies, CVF concentrations of IL-8 have been reported to increase from the first to third trimester (K. B. Ashford et al., 2018). Sandoval-Colin found that CVF cytokines concentrations (IL-8, IL-1β and IL-6, but also IFN-γ and IL-10) varied between the stages of labour, with higher concentrations in women sampled during advanced labour (Sandoval-Colin

et al., 2023). Elevated levels of CVF IL-1β in the mid-trimester are associated with subsequent sPTB (Amabebe et al., 2018). More recently, there has been a growing interest identifying host immune-microbial interactions in CVF, detecting inflammatory cytokines in women with vaginal dysbiosis and adverse vaginal microbial composition (Chan et al., 2022; Florova et al., 2021; Kindinger et al., 2016).

Finally, the role of cytokines as mediators of inflammation in pregnancy and labour is also evident from immunohistochemistry in the fetal membranes and maternal decidua, myometrium and cervix. Labour was characterised by an increased expression of IL-1β, IL-6 and IL-8 in the cervix and myometrium; and IL-6 and IL-8 are increased in decidua and amnion (Osman et al., 2003). Furthermore, Young immunolocalised these cytokines within leukocytes in myometrium and cervix. In the cervix, IL-6 and IL-8 were also detected in the epithelium (Young et al., 2002).

1.5.2. Adaptive immune system

The adaptive immune system (also referred to as the acquired immune system) is more specific than the innate immune system since lymphocytes acquire unique structurally receptors against specific antigens during development, leading to a repertoire of individualised receptors. The adaptive immune system can be subdivided into the cellular and humoral response.

1.5.2.1. Cellular response

T cells can express either surface markers CD4 or CD8, that will allow further recognition and bind to MHC class II or I, respectively.

The $CD4^+$ T phenotype is also known as the T helper cell. There are two types of T-helper (Th) cells: Th1, involved in cellular immunity; and Th2, involved in humoral immunity. During pregnancy, a Th2 cell profile is predominant; and Th1 immunity is supressed. Type 2 $CD4^+$ T cells produce IL-4, IL-5, IL-1β, IL-13, IL-10 and IL-6. A Th2 response is related to a strong antibody response, whereas the Th1 response is responsible for the production of proinflammatory cytokines (IFN-γ, IL-2, and TNF-α) that can promote the production of complement-fixing antibodies involved in antibody-dependent cell cytotoxicity. During labour, the ratio of cytokines is reversed and the Th1 response increases (Challis et al., 2009; Saito et al., 2010; Sykes et al., 2012).

T regulatory cells (T regs) are a subset of $CD4^+$ T cells that oversee the regulation of the inflammatory process. They are essential to avoid the recognition of paternal semi-allogenic tissues by the maternal immune system (La Rocca et al., 2014).

1.5.2.2. Humoral response

B cells produce immunoglobulins. B cell counts in the 3rd trimester of pregnancy are reduced compared to non-pregnant women, and levels further decrease upon delivery (Lima et al., 2016). B cells are generated in the bone marrow and then migrate to the spleen. The initial diversity of the B cell receptor (BCR) repertoire is the result of a somatic recombination process (VDJ recombination). Upon encountering a specific antigen (usually dependent on $CD4^+$ Th cells), B cells become activated and differentiate into plasma cells that will secrete immunoglobulins, or memory B cells for a more rapid response if there is a reinfection with the same pathogen. Immunoglobulins (Ig) secreted from mature B cells have undergone a selection process which leads to expression of a single antigen (Ag) specific pattern (Hoehn et al., 2016; Jackson and Elsawa, 2015).

In humans there are five immunoglobulin (Ig) classes: IgG, IgM, IgA, IgD and IgE. IgG comprises four subclasses (IgG1, IgG2, IgG3 and IgG4); and IgA two subclasses (IgA1, IgA2). The basic structure of Igs consists of four chains (two light and two heavy) connected by disulphide bridges (Figure 1.13.A). The light chains contain one variable and one constant region, whereas the heavy chains are composed of 3 (IgG, IgA, IgD) or 4 constant regions (IgM, IgE) (Figure 1.13.B). It is possible to discriminate the Fab fragment in the N-terminal variable domain, known as the Ag binding site. The Fc fragment is the remaining constant domain. (Mix et al., 2006).

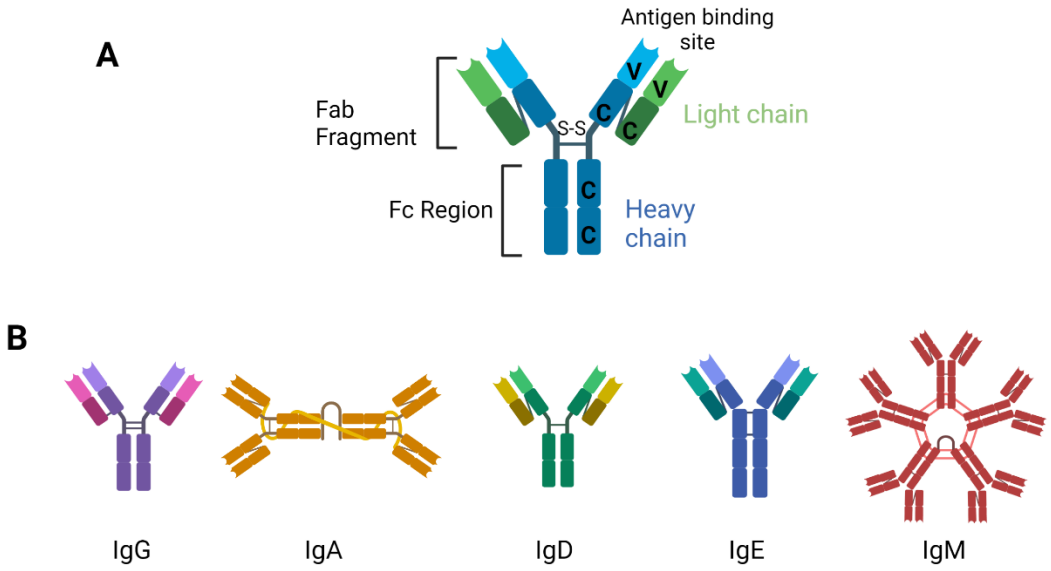


Figure 1.13. Antibody structure. A) Antibodies are composed of two light chains (green) and two heavy cHains (blue). The constant (C) and variable (V) regions are depicted for each chain. Chains are connected by disulphide bridges (S-S). The Fc region is the constant domain. The Fab fragment is the antigen binding site. **B)** Antibody classes vary in the number of heavy chains; IgG, IgA and IgD are composed of 3, whereas IgM and IgE have 4 heavy chains. The predominant and soluble form of IgM is the pentamer. Secretory IgA is a dimer antibody.

The different immunoglobulin isotypes are selectively distributed in the body and perform different functions. IgG is the predominant isotype in the body, with the longest serum half-life. The first three subclasses (IgG1, IgG2 and IgG3) can activate the complement cascade. Different subclasses are also induced depending on the secondary antibody response: IgG1 and IgG3 are usually induced in response to protein antigens, whereas IgG2 and IgG4 are predominant against polysaccharide antigens. The next most abundant Ig in serum is IgA. IgA levels are higher than IgG at mucosal surfaces and in secretions, where IgA forms a dimer, termed secretory IgA (sIgA). IgA has been associated with protection of mucosal interfaces from toxins, viruses and bacteria directly (neutralisation) and indirectly (preventing binding to the mucosal surface). IgE has the lowest concentration in serum, but it can be very potent; it has been associated with hypersensitivity and allergic reactions, as well as parasitic infections (Schroeder and Cavacini, 2010).

Lower concentrations of IgG, IgA and IgM were observed in pregnant women in the third trimester of pregnancy (>27 weeks of gestation), compared to non-pregnant controls (Benster et al 1970). Igs can be measured in cervicovaginal secretions (Jalanti and Isliker, 1977). Chan et

al found higher concentration of IgM and IgG1-4 in pregnant women with a CST IV microbial composition (Chan et al., 2022).

1.6. The potential role of neutrophils in microbial-driven preterm birth

Despite their key role in mediating inflammatory and/or microbial-driven responses, the role of neutrophils in microbial-driven preterm birth is poorly understood. This section provides additional background on neutrophils and is based on the recently published review The Role of Neutrophils in Pregnancy, Term and Preterm Labour. *Life* (2022). Belen Gimeno-Molina, Ingrid Muller, Pascale Kropf & Lynne Sykes.

1.6.1. Neutrophils: what are they and what do they do

The primary effector cells during infection and inflammation are polymorphonuclear leukocytes. Neutrophils are polymorphonuclear leukocytes that participate in acute inflammation, as they are recruited into sites of infection to clear infections (Kolaczkowska and Kubes, 2013). Neutrophils activate proinflammatory responses via PRRs that are triggered by LPS and other bacterial and viral products. Neutrophils are in circulation until they are recruited into sites of infection, when they transmigrate, via a selectin- and integrin- dependent process, into tissue, following a chemokine gradient (Kolaczkowska and Kubes, 2013). Neutrophils can perform a huge variety of effector functions (Figure 1.13) (Gimeno-Molina et al., 2022b), including phagocytosis (Peiser, 2016), degranulation (Table 1.5) (Faurschou and Borregaard, 2003; Othman et al., 2022; Segal, 2005), release of reactive oxygen species (ROS) via activation of respiratory burst (Nguyen et al., 2017; Segal, 2005) and NETosis (release of Neutrophil Extracellular Traps or NETs) (Brinkmann et al., 2004).

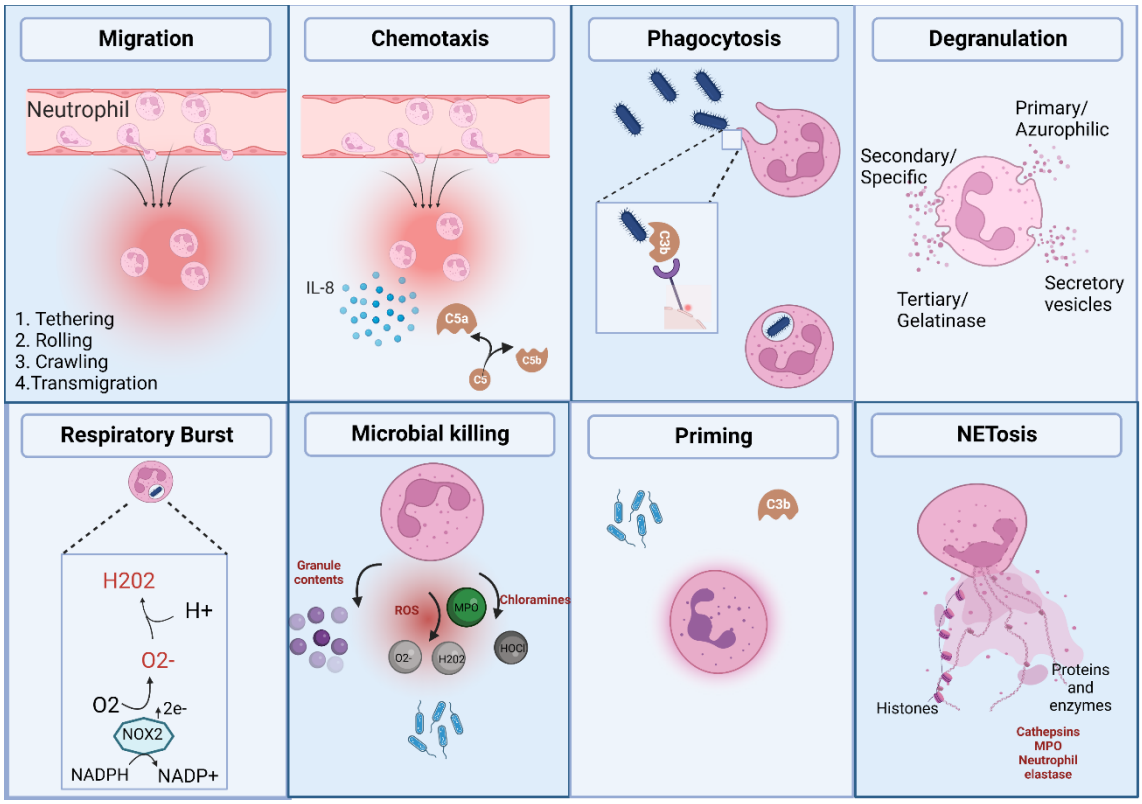


Figure 1.14. Neutrophils and their effector functions. Neutrophils are the first line of defence and the migrate into tissue following a chemotactic gradient. Neutrophils can phagocytose pathogens aided by opsonins. They are implicated in microbial killing thanks to the antimicrobial properties of the granules that are released via degranulation and the products of the respiratory burst activation. Neutrophils can also be primed when encountering bacterial products and products of inflammation, potentiating their activation status. Finally, neutrophils are able to release Neutrophil Extracellular Traps (NETs). Source: Gimeno-Molina et al., 2022b.

Table 1.5. Content of neutrophil granules and secretory vesicles. Reproduced from Gimeno-Molina et al., 2022b.

Function	Primary/ Azurophilic	Secondary/ Specific	Tertiary/ Gelatinase	Secretory vesicles
Microbicidal	Myeloperoxidase Lysozyme Defensins Cationic proteins Azurocidin Cap57	Lysozyme Lactoferrin Pentraxin 3 Lipocalin 2 Haptoglobin	Cathelicidin Lysozime	
Serine proteases	Neutrophil elastase Cathepsins			
Metalloproteinases	Proteinases	Collagenase (MMP-8)	Gelatinase B (MMP-9) Leukolysin (MMP-25) Collagenase (MMP-8)	Leukolysin (MMP-25) Proteinase 3 (myoblastin)
Acid hydrolases	Cathepsins B β-glucuronidase Glycerophosphatase N-acetylglucosaminidase α-mannosidase		β2-microglobulin	
Others	Heparin binding protein (HBP) Sialidase Presenilin Granulophysin α1-antitrypsin	Histaminase Heparanase Stomatin B12 binding protein Cytochrome b C3b receptor	C3bi receptor	Heparin-binding protein (HBP) Plasma proteins (including albumin) Alkaline phosphatase

1.6.2. Neutrophils in pregnancy and labour

The contribution of neutrophils to pregnancy and parturition has generally been described in section 5.1.1. Overall, there is an increase in number and activation of granulocytes in the periphery. In addition, there is histochemical evidence of neutrophil populations infiltrating in the cervical stroma in both term and preterm deliveries (Bokström et al., 1997; Osman et al., 2003; Whitworth et al., 2007). However, any assessment of the potential role of cervical leukocytes, especially in the context of spontaneous preterm birth in the human, remained unknown until the work of Hunter et al. They found that the main leukocyte population at the cervical-vaginal interface are polymorphonuclear cells (PMN), predominantly neutrophils (Hunter et al., 2016). Interestingly, early preterm births were associated with absence and lower levels of PMN (Hunter et al., 2016). It is known that early preterm births are more likely to be driven by infectious aetiologies, however this work was published prior to wider outburst of studies exploring the vaginal microbiota using next generation sequencing.

More recently, Mohd Zaki et al. also evaluated the leukocyte subsets present in the cervical milieu. As expected, neutrophils were the predominant population in the cervix. In a subset of samples (n=10) they correlated the transcriptome of cervical neutrophils with the vaginal microbial composition and found some clusters of genes implicated in the immune response and effector functions of neutrophils that were negatively correlated with *G. vaginalis* abundance and with *L. iners* abundance (Mohd Zaki et al., 2022). These studies support the growing interest in the key role that neutrophils might play in pregnancy and parturition. With the paucity of studies reporting on the potential role and immunophenotype of neutrophils in the cervical milieu, this study aims to deepen our understanding of how neutrophils may play a role in microbial driven preterm birth.

1.7. Hypothesis and aims

1.7.1. Hypothesis

The innate and adaptive immune response at the cervicovaginal interface play a key role in microbial driven cervical shortening and preterm birth and can provide novel targets for preterm birth prevention.

1.7.2. Aims

This project will investigate the role of the local immune response in cervical shortening and microbial driven preterm labour.

There are three specific aims to this project:

1. To determine if the immune milieu modulates the risk of spontaneous preterm birth in women who have a high-risk microbial signature

2. To determine if the immunophenotype of cervical neutrophils influences the local immune milieu in response to a high-risk microbial signature, cervical shortening and preterm labour

3. To determine if the recognition of vaginal microbiota by opsonins differs by microbial composition, and in cases of cervical shortening and preterm birth

1.7.3. Objectives

Aim 1: To determine if the immune milieu modulates the risk of spontaneous preterm birth in women who have a high-risk microbial signature

- To determine levels of local immune mediators (cytokines, complement proteins, matrix metalloproteinases) in CVF samples collected from women at high-risk of cervical shortening and preterm birth
- To characterise the relationship between local immune mediators and microbial composition in the context of cervical shortening, and pregnancy outcome

Aim 2: To determine if the immunophenotype of cervical neutrophils influences the local immune milieu in response to a high-risk microbial signature, cervical shortening and preterm labour

- To optimise methodologies for the assessment of peripheral and cervical neutrophils
- To characterise the immunophenotype of cervical neutrophils and compare with matched peripheral blood neutrophils
- To correlate the activation status and functional capacity of cervical and peripheral neutrophils with pregnancy outcomes
- To compare the immunophenotype of neutrophils with vaginal microbial composition
- To correlate the presence and activation status of cervical and peripheral neutrophils with the presence of local immune mediators

Aim 3: To determine if the recognition of vaginal microbiota by opsonins differs by microbial composition, and in cases of cervical shortening and preterm birth

- To optimise bacterial opsonisation protocols for the assessment of opsonisation of cervicovaginal bacteria
- To determine the percentage of opsonised cervicovaginal bacteria
- To determine if the degree of opsonisation influences the local immune milieu
- To determine if the percentage of opsonisation is altered depending on local microbial composition, cervical shortening and preterm birth

2. MATERIALS AND METHODS

2.1. Study design

This was a prospective study designed to investigate the role of the complement system and neutrophils at the cervicovaginal interface in women who undergo cervical shortening and who deliver preterm.

Pregnant women at high risk of preterm birth were recruited from Imperial College Healthcare NHS Trust maternity hospitals: St Mary's Hospital (SMH), Queen Charlotte's and Chelsea Hospital (QCCH) and Chelsea and Westminster Hospital (CWH); as well as from University College London Hospital (UCLH). Patients were recruited and samples collected from June 2018 to June 2022.

The study was performed under the Ethics approval REC 14/LO/0328 as part of the Vaginal Microbiome and Metabonome in Pregnancy (VMET 2) Research Study and approved by the NHS Health Research Authority (London -Stanmore Research Ethics Committee). Patients from the FLIP-1 Study: Vaginal Lactobacillus Supplementation in Women at High Risk of Preterm Birth (ClinicalTrials.gov Identifier: NCT03992534, registration date 20th June 2019) were also included in the study only before they received vaginal supplementation with LACTIN-V. The study was granted by the London Surrey Borders Research Ethics Committee (IRAS 262987). Health and Care Research Wales Approval was given (REC 19/LO/1018).

2.2. Patient recruitment

Pregnant women who were identified as being at risk of preterm labour were recruited from the Preterm Birth Prevention and Surveillance clinic, or prior to receiving an emergency cervical cerclage. Pregnant women are referred to the clinic at the time of their booking appointment by 12 weeks if they have a history of previous preterm birth (<34 weeks), previous preterm prelabour rupture of membranes (<34 weeks), previous mid-trimester loss (16-23 weeks), a history of cervical trauma (either cervical treatment including surgery such as previous cone biopsy and large loop excision of the transformation zone) or a uterine anomaly. Women at intermediate risk (history of cervical cerclage in a previous pregnancy or previous fully dilated caesarean section) were also seen in clinic. Women were also referred from routine antenatal care if a short cervix and/or bulging membranes is detected at the time of the routine anomaly scan (18-22 weeks). All patients provided written informed consent to donate specimens. Samples were collected at three different gestational timepoints: A: 12-16 weeks; B: 20-24 weeks; and C: 30-34 weeks.

Exclusion criteria included: sexual activity within 72 hours of sampling, vaginal bleeding in the preceding week, HIV positive women, hepatitis B positive serology, and women under the age 18.

Detailed metadata was collected for women participating in the study, including maternal age, body mass index (BMI), ethnicity, blood group, smoking status, gestational age at sample collection and subsequent interventions to prevent PTB (cerclage and/or progesterone). Cervical length (CL) was carried out routinely on every patient visit to clinic, using a transvaginal ultrasound by trained clinicians, using a consistent technique of measuring the closed length (mm) with a straight line between the internal os and the external os (Figure 2.1.). Three measurements were taken, and the shortest was recorded in the database. At every visit, the use of any medication and antibiotics in the preceding 2 weeks was also recorded, together with any use of progesterone pessary. Metadata also included delivery details (occurrence of PPROM (preterm prelabour rupture of membranes), onset of labour, mode of delivery, gestational age of delivery, antenatal and intrapartum complications) and neonatal outcomes (birth weight, gender, admission to Neonatal Intensive Care Unit (NICU) or Special Care Baby Unit (SCBU)).

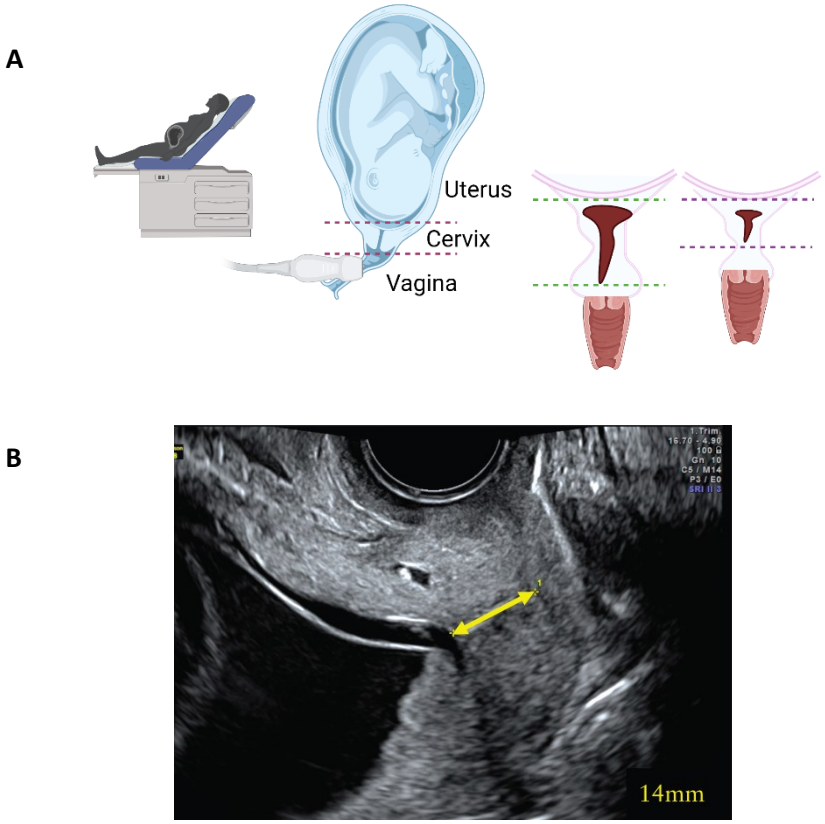


Figure 2.1. Cervical length measurement. A) Transvaginal ultrasound was used in this study to measure cervical length, with a normal length cervix and short cervix (≤25mm) depicted in the diagram. Image created with Biorender. **B)** Example of a cervical length measurement using transvaginal ultrasound. Retrieved from (Kagan and Sonek, 2015).

2.3. Cohort descriptions

Recruited women received standard care and were followed up across their pregnancy. Upon pregnancy completion, outcome data was retrieved, and patients were separated into one of the following cohorts:

Term uncomplicated (TU) delivery

Women described as at risk of delivering preterm attending the Preterm Birth Surveillance clinic that ultimately delivered at term and did not receive any type of intervention to prevent preterm birth (neither vaginal progesterone nor cervical cerclage) at any point during their pregnancy.

Spontaneous preterm (PT) delivery

Women who delivered before completing 37 weeks of gestation. It includes cases of preterm labour and PPROM. This cohort category does not include indicated preterm deliveries for maternal or fetal conditions.

Term intervention (TI) delivery

Women who delivered at term following intervention, including cervical cerclage (pre-pregnancy, elective or ultrasound indicated) and/or progesterone.

2.4. Sampling

Samples were processed at the Institute of Reproductive and Developmental Biology (IRDB) in Imperial College London Hammersmith Campus and in the School of Medicine in Imperial College London St Mary's campus.

2.4.1. Blood sample collection and processing for peripheral blood neutrophils

Peripheral venous blood (5mL) was collected by venepuncture into Lithium Heparin (LiH) collection tubes. Samples were processed within 20 minutes of collection. Two different methodologies were employed to isolate peripheral blood neutrophils during the protocol optimisation (see Section 4.1.5). After optimisation of sample collection, Method 2 (Histopaque) was selected to analyse peripheral blood samples to maximize timings of procedures.

Method 1- Ficoll-Paque

Peripheral blood was layered over Ficoll-Paque (Cat# 17-1440-02, GE Healthcare Biosciences) and centrifuged at 765 x g, at room temperature for 30 minutes, without break. After density gradient centrifugation (Figure 2.2.), the layers of plasma, PBMC and Ficoll interphase were discarded; and normal density granulocytes were then isolated from the erythrocyte fraction. The remaining fraction was resuspended in 7mL of PBS (phosphate-buffered saline) and 7mL of 3% dextran (Cat# 31392-10G, Sigma) solution and incubated at room temperature for 20 minutes (Figure 2.3.).

The upper phase was harvested and transferred into a new tube. The fraction was centrifuged (1200rpm, 8 minutes) and the cell pellet was resuspended in 7mL of erythrocyte lysis buffer (155mM NH_4Cl; 10mM $KHCO_3$; 0.1mM EDTA) for 15 minutes. The sample was centrifuged (1200rpm, 8 minutes) and the supernatant was discarded. The remaining pellet containing granulocytes was washed with PBS (centrifugation, 1200rpm, 8 minutes).

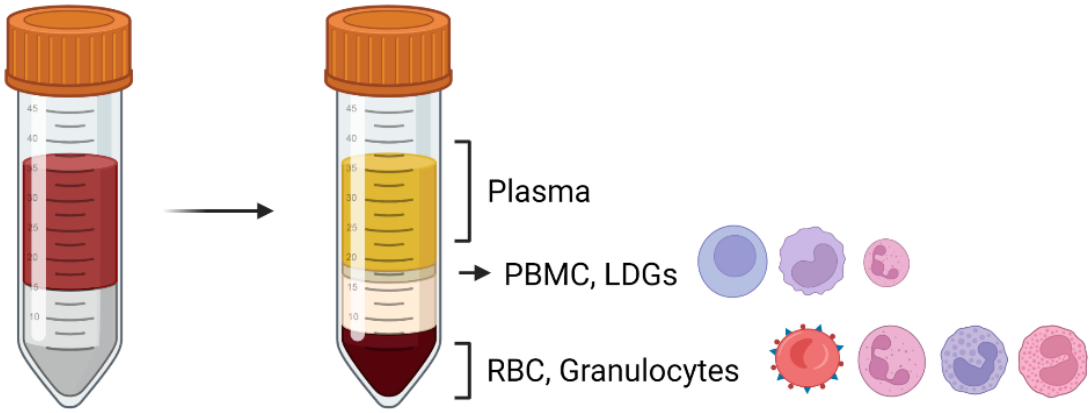


Figure 2.2. Isolation of peripheral neutrophils. Blood fractioning and separation by density gradient centrifugation. Neutrophils were isolated from the bottom layer containing red blood cells (RBC) and granulocytes. From Gimeno-Molina et al., 2022b. Image created with Biorender.com.

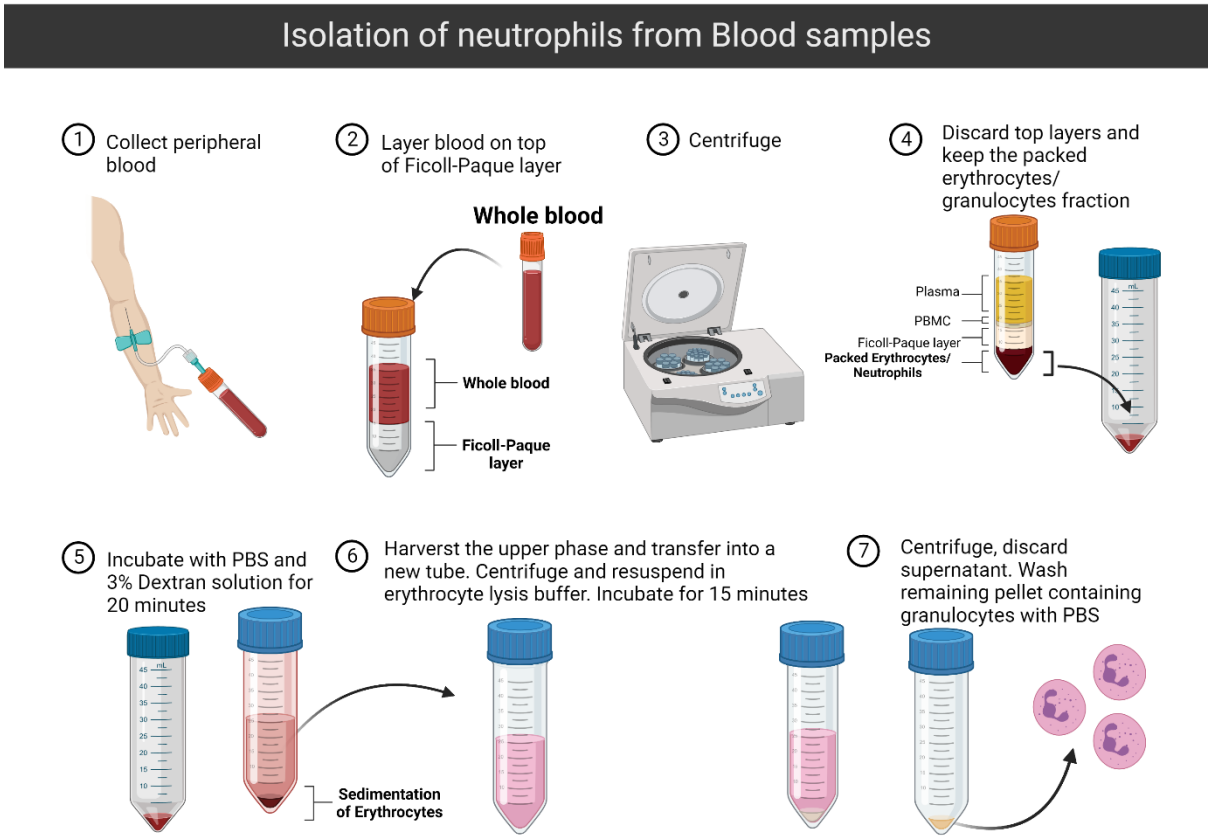


Figure 2.3. Isolation of neutrophils from Blood Samples-Method 1-Ficoll Paque. Neutrophils were retrieved from peripheral blood by density gradient centrifugation over a Ficoll-Paque layer. The granulocytes fraction was incubated with PBS and 3% Dextran solution. After 20 minutes of incubation, the upper phase was resuspended in erythrocyte lysis buffer and incubated for 15 minutes. Neutrophils were retrieved by centrifuging and discarding the supernatant. The remaining pellet containing the granulocytes was washed with PBS. Image created with Biorender.com.

Method 2-Histopaque double gradient

Histopaque – 1119 (Cat# 11191, Sigma-Aldrich) was used in conjunction with Histopaque – 1077 (Cat#, 10771, Sigma-Aldrich), following manufacturer's instructions to facilitate the recovery of granulocytes. Briefly, a double gradient is formed by layering whole blood onto a layer of 3mL of Histopaque – 1119 and 3mL Histopaque – 1077. A total of 5mL of whole blood was carefully layered on top of the two histopaques. After centrifugation (765 x g, 30 minutes, room temperature, without break), two distinct opaque layers are observed: peripheral blood mononuclear cells and granulocytes (Figure 2.4.). The protocol was optimised to include an incubation with erythrocyte lysis buffer (15 minutes) and washing with PBS, twice.

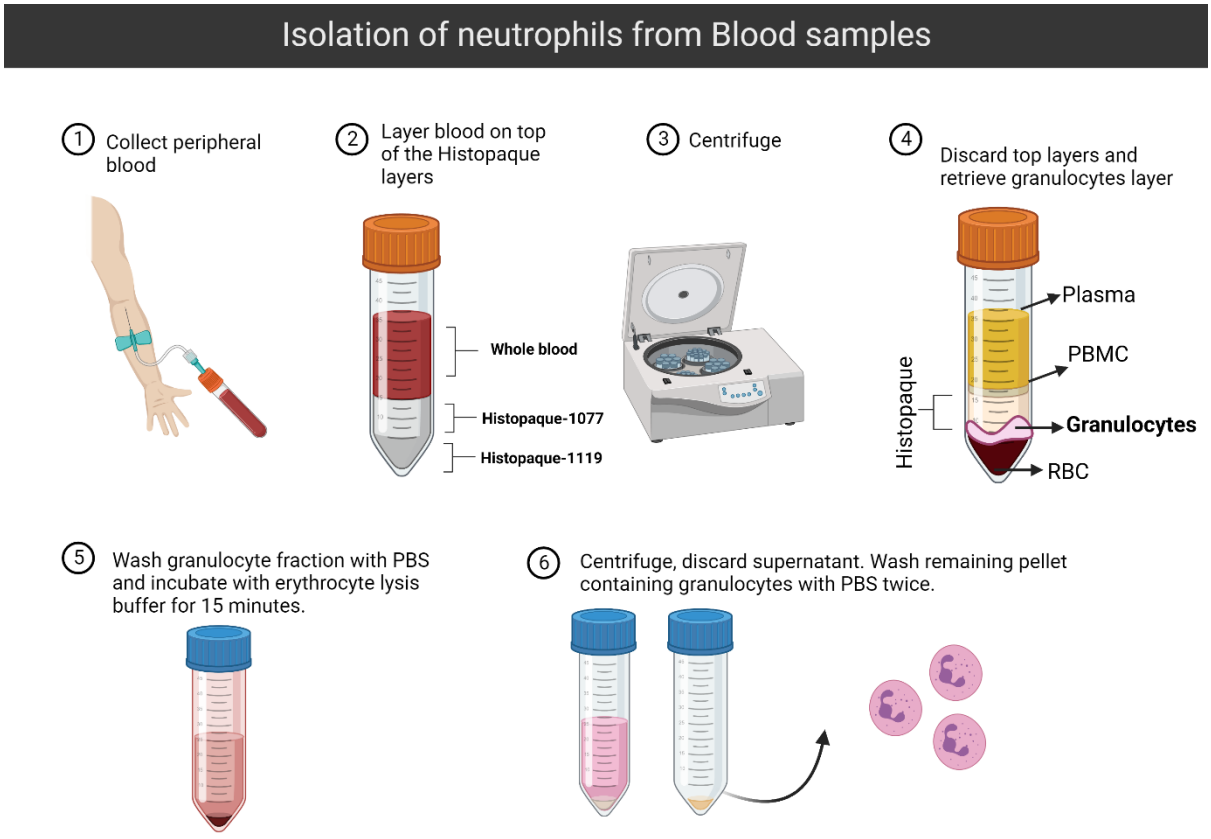


Figure 2.4. Isolation of neutrophils from Blood Samples-Method 2-Histopaque double gradient. Whole blood was layered onto a fraction with Histopaque-1077 and Histopaque-1119. After density gradient centrifugation, granulocytes fraction could be retrieved straight from the layer within the Histopaque and RBC. Granulocytes were washed with PBS and incubated with erythrocyte lysis buffer. Finally, granulocytes were washed with PBS twice. Image created with Biorender.

2.4.2. Cytobrush collection and processing for cervical neutrophils

A speculum was inserted into the vaginal canal and the cervical os exposed by a trained clinician. A cervical cytobrush (Cat #720-2068, Cervical brush Q Path ® NovaBrush ®) was used to make 10 gentle brush strokes over the anterior lip of the cervix. The brush was placed in a 50mL conical tube containing 5mL of phosphate buffered saline (PBS).

Cytobrush samples were passed through a sterile 40µm cell strainer (Cat# 11587522, Fisherbrand™) to remove mucus and epithelial content. The sample was then centrifuged (500x *g*, 2000rpm) for 5 minutes at room temperature and resuspended in ~1mL PBS (to achieve a minimum of $1x10^7$ cells/mL if possible). Samples were processed within 30 minutes of collection.

2.4.3. Cervicovaginal fluid collection and processing

At each visit, cervicovaginal fluid (CVF) was collected from participants, using a BBL™ CultureSwab™ MaxV Liquid Amies swab (Becton, Dickinson and Company, Oxford UK)). Samples were collected from the posterior fornix of the vagina under direct visualisation by trained personnel. Two vaginal swabs were collected per participant, and immediately stored at -80°C, until further analysis: one vaginal swab was dedicated for microbiome studies; and the other matched swab was reserved for bacterial opsonisation studies. Supernatants from these swabs were used for immunophenotyping, whilst the pellets were used for microbial composition studies. A third swab (Transwab MW170 with rayon blue lid, Medical Wire & Equipment, Corsham, UK), was collected for immunophenotyping (Luminex Multiplex Immunoassays), also stored at -80°C until further analysis.

2.5. Flow cytometry of neutrophils by Aurora Cytek™

Peripheral neutrophils isolated from blood and cervical neutrophils collected with the cytobrush were resuspended in 100µL of PBS and stained with 2µL of Fc Blocker (Cat# 564220, BD Biosciences) for 10 minutes. Cells were stained with 2µL of 7-AAD viability staining solution (Cat# 420403, BioLegend) and anti-human antibodies (Table 2.1) for 15 minutes in the dark at room temperature. The adequate volume per antibody was determined after titration of antibodies (details available in section 4.5.2.). After incubation, cells were washed with 1mL of staining buffer (PBS, 1% BSA (A7906, Sigma), 0.1% sodium azide (Cat# 71289-50G, Sigma)). The supernatant was discarded, and the cell pellet was resuspended in 500µL of 0.25% formaldehyde PBS prior data acquisition with Cytek™ Aurora (Cytek

Biosciences) at the LMS NIHR Flow Facility at Imperial (MRC-LMS Imperial College, Hammersmith Campus).

Table 2.1. Multicolour flow cytometry panel. Anti-human monoclonal antibodies for flow cytometry.

Anti-human antibodies	Clone	Fluorochrome	Purpose	Catalogue Number	Company (Brand)	Control	Volume
CD45	2D1	PerCP/ Cyanine 5.5	Immune cells (Leukocyte Common Antigen)	368503	BioLegend	Cells	1.7µL
CD15	W6D3	Brilliant Violet 785™	Granulocytes	323044	BioLegend	Cells	0.5µL
CD14	61D3	PerCP-eFluor™ 710	Macrophages/monocytes	46014942	ThermoFisher (eBioscience™)	Beads	0.5µL
CD16	CB16	eFluor™ 450	Fc gamma receptor III Macrophages/neutrophils	48-016842	ThermoFisher (eBioscience™)	Cells	0.5µL
CD63	H5C6	APC	Degranulation marker	353007	BioLegend	Cells	0.5µL
CD66b	G10F5	PE/Dazzle™ 594	Phagocytosis	305122	BioLegend	Beads	0.5µL
CD62L	DREG-56	BV480	L-selectin Migration	566111	BD Biosciences (BD Horizon™)	Beads	0.5µL
CD11b	ICRF44	Alexa Fluor ® 700	Integrin Migration	301356	BioLegend	Cells	0.5µL
CD10	HI10a	APC/Cyanine 7	Maturation	312212	BioLegend	Beads	0.5µL
CD33	P67.6	Alexa Fluor ®647	Maturation/Immune regulation	366626	BioLegend	Beads	0.5µL
CD55	IA10	Brilliant Violet 510	Complement	742678	BD Biosciences (BD OptiBuild™)	Beads	0.5µL
CD35	E11	FITC	C3b/C4b receptor, complement receptor type 1 (CR1)	333404	BioLegend	Beads	0.5µL
CD88	D53-1473	BV421	Complement C5a Receptor	742315	BD Biosciences (BD OptiBuild™)	Beads	0.5µL

Several controls were included. The unstained control was prepared for both peripheral and cervical neutrophils. Single colour stain, with bead or cell controls were recorded on SpectroFlo software (Table 2.1.). Cell controls were preferred over bead controls; however, beads were required for cell markers with lower expression or to ensure detection and accuracy. If possible, a minimum of 100000 events were acquired on the granulocytes gate. Raw spectral data was acquired and unmixed using Spectroflo Software (Cytek Biosciences) with autofluorescence extraction. Further spectral analysis was performed with FlowJo v10 Software (FlowJo).

The manual gating strategy was used to identify peripheral and cervical neutrophils (details available in section 4.5.5.). After gating on the peripheral and cervical neutrophils subsets, MFI (Median Fluorescence Intensity) of different activation markers were obtained.

2.5.1. Aurora Cytek®: The instrument

Flow cytometry is a methodology that allows the characterisation of single-cell protein expression, using fluorochrome-labelled antibodies that bind to a protein of interest. The traditional flow cytometry instrument consists of a combination of lasers, optic systems (mirrors and band-pass filters) and detectors (known as photomultiplier tubes, PMTs). The development and evolution of this technique has led to a high-throughput technology, thanks to the addition of extra lasers and detectors, moving away from conventional flow cytometry, towards spectral flow cytometry (Figure 2.5.) (Nolan and Condello, 2013).

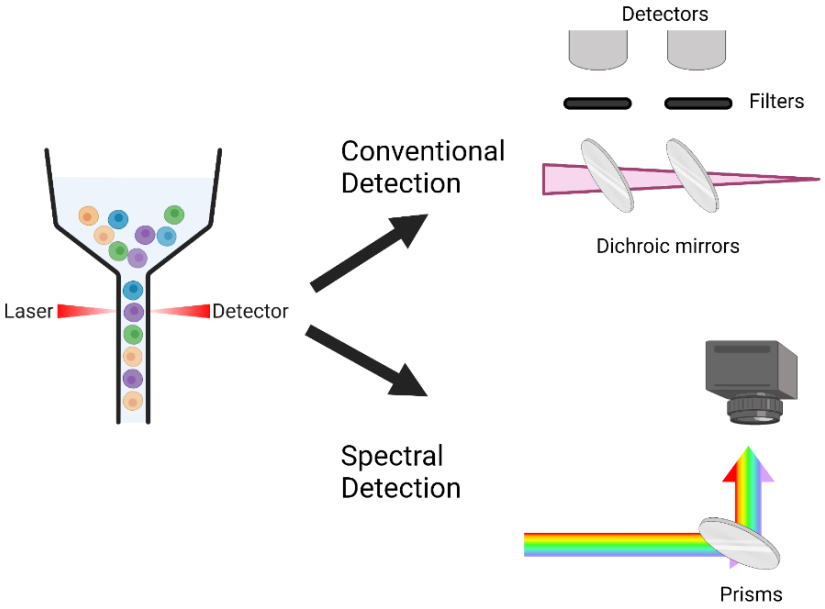


Figure 2.5. Evolution of flow cytometry techniques. Conventional detection that relies on dichroic mirrors and filter for detection on PMTs. Spectral detection captures the dispersed light within a detector array. Image created with Biorender. Adapted from Nolan & Condello (2013).

One of the main differences between these techniques (Table 2.2.) is the optic system. While conventional flow cytometry uses mirrors to block/reflect the light, spectral flow cytometers disperse photons according to wavelength via prisms and gratings (Nolan and Condello, 2013). Also, the latter has the advantage of being able to characterise and immunophenotype greater numbers of markers per cell, since it is possible to combine more antibodies/fluorophores per reaction. In this case, the instrument acquires the unique spectral signature of each fluorophore, across all laser lines (Figure 2.6.). Therefore, it becomes possible to analyse multiparametric panels, even if the peak emissions of fluorochromes included in the panel are similar (Park et al., 2020). Regardless, the core concept of flow cytometry is maintained, and the sample preparation and uptake of single-cell suspension via a continuous sheath fluid is maintained.

Table 2.2. Characteristics of conventional and spectral flow cytometry

	Conventional flow cytometry	Spectral flow cytometry
Fluorophore detection	Limited	Multiple
	One section is collected	Detail unique spectral signature
Spillover correction method	Compensation	Unmixing
Fluorophore selection	Limited depending optical configuration	Limited fluorophore spectral signature
Autofluorescence extraction	No	Yes

Adapted from (Thermofisher)

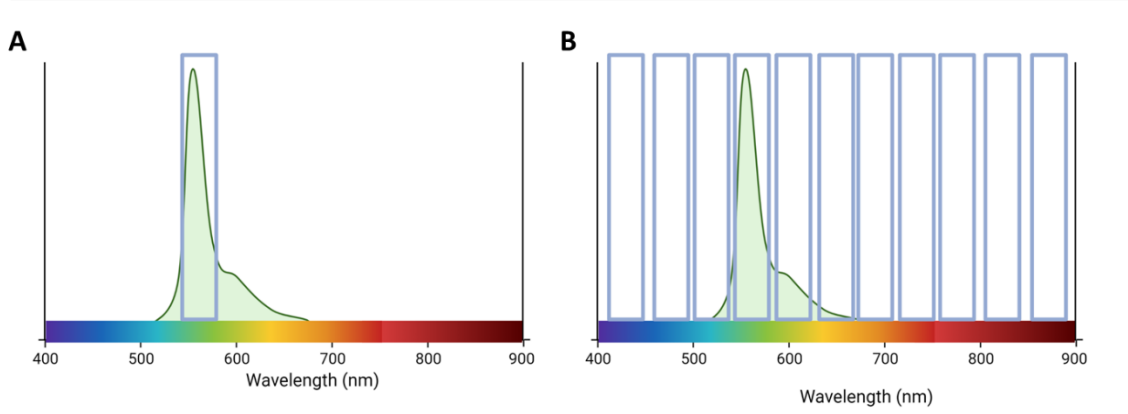


Figure 2.6. Fluorophore detection in conventional and spectral flow cytometry. A) Conventional cytometers use a single detector to collect fluorescence emission from one primary fluorophore, corresponding to the peak of intensity (only a section of emission is collected). **B)** Spectral unmixing uses multiple detectors to collect the full spectrum fluorescence of emission. Created with Biorender.com.

The use of spectral flow cytometry allows for the maximisation of data that can be retrieved using a single sample. The opportunity to evaluate more parameters facilitates a larger and more complete immunophenotyping, which is particularly relevant when working with limited samples, as in research studies and clinical trials.

The Cytek® Aurora instrument was used to perform the immunophenotype of neutrophils. This instrument is a compact benchtop cytometer with a 3-laser configuration (405nm, 488nm, 640nm), that includes SpectroFlo® software for unmixing. The fluorescence detector enables spectrum capture within the range of 365-829nm. The optical configuration consists of a violet, blue and red detector module with 16, 14, and 8 channels, respectively, covering the range of spectrum capture (Cytek). Cytek ® SpectroFlo QC Beads (SKU B7-10001) were routinely used to ensure performance of instrument.

2.6. Extraction of DNA for microbiota analysis

2.6.1. DNA Extraction

DNA extraction was performed as previously described (MacIntyre et al., 2015). Firstly, swabs were thawed on ice and resuspended in Amies Liquid transport medium by vigorous vortex. Contents were transferred to a 2mL Eppendorf and centrifuged (7000 rpm, 10 minutes, 4°C). At this stage, supernatant was stored in aliquots at -20°C for further analysis with Luminex immunoassays, and the remaining pellet was resuspended in 300µL of lysis enzyme master mix (Table 2.3.) and incubated at 37°C for 1h in a water bath. After the incubation, samples were oscillated with 100mg of acid washed 0.1mm glass beads (BBI-8541400, Sartorius Stedim) for 1 minute at 25Hz in a Tissue Lyser LT (Qiagen). Finally, DNA was extracted using QiAamp DNA Mini Kit (Qiagen), following manufacturer's instructions. The lysate was incubated at 56°C with Proteinase K and AL buffer. After this incubation, the lysate was mixed with 200µL of 100% ethanol and transferred to a spin column. The columns were centrifuged at 8000rpm for 1 minute. Buffer AW1 was added to the column, which was centrifuged under the same conditions (8000rpm, 1 minute). Later, buffer AW2 was added and centrifuged at 14000 rpm (first for 2 minutes, and an extra centrifugation for 1 minute). Collection tubes were discarded and replaced after every centrifugation step. Finally, DNA was eluted in 100 µL of Elution Buffer. Eluted DNA was stored at -20°C.

Table 2.3. Lysis enzyme mastermix for DNA extraction

Reagent	Volume
Lysozyme (10mg/mL) (Sigma L6876)	50µL
Mutanolysin (25000U/mL) (Sigma M9901/10Ku)	6 µL
Lysostaphin (4000U/mL) (Sigma L9043)	3 µL
TE_{50} (10Mm TRIS/50Mm EDTA)	41 µL
12% Triton	30 µL
PBS	170 µL

2.6.2. Polymerase Chain Reaction amplification

The presence of bacterial DNA in samples extracted was confirmed by Polymerase Chain Reaction (PCR), using 16S rRNA Universal Primers:

- Forward: 27F-5'AGAGTTTGATCCTGGCTCAG-3'
- Reverse: 338R-5'GCTGCCTCCCGTAGGAGT-3'

Five microliters of DNA template were mixed with 45 µL of PCR mastermix (Table 2.4.), and further run on a thermocycler (Tetrad 2, or BioRad T100) under the conditions described in Table 2.5.

Table 2.4. PCR MasterMix Formulation for a single PCR reaction

PCR MIX	Volume (µL/sample)
One Taq ® DNA Polymerase (M0480L, New England BioLabs)	0.25 µL
1mM Deoxynucleotide Mix (D7295, Sigma-Aldrich)	1 µL
One Taq ® Standard Reaction Buffer (New England BioLabs)	10 µL
Forward Primer (10pmol/1µL)	1 µL
Reverse Primer (10pmol/1µL)	1 µL
DEPC-Treated water (AM9906, Invitrogen)	31.75 µL

Table 2.5. PCR steps and cycle

Step	Temperature	Time (00m: 00s)	Cycles
Initial Denaturation	95°C	05:00	1x
Denaturation	95°C	00:30	30x
Annealing	55°C	00:30	
Extension	72°C	01:30	
Final Extension	72°C	07:00	1x

The analysis of PCR products was assessed via agarose gel electrophoresis. First, 1.5% agarose gels (BP1356-100, Fisher BioReagents) were prepared in 1X TBE. SYBR™ Safe DNA Gel stain (S33102, Invitrogen) was added (8 µL per 100mL of agarose solution), and the gel was left at room temperature to set. The gel was loaded with 5 µL of GeneRuler 100bp DNA Ladder (SM0241, Thermo Scientific); and 20 µL of each PCR product (previously mixed with 6X DNA loading dye TriTrack Dna Loading Dye (6X) (R1161, ThermoScientidic)) and run at 140 V for 20-30 minutes (Bio-rad Power Pac 300). The gel was visualised on a transilluminator, and images acquired with UVITEC Cambridge Firereader (Uvitec Cambridge) (Figure 2.7.). In each PCR reaction, a positive control and 4 negative controls (one blank swab, one pure water, one pure PBS, one pure elution buffer) were included in the gel run to ensure there was no DNA contamination during the different extraction steps of the process.

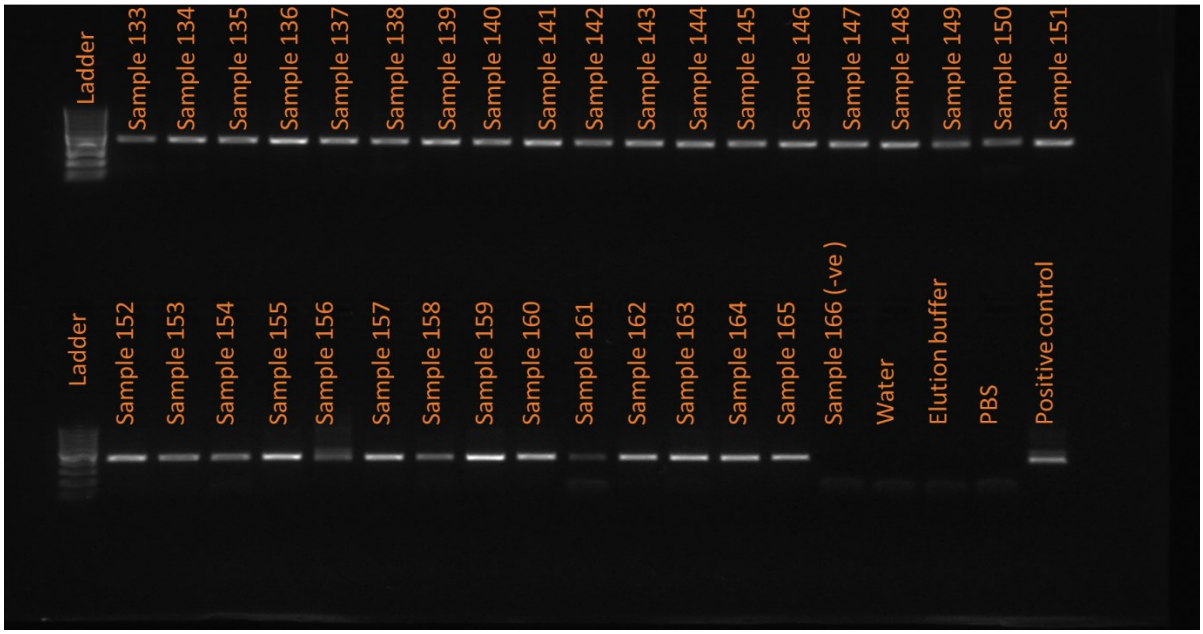


Figure 2.7. Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using 16S rRNA Universal Primers. Ladder: 100bp DNA ladder. Samples 133-65 correspond to patient samples. Sample 166: negative control/swab. Negative controls: pure water, pure elution buffer, pure PBS. Positive control: sample previously extracted. Samples and positive control had detectable bacterial DNA amplicons at 400bp (expected molecular weight).

2.6.3. DNA sequencing

Whole genomic DNA extracted from each swab was sequenced for the V1-V2 hypervariable regions of 16S rRNA on Illumina MiSeq platform (Illumina, Inc. Sand Diego, California) using forward and reverse 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 of 388R GCTGCCTCCCGTAGGAGT. Sequencing was performed at Research and Testing Laboratories (RTL Genomics, Texas, USA).

2.7. Luminex® Multiplex Immunoassays

Luminex ® Multiplex Immunoassay is a bead-based platform relying on fluorescent-coded magnetic bead microspheres or microparticles coupled to specific antibodies against an analyte of interest. Each bead has a unique bead region assigned, which allows targeting of multiple biological analytes on a single multiplex assay, maximising the opportunities where limited sample is available. Using the same sandwich principle from traditional ELISAs, after incubating the sample with the magnetic beads (Figure 2.8.), biotinylated detection antibodies specific to the analyte were added to the mixture, as

well as a final incubation with phycoerythrin (PE)-conjugated streptavidin. Finally, data was acquired using MAGPIX® Instrument with xPONENT4.3 software.

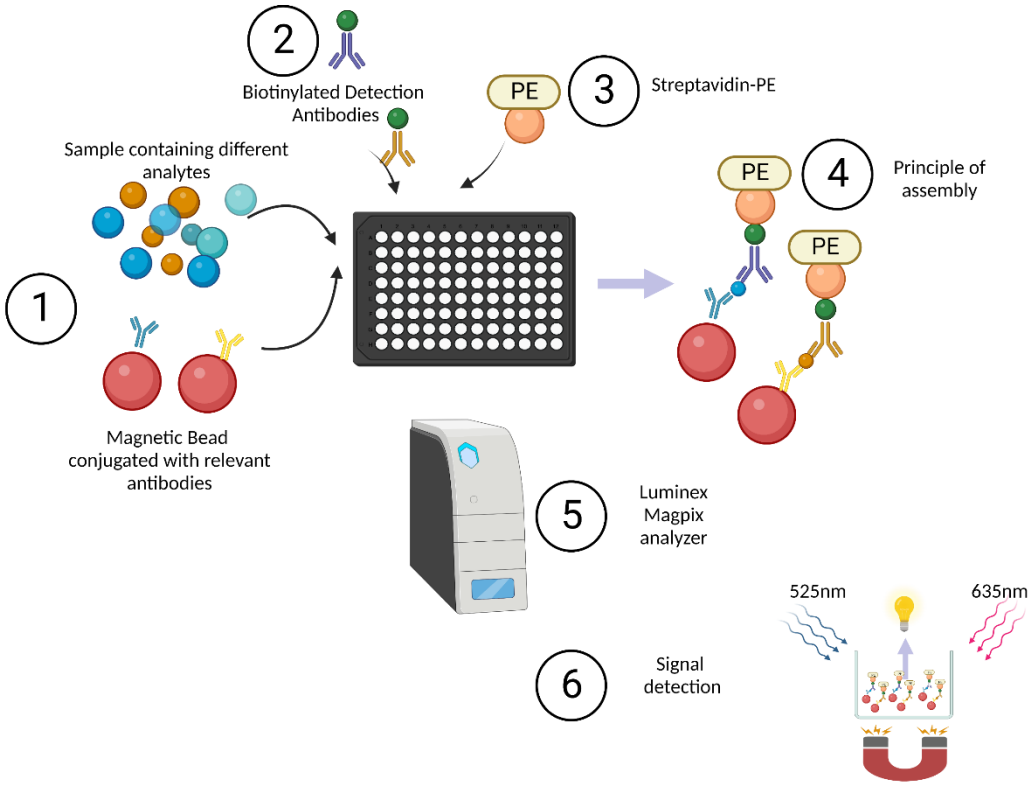


Figure 2.8. Luminex ® Immunoassays principle. Sample containing different analytes is incubated with magnetic beads conjugated with relevant antibodies against the analytes of interest. Secondly, biotinylated detection antibodies are added to the mixture, following same sandwich principle from traditional ELISAs. The last component is the addition of phycoerythrin-streptavidin. Data was acquired on a Magpix ® analyser. The instrument contains a chamber where a magnet holds the magnetic beads in a monolayer. One light-emitting diode identifies the bead/analyte; the other one determines the magnitude of the PE-signal, which is directly proportional to the contraction of the specific analyte.

2.7.1. Analyte extraction from cervicovaginal fluid samples

Two different vaginal swabs were processed to measure the concentration of several analytes in CVF. As previously outlined in the DNA extraction protocol (section 2.6.1.) from BBL™ CultureSwab™ MaxV Liquid Amies swabs, after vortexing the swabs, and centrifuging the samples, the supernatant was stored at -20°C for this purpose.

Separately, a Transwab was also processed. Swabs were thawed on ice and resuspended in 200 µL of PBS with protease inhibitor (PI) (5µL PI/1mL PBS, P8230, Sigma-Aldrich). After gentle vortex, samples were centrifuged (13000rpm, 10 minutes, 4°C) and the supernatant was collected. The swabs were

then transferred and inverted into a new collection tube, and further centrifuged (13000rpm, 5 minutes, 4°C). Both supernatants' fractions were then combined and stored at -20°C until further use.

Cytokine assays and MMPs assays were performed with samples collected using Amies-buffer containing swabs. Similarly, complement assays were also acquired with Amies-buffer containing swabs with the exception of the Human Complement Panel 2 containing C1q and Factor B analytes (Table 2.6.) which was performed using supernatants collected from Transwabs. Different swabs were employed for different panels to ensure optimal concentration to detect the analytes of interest.

2.7.2. Cytokine Assays

Cytokines are intercellular signalling proteins, that can be released from a wide variety of cells and tissue, playing a key role in inflammatory responses. The panels were designed to maximise the cytokine information related to inflammatory responses in preterm birth, cervical shortening and/or response to vaginal microbiota. One panel included: IL-6, IL-1β and IL-10 (3-plex) (LXSAHM-03, R&D Systems) and samples were analysed neat. The second panel included only IL-8 (1-plex) (LXSAHM-01, R&D Systems), that required a 1:10 dilution, using Calibrator Diluent RD6-52.

Assays were performed following the manufacturer's instructions. Samples were thawed on ice and centrifuged (13000 rpm, 5 minutes) prior to dilution and use. Each unique standard cocktail was resuspended in 100 µL of Calibrator Diluent RD6-52. After allowing the standard to sit for minimum 15 minutes, Standard 1 (S1) was created combining 100 µL of each cocktail into a final volume of 1000 µL with Calibrator Diluent RD6-52. A 3-fold dilution series was created to produce the rest of the standards (S2-S6), also using Calibrator Diluent RD6-52. Calibrator Diluent RD6-52 was used as a blank. A pool sample was created to include an internal calibrator in all plates.

The procedure included a 2-hour incubation of 50 µL of standard or sample per well with the microparticle cocktail, at room temperature on a horizontal orbital microplate shaker. Standards, samples, and blanks were assayed in duplicate. Next, the plate was washed using a magnetic device, and incubated with Biotin-Antibody cocktail for 1-hour. After re-washing the plate, Streptavidin-PE was added to each well and further incubated for 30 minutes. Finally, the plate was washed and the microparticles were resuspended with 100 µL of Wash Buffer. The microparticles were resuspended on the plate shaker immediately prior to acquisition with Magpix analyser.

2.7.3. Complement Assays

The complement system comprises more than 50 proteins that are part of the immune system. The complement system plays a key role in the clearance of pathogens by facilitating phagocytosis and antibody dependent clearance. Activation of the complement cascade results in an amplification response and activation of the MAC (membrane attack complex).

Different commercially available panels (Table 2.6.) were assayed to cover the different components of the complement cascade. All samples were centrifuged (13000 rpm, 5 minutes) prior to use and analysed neat.

Table 2.6. Complement panels assessed with Luminex ® technologies

PANEL	ANALYTES	BRAND	CATALOGUE NUMBER	SUPERNATANT ORIGIN
Human Complement Magnetic Bead Panel 1	C2, C4b, C5, C5a, Factor D, MBL, Factor I	Milliplex ® Millipore ®	HCMP1MAG-19K	from BBL™ CultureSwab™ MaxV Liquid Amies swab
Human Complement Magnetic Bead Panel 2	C3, C3b/iC3b, C4, Factor H	Milliplex ® Millipore ®	HCMP2MAG-19K	from BBL™ CultureSwab™ MaxV Liquid Amies swab
C3a Human ProcartaPlex™ Simplex Kit	C3a	Invitrogen™	EPX010-12282-901	from BBL™ CultureSwab™ MaxV Liquid Amies swab
Human Complement Magnetic Bead Panel 2	C1q, Factor B	Milliplex ® Millipore ®	HCMP2MAG-19K	from Transwab

Human Complement Magnetic Bead Panel 1 and 2 (Milliplex ® Millipore ®) reagents and assays were prepared following manufacturer instructions. The standard cocktail was resuspended with 250 µL of deionized water and left at room temperature for 10 minutes. A 3-fold dilution series was created with Assay Buffer to prepare the rest of standards. Quality Control 1 (QC1) and Quality Control 2 (QC2) were also resuspended in 250 µL of deionized water and allowed to sit for 10 minutes; QC1 and QC2 were also re-purposed as an internal calibrator to assay on the whole set of plates required.

The plate was initially pre-washed with 200 µL of Wash buffer and shaken for 10 minutes at room temperature. Contents were decanted prior the addition of 25 µL of standards, quality controls, background (using Assay Buffer) and samples per well, together with 25 µL of Assay Buffer. All samples were assayed in duplicate. After the addition of beads to all wells, the plate was sealed and incubated

for 2 hours at room temperature on a plate shaker. Next, the plate was washed, and detection antibodies were added. After incubation for 1 hour, the contents were not removed, and Streptavidin-Phycoerythrin was added to the mixture, for a further incubation of 30 minutes. Finally, the plate was washed, and beads resuspended in 150 µL of Drive Fluid (MAGPIX® Drive Fluid PLUS, 4050030, Invitrogen™) per well. The plate was run on Magpix ®.

C3a HumanProcataPlex™ Simplex Kit (Invitrogen™) was used in conjunction with ProcartaPlex Human Basic Kit (EPX010-10420-901, Invitrogen™), following manufacturer instructions. The antigen standard was reconstituted using Universal Assay Buffer, incubated on ice for 10 minutes. Vial contents were filled up to a final volume of 250 µL with Universal Assay Buffer. Assay Buffer was also used to prepare a 4-fold serial dilution to generate the rest of the standards, as well as serving as a blank. The protocol consisted of firstly, adding and washing the magnetic beads. Secondly, Universal Assay Buffer was added to each well, followed by samples, standards and blanks. After sealing, the plate was shaken for 2-hours at room temperature. Thirdly, after washing the plate, Detection Antibody Mixture was added, and left incubating for 30 minutes on a plate shaker, followed by another plate wash. Fourthly, SAPE was added, and plate was incubated for another 30 minutes. Lastly, the plate was washed and sample/microparticle mixture was resuspended in 120 µL of Reading Buffer, with 5 minutes incubation on a shaker prior acquisition on Magpix ® instrument.

2.7.4. Matrix Metalloproteinases Assays

Matrix metalloproteinases (MMPs) are proteases capable of degrading the extracellular matrix, among other biological functions. The panel was designed to maximise the information related to inflammatory responses in preterm birth and cervical shortening. The panel included: MMP-2, MMP-8 and MMP-9 (3-plex) (LXSAHM-03, R&D Systems) and samples required a 1:5 dilution, using Calibrator Diluent RD6-52. Assays were performed following manufacturer instructions. Samples were thawed on ice and centrifuged (13000 rpm, 5 minutes) prior to dilution and use. Each unique standard cocktail was resuspended in 100 µL of Calibrator Diluent RD6-52 and a 3-fold dilution series was created to produce the rest of the standards (S2-S6), also using Calibrator Diluent RD6-52. Calibrator Diluent RD6-52 was used as a blank. A pool sample was created to include an internal calibrator in all plates.

The procedure included a 2-hour incubation of 50 µL of standard or sample per well with the microparticle cocktail. After washing the plate three times, the sample-microparticle mixture was incubated with Biotin-Antibody cocktail for 1-hour. After re-washing the plate, Streptavidin-PE was added to each well and incubated for 30 more minutes. Finally, the plate was washed and the

microparticles were resuspended with 100 µL of Wash Buffer. The microparticles were resuspended on the plate shaker immediately prior acquisition with Magpix® analyser.

2.7.5. Raw data examination

Raw data from the multiplex assay was primarily analysed with xPONENT ® software, the software package control of the MAGPIX ® instrument. Routine procedures were performed following manufacturer instructions, including minimum of a weekly calibration (MPXCALK25, Invitrogen™) and daily (up to 24 hours) verification (MPXPVERK25, Invitrogen™).

xPONENT ® software was used as the primary source of raw data examination. Firstly, it was annotated if there were any problems during the acquisition stage (such as slow bead acquisition) or post-acquisition (low bead count). Next, the standard curve was examined with a visual inspection to see the fit of the curve and to look at potential duplicate standard point separation (if plotted separately) or high % CVs (coefficient of variation). Before validation of the standard curve, the raw data was examined for points with poor replication or outliers, that can be assessed inspecting the %CV of replicates [%CV = STDev/Mean) *100]; as well as the % Recovery [%Recovery = (Observed Standard Value/Expected Standard Value) * 100]. If necessary (high percentages of CV or Recovery), the standard curve could be edited by invalidating one of the standard point replicates and re-analysing). Finally, the R^2 values were annotated to keep record for the quality of fit, given that data yields R^2>0.985.

Separately, the proportion of detectable and out of range concentration was recorded across panels. Low out of range concentration were assigned half of the sensitivity of detection of the assay.

Finally, the coefficient of variation was calculated across all plates for standards and the internal pool calibrator to assess the potential plate-to plate variation.

2.8. Flow cytometry-based assessment of bacterial opsonisation

2.8.1. Preparation of samples for bacterial flow cytometry

The second matched cervicovaginal fluid (CVF) BBL™ CultureSwab™ MaxV Liquid Amies swabs were thawed on ice and resuspended in Amies Liquid transport medium by vigorous vortex. Contents were transferred to a 2mL Eppendorf and centrifuged (10000 rpm, 10 minutes, 4°C). Supernatant was stored at -80°C for potential future work (e.g., expand immunophenotyping assays). The pellet was resuspended in 500 µL of 3.6% paraformaldehyde in filtered sterile PBS and left at 4°C overnight.

The following day, samples were centrifuged (10000rpm, 10 mins, 4°C) and resuspended in 1mL of filtered sterile PBS, twice. Samples were centrifuged again and resuspended in 1mL of 5% BSA (A7906, Bovine Serum Albumin, Sigma-Aldrich). 100 µL of cells were stained with 0.5 µL of SYTO™ BC Green Fluorescent Nucleic Acid Stain (B7277, Invitrogen™) and the following anti-human antibodies (Table 2.7.) for 30 minutes, in the dark, at room temperature. Antibodies were titrated prior to use (further details available in chapter 5, section 5.5.). After incubation, cells were washed twice with 300 µL of PBS; and finally resuspended in 500 µL of PBS for flow cytometry acquisition on a BD FACSymphony™ A3 Cell Analyzer (BD Biosciences), where a minimum of 50000 events were collected. BD FACSDiva and Flowjo v10 software were used for instrument setup and data analysis.

Table 2.7. Monoclonal anti-human antibodies for opsonization of bacteria by flow cytometry

Antibody	Fluorochrome	Clone	Brand/ Catalogue Number	Isotype Control	Brand/ Catalogue number/Clon
Anti- IgG	APC	IS11-3B2.2.3	Milteny Biotec/ 130-119-772	Mouse IgG1κ	eBioscience 17-4714-42
Anti- IgA	APC	IS11-8E10	Milteny Biotec/ 130-113-998	Mouse IgG1κ	
Anti- C3b	Alexa Fluor-647*	10C7	Invitrogen/ MA1-70054	Mouse/IgG1	BD Pharmigen™ 566011 MOPC-31C

*In house conjugation using AF647 NHS Ester (APC-005-1, Jena Bioscience). Protocol provided by Dr Alexiane. Decout. Antibody was incubated with label for 1 hour, at room temperature in the dark. After, unbound fluorescent label was removed using Zeba™ Spin Desalting Columns (89882, Thermofisher Scientific). Briefly, columns were prepared washing with 300 µL of PBS the top resin bed of the column for 3 times (centrifuge at 1500 x g, 1 minute). Later, the column was placed in a new collection tube; and sample was applied at the top of the compact resin bed. Finally, a centrifuge (1500 x g, 2 minutes) was undertaken to collect the sample.

During the protocol optimisation, Cell Trace™ Far Red Cell stain (Invitrogen™, C34564) was also used. To establish the flow cytometry settings, 1µm polystyrene beads (Invitrogen, A37362) and 6µm polystyrene beads (Invitrogen, B7277B part of the bacterial counting kit (B7277)) were used as a size reference (details of protocol optimisation in Chapter 5, section 5.5.).

2.8.2. Bacterial cultures for controls

Pure bacterial cultures were used both as positive (bacterial detection) and negative (no binding to immunoglobulins or opsonins) controls (see protocol optimisation in chapter 5, section 5.5.). Bacterial

pellets from liquid cultures were stained and analysed under the same conditions. Three bacterial cultures representative of the vaginal microbiota composition were provided by Lauren Roberts (Marchesi lab): *L. crispatus* (DSM 20584), *L. iners* (DSM 13335) and *G. vaginalis* (DSM4944). Briefly samples were plated onto agar plates and incubated overnight in an anaerobic chamber (10% CO_2, 10% H_2, 80% N_2) 37°C and 70% humidity. Next, the culture was placed into degassed medium and incubated overnight again. 1mL of this liquid culture suspension was provided. Samples were prepared for flow cytometry under the same conditions as previously described.

2.9. Imaging techniques

2.9.1. Giemsa smear

The Giemsa stain was used to detect neutrophils in cytobrush samples. After cytobrush sample processing (Section 2.4.2), a drop (~10µL) was placed on to a microscope slide. Using another slide, the sample was smeared over the slide creating a very thin film (Figure 2.9). Methanol was used to cover the slide and fix the sample (2 minutes of incubation). After removing the excess methanol, Giemsa solution (1:10) was added to cover the whole slide. After 20 minutes of incubation, slides were rinsed with water and left to dry. Slides were observed on a brightfield microscope.

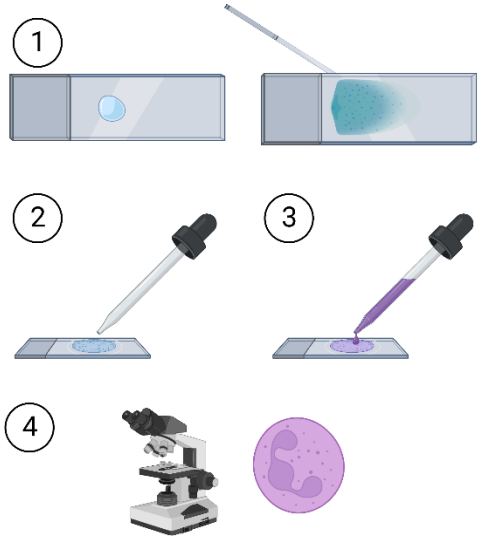


Figure 2.9. Giemsa smear for detection of neutrophils in cytobrush samples. 1) A thin smear was created over a slide. Then, the sample was 2) fixed with methanol (2 minutes) and covered with 3) Giemsa solution (20 minutes). 4) Under direct visualisation on a brightfield microscope it is possible to recognise neutrophils by detecting multinucleated morphology in purple.

2.9.2. Confocal microscopy of cytobrush samples

After cytobrush sample processing (Section 2.4.2.), 100µL were stained as previously described (section 2.5.). Cell surface staining was selected to identify structures of relevance for immunocytochemical characterisation, including anti-CD15–FITC (clone H198, Cat#2007711, Invitrogen); anti-CD16–AlexaFluor450 and anti-CD63–APC (Table 2.1). After incubation and washing with PBS, samples were immobilised on agar slides (1% agarose), and ~10 µL were placed onto the centre of the slide. A cover slip was applied, and the sample was allowed to dry. Samples were analysed using Leica SP5 confocal microscope.

2.9.3. Confocal microscopy of bacterial liquid cultures

Bacterial liquid cultures *L. crispatus* (DSM 20584), *L. iners* (DSM 13335) and *G. vaginalis* (DSM4940) (section 2.8.2.) were fixed overnight and washed with PBS twice, to finally resuspend samples with 1mL of 5% BSA (section 2.8.1.). 100µL of each bacterium was stained with 0.5 µL of SYTO nucleic acid stain for 30 minutes. After washing the cells with PBS, bacterial pellet was resuspended in 1% agarose solution and 20 µL were placed on the centre of a slide and covered with a cover slip. Samples were analysed using Leica SP5 confocal microscope.

2.10. Data and Statistical analysis

2.10.1. Bioinformatics for Microbiome Analysis

The following bioinformatics section was performed by in house bioinformaticians Dr Samit Kundu and Dr Sherrianne Ng, unless otherwise stated. Cutadapt was used for primer sequences trimming (Martin, 2011). ASV (amplicon sequence variant) counts per sample were calculated using the QIIME2 bioinformatics pipeline (version2022.2.1) (Bolyen et al., 2019). DADA2 (version 2022.2.0) (Callahan et al., 2016) was utilised for denoising. Finally, the STIRRUPS database (Fettweis et al., 2012) was used for taxonomic classification of ASVs.

At species level, samples were classed using the VALENCIA centroid classification algorithm (France et al., 2020) into five community state types. Four of them were dominated by a single species of *Lactobacillus*: CST I (*L. crispatus*), CST II (L. *gasseri*), CST III (*L. iners*) and CST V (*L. jensenii*). The last one was characterised by reduced lactobacilli and increased diversity: CST IV (heterogeneous).

Transitions of the vaginal microbiota groups across pregnancy was visualised using a Sankey Diagram created with (SankeyMATIC) (https://sankeymatic.com/). Further analysis to compare proportional

differences was performed with Graphpad Prism 9. Data visualization and calculation of diversity (Shannon Index, species observed) was performed in Microbiome Analyst 2.0 (Chong et al., 2020) (https://www.microbiomeanalyst.ca/).

2.10.2. Immunophenotyping

Luminex Multiplex Immunoassays were acquired with a Magpix ® analyser and xPONENT ® software was used as the primary source of raw data examination. Coefficient of variation between internal calibrators was analysed using Microsoft Excel (v2211). Statistical analysis of cytokines, complement and matrix metalloproteinases analytes from CVF were performed using Graphpad Prism 9, to compare differences between groups, as well as to establish correlations between concentrations of different analytes.

2.10.3. Flow cytometry data

Flow cytometry data for assessment of the immunophenotype of peripheral and cervical neutrophils was acquired with the Cytek® Aurora flow cytometer together with the SpectroFlo® software package. Further analysis was performed using FlowJo software (Flowjo, LLC). Flow Cytometry data for evaluation of the percentage of bacteria coating was acquired with BD FACS Symphony™ A3 Cell analyser together with BD FACSDiva software. Further analysis was performed using Flowjo software.

Statistical analysis using Graphpad Prism 9 was used to compare markers of expression (MFI) and percentages of bacterial coating between groups.

2.10.4. Statistical analyses

Statistical analyses were performed with Graphpad Prism 9. Differences between two groups were analysed with a t-test or a Mann Whitney test, depending on if the data was normally distributed or non-parametric, respectively. Differences between three or more groups were analysed with one-way ANOVA test or Kruskal-Wallis, also depending on the distribution of the data. For proportional differences, the Fisher's exact test or Chi square test was used. To correlate concentration of different analytes and parameters, Spearman correlation was used. Finally, in all cases, a p-value <0.05 was taken as the level of statistical significance.

Details on statistical analysis are also specified and summarised in each results section.

3. LOCAL CERVICOVAGINAL MICROBIAL-DRIVEN IMMUNE RESPONSE IN HEALTHY PREGNANCY AND IN WOMEN WHO DELIVER PRETERM

3.1. Chapter Summary

Introduction

Cervical shortening during pregnancy and preterm birth are associated with an adverse vaginal microbial composition, which is typically characterised by *Lactobacillus* species depletion and increased bacterial diversity. While most studies have focused on establishing associations between the vaginal microbial composition and pregnancy outcome, there is growing evidence that host immune response to specific vaginal microbial compositions is a key determinant of preterm birth risk. Despite this, the mechanism underpinning microbial-driven inflammation and preterm birth is not fully understood.

Hypothesis

- There is a dysregulated immune response in women who undergo cervical shortening and in women who deliver preterm
- The local cervicovaginal microbial composition modulates the local immune response
- The complement system plays a role in microbial driven cervical shortening and preterm birth

Aims

- To describe the vaginal microbial composition in women who undergo cervical shortening and women who deliver preterm
- To determine levels of local immune mediators (cytokines, complement proteins, matrix metalloproteinases) in CVF samples collected from women at high-risk of cervical shortening and preterm birth
- To characterise the relationship between local immune mediators and microbial composition in the context of cervical shortening, and pregnancy outcome

Methods

Cervicovaginal fluid was collected using swabs from 51 pregnant women at two timepoints in pregnancy: 12-16 weeks, and 20-24 weeks. Study participants were divided by outcome into women

who had a spontaneous preterm birth (n=17) or women who had a term birth without intervention (n=34). CVF samples were assessed for microbial composition by sequencing the V1-V2 region of bacterial 16S rRNA genes using an Illumina MiSeq; and supernatant was used for cytokines, complement protein and matrix metalloproteinases analysis with Luminex Multiplex immunoassays.

Results

Women who experienced cervical shortening (≤25mm) were more likely to have a high-risk microbial composition (54% CST III or CST IV), compared to women that maintained a normal cervical length (>25mm) throughout pregnancy (36%) (p=0.2313). At timepoint A, women who experienced cervical shortening exhibited significantly increased concentrations of IL-1β (p<0.0001), IL-6 (p=0.0046) and IL-8 (p<0.0001), compared to women who maintained a normal cervical length. An increased concentration of complement proteins was also seen in women who developed a short cervix via the classical (C1q, p=0.0038 at timepoint A, p=0.0009 at timepoint B; C2, p=0.0043 at timepoint A, p=0.0173 at timepoint B; and C4, p=0.0003 at timepoint A, p=0.0099, at timepoint B), lectin (MBL at timepoint B p=0.0084) and alternative pathways (Factor D at timepoint A, p=0.0460). Downstream complement proteins (C3a at timepoint A, p=0.0046; C3b at timepoint A, p=0.0192 and timepoint B p=0.0114; C5a, at timepoint A p=0.0028 and at timepoint B, p=0.0270) and inhibitors (Factor H and Factor I, p<0.05) were also increased. Additionally, MMP-2, MMP-8 and MMP-9, enzymes involved in tissue remodelling, were elevated in women who experienced cervical shortening compared to women who maintained a normal cervical length (p=0.0001, p=0.0061, p=0.0257, respectively at timepoint A).

There was a higher proportion of women who delivered preterm that had a high-risk microbial composition (35% CST III, 17% CST IV) compared to women who delivered at term (29% CST III, 8% CST IV) at timepoint B (p=0.6708). A strong local inflammatory response, characterised by increased levels of cytokines, complement proteins and MMPs was seen in women who delivered preterm. The concentration of IL-1β was higher in women who delivered preterm compared to women who had a term pregnancy as early as timepoint A (p<0.0001), as well as IL-6 (p<0.001) and IL-8 (p<0.05). The concentration of several complement proteins was also increased in women who delivered preterm, including C1q (p<0.05 at timepoint A, p<0.01 at timepoint B), C4 (p<0.001), MBL (p<0.05 at timepoint A), C3a and C3b (p<0.05). All MMPs were significantly increased in women who deliver preterm at timepoint A, especially MMP-9 (p<0.001).

A diverse microbial composition (CST IV) was associated with increased concentrations of complement proteins compared to CST I, such as C1q and C4 ($p<0.001$), Factor B and Factor D ($p<0.0001$), C3 ($p<0.001$) and C3b ($p<0.0001$). The concentration of MMPs did not differ according to CST vaginal composition. In contrast to expectations, preterm birth and cervical shortening in women with CST I compositions were also associated with an increased inflammatory response in a proportion of women.

Conclusion

There is an augmented inflammatory response involving cytokines, complement proteins and matrix metalloproteinases in the cervicovaginal milieu in women who undergo cervical shortening and in women who delivered preterm. These data support the role of an adverse microbial composition and augmented immune response in the process of cervical shortening and preterm birth. However, these data also reveal local heightened inflammation in the context of a low-risk microbial composition in a proportion of women with adverse pregnancy outcome, alluding to the possibility of a non-microbial driven inflammatory mechanism.

3.2. Introduction

Spontaneous preterm birth is a syndrome with multiple aetiologies, although cervical insufficiency and microbial-induced inflammation are the two most commonly described mechanisms leading to spontaneous preterm birth (Romero et al., 2014a). The immunological processes during pregnancy and physiological term labour are very complex and tightly regulated in order to achieve a successful pregnancy, with contributions and adaptations of both the innate and adaptive immune responses. There is an obvious overlap in the processes and events required for both term and preterm labour, although the latter is triggered earlier, and is likely to be associated with a dysregulated inflammatory response.

Cervicovaginal inflammation has been long associated with preterm birth, especially in the context of chorioamnionitis, intraamniotic infection, and PPROM (Amabebe et al., 2018; Jun et al., 2000; Rizzo et al., 1996; Sandoval-Colin et al., 2023; Tanaka et al., 1998). Higher concentrations of cytokines and chemokines in CVF such as IL-1β, IL-6, IL-8 and TNF-α have been described compared to women with uncomplicated pregnancies. Cervicovaginal fluid contains a vast number of proteins apart from cytokines, that can be implicated in the immune response, such as granulocyte elastase (Nakai et al., 2005), sialidases (Andrews et al., 1999), defensins (Elovitz et al., 2019), matrix metalloproteinases (Nguyen et al., 2023) and complement proteins (Livson et al., 2022, 2021).

Matrix metalloproteinases (MMPs) are enzymes that degrade collagen and are implicated in the remodelling of the extracellular matrix. Therefore, they have been implicated in the physiological and pathophysiological processes of ruptures of membranes (Maymon et al., 2000; Nguyen et al., 2023) and cervical ripening and dilatation (Amabebe et al., 2022b; Choi et al., 2009; Heng et al., 2012; Sennström et al., 2003; Stygar et al., 2002). In general, the equilibrium between expression of MMPs and TIMPs will tip the balance towards stability or degradation of ECM. As reviewed by Geng et al., rupture of membranes in normal parturition and preterm birth is associated with an upregulation of MMP-1, MMP-2, MMP-8 and MMP-9, together with downregulation of TIMP-1, and -2. Several MMPs are also implicated with cervical ripening (upregulation of MMP-1, -2, -3 -7, -8 and -9) (Geng et al., 2016).

The complement system acts as a bridge between the innate and the adaptive immune system. It is highly complex and comprises more than 30 soluble proteins. Activation of these proteins occur sequentially, forming a cascade of events. As its name indicates, its role is to “complement” and enhance the ability of phagocytic cells and antibodies to clear infection. The complement system has been well studied as a major contributor of autoimmune diseases such as systemic lupus erythematosus (SLE) (Macedo and Isaac, 2016), rheumatoid arthritis, and anti-phospholipid antibody

syndrome (Chen et al., 2010). It is well established that women with these conditions have a higher risk of pregnancy complications such as miscarriage and preterm birth (Chighizola et al., 2020). Complement proteins have been detected in fetal membranes (Lappas et al., 2012; Segura-Cervantes et al., 2015) and have been implicated in key important processes such as implantation, placentation, and spiral artery vascularization (Dunn et al., 2017; Regal et al., 2015). Circulating complement proteins have also been studied in pregnancy, and pregnancy complications like preeclampsia (Lynch et al., 2008b), and preterm birth (Soto et al., 2005). More recently there has been growing interest in the potential role of complement in local microenvironments such as amniotic fluid (Kim et al., 2018) and cervicovaginal fluid (Chan et al., 2022; Livson et al., 2022, 2021). A summary of key findings is available in Table 1.4. (Introduction). To date, the study of complement activation in CVF has been limited to only a few proteins. Livson et al. reported activation of C3 (Livson et al., 2021) and Factor B (Livson et al., 2022) in CVF using western blotting techniques, detecting higher levels closer to and in labour, compared to non-pregnant woman. Chan et al. started to reveal the implications of the complement system in the context of vaginal microbial composition measuring several complement proteins (MBL, C5, C5a, C3b) in CVF in a longitudinal cohort of women at high-risk of delivering preterm. They detected higher levels of MBL, C3b and C5 in women with a high-risk microbial composition (defined as CST III or *Lactobacillus* deplete communities). Further, elevated concentrations of these proteins were detected in women who delivered preterm, compared to term (Chan et al., 2022).

There is growing evidence that the vaginal microbiota plays an important role in shaping preterm birth risk (Introduction, Table 1.2), with consistent reports of *Lactobacillus* spp. being protective and associated with term delivery, especially when the niche is dominated by *L. crispatus* (CST I). In contrast, *L. iners* (CST III) and high-diversity compositions enriched with heterogenous anaerobic bacteria (CST IV), are associated with increased risk of PTB. However, only a few recent reports have linked vaginal microbial composition with inflammatory response in the context of pregnancy outcome, and these have mainly been limited to reports on cytokine and chemokines (Elovitz et al., 2019; Fettweis et al., 2019; Florova et al., 2021; Kindinger et al., 2016).

Early cervical shortening during pregnancy is a major risk factor for preterm birth (Iams et al., 1996; Wang et al., 2021). Cervical shortening during pregnancy is also associated with adverse vaginal composition, specifically with communities dominated by *L. iners* (CST III) and with reduced abundance of *Lactobacillus* spp. (Chan et al., 2022; Di Paola et al., 2020; Kindinger et al., 2017). Furthermore, women with a CST III who developed a short cervix had a stronger inflammatory response (higher concentrations of IgM, C3b, C5, IL-6) than women with the same vaginal microbial composition who did not shorten (Chan et al., 2022).

In this chapter data describing the local innate immune response in cervicovaginal fluid during pregnancy, with detailed evaluation of the complement cascade and matrix metalloproteinases, and its associations with the vaginal microbial composition and pregnancy outcome will be presented.

3.3. Study design

Pregnant women at high-risk of delivering preterm were recruited from several preterm birth prevention clinics from different UK hospitals (Queens Charlotte's & Chelsea Hospital, St Marys Hospital, University College London Hospital, Chelsea and Westminster Hospital). Women were recruited between June 2018 and March 2021. Women who provided matched samples across pregnancy (timepoint A: 12-16 weeks of gestation; and timepoint B: 20-24 weeks of gestation) were included in the study. A total of 17 women who experienced spontaneous preterm birth matched these criteria, and 34 women with term uncomplicated pregnancies with similar risk factors were selected to serve as controls at a ratio of 1:2. Detailed maternal metadata was collected from all the participants.

Bacterial DNA was extracted from vaginal swabs for 16S rRNA gene sequencing of V1-V2 hypervariable regions, as described in Chapter 2, Section 2.6.

Luminex Multiplex Assays was performed on supernatant from vaginal swabs to measure cytokines, complement proteins and matrix metalloproteinases as described in Chapter 2, Section 2.7.

3.4. Statistical analysis

Statistical analysis was performed using Graphpad Prism 9. Comparisons between two groups of normally distributed data (e.g., gestational age at delivery, gestational age at sampling, maternal age) was analysed with an unpaired t-test. All immunological data was not normally distributed, therefore non-parametric tests were used. Differences between two groups were analysed using the Mann Whitney test. Differences between three or more groups were analysed using the Kruskal Wallis test with the Dunn's post hoc test. To analyse proportional differences, the Fisher's exact or Chi square test were used. In all cases, statistical significance was accepted with p-values < 0.05. Data was reported and plotted as median with interquartile range (IQR), or median with standard deviation, depending on if the data was normally or not-normally distributed, respectively.

3.5. Standardisation techniques for Multiplex Luminex Immunoassays

A total of 102 CVF samples were assayed on duplicates across three 96-well plates. The coefficient of variation between plates was calculated for all standards and internal calibrator (Table 3.1., Table 3.2., Table 3.3.). Overall, the level of variation between plates was low. The variation was greater in the less concentrated standards and controls. The standards were also plotted for comparison across different plates for each cytokine, complement protein and MMP analyte (Appendix 3.1., Appendix 3.2., and Appendix 3.3., respectively), to confirm kit reproducibility. Quality Controls from the Human Complement panels were within the minimum and maximum expected by manufacturer's instruction, except for C1q, that was consistently just below the range, and C3 that was consistently just outside the upper range (Appendix 3.4. and 3.5.). All standard curves generated had R^2 value greater than 0.99. (Table 3.4.).

Table 3.1. Coefficient of variation for each cytokine analyte

	Cytokines Panel		
	IL-1β	**IL-6**	**IL-8**
S1	3.31	1.77	2.52
S2	5.44	4.75	5.98
S3	4.65	4.10	4.12
S4	3.38	2.69	4.36
S5	2.10	0.96	3.31
S6	1.63	0.43	1.26
Calibrator	12.89	14.53	11.68

S: Standard. S1-S6 represents each standard after serial dilution, being S1 the most concentrated.

Table 3.2. Coefficient of variation for each complement analyte

A) Human Complement Panel 1

	Complement Panel 1						
	C2	**C4b**	**C5**	**C5a**	**Factor D**	**MBL**	**Factor I**
S1	1.74	1.96	4.99	4.46	2.28	1.04	9.04
S2	2.58	2.80	3.74	2.33	2.44	1.97	4.12
S3	4.88	7.12	4.49	4.07	4.42	6.43	6.63
S4	3.44	2.54	2.10	2.70	3.51	5.35	3.23
S5	5.26	4.53	4.46	3.49	3.96	2.32	2.69
S6	3.60	4.60	7.25	3.39	3.71	2.71	1.86
S7	2.83	1.57	1.85	1.62	0.22	0.85	1.15
QC1	12.57	24.66	6.89	16.56	4.62	12.33	15.30
QC2	3.40	18.64	3.19	6.59	4.27	0.82	15.71

S: Standard. S1-S7 represents each standard after serial dilution, being S1 the most concentrated. QC1,2: Quality control provided by manufacturer.

B) Human Complement Panel 2

	Complement Panel 2					
	Subset A				Subset B	
	C3	**C3b**	**C4**	**Factor H**	**C1q**	**Factor B**
S1	6.04	n.a.	0.84	1.08	0.90	0.15
S2	3.61	11.43	0.23	1.04	0.86	1.04
S3	2.12	6.31	1.54	2.81	1.39	1.13
S4	2.43	3.62	2.29	2.58	1.60	1.07
S5	2.48	1.23	2.90	2.81	3.54	2.06
S6	1.75	2.19	2.45	2.08	2.81	0.72
S7	0.59	0.19	0.53	0.73	0.54	1.28
QC1	12.18	2.88	3.30	11.03	13.90	7.89
QC2	2.43	5.41	1.64	4.22	8.49	6.75

S: Standard. S1-S7 represents each standard after serial dilution, being S1 the most concentrated. QC1,2: Quality control provided by manufacturer.

C) C3a simplex panel 3

	C3a
S1	n/a
S2	14.05
S3	12.72
S4	4.31
S5	2.34
S6	0.62
S7	0.92
Calibrator	9.61

n/a.: not aplicable. S: Standard. S1-S7 represents each standard after serial dilution, being S1 the most concentrated.

Table 3.3. Coefficient of variation for each matrix metalloproteinase analyte

	MMPs Panel		
	MMP-2	**MMP-8**	**MMP-9**
S1	1.26	0.47	1.55
S2	0.59	0.43	0.27
S3	0.75	0.57	0.18
S4	0.58	0.41	0.74
S5	0.59	0.18	0.84
S6	0.17	0.14	0.08
Calibrator	33.78	28.34	13.24

S: Standard. S1-S6 represents each standard after serial dilution, being S1 the most concentrated.

Table 3.4. Evaluation of R^2 values for each analyte across three different plates

Values highlighted in grey indicate final value after required invalidation of points

		Plate 1	Plate 2	Plate 3
Cytokines	IL-1	0.994987	0.985987	0.988824
	IL-6	0.996225	0.993813	0.99671
	IL-8	0.99234	0.990034	0.993481
Complement analytes	C3a	0.998619	0.996927	0.996672
	C2	0.989986	0.986096	0.993413
	C4b	0.991156	0.997178	0.987737
	C5	0.990832	0.995366	0.985575
	C5a	0.991113	0.997851	0.988283
	Factor D	0.996729	0.997403	0.993116
	MBL	0.994618	0.994018	0.990824
	Factor I	0.992992	0.996192	0.987614
	C3	0.997256	0.992994	0.997604
	C3b	0.997633	0.994115	0.99757
	C4	0.998142	0.994332	0.996853
	Factor H	0.995463	0.993695	0.993899
	C1q	0.997096	0.997253	0.995543
	Factor B	0.999154	0.997953	0.992479
MMPs	MMP-2	0.998468	0.99771	0.999101
	MMP-8	0.997809	0.996317	0.998339
	MMP-9	0.998707	0.998157	0.999301

A total of 102 swabs were included in this study, except for the C1q and Factor B panel, where only 100 swabs were analysed as two women only provided a sample at one timepoint; and the matrix metalloproteinases panel, since not enough material was available.

All cytokines, complement and MMPs analytes were detectable except IL-10. Samples were considered detectable when the concentration fell within the standard curve or were automatically given an extrapolated value. Samples that were out of range (OOR) because their concentration was lower than the sensitivity of the assay were assigned half of the sensitivity value and included for further analysis. Samples that were out of range from the upper limit of the standard curve were further diluted and included for further analysis.

The detection ranges of cytokines analytes are described in Tables 3.5. The most detectable cytokine was IL-8 (92% within the standard curve, ~8% extrapolated, no values out of range). Two samples required a 1:100 dilution to obtain results within the IL-8 assay range. The least detectable cytokine was IL-6 (28% of the samples were out of range and almost half of the samples were extrapolated).

Table 3.5. Detection ranges of cytokine analytes in CVF samples

Cytokine	Standard Curve Range		Sensitivity	Units	Assigned OOR value	Samples within Standard Curve N (%)	Extrapolated samples N(%)	OOR N (%)
	Max	Min						
IL-1β	3830	15.76	0.8	pg/mL	0.4	69 (67.65%)	24 (23.53%)	9 (8.82%)
IL-6	1030	4.24	1.7	pg/mL	0.85	23 (22.55%)	50 (49.02%)	29 (28.43%)
IL-8	930	3.83	1.8	pg/mL	0.9	94 (92.16%)	8 (7.84%)	0 (0.0%)

OOR: Out of range. Value established as half of the sensitivity of detection

The detection ranges of complement analytes are described in Table 3.6. The most detectable complement protein was C3a (none of the samples were out of range), followed by C2, C4, C1q and MBL. For all the complement proteins measured, at least half of the samples were within range or extrapolated, being Factor I (50%), C3 (48%), Factor B (48%) and C4b (44.12%) the analytes with more samples out of range.

Table 3.6. Detection ranges of complement analytes in CVF samples

Complement analyte	Standard Curve Range		Sensitivity	Units	Assigned OOR value	Samples within Standard Curve N (%)	Extrapolated samples N (%)	OOR N (%)
	Max	Min						
C2	1000	1.37	0.34	ng/mL	0.17	84 (82.35%)	17 (16.67%)	1 (0.98%)
C4b	1000	1.37	0.33	ng/mL	0.165	10 (9.80%)	47 (46.08%)	45 (44.12%)
C5	2000	2.74	0.8	ng/mL	0.4	12 (11.76%)	64 (62.75%)	26 (25.49%)
C5a	3000	4.12	0.0035	pg/mL	0.00175	68 (66.67%)	24 (23.53%)	10 (9.80)
Factor D	50	0.07	0.027	ng/mL	0.0135	33 (32.35%)	36 (35.29%)	33 (32.35%)
MBL	100	0.14	0.033	ng/mL	0.0165	49 (48.04%)	48 (47.06%)	5 (4.90%)
Factor I	500	0.69	0.18	ng/mL	0.09	27 (26.47%)	23 (22.55%)	52 (50.98%)
C3	200	0.27	0.22	ng/mL	0.11	17 (16.67%)	36 (35.29%)	49 (48.04%)
C3b	6000	8.20	4.775	ng/mL	2.3875	41 (40.2%)	22 (21.57%)	39 (38.24%)
C4	400	0.55	0.146	ng/mL	0.073	88 (86.27%)	13 (12.75%)	1 (0.98%)
Factor H	300	0.41	0.092	ng/mL	0.046	99 (97.06%)	2 (1.96%)	1 (0.98%)
C1q	60	0.08	0.165	ng/mL	0.0825	72 (72%)	26 (26%)	0 (0.00%)
Factor B	60	0.08	0.022	ng/mL	0.011	28 (28%)	22 (22%)	48 (48%)
C3a	142800	35.00	0.6	pg/mL	n.a.	93 (92.16%)	9 (8.82%)	0 (0.00%)

OOR: out of range. n.a.: not applicable

Finally, Table 3.7. describes the detection ranges of MMPs analytes. The less detectable MMP was MMP-2 (55% of samples were out of range), and MMP-9 was the most detectable (almost 91% of samples were within the standard curve range). MMP-9 was the only analyte that had samples out of range above the standard curve; but lack of space within the given plates made it not possible to re-run by dilution and re-analyse; therefore 4 samples (4%) were not included for further analysis for the purpose of this PhD. The samples will be run on future plates for the purpose of future publication.

Table 3.7. Detection ranges of matrix metalloproteinases in CVF samples

MMP Analyte	Standard Curve Range		Sensitivity	Units	Assigned OOR value	Samples within Standard Curve N (%)	Extrapolated samples N (%)	OOR N (%)
	Max	Min						
MMP-2	67000	276.00	108	pg/mL	54	30 (30.3%)	15 (15%)	54 (54.55%)
MMP-8	55000	226.00	34.2	pg/mL	17.1	85 (85.86%)	10 (10%)	4 (4.04%)
MMP-9	30000	123.00	13.6	pg/mL	n.a.	90 (90.91)	5 (5%)	4 (4.04%)

OOR: out of range. n.a. not applicable.

3.6. Results

3.6.1. Study population characteristics

Fifty-one women from the VMET-2 Research Study were retrospectively included in this study. Based on the pregnancy outcome, women were divided into 2 groups: women who had a preterm delivery before 37 weeks of gestation (PT, N=17, 33.33%) and women who had an uncomplicated term delivery without requiring any type of intervention (neither cervical cerclage and/or progesterone) (TU, N=34, 66.66%). The mean gestation at delivery was 31+5 for the PT group, and 39+5 for the TU group ($p<0.0001$). From the 17 PT deliveries, 9/17 were classified as early preterm (52.9%) (<34 weeks) and 9/17 as late preterm (47%) (≥34 weeks). Only women from the PT group received any type of intervention (cervical cerclage and/or progesterone). The most common intervention was cervical cerclage (7/17, 41%), and only 3/17 (17.6%) received progesterone only. Details about the clinical characteristics from both groups are fully detailed in Table 3.8., including type of intervention and pregnancy risk factor.

Table 3.8. Study cohort demographics and clinical description

	Preterm (PT)	Term (TU)	p-value
N (%)	17 (33.33%)	34 (66.67%)	n.a.
Gestation at delivery			Unpaired t test
Mean	31+5	39+5	<0.0001
Range	24+3-36+5	37+1-42+2	****
Early preterm ≤ 33+6 N (%)	9 (52.94%)	n.a.	
Late preterm ≥ 34+0 N (%)	8 (47.06%)	n.a.	
Age			Unpaired t test
Mean	35.59	33.91	p=0.209
Range	23-42	25-41	
BMI			Mann-Whitney
Median	29.4	23.5	p=0.0048
Range	20.00-47.60	18.20-43.00	**
Ethnicity			Chi-square [a]
White	10 (58.82%)	23 (67.65%)	p=0.6420
Black	3 (17.65%)	3 (8.82%)	
Other	4 (23.53%)	8 (23.53%)	
Interventions			
Cervical cerclage	7		
History indicated (material)	2 (1 mersilene, 1 nylon)	n.a.	
USS indicated (material)	2 (1 nylon, 1 unk)	n.a.	
Other	3 (1 pregnancy, 1 abdominal, 1 Hx + USS)[c]	n.a.	
Progesterone only	3	n.a.	
Risk factor [b]			
Cx treatment	1	14	
Cx treatment + PTB	0	1	
Cx treatment +MTL	1	0	
Cx treatment + MTL + PPROM	0	1	
Uterine anomaly + PTB	1	0	
MTL	2	2	
MTL + PTB	0	0	
MTL+PPROM	1	6	
PTB	5	1	
PTB + PPROM	3	8	
PTB+MTL+PPROM	2	0	
PTB + PPROM + Hx cerclage	0	1	
PPROM	1	0	
Gravida			Mann-Whitney
Median	4	2	p=0.0042
Range	1-11	1-10	**
Parity			Mann-Whitney
Median	1	1	p=0.0394
Range	0-7	0-4	*

Table 3.8. Continuation

	Preterm (PT)	Term (TU)	p-value
Gestation at sampling			Unpaired t test
Timepoint A			
Mean	15+3	15+1	p=0.5784
Range	13+4-16+5	12+6-16+6	
Timepoint B			
Mean	21+6	22	p=0.7682
Range	19+6-24+5	19+5-24+0	

n.a. not applicable; USS: ultrasound; unk: unknown.

[a] Approximate chi-square calculations, since conditions (all expected values should be greater than 1.0 and at least 20% of the expected values greater than 5) have not been met. Ethnicity differences were also assessed combining groups and calculating Fisher's exact tests. No significant differences were observed.

[b] Risk Factors. Cx treatment: cervical treatment (e.g., LLETZ, cone biopsy, etc.). PTB: spontaneous preterm birth. MTL: mid-trimester loss. PPROM: preterm premature rupture of membranes. Hx: history

[c] One patient received two cervical cerclages: one history indicated in early pregnancy; one ultrasound indicated in mid-pregnancy.

No differences were observed regarding ethnicity between PT and TU participants (p<0.5510). Most of the participants in both groups were white (58.8% and 67.6%, respectively). The only differences observed between PT and TU groups was a higher BMI median on the former (29.4 vs 23.5; p=0.0048), as well as a higher median gravidity (4 vs 2, p=0.0042) and parity (p=0.0394).

Women from this cohort were also grouped based on the detection of cervical shortening (≤25mm) during their pregnancy. At total of 11 women (21.57%) experienced cervical shortening while 40 women (78.43%) maintained a normal cervical length during the surveillance period of pregnancy. The difference of the median shortest cervical length was statistically significant between both groups (14mm vs 31.5mm, p<0.0001). The majority of women who experienced cervical shortening delivered preterm (9/11, 81%). Table 3.9. shows the clinical characteristics of the women according to the detection of cervical shortening.

Table 3.9. Clinical characteristics of the study population according to the detection of cervical shortening

	Cervical shortening	**Normal cervical length**	**p-value**
N (%)	11 (21.57%)	40 (78.43%)	n.a.
Shortest cervical length (mm)			Unpaired t-test
Median	14	31.5	****
Range	5-25	26-40	<0.0001
Gestational age at shortening			
Mean	23+2	n.a.	n.a.
Range	14+6-32+2	n.a.	
Latency delivery			
Mean	8+6 (63 days)	n.a.	n.a.
Range	2+3 (17 days)-17+6 (125 days)	n.a.	
Cervical cerclage			
Gestation insertion			
Mean	16+2	15+2	n.a.
Range	12+5-22+6	n.a.	
History indicated (material)	2 (1 nylon, 1 mersilene)	1 (1 nylon)	
USS indicated (material)	2 (1 nylon, 1 unk)	0	
Other	3 (1 pregnancy, 1 abdominal, 1 Hx+ USS)	0	
Gravida			Mann-Whitney
Median	3	2.5	p=0.1722
Range	1-11	1-11	
Parity			Mann-Whitney
Median	1	1	p=0.1749
Range	0-4	0-7	
Outcome			Fisher's exact
Preterm	9 (81.81%)	8 (20%)	***
Term	2 (18.18%)	32 (80%)	0.0003
Gestation at sampling			
Timepoint A			Unpaired t-test
Median	15+5	15+3	0.0765
Range	14+4-16+6	12+6-16+5	
Timepoint B			Unpaired t-test
Median	21+6	22+1	0.4059
Range	20+6-22+6	19+6-24+5	

n.a. not applicable; USS: ultrasound; unk: unknown

3.6.2. Cervicovaginal microbial composition

A total of 102 vaginal swabs were obtained from 51 women through pregnancy at two different timepoints (A: 12-16 weeks; B: 20-24 weeks). 16S rRNA sequencing was performed to species taxonomic levels and samples were classified to different Community State Types (CSTs): CST I: *Lactobacillus crispatus*, CST II: *Lactobacillus gasseri*, CST III *Lactobacillus iners,* CST IV: diverse species or CST V*: Lactobacillus jensenii*. The most common CST in the overall population was CST I *L. crispatus* (N=51/102, 50%), followed by CST III (*L. iners*, N=27/102, 26.5%) and CST IV (N=15/102, 14.7%). Samples were less frequently classified as CST V *L. jensenii* (N=5/102, 4.9%) and CST II *L. gasseri* (N=4/102, 3.9%) (Figure 3.1.A).

The alpha diversity was measured by the Shannon Index. Samples classified as CST IV had higher alpha diversity (median Shannon Index 0.86) compared to samples classified as CST I *L. crispatus* (median 0.036) and CST III *L. iners* (median 0.048) (Kruskall-Wallis test, p=0.0002, p=0.0079, respectively) (Figure 3.1B). The richness (species observed) in the total population (Figure 3.1.C) followed a similar trend; samples classified as CST IV had a higher median of species observed, compared to CST I and CST III (Kruskal-Wallis, p=0.0002).

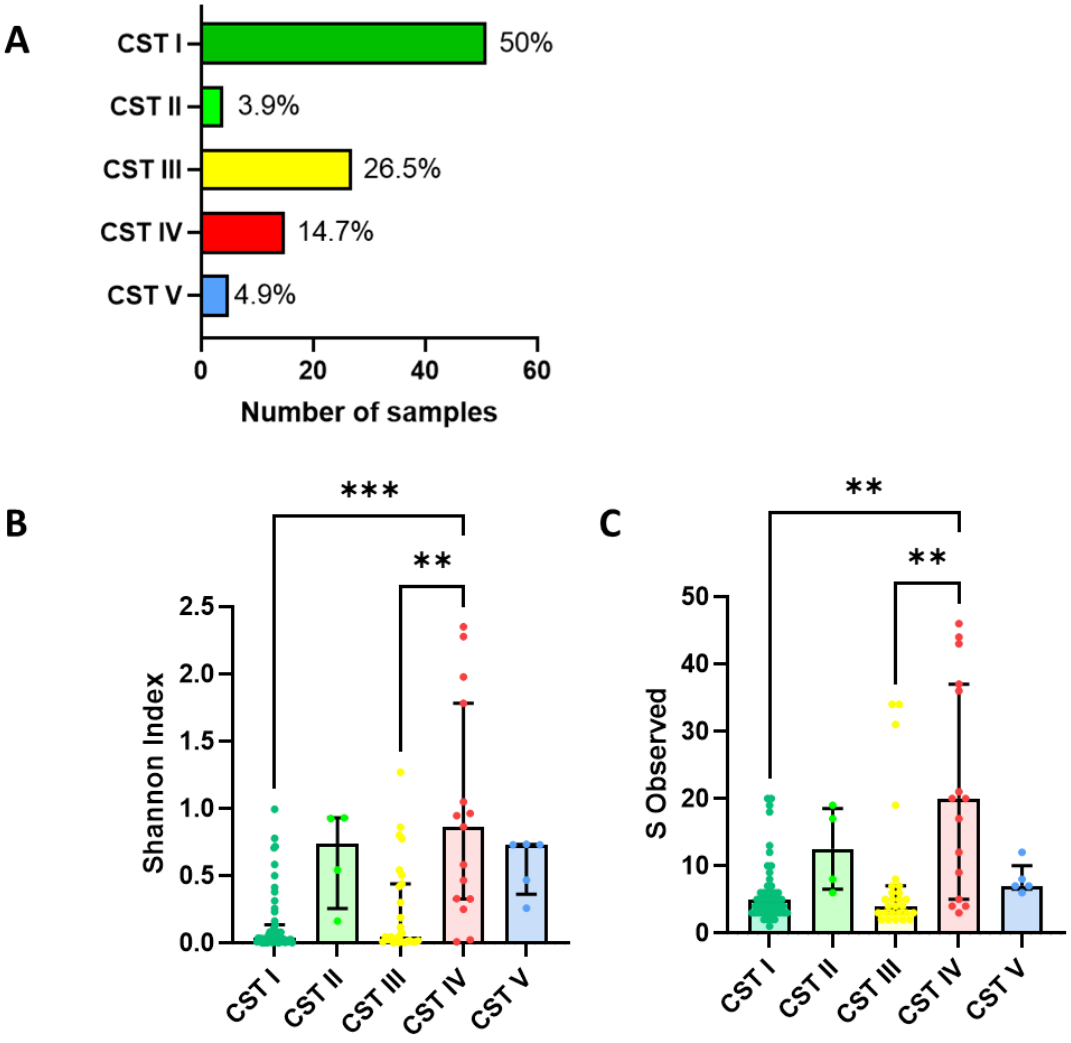


Figure 3.1. Vaginal microbial composition and structure. CVF samples were assigned to CST I (dark green; dominated by *Lactobacillus crispatus*) (n=51/102), CST II (light green, dominated by *L. gasseri*) (n=4/102), CST III (yellow, dominated by *L. iners*) (n=27/102), CST IV (red, heterogenous anaerobic bacteria) (n=15/102) or CST V (blue, *L. jensenii*) (n=5/102). **A)** Frequency of assigned Community State types (CSTs) of 102 samples from 51 pregnant women. **B)** Shannon diversity index and **C)** richness (Species observed) in the total population. Statistical analysis performed with Kruskal-Wallis test and Dunn's Multiple Comparison post hoc test. **p<0.01; ***p<0.001. Data is presented as median values with interquartile range.

The local vaginal microbial composition was divided by sampling timepoint. The most common CST at both timepoints A and B was still CST I (51% and 49%, respectively), followed by CST III (21.6% and 31.4%) and CST IV (17.7% and 11.7%) (Figure 3.2. A, B). Most women had a stable vaginal microbial composition between early and mid- pregnancy (Figure 3.2.C), and only 5 women transitioned between timepoint A and B. All samples transitioned towards a CST III vaginal composition, from CST I (n=1), CST IV (n=3) and CST V (n=1). No differences were observed in the Shannon Index (Figure 3.2.D) or the richness (species observed, Figure 3.2.E) between timepoint A and B (Mann-Whitney test, p=0.8520, p=0.8751, respectively).

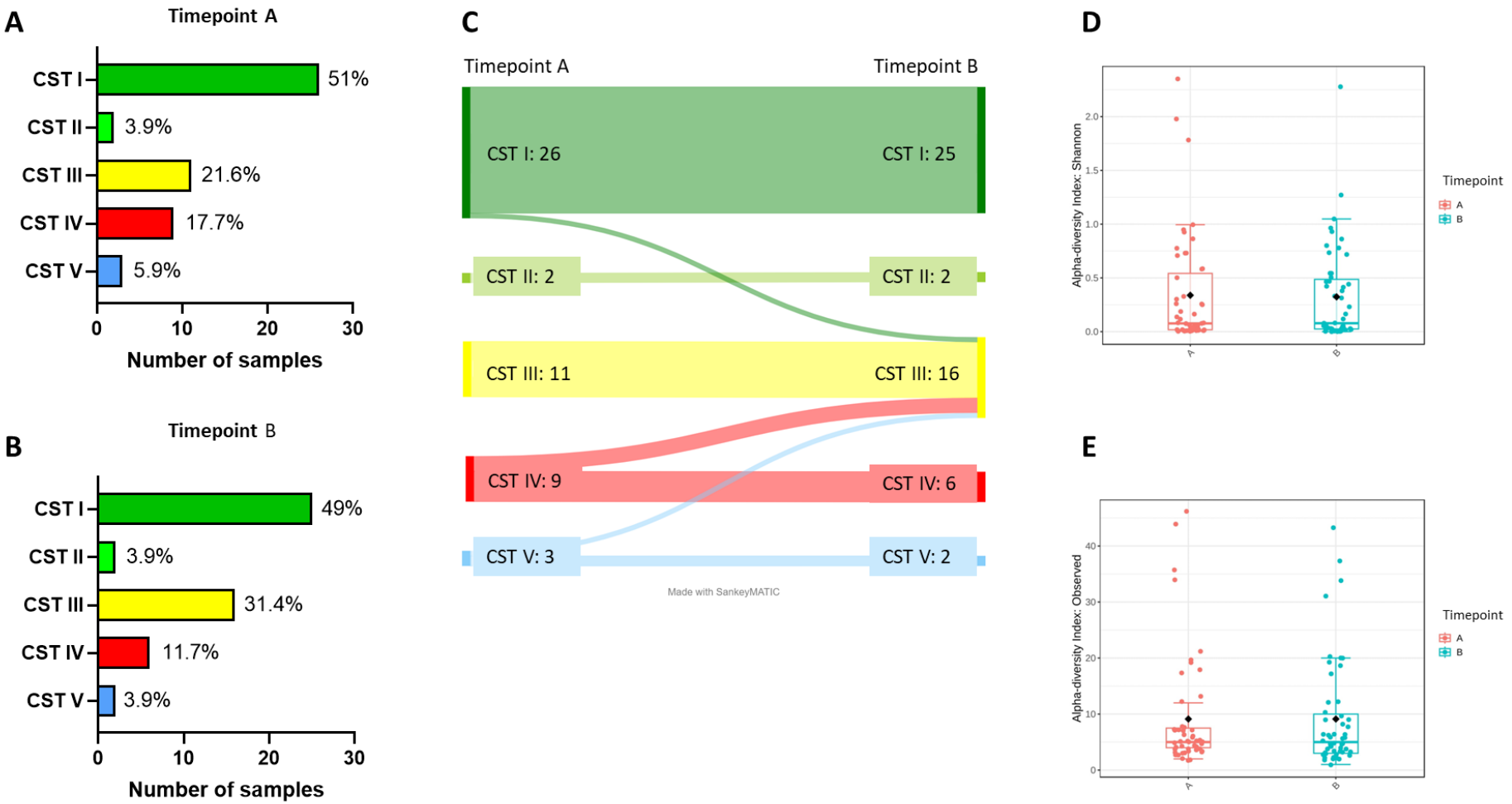


Figure 3.2. Vaginal microbial composition and structure by gestational age. A) Frequency of assigned Community State Types (CSTs) at **A)** timepoint A (12-16weeks) (n=51 samples) and **B)** timepoint B (20-24 weeks) (n=51 samples) of N=102 samples from 51 pregnant women. **C)** Sankey diagram depicting transitions between CST from timepoints A to B. Image created with sankeymatic.com. **D)** Diversity (Shannon Index) and **E)** richness (species observed) between timepoint A and B. Data is presented as median values with interquartile range.

3.6.3. Cervicovaginal microbial composition and immune response in women who undergo cervical shortening

Cervical remodelling and shortening that occurs early in pregnancy, and in the absence of contractions is pathological, and places women at higher risk of preterm birth. This is in contrast to cervical shortening and remodelling in term birth, which is a physiological process, and usually occurs in the presence of painful contractions, and after 37 weeks. From the cohort of 51 women included in the study, 11 women experienced cervical shortening (cervical length ≤25mm) at some point during the surveillance period in the preterm birth prevention clinic (N=11/51, 21.5%). From all the women who experienced cervical shortening, almost all (81%) had a preterm delivery (9/11), whereas only 20% of women with a normal cervical length (8/40) ultimately delivered preterm (p=0.0003). The mean gestational age at which cervical shortening event occurred was 23+2 and the mean latency until delivery was 63 days (Table 3.9.).

Cervicovaginal microbial composition in cervical shortening

There was a higher proportion of women with CST III (36%) and CST IV (18%) in women who experienced cervical shortening compared to women who maintained a normal cervical length (CST III, 23%; CST IV, 13%; the majority being CST I, 53%) (Figure 3.3.A). Women who experienced cervical shortening had an increased bacterial alpha diversity (n=11; median Shannon index 0.54) compared to woman who maintained a normal cervical length (n=40, median Shannon index 0.28) (p=0.045) (Figure 3.3.B). No differences were observed in richness (species observed) between the two groups (p=0.0901) (Figure 3.3.C).

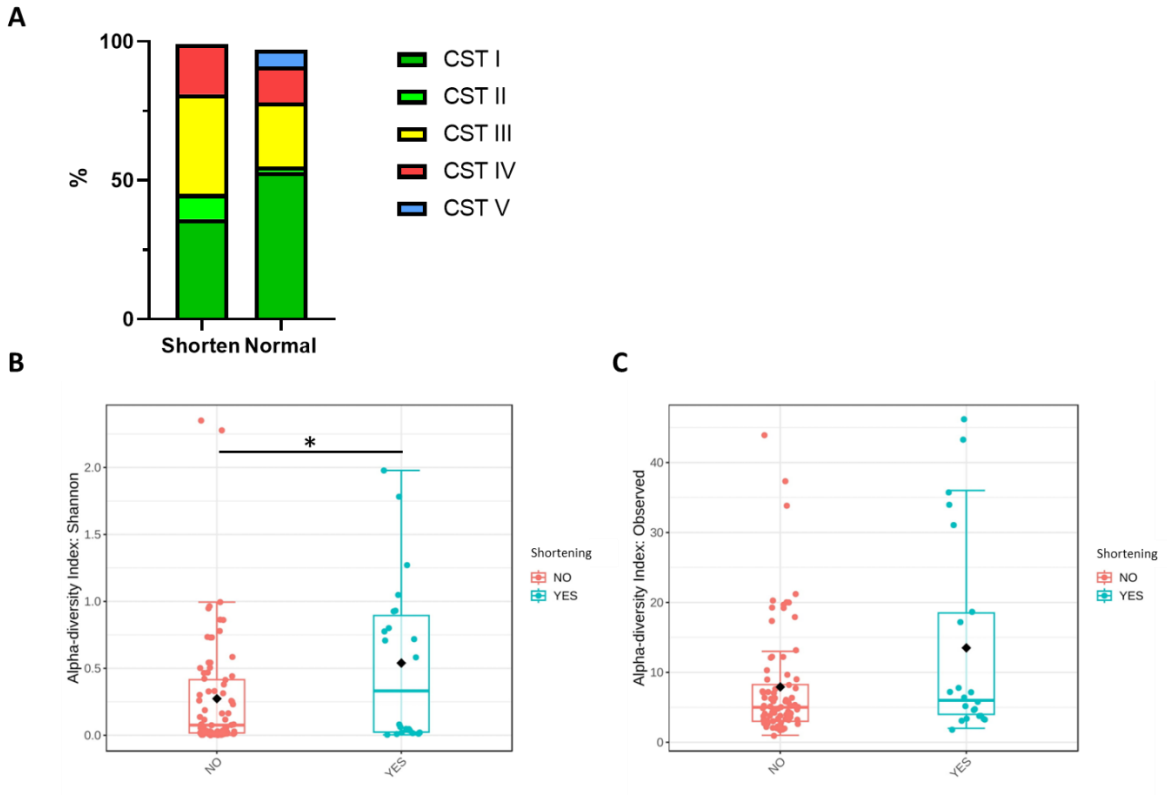


Figure 3.3. Vaginal community state types (CST) in women who experience cervical shortening. CVF samples were assigned to CST I (dark green; dominated by *Lactobacillus crispatus*), CST II (light green, dominated by *L. gasseri*), CST III (yellow, dominated by *L. iners*), CST IV (red, heterogenous anaerobic bacteria) or CST V (blue, *L. jensenni*). **A)** Comparison of proportion of CSTs in women who experienced cervical shortening (≤25mm) (n=11) compared to woman who maintained a normal cervical length (>25mm) (n=40). Statistical analysis was performed with Chi-square test. **B)** Diversity (Shannon Index) and **C)** richness (species observed) in women who experience cervical shortening. Statistical analysis between two groups was performed with Mann-Whitney one-tailed test. * p<0.05. Data is presented as median values with interquartile range.

Cervicovaginal cytokine profile in cervical shortening

Three proinflammatory cytokines (IL-1β, IL-6 and IL-8) were quantified from CVF samples obtained during early pregnancy (timepoint A: 12-16 weeks of gestation) and mid-pregnancy (timepoint B: 20-24 weeks of gestation). Women who shortened had higher median concentrations of the following cytokines compared to women who had a normal cervical length at both timepoint A: IL-1β (735.2 vs 33.56 pg/mL, p<0.0001), IL-6 (6.253 vs 0.8500 pg/mL, p=0.0046) and IL-8 (7801 vs 689 pg/mL, p=0.0008); and at timepoint B: IL-1β (201 vs 59.94 pg/mL, p=0.0323), IL-6 (1.344 vs 0.8500 pg/mL, p=0.0526) and IL-8 (2250 vs 705 pg/mL, p=0.0207) (Figure 3.4.).

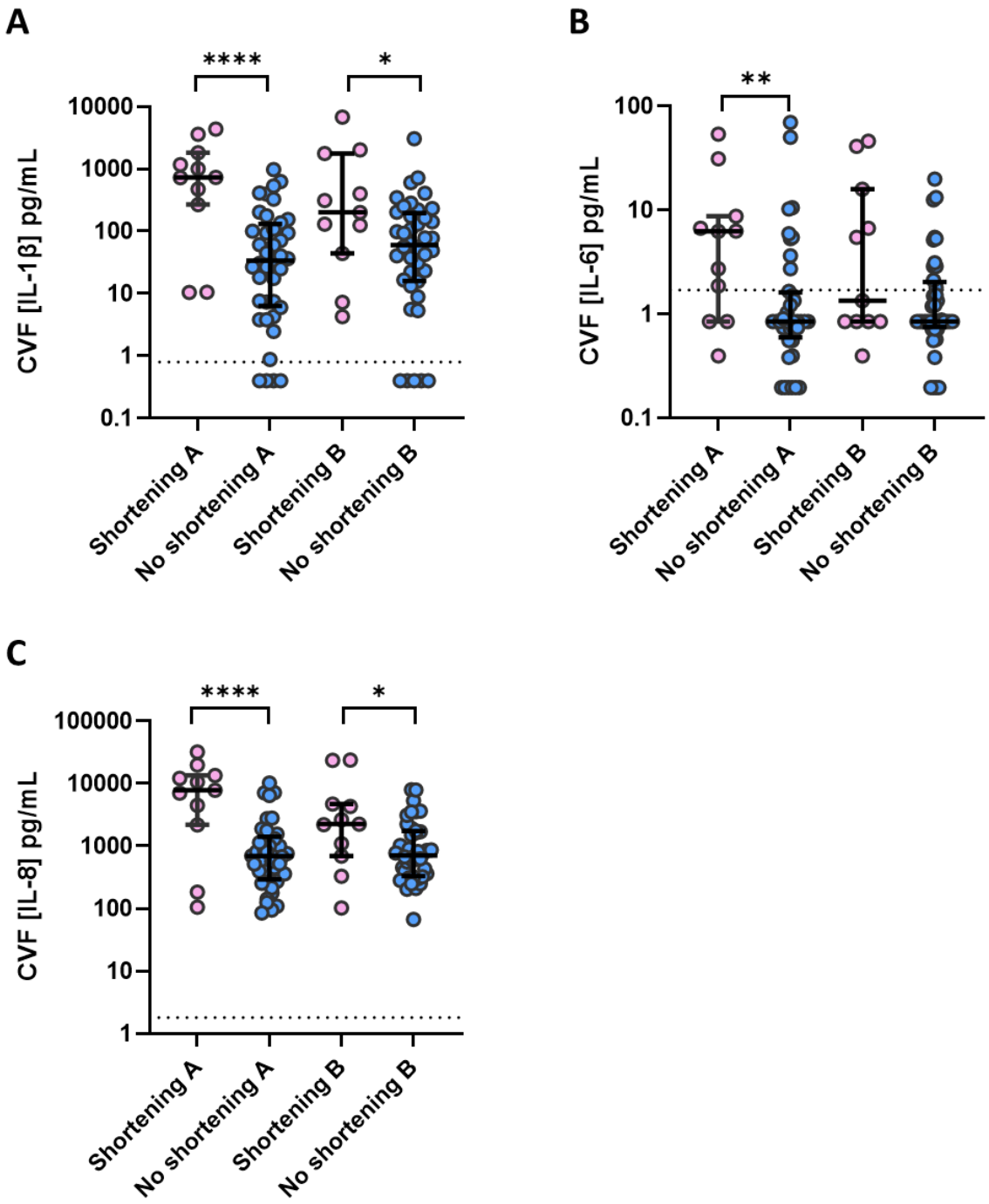


Figure 3.4. Cervicovaginal fluid cytokines concentrations in women who experienced cervical shortening. A) Concentration of IL-1β in CVF. **B)** Concentration of IL-6 in CVF. **C)** Concentration of IL-8 in CVF. Statistical analysis between women who experienced cervical shortening (≤25mm) at some point during their pregnancy (n=11), compared to women who maintained cervical length (n=40), was assessed using a Mann-Whitney one-tailed test. Timepoint A (12-16 weeks of gestation); timepoint B (20-24 weeks of gestation). All cytokines measured (IL-1β, IL-6, IL-8) were higher in CVF of women who experienced cervical shortening. *p<0.05; **p<0.01; ****p<0.0001. Data is presented as median values with interquartile range.

Cervicovaginal complement profile in cervical shortening

The complement system can be initiated via three different pathways. The classical pathway requires the activation of the C1-complex. C1q is one of the molecules that conform the C1-complex. The concentration of C1q was higher in women that experienced cervical shortening, compared to woman who maintained a normal cervical length (A: median 0.5369 vs 0.1215 ng/mL, p=0.0038; B: median 0.9468 vs 0.1222, p=0.0009) (Figure 3.5.A).

The activated C1-complex can split C4 and C2 into fragments C4a, C4b, C2a and C2b. The concentration of C2 was also higher in women that shorten compared to woman that did not shorten (A, p=0.0043; B, p=0.0173) (Figure 3.5.B). The median concentration in CVF of C4, but not C4b, was also statistically significantly higher in women with a short cervix (Figure 3.5. C, D). At timepoint A, the median concentration of C4 was significantly higher in women who shorten (72.24 ng/mL) compared to women who did not shorten (median 9.085 ng/mL) (p=0.0003); as well as timepoint B (median 69.97 vs 11.17 ng/mL, p=0.0099). For C4b, the increased median concentration in women who shorten was not statistically significant (A, p=0.0544; B, p=0.0812).

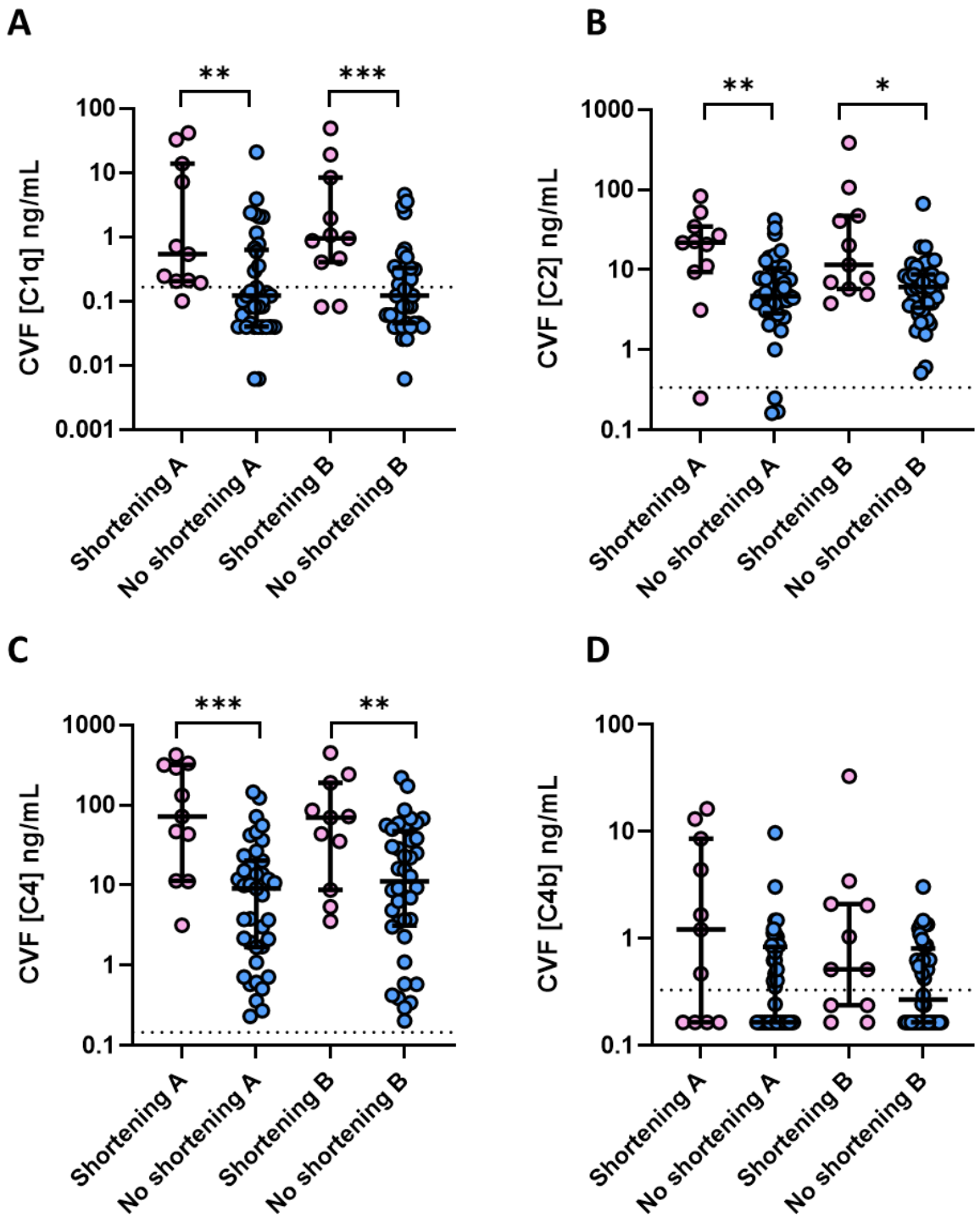


Figure 3.5. Cervicovaginal fluid complement proteins concentrations from the classical pathway in women who experienced cervical shortening. A) Concentration of C1q in CVF. **B)** Concentration of C2 in CVF. **C)** Concentration of C4 in CVF. **D)** Concentration of C4b in CVF. Statistical analysis between women who experienced cervical shortening (≤25mm) at some point during their pregnancy (n=11), compared to women who maintained cervical length (n=40), was assessed using a Mann-Whitney two-tailed test. Timepoint A (12-16 weeks of gestation); timepoint B (20-24 weeks of gestation). *p<0.05; **p<0.01; ***p<0.001. Data is presented as median values with interquartile range.

Mannose-Binding Lectin (MBL) is an opsonin that can bind to the surface of pathogens and activate the complement system via the lectin pathway. At timepoint A, the median concentration of MBL in CVF was higher in women who experienced cervical shortening at some point during their pregnancy (median 1.019 ng/mL) compared to women who did not shorten (median 0.1660 ng/mL) (p=0.0084). Differences were not statistically significant at timepoint B (median 0.3939 vs 0.1812 ng/mL, p=0.1742) (Figure 3.6).

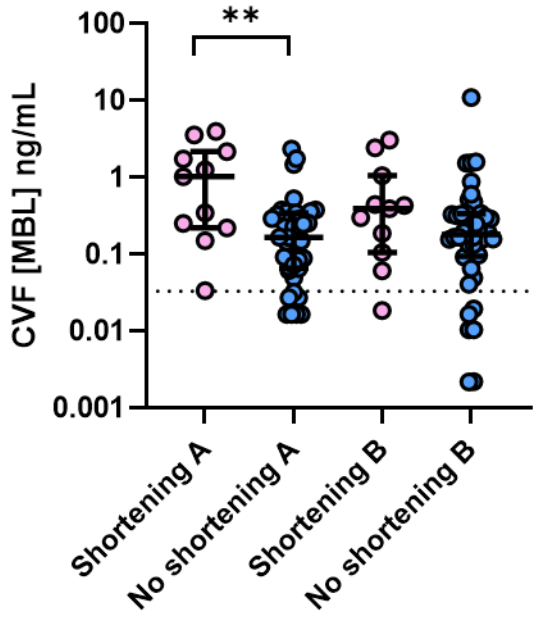


Figure 3.6. Cervicovaginal fluid complement proteins concentrations from the lectin pathway in women who experienced cervical shortening. Concentration of MBL in CVF. Statistical analysis between women who experienced cervical shortening (≤25mm) at some point during their pregnancy (n=11), compared to women who maintained cervical length (n=40), was assessed using a Mann-Whitney two-tailed test. Timepoint A (12-16 weeks of gestation); timepoint B (20-24 weeks of gestation). **p<0.01. MBL: mannose-binding lectin. Data is presented as median values with interquartile range.

Finally, the other pathway described for the activation of the complement system is the alternative pathway. This pathway is activated via spontaneous hydrolysis of component C3. When C3b is available it can bind to factor B to form C3bB. The median concentration of Factor B was higher in women who experienced cervical shortening compared to women who maintained a normal cervical length, but this did not reach statistical significance (at timepoint A, p=0.1362; at timepoint B, p=0.0946) (Figure 3.7.A).

Factor D activity is to cleave the previous complex C3bB into Ba and Bb, to form the convertase C3bBb. The median concentration of Factor D was higher in women with a short cervix, compared to women who did not shorten, specially at timepoint A (median 0.1634 vs 0.0135 ng/mL, p=0.0460) (Figure 3.7.B).

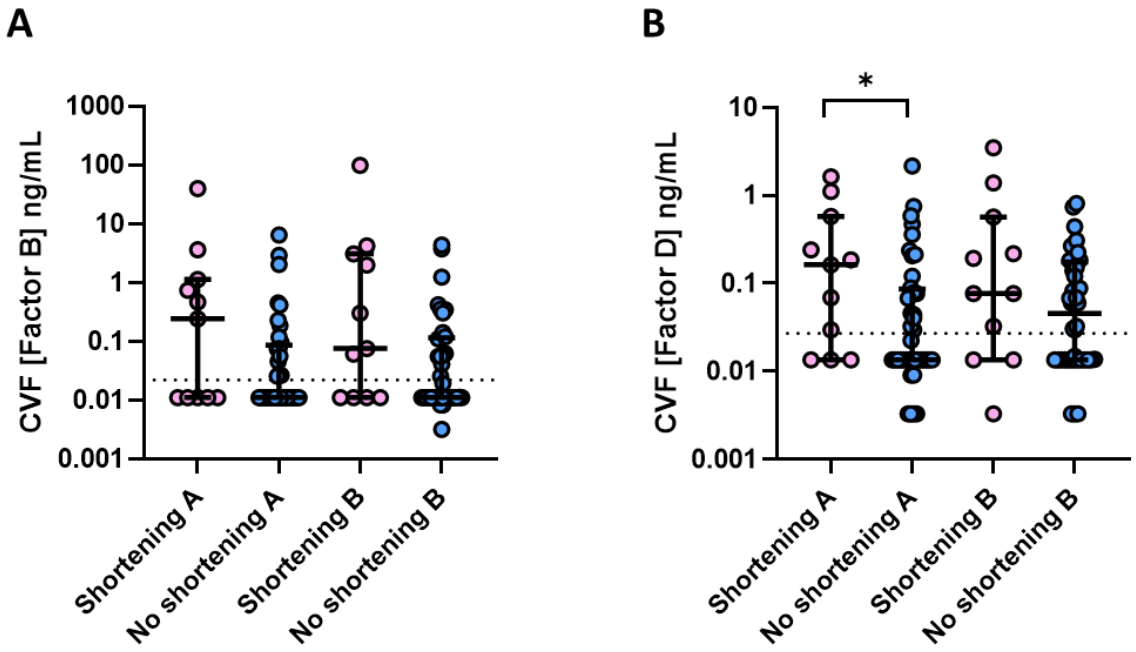


Figure 3.7. Cervicovaginal fluid complement proteins concentrations from the alternative pathway in women who experienced cervical shortening. A) Concentration of Factor B in CVF. **B)** Concentration of Factor D in CVF. Statistical analysis between women who experienced cervical shortening (≤25mm) at some point during their pregnancy (n=11), compared to women who maintained cervical length (n=40), was assessed using a Mann-Whitney two-tailed test. Timepoint A (12-16 weeks of gestation); timepoint B (20-24 weeks of gestation). The median concentration of Factor D was higher in CVF of women who experienced cervical shortening at timepoint A. *p<0.05. Data is presented as median values with interquartile range.

The three pathways converge on the formation of the C3 convertase (C4b2a from the classical and lectin pathways, and C3bBb from the alternative pathway). C3 convertases target component C3 that will be fragmented into C3a and C3b. At timepoint A, the median concentration of C3 was significantly increased in women who shorten (median 0.2788 vs 0.1100 ng/mL, p=0.0400). However, no differences were observed on the concentration of C3 in CVF at timepoint B (p=0.6638) (Figure 3.8.A). For the C3 activation subproducts, the concentration of C3a and C3b dramatically increased in women who experienced cervical shortening compared to women who maintained a normal cervical length (median C3a 1176 vs 268.5 pg/mL; p=0.0046, at timepoint A) (median C3b 30.83 vs 2.761 ng/mL; p=0.0192, at timepoint A) (median C3b 30.69 vs 7.892 ng/mL; p=0.0114, at timepoint B) (Figure 3.8. B and C).

The released fragment C3b activates C5 convertase, the next common step of the complement cascade. Similarly, C5 convertase will fragment C5 into C5a and C5b. In our panel, CVF concentrations of C5 and C5a were measured. No statistically significant differences were observed in the median concentration of C5 at timepoint A (median 0.9258 vs 0.8674, ng/mL; p=0.1579) nor timepoint B (median 1.845 vs 0.9258 ng/mL, p=0.1946). However, the median concentration of C5a was more than 3-times higher in women who experienced cervical shortening at timepoint A (median 49.19 vs 13.29 pg/mL, p=0.0028) and at timepoint B (median 30.89 vs 10.07 pg/mL, p=0.0270) (Figure 3.8. E).

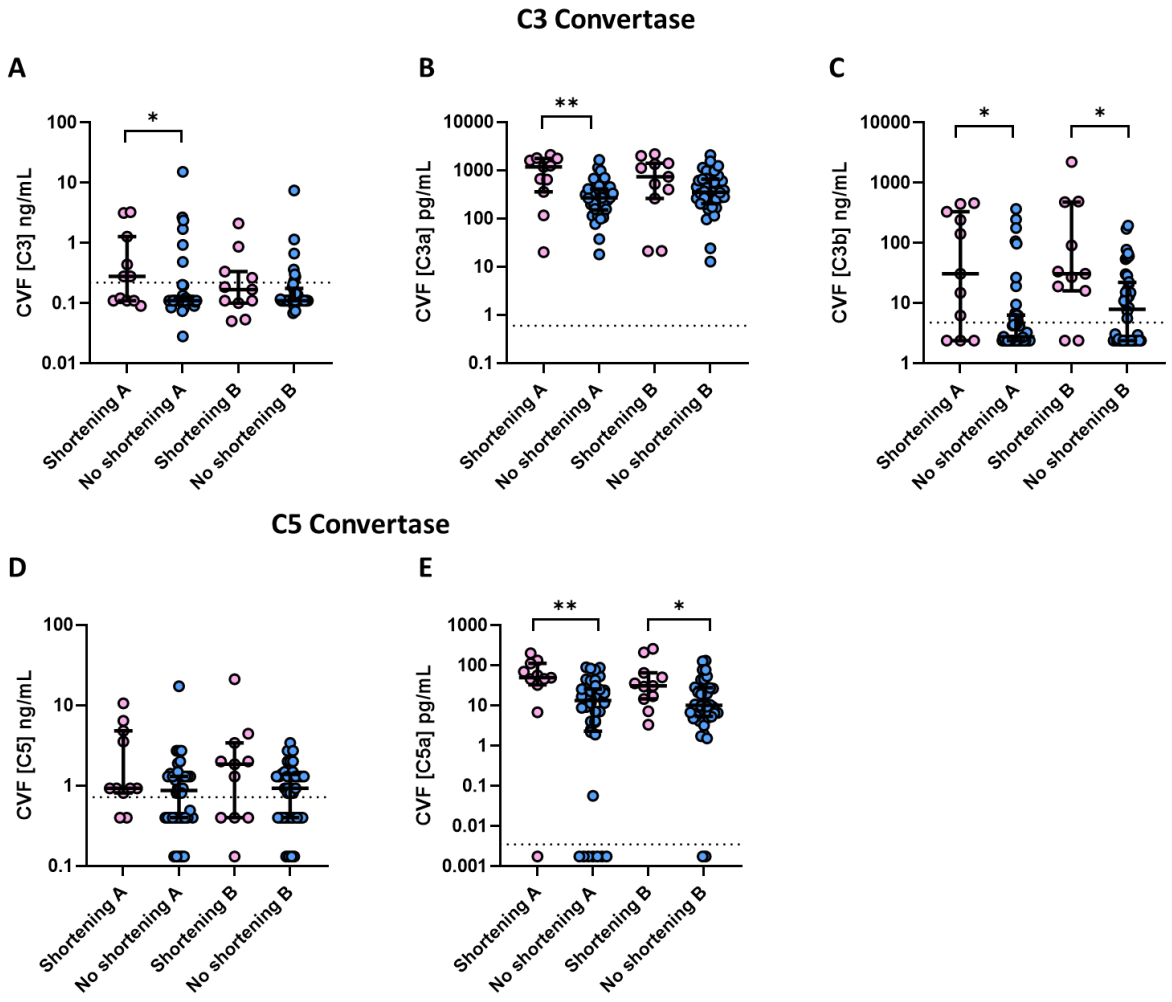


Figure 3.8. Cervicovaginal fluid complement proteins concentrations from the convergence of pathways in activation of C3 and C5 convertases. A) Concentration of C3 in CVF. **B)** Concentration of C3a in CVF. **C)** Concentration of C3b in CVF. **D)** Concentration of C5 in CVF. **E)** Concentration of C5a in CVF. Statistical analysis between women who experienced cervical shortening (≤25mm) at some point during their pregnancy (n=11), compared to women who maintained cervical length (n=40), was assessed using a Mann-Whitney two-tailed test. Timepoint A (12-16 weeks of gestation); timepoint B (20-24 weeks of gestation). At timepoint A, the median concentration of C3, C3a, C3b and C5a was higher in women who experienced cervical shortening. At timepoint B, C3b and C5a median concentrations were also higher in women who shorten. *p<0.05; **p<0.01. Data is presented as median values with interquartile range.

Factor H and Factor I are regulators of the complement cascade. The concentration of both proteins was higher in women who experienced cervical shortening, compared to women who did not shorten (Factor H timepoint A, p<0.0001; timepoint B, p=0.0011) (Factor I timepoint A, p=0.0274; timepoint B, p=0.0303) (Figure 3.9.).

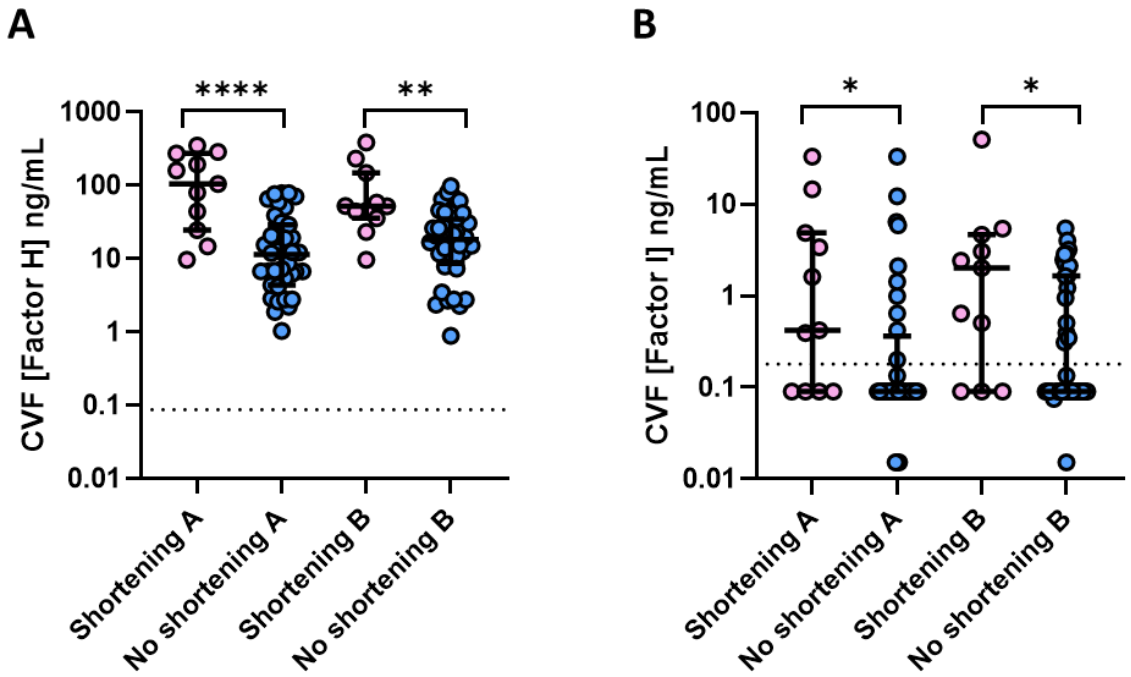


Figure 3.9. Cervicovaginal fluid complement regulators concentrations in women who experienced cervical shortening. A) Concentration of Factor H in CVF. **B)** Concentration of Factor I in CVF. Statistical analysis between women who experienced cervical shortening (≤25mm) at some point during their pregnancy (n=11), compared to women who maintained cervical length (n=40), was assessed using a Mann-Whitney two-tailed test. Timepoint A (12-16 weeks of gestation); timepoint B (20-24 weeks of gestation). The concentration of Factor H and Factor I was higher in CVF of women who experienced cervical shortening. *p<0.05; **p<0.01; ****p<0.0001. Data is presented as median values with interquartile range.

Cervicovaginal matrix metalloproteinases profile in cervical shortening

Matrix metalloproteinases are proteases capable of degrading extracellular matrix components. Three different MMPs were quantified in CVF. At timepoint A, the median concentration of MMP-2 (Figure 3.10.A.) was considerably increased in women with a short cervix (median 4563 pg/mL) compared to women with a normal cervical length (median 54 pg/mL) (p=0.0001). These differences were not statistically significant at timepoint B (median 1317 vs 54 pg/mL, p=0.1156).

At timepoint A, women with a short cervix also had higher concentrations of MMP-8 (median 50953 vs 5437 pg/mL; Figure 3.10.B.) and MMP-9 (median 101695 vs 22310 pg/mL; Figure 3.10.C.) (p=0.0069, p=0.0136, respectively).

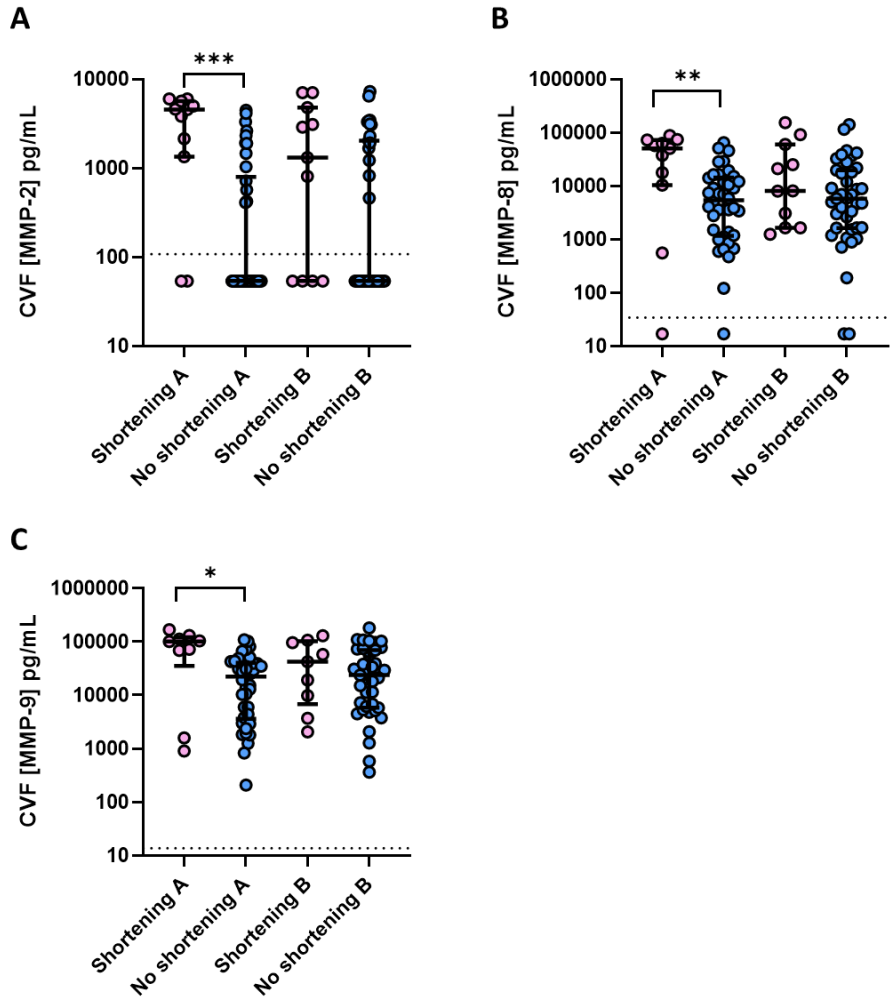


Figure 3.10. Cervicovaginal fluid matrix metalloproteinases concentrations in women who experienced cervical shortening. A) Concentration of MMP-2 in CVF. **B)** Concentration of MMP-8 in CVF. **C)** Concentration of MMP-9 in CVF. Statistical analysis between women who experienced cervical shortening (≤25mm) at some point during their pregnancy (n=11), compared to women who maintained cervical length (n=40), was assessed using a Mann-Whitney two-tailed test. Timepoint A (12-16 weeks of gestation); timepoint B (20-24 weeks of gestation). All MMPs measured (MMP-2 MMP-8, MMP-9) were higher in CVF of women who experienced cervical shortening. *p<0.05; **p<0.01; ***p<0.001. Data is presented as median values with interquartile range.

3.6.4. Cervicovaginal microbial composition and immune response in women who delivered preterm

A 102 CVF samples were obtained from 51 pregnant women. As previously described (Table 3.8.), in the overall population 34 women (66%) had a term uncomplicated (TU) delivery, whereas 17 women had a spontaneous preterm delivery (33%), of which 9/17 were early preterm deliveries (≤33+6) and 8 were late preterm deliveries (≥34+0). The mean gestational age at delivery was 31+5 for the PT group and 39+5 for the TU group (p<0.0001).

Women provided samples at two different timepoints across pregnancy. The mean gestational age at sampling for the preterm and the term groups were 15+3 and 15+1 at timepoint A (p=0.5784), and 21+6 and 22+0 for the timepoint B (p=0.7682).

Microbial composition by pregnancy outcome

The local vaginal microbial composition was divided by sampling timepoint and compared according to pregnancy outcome (preterm, PT; term uncomplicated, TU) (Figure 3.11. A and B). There was a higher proportion of CST I *L. crispatus* in women who had a TU vaginal delivery (59% at timepoint A, 56% at timepoint B), compared to women who delivered preterm (35% at both timepoints). At timepoint A, there was a higher proportion of women who had a CST III (30%) or CST IV (24%) vaginal dominance in the PT group compared to women who delivered at term (18% and 15%, respectively). These differences did not reach statistical significance (at timepoint A, p=0.6078; at timepoint B, p=0.6708).

Transitions of the vaginal microbiota groups across pregnancy according to pregnancy outcome were visualised using a Sankey plot (Figure 3.11. C and D). In the preterm cohort, only one woman transitioned from a CST IV vaginal composition to a CST III. In the term cohort, a total of 4 women transitioned towards a CST III as well. Proportional differences of stable and transitioning communities were not significant between groups (p=0.6536).

Finally, diversity was evaluated between pregnancy outcomes. Women who ultimately delivered preterm had an increased bacterial alpha diversity (n=17; median Shannon index 0.26) compared to woman with a term delivery (n=41, median Shannon index 0.08) (p=0.0493) (Figure 3.11. E). Women who delivered preterm also had higher richness (species observed) compared to women who delivered at term, but this did not reach statistical significance (p=0.0524) (Figure 3.11. F).

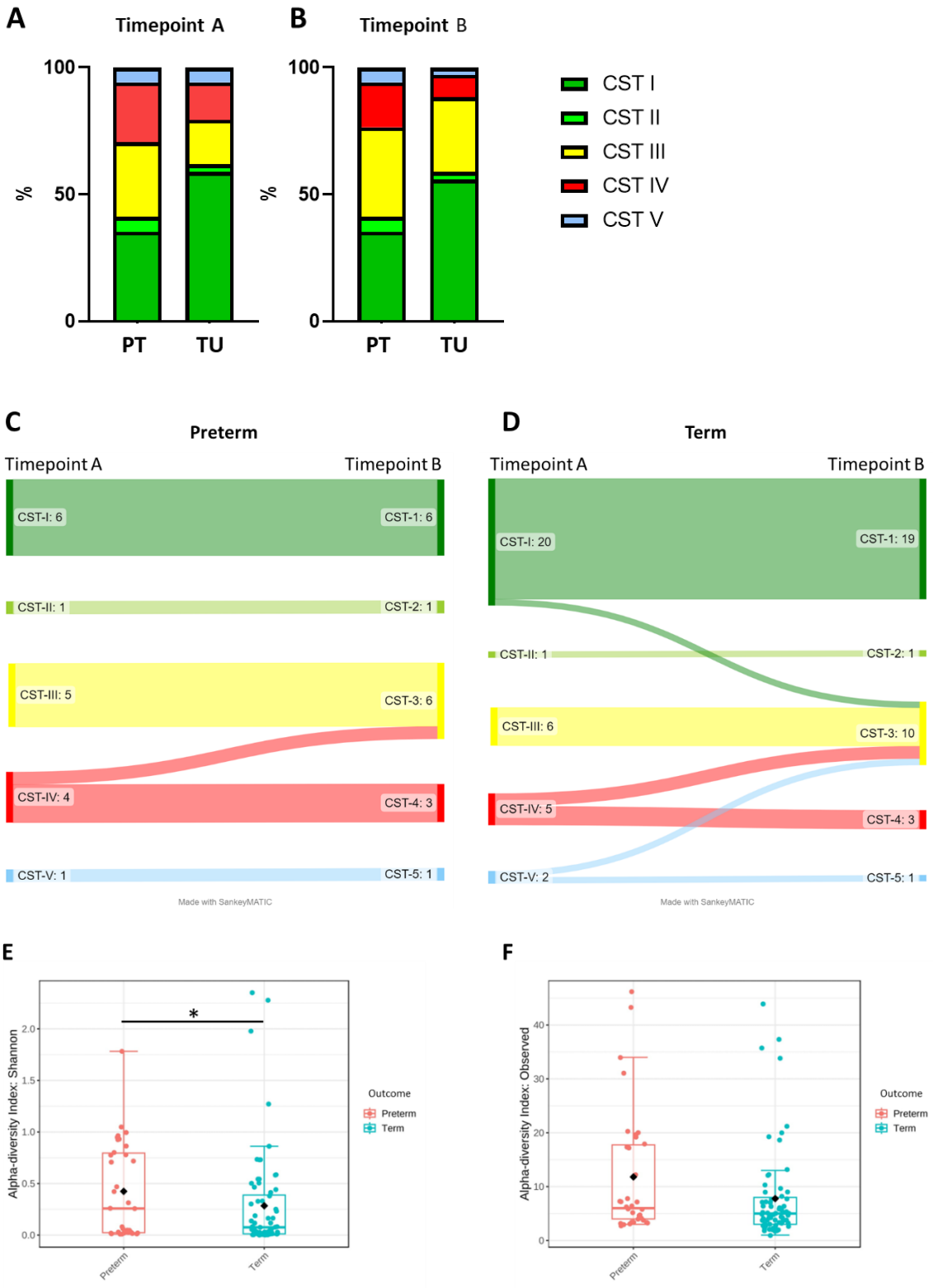


Figure 3.11. Vaginal community state types (CST) throughout pregnancy in preterm and term deliveries. Comparison of proportion of CSTs by pregnancy outcome (PT: preterm, n=17; TU: term, n=34) of samples from **A)** timepoint A (collection at 12-16 weeks of gestation) and **B)** timepoint B (collection at 20-24 weeks of gestation). Sankey diagram depicting transitions between CST throughout timepoints A and B in **C)** women who delivered preterm and **D)** women who delivered at term. Image created with sankeymatic.com. **D)** Diversity (Shannon Index). **E)** Richness (species observed). *p<0.05. Data is presented as median values with interquartile range.

The local cervicovaginal microbial composition was described according to pregnancy outcome (Figure 3.12.). Overall, no statistically significant differences were observed between timepoint A and B for any outcome classification. In the early preterm birth group (<34 weeks of gestation) (Figure 3.12.A) 33% of the women had a high-risk vaginal composition (CST III or IV, 3/9). In the late preterm group (34+0-36+6, Figure 3.12.B), CST III *L. iners* was the most predominant vaginal composition observed (n=4/8, 50%). Three out of those women had a cervical cerclage, one with mersilene material, one with nylon, and one unknown. The majority of women that delivered at term had CST I *L. crispatus* dominance (at timepoint A, 59%; at timepoint B, 56%) (Figure 3.12.C).

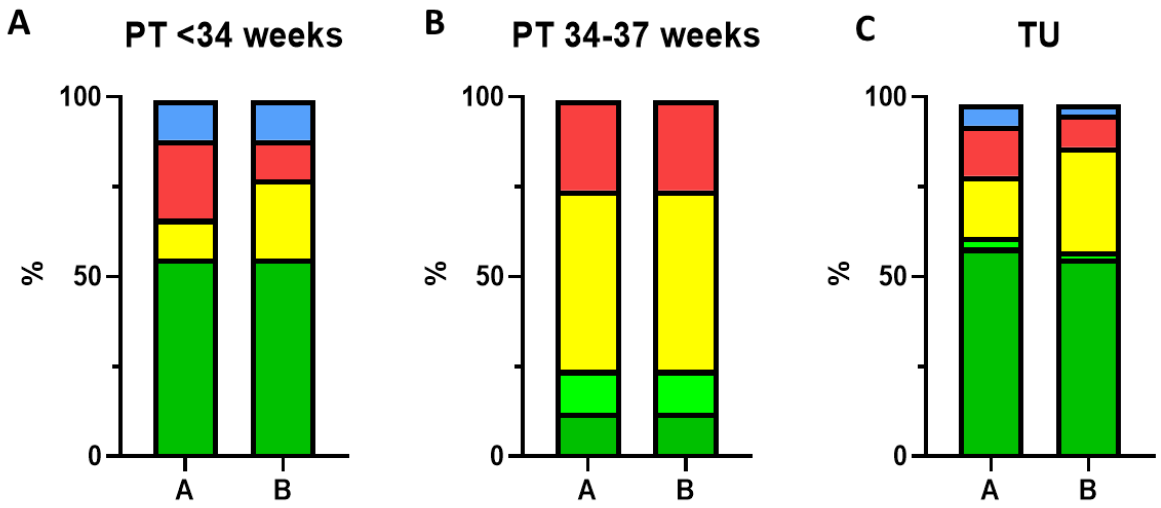


Figure 3.12. Vaginal community state types (CST) according to pregnancy outcome. Longitudinal samples were assigned to CST I (dark green; dominated by *Lactobacillus crispatus*), CST II (light green, dominated by *L. gasseri*), CST III (yellow, dominated by *L. iners*), CST IV (red, heterogenous anaerobic bacteria) or CST V (blue, *L. jensenni*). Proportion of CSTs at timepoint A and timepoint B in women who had an **A)** early preterm birth (≤33+6 weeks of gestation, n=9), **B)** late preterm birth (34+0-36+6 weeks of gestation, n=8) and **C)** term uncomplicated delivery (>37 weeks, n=34).

Cervicovaginal cytokine profile by pregnancy outcome

Proinflammatory cytokines IL-1β, IL-6 and IL-8 were quantified from CVF samples obtained during early pregnancy (timepoint A: 12-16 weeks of gestation) and mid-pregnancy (timepoint B: 20-24 weeks of gestation) and comparisons were made between women who delivered preterm (PT) and women who delivered at term without intervention (TU).

A heightened local proinflammatory response was detected in women who delivered preterm regardless of timepoint (Figure 3.13.). As early as 12-16 weeks, compared to the TU group, women who delivered PT had higher concentrations of IL-1β (median 475.5 vs 25.91 pg/mL, $p<0.0001$), IL-6 (5.485 vs 0.85 pg/mL, $p=0.0004$) and IL-8 (median 1885 vs 641.3 pg/mL, $p=0.0292$). Similarly, at timepoint B (20-24 weeks of gestation), women who delivered preterm also had higher median concentrations of IL-1β (median 312 vs 46.37 pg/mL, $p=0.0021$), IL-6 (median 1.5 vs 0.85 pg/mL; $p=0.0137$) and IL-8 (median 1093 vs 652 pg/mL; $p=0.0178$), compared to women who delivered at term.

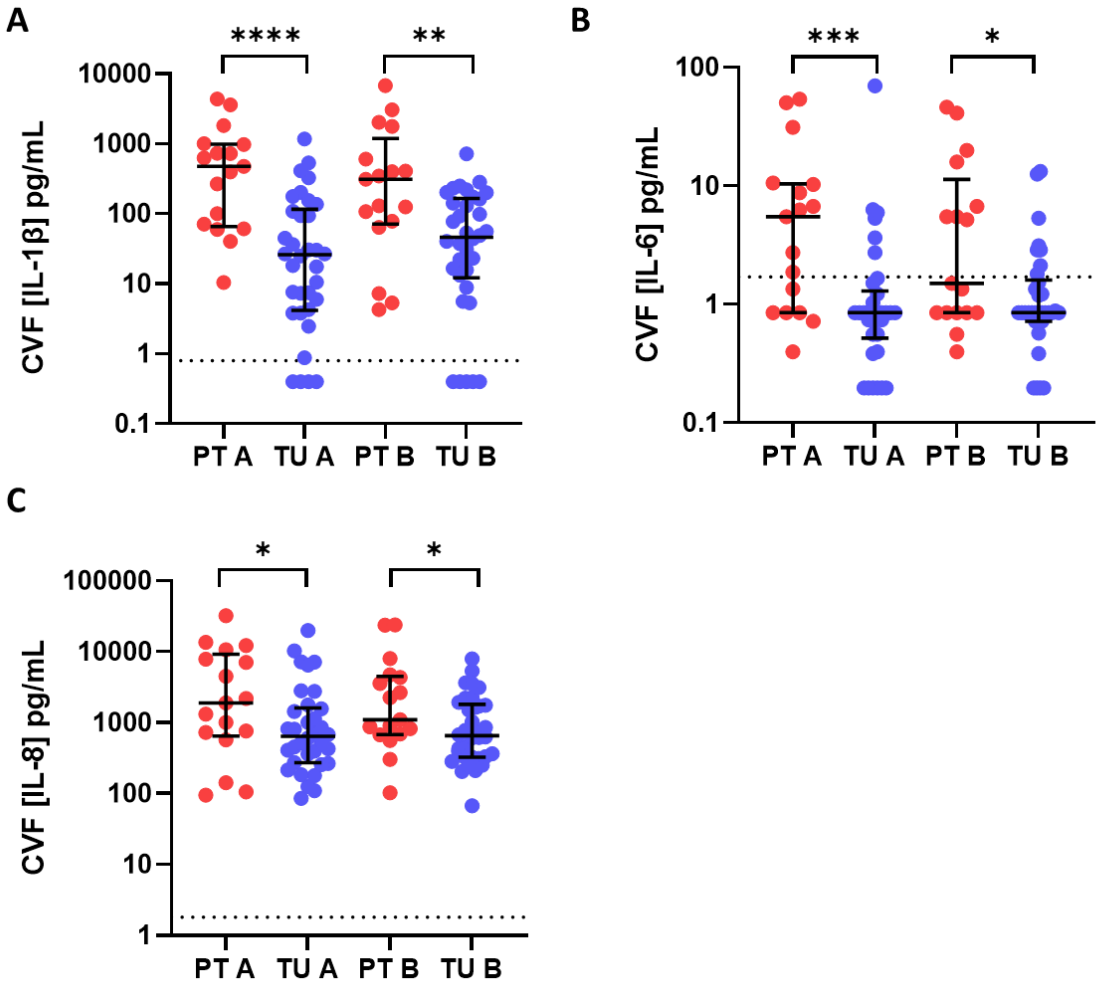


Figure 3.13. Comparison of cervicovaginal fluid cytokines' levels according to pregnancy outcome. A) Concentration of IL-1β in CVF. **B)** Concentration of IL-6 in CVF. **C)** Concentration of IL-8 in CVF. Statistical analysis was performed between preterm (PT, n=17) and term (TU, n=34) for each collection timepoint (A vs A; B vs B) using a Mann-Whitney one-tailed test. A: collection between 12-16 weeks of gestation. B: collection between 20-24 weeks of gestation. Overall, samples collected from women who delivered preterm had higher concentration of cytokines assessed. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Data is presented as median values with interquartile range.

The concentration of all of the cytokines measured had a significant positive correlation between each other: IL-1β and IL-6 (r=0.6050, p<0.0001), IL-1β and IL-8 (r=0.7075, p<0.0001), IL-6 with IL-8 (r=0.4929, p<0.0001) (Figure 3.14.).

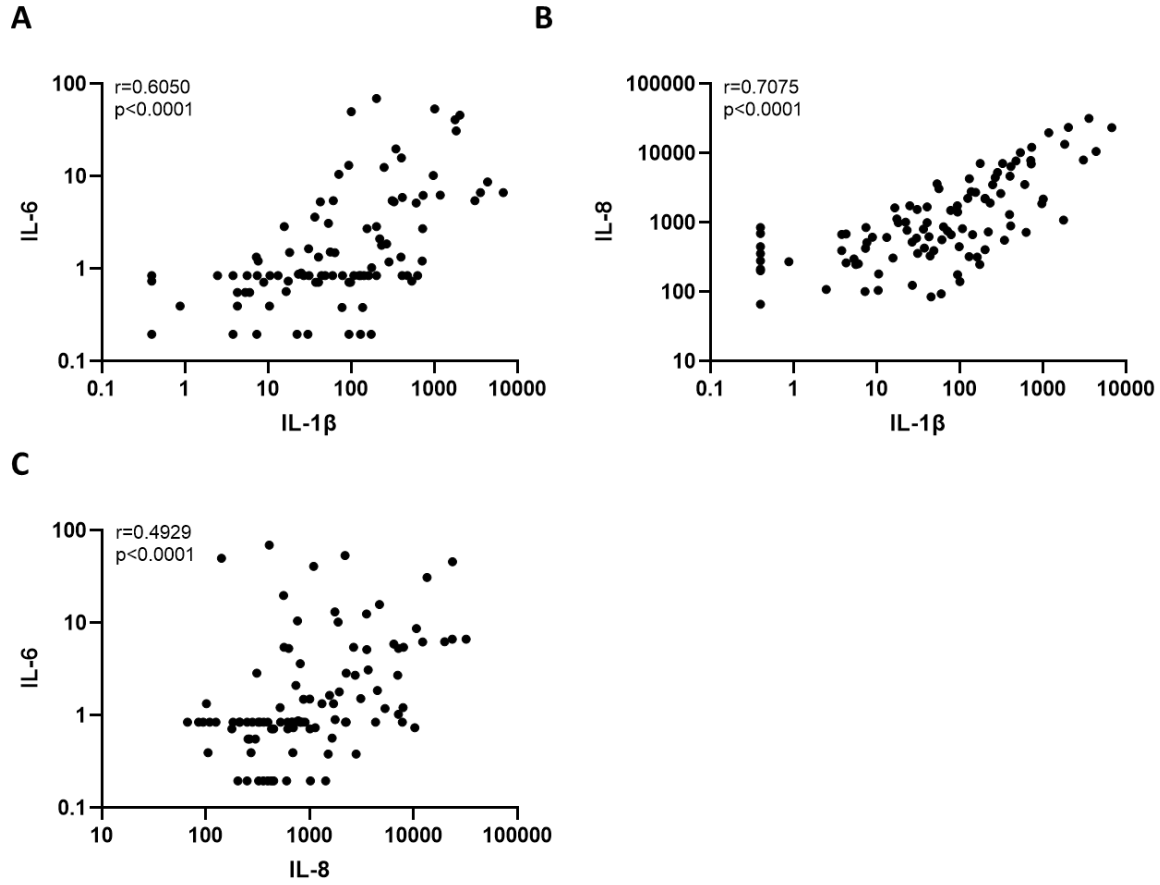


Figure 3.14. Correlation between cytokines concentration in cervicovaginal fluid. Statistical analyses were performed with Spearman correlation between concentration of **A)** IL-6 and IL-1β, **B)** IL-8 and IL-1β and **C)** IL-6 and IL-8.

Cervicovaginal complement profile by pregnancy outcome

Concentrations of C1q were higher in the preterm cohort at both timepoint A and B compared to women who delivered at term (at timepoint A, median 0.2949 vs 0.1215 ng/mL, p=0.0126; at timepoint B, median 0.4638 vs 0.1222 ng/mL, p=0.0072) (Figure 3.15.A). For complement protein C2, non-statistically significant differences were observed (p=0.0796 at timepoint A; p=0.1175 at timepoint B), however women who delivered preterm had higher median concentrations, particularly at timepoint A (median 9.334 ng/mL vs 4.977 ng/mL) (Figure 3.15.B). The concentration of C4 was extremely distinctive between the preterm and term groups. At timepoint A, the PT median was 43.33 ng/mL compared to the considerably reduced 3.758 ng/mL in TU group (p<0.0002). At timepoint B the concentration was almost 6-times higher in women who delivered preterm (median 53.61 vs 9.015 ng/mL, p=0.0034) (Figure 3.15.C). No differences were observed in the CVF concentration of C4b at any timepoint (timepoint A, p=0.2831; B, p=0.5915) (Figure 3.15.D).

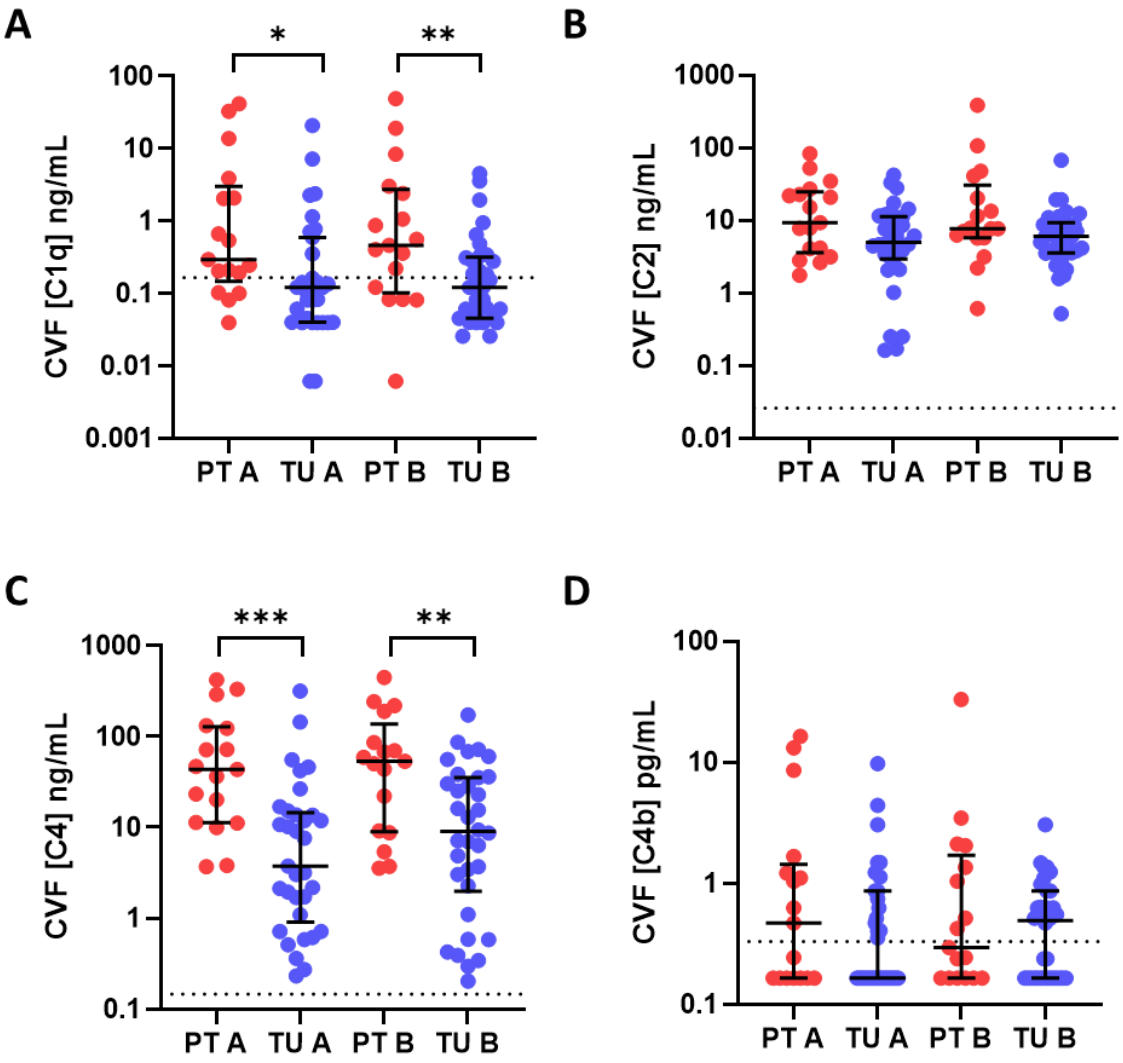


Figure 3.15. Comparison of concentrations of cervicovaginal fluid complement proteins from the classical pathway according to pregnancy outcome and timepoint of collection. A) Concentration of C1q in CVF. **B)** Concentration of C2 in CVF. **C)** Concentration of C4 in CVF. **D)** Concentration of C4b in CVF. Statistical analysis was performed between preterm (PT, n=17) and term (TU, n=34) samples for each collection timepoint (A vs A; B vs B) using a Mann-Whitney two-tailed test. A: collection between 12-16 weeks of gestation. B: collection between 20-24 weeks of gestation. Concentrations of C1q and C4 were higher in women who delivered preterm at both timepoint A and timepoint B. * $p<0.05$; ** $p< 0.01$; *** $p<0.001$. Data is presented as median values with interquartile range.

With reference to the lectin pathway, the median CVF concentration of MBL (Figure 3.16) was higher in women who delivered preterm at both timepoints but was only statistically significant at timepoint A (median 0.3468 vs 0.1740 ng/mL at term, p=0.0422) (timepoint B median 0.3292 vs 0.1671 ng/mL, p=0.1073).

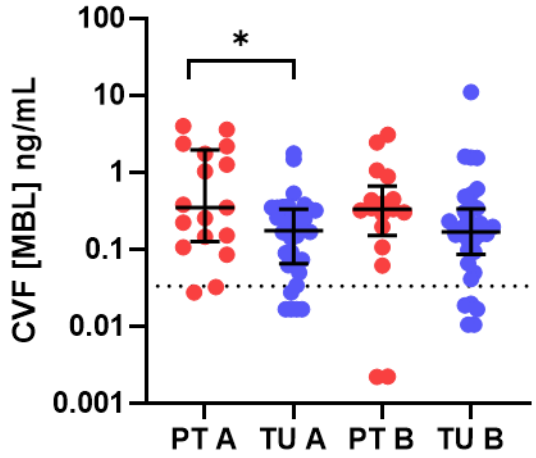


Figure 3.16. Comparison of concentrations of cervicovaginal fluid complement proteins from the lectin pathway according to pregnancy outcome and timepoint of collection. Concentration of MBL in CVF. Statistical analysis was performed between preterm (PT, n=17) and term (TU, n=34) samples for each collection timepoint (A vs A; B vs B) using a Mann-Whitney two-tailed test. A: collection between 12-16 weeks of gestation. B: collection between 20-24 weeks of gestation. At timepoint A, concentration of MBL was higher in women who delivered preterm. * $p<0.05$. Data is presented as median values with interquartile range.

The concentration of complement proteins participating in the alternative pathway Factor B (Figure 3.17. A) and Factor D (Figure 3.17. B) did not significantly differ between outcome groups at either timepoint. The spread of the data points shows great variation within each cohort, with a high percentage of samples below the sensitivity of detection of the assay for Factor B and H in both women who delivered at term and preterm.

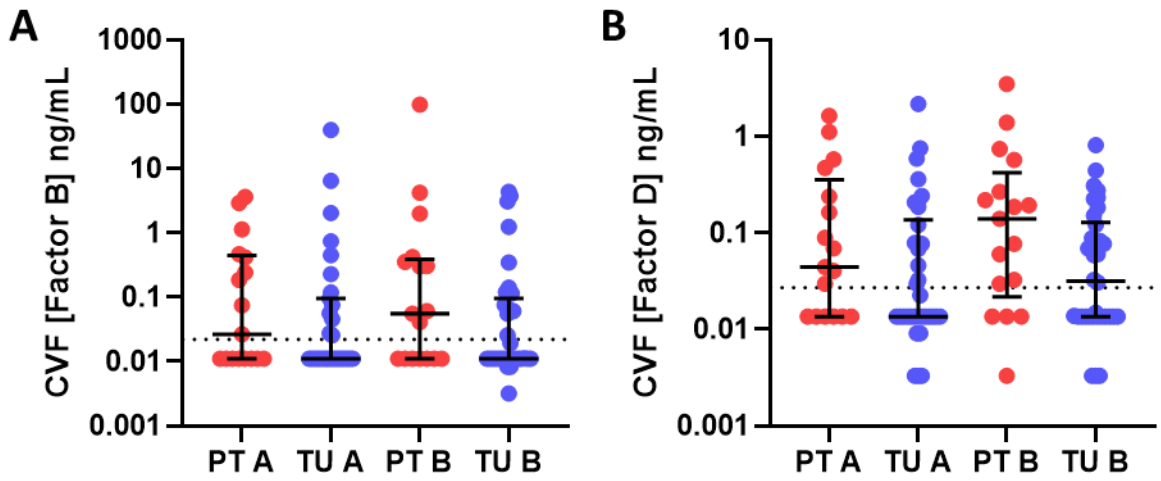


Figure 3.17. Comparison of concentrations of cervicovaginal fluid complement proteins from the alternative pathway according to pregnancy outcome and timepoint of collection. A) Concentration of Factor B in CVF. **B)** Concentration of Factor D in CVF. Statistical analysis was performed between preterm (PT, n=17) and term (TU, n=34) samples for each collection timepoint (A vs A; B vs B) using a Mann-Whitney two-tailed test. A: collection between 12-16 weeks of gestation. B: collection between 20-24 weeks of gestation. No statistically significant differences were observed between women who delivered preterm or at term. Data is presented as median values with interquartile range.

As previously mentioned, the three different complement pathways converge on C3. No significant differences were observed for the concentration of C3 (Figure 3.18. A), and many of the samples were below the level of detection (48% of total 102 samples were OOR), perhaps reflecting consumption or instability. However, activation of the complement cascade was detected due to the higher levels of the C3 subproducts. At timepoint B, women who delivered preterm had a significantly higher median concentration of C3a (729.7 pg/mL) compared to term (331.5 pg/mL) (p=0.0220) (Figure 3.18. C). The concentration of C3b was also higher in the preterm group at both timepoint A (median 6.214 vs 2.695 ng/mL, p=0.0382) and timepoint B (median 20.90 vs 7.005 ng/mL, p=0.0262).

C5 convertase leads to the defragmentation of C5 into C5a and C5b. At timepoint A, there were no differences in the median concentration of C5 between women who delivered preterm or term (median 0.9258 ng/mL in both groups, p=0.9641). Despite not statistically significant, the median C5 increased at timepoint B in the preterm group compared to women who delivered at term (median 1.299 vs 0.9258 ng/mL, p=0.3125) (Figure 3.18. D). For the activation subproduct C5a, the median concentration was higher at both timepoint A (median 32.70 vs 16.75 pg/mL) and timepoint B (median 19.91 vs 10.07 pg/mL) (p=0.1043, p=0.4428, respectively) (Figure 3.18. E).

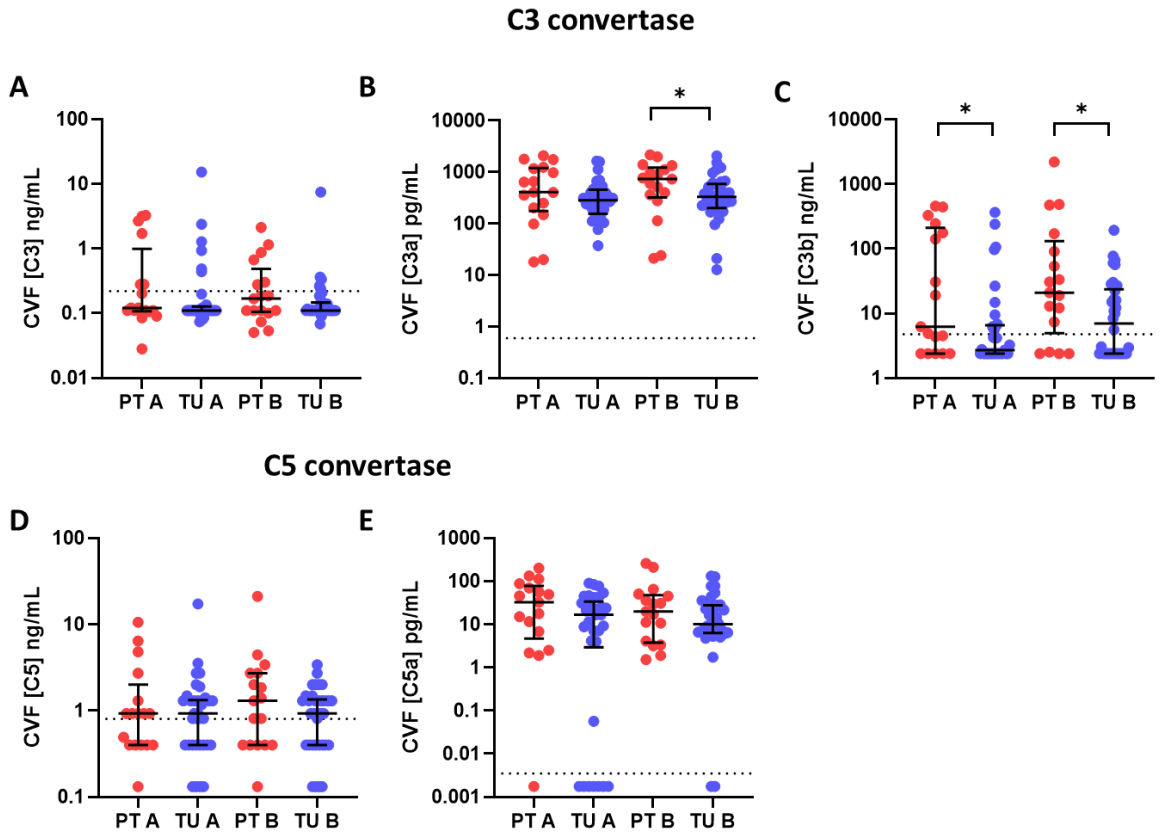


Figure 3.18. Comparison of cervicovaginal fluid complement proteins concentrations from the convergence of pathways in activation of C3 and C5 convertases according to pregnancy outcome and timepoint of collection. **A)** Concentration of C3, **B)** C3a, **C)** C3b, **D)** C5 and **E)** C5a in CVF. Statistical analysis was performed between preterm (PT, n=17) and term (TU, n=34) samples for each collection timepoint (A vs A; B vs B) using a Mann-Whitney two-tailed test. A: collection between 12-16 weeks of gestation. B: collection between 20-24 weeks of gestation. At timepoint A, women who delivered preterm had a higher concentration of C3b. At timepoint B, women who delivered preterm had higher concentrations of C3a and C3b than women who delivered at term. *p<0.05. Data is presented as median values with interquartile range.

Finally, complement regulators were also assessed by pregnancy outcome (Figure 3.19.). The concentration of Factor H was higher both at timepoint A (median 44.07 vs 9.666 ng/mL, p=0.0019) and timepoint B (median 44.17 vs 15.94 ng/mL, p=0.0077) compared to women who delivered at term (Figure 3.19. A). In contrast, no differences were observed in the concentration of Factor I between groups (Figure 3.19.B). For Factor I, there was a great variation and data spread within each cohort, with a high percentage of samples below the range of the assay.

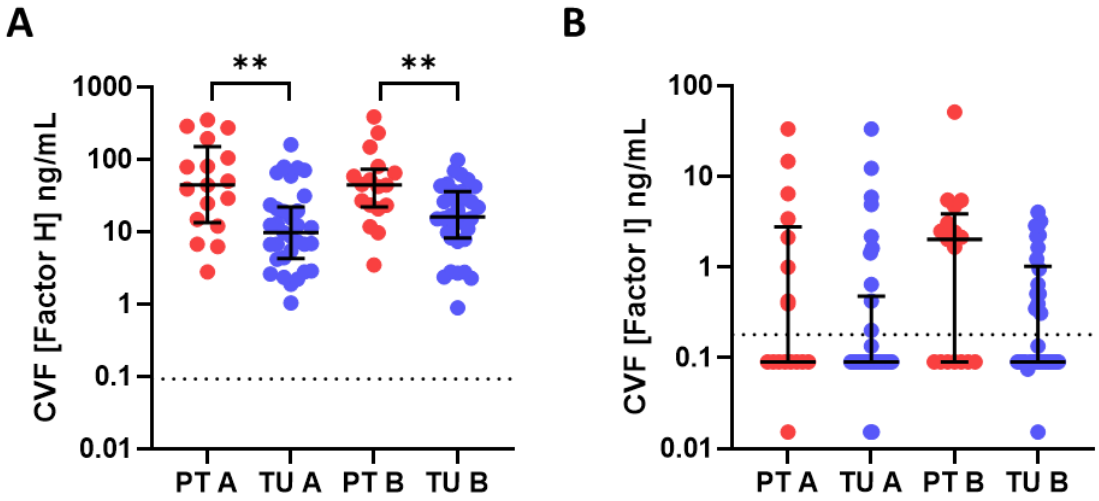


Figure 3.19. Comparison of cervicovaginal fluid complement regulators concentrations according to pregnancy outcome and timepoint of collection. A) Concentration of Factor H and **B)** Factor I in CVF. Statistical analysis was performed between preterm (PT, n=17) and term (TU, n=34) samples for each collection timepoint (A vs A; B vs B) using a Mann-Whitney two-tailed test. A: collection between 12-16 weeks of gestation. B: collection between 20-24 weeks of gestation. Women who delivered at term had a higher Factor H concentration at both timepoint A and B than women who delivered at term. **p<0.01. Data is presented as median values with interquartile range.

Cervicovaginal matrix metalloproteinases profile by pregnancy outcome

The other panel that was evaluated on the supernatant of the study cohort were matrix metalloproteinases (Figure 3.20.). The concentration of MMP-2 was higher in women who delivered preterm compared to women who delivered at term at both timepoint A (median 1466 vs 54 pg/mL, p=0.0070) and timepoint B (median 1317 vs 54 pg/mL, p=0.0325). In all groups there was a clear bimodal distribution, that was even more evident in women who delivered at term. In fact, there was a higher proportion of women with OOR values in the term group (44/67, 65%) than in the preterm group (10/32, 31%) (p=0.0051) (Figure 3.20.A).

The median concentration of MMP-8 was also higher in women who delivered preterm (15142 pg/mL vs 4566 pg/mL at timepoint A; 8144 vs 5057 pg/mL at timepoint B). However, this was only significant in the early pregnancy timepoint (A, p=0.0125; B, p=0.1145) (Figure 3.20.B). Finally, the concentration of MMP-9 also increased dramatically in women who delivered preterm. At timepoint A, the median concentration was almost five times higher in the preterm group (median 60112 vs 12983 pg/mL, p=0.0009), and three times higher at timepoint B (median 58042 vs 18233, p=0.0713) (Figure 3.20.C).

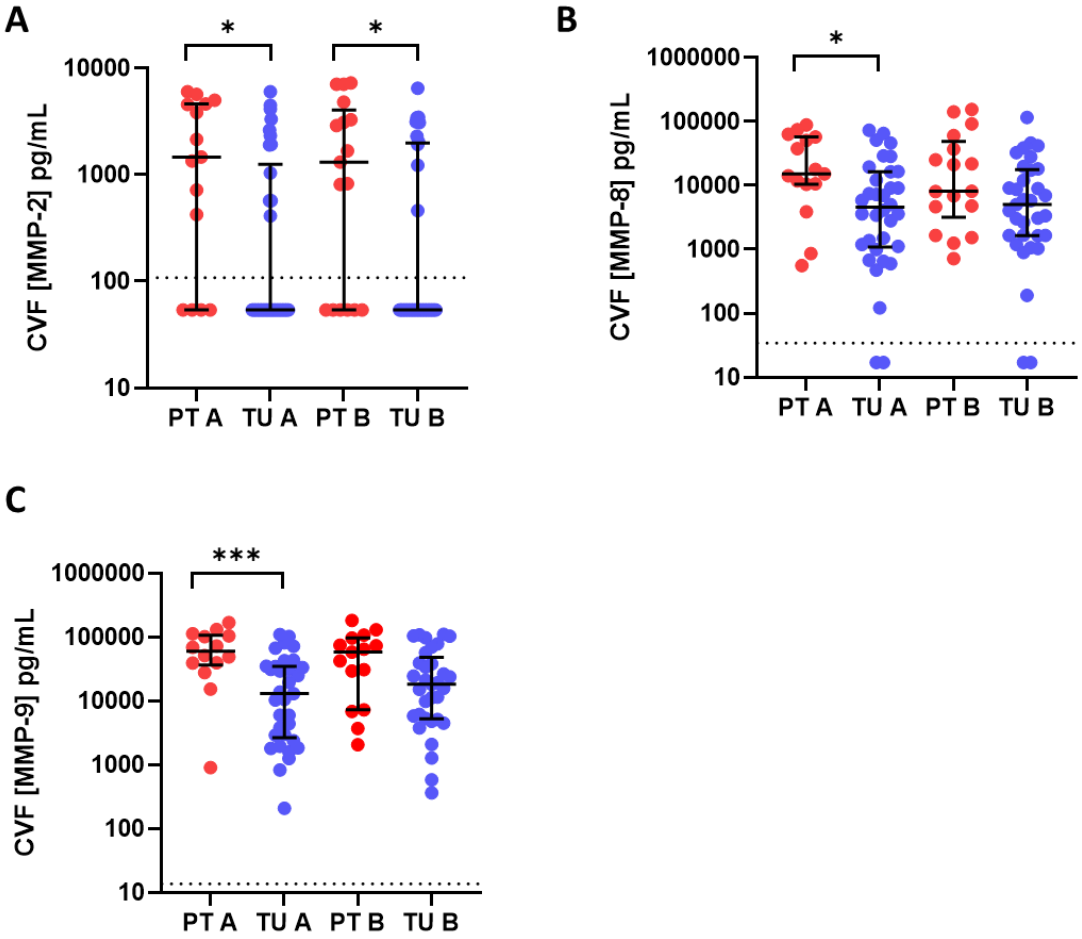


Figure 3.20. Comparison of cervicovaginal fluid matrix metalloproteinases levels according to pregnancy outcome and timepoint of collection. A) Concentration of MMP-2 in CVF. **B)** Concentration of MMP-8 in CVF. **C)** Concentration of MMP-9 in CVF. Statistical analysis was performed between preterm (PT, n=17) and term (TU, n=34) samples for each collection timepoint (A vs A; B vs B) using a Mann-Whitney two-tailed test. A: collection between 12-16 weeks of gestation. B: collection between 20-24 weeks of gestation. Overall, samples collected from women who delivered preterm had higher concentration of complement proteins assessed. *p<0.05; *** p<0.001. Data is presented as median values with interquartile range.

Furthermore, the concentration of all MMPs concentrations were strongly correlated between each other (Figure 3.21.).

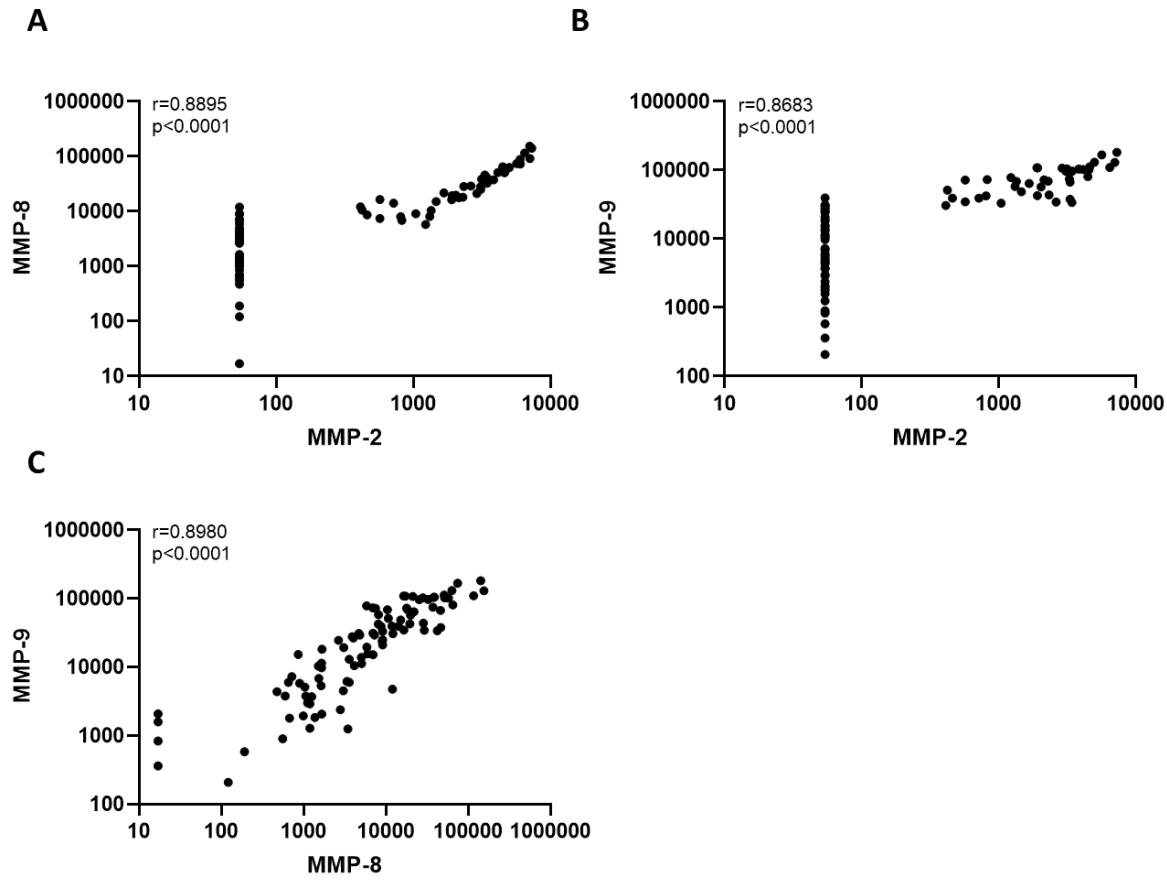


Figure 3.21. Correlation between matrix metalloproteinases in CVF. Statistical analyses were performed with Spearman correlation between concentrations of **A)** MMP-2 and MMP-8; **B)** MMP-2 and MMP-9; and **C)** MMP-8 and MMP-9.

Since some of the complement analytes measured had a great proportion of samples out of range (for example, between 40-50% for C4b, Factor B, C3 and Factor I, MMP-2), and could impact the accuracy to detect distinctive concentration patterns across groups, proportional differences of detectable, extrapolated and OOR samples between preterm and groups were evaluated with a Chi-square test unless otherwise stated (Table 3.10., 3.11., 3.12.). Quantitative interpretation between groups for the aforementioned analytes should be made carefully, however the detectable ranges were highly dependent on the pregnancy outcome, suggesting indeed a considerable decrease in inflammatory mediators such as IL-1β, IL-6, C1q, C3, C3b, C5, C5a and MMP-2 in women who delivered at term.

Table 3.10. Detectable ranges of cytokines analytes according to pregnancy outcome

	IL-1β		**IL-6**		**IL-8**	
	PT	TU	PT	TU	PT	TU
Detectable	28	41	16	7	28	66
Extrapolated	6	18	10	40	6	2
OOR	0	9	8	21	0	0
p-value	0.0321	*	0.0001	***	0.0157[a]	*

[a] Fisher's exact test. OOR: out of range. Highlighted values indicated statistical proportional differences.

Table 3.11. Detectable ranges of complement proteins analytes according to pregnancy outcome

	C1q		**C2**		**C4**		**C4b**		**MBL**		**Factor B**		**Factor D**	
	PT	TU	PT	TU	PT	TU	PT	TU	PT	TU	PT	TU	PT	TU
Detectable	32	44	27	57	32	56	6	4	17	32	12	20	14	19
Extrap	2	24	7	10	2	11	15	32	17	31	7	15	11	25
OOR	0	0	0	1	0	0	13	32	0	5	15	33	9	24
p-value	0.0014[a] **		0.5992		0.2095[a]		0.1618		0.2679		0.8326		0.3872	

[a] Fisher's exact test.

	C3		**C3a**		**C3b**		**C5**		**C5a**		**Factor H**		**Factor I**	
	PT	TU	PT	TU	PT	TU	PT	TU	PT	TU	PT	TU	PT	TU
Detectable	8	9	30	64	20	21	8	4	19	49	32	67	13	14
Extrap	18	18	4	4	6	16	16	47	14	10	2	0	6	17
OOR	8	41	0	0	8	31	10	16	1	9	0	0	15	37
p-value	0.0021 **		0.4355[a]		0.0218 *		0.0179 *		0.0064 **		0.1111[a]		0.1587	

[a] Fisher's exact test. Extrap: Extrapolated. OOR: out of range. Highlighted values indicated statistical proportional differences.

Table 3.12. Detectable ranges of matrix metalloproteinases analytes according to pregnancy outcome

	MMP-2		**MMP-8**		**MMP-9**	
	PT	TU	PT	TU	PT	TU
Detectable	14	16	31	57	27	64
Extrapolated	8	7	3	7	2	3
OOR	10	44	0	4	3	1
p-value	0.0051	**	0.3342		0.1516	

OOR: out of range. Highlighted values indicated statistical proportional differences.

3.6.5. Microbial-host immune responses: associations between the local cervicovaginal microbial composition, the immune response and pregnancy outcome

Cytokine profile

The concentration of the three proinflammatory cytokines IL-1β, IL-6 and IL-8 (Figure 3.22., Figure 3.23. and Figure 3.24., respectively) was compared according to vaginal microbial composition categorised using the community state type (CST) classification. In addition, the cytokine concentrations were compared depending on CST I, CST III, and CST IV subgroups between women who developed cervical shortening and those who maintained cervical length, and between women who delivered preterm compared women who delivered at term. The proportion of samples assigned to the different CSTs in women who shorten and women who delivered preterm is described in Table 3.13.

Table 3.13. Distribution of samples according to CST classification in women who shorten and in women who delivered preterm

CST	Women who shorten		Women who delivered preterm	
	11/51 (21.6%)		17/51 (33.33%)	
	A	**B**	**A**	**B**
I - *L. crispatus*	4 (36%)	4 (36%)	6 (35%)	6 (35%)
II - *L. gasseri*	1 (9%)	1 (9%)	1 (6%)	1 (6%)
III - *L. iners*	3 (27%)	5 (45%)	5 (29%)	6 (35%)
IV - Heterogeneous	3 (27%)	1 (9%)	4 (23%)	3 (18%)
V - *L. jensenni*	0 (0%)	0 (0%)	1 (6%)	1 (6%)

Firstly, the concentration of IL-1β was significantly increased in women with a CST III vaginal composition (median 137.1 pg/mL), compared to women with a CST I (median 30.22 pg/mL) dominating vaginal composition (p=0.0198). The median concentration of women classified as CST IV was also higher, but not statistically significant, than in women with CST I (median 98.37 vs 30.22 pg/mL, p=0.0981) (Figure 3.22.A). When stratifying women by microbial composition and cervical length (those who shortened compared to those who maintained a normal cervical length) (Figure 3.22.B.), women who experienced cervical shortening exhibited higher concentrations of IL-1β than women with a normal cervical length in the presence of CST I (p<0.0001) and CST III (p=0.0292) vaginal composition (Figure 3.22.B). Women who delivered preterm had a higher concentration of IL-1β compared to women who delivered preterm if they had a vaginal microbial composition of CST I (p <0.0001) and CST IV (p=0.6943) (Figure 3.22. C).

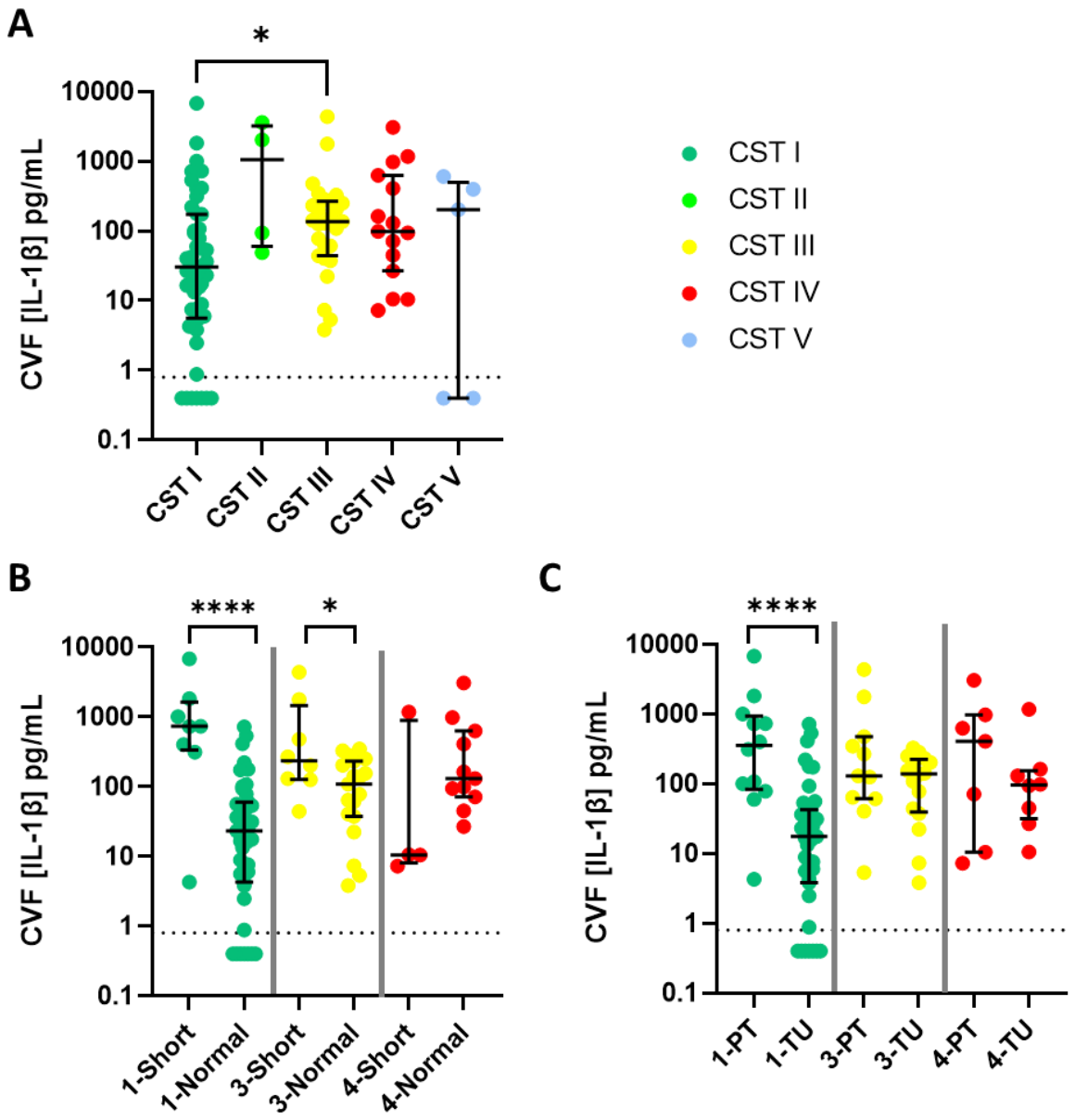


Figure 3.22. Cervicovaginal fluid concentration of IL-1β according to CST vaginal composition. A) Concentration of IL-1β according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of IL-1β was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney one tailed-test. * p<0.05; ****p<0.0001. Data is presented as median values with interquartile range.

In the case of IL-6, no differences were observed based on CST microbial composition (p=0.9275) (Figure 3.23.A). When further subcategorising the cohort by CST and cervical shortening, women classified as CST I that experienced cervical shortening at some point during their pregnancy had higher median IL-6 concentration (median 6.483 pg/mL) than the respective counterpart that did not shorten (median 0.8500 pg/mL) (p=0.0006). This was not observed though in women with CST III (p=0.4196) and CST IV (p=0.4520). (Figure 3.23.B). In women who delivered preterm a higher median concentration was observed in women with a CST I (p=0.0041) and in women with a CST IV (p=0.1333) (Figure 3.23.C).

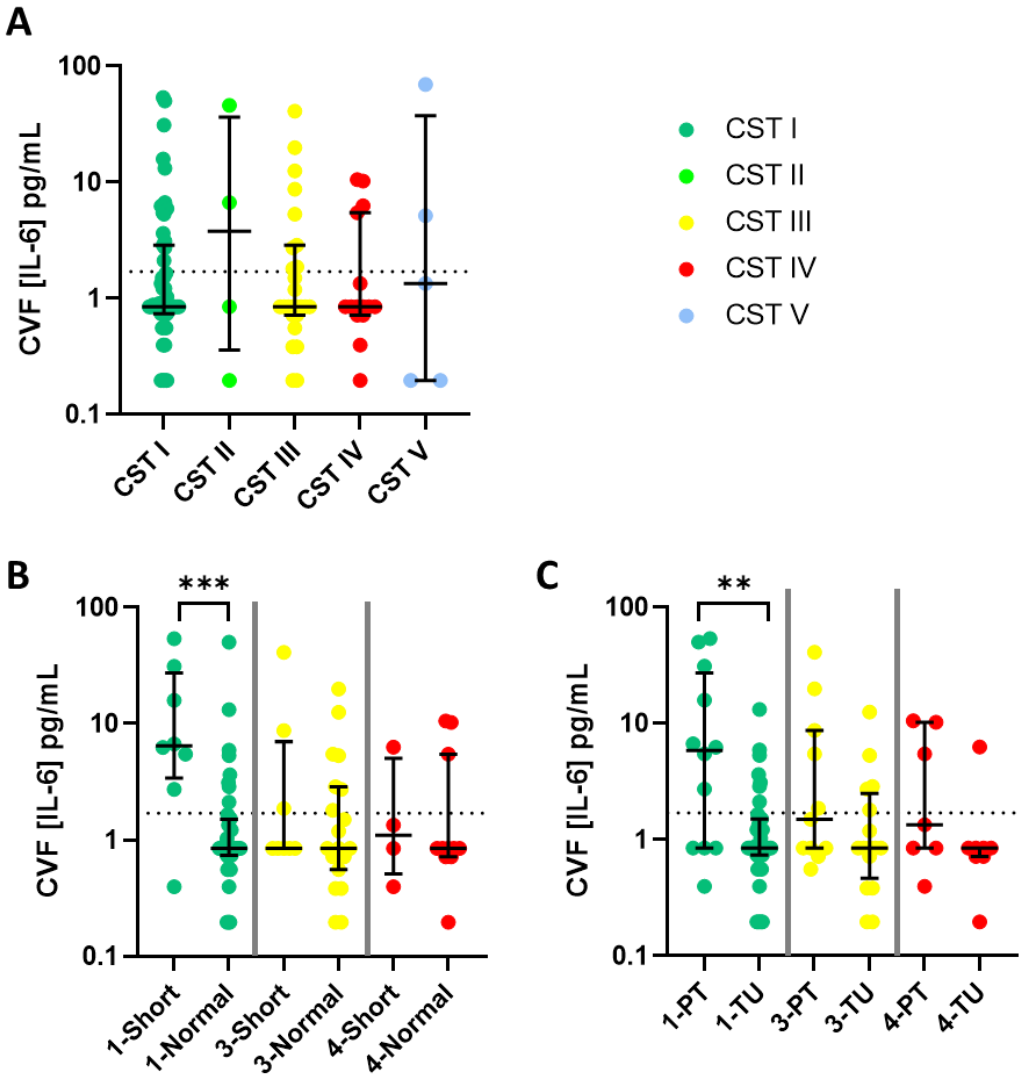


Figure 3.23. Cervicovaginal fluid concentration of IL-6 according to CST vaginal composition. A) Concentration of IL-6 according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of IL-6 was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney one tailed-test. * p<0.05; ** p<0.01; ***p<0.001. Data is presented as median values with interquartile range.

Finally, the median concentration of IL-8 did not significantly differ between women with a CST III or CST IV and women with a CST I (p=0.5784, p=0.7682, respectively). However, women with a CST III had a higher median IL-8 than women with CST IV vaginal composition (p=0.0144) (Figure 3.24.A). In contrast to what was expected, the highest IL-8 median concentration was seen in women with cervical shortening and preterm birth in women who had a vaginal microbial composition dominated by CST I *L. crispatus*. In cases of cervical shortening (Figure 3.24.B), IL-8 was higher compared to women who maintained their cervical length if the microbial composition was CST I (p<0.0001) or CST III (p=0.0374), but not CST IV (p=0.2447) (Figure 3.24.B). In women who delivered preterm, median concentration of IL-8 was significantly higher than women who delivered at term within the CST I group (median 2421 vs 630.3. pg/mL, p=0.0268), however no significant differences were seen within CST III (p=0.3763) and IV (p=0.1678) (Figure 3.24.C).

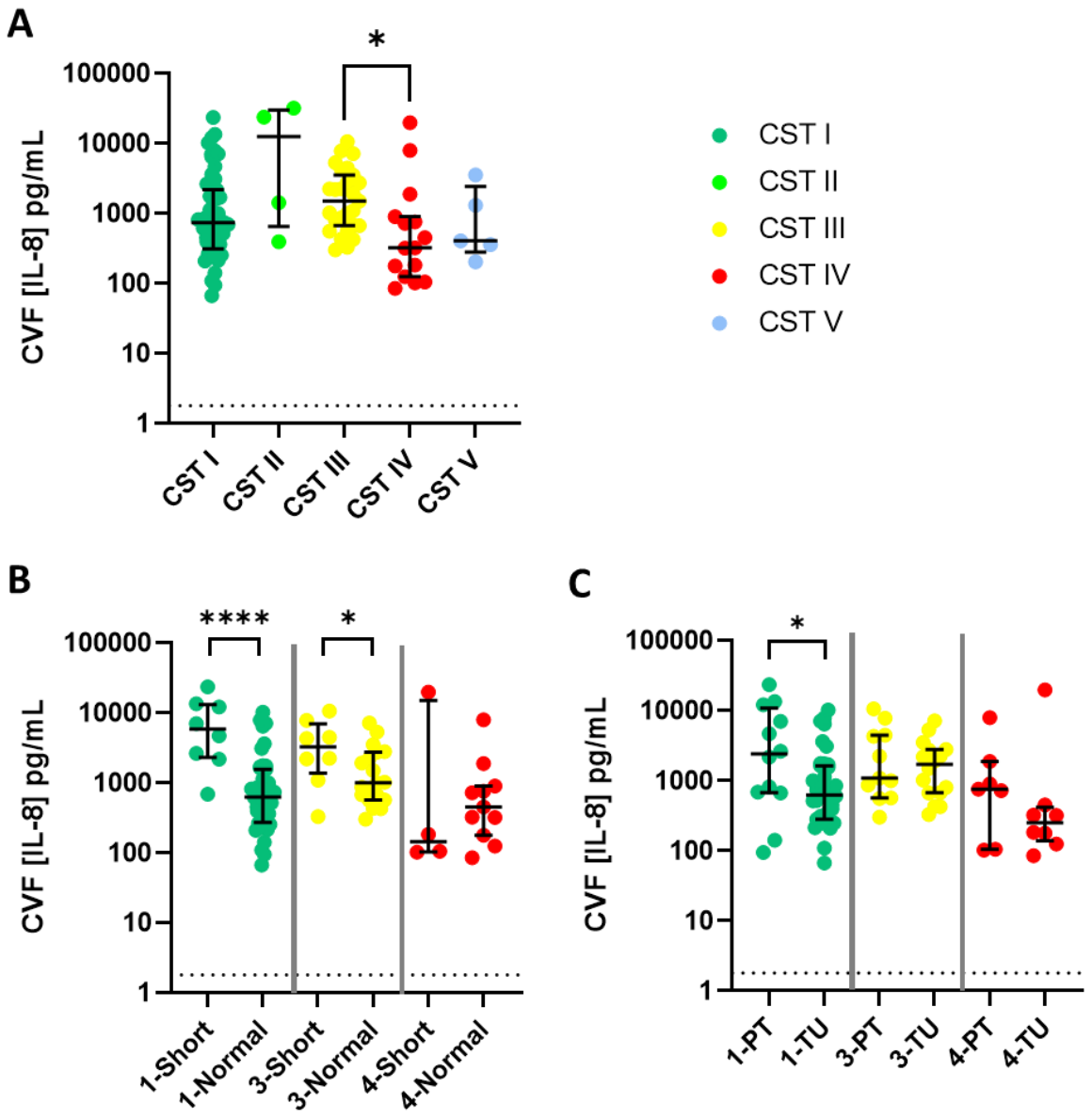


Figure 3.24. Cervicovaginal fluid concentration of IL-8 according to CST vaginal composition. A) Concentration of IL-8 according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of IL-8 was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney one tailed-test. * $p<0.05$; ****$p<0.0001$. Data is presented as median values with interquartile range.

Complement profile

Cervicovaginal fluid complement proteins were also assessed in association with the local cervicovaginal microbial composition and pregnancy outcomes. Complement proteins from the classical pathway will be described first (Figures 3.25.-3.28.).

The classical pathway

The median concentration of C1q was significantly higher in women with a CST IV microbial composition (median 2.177 ng/mL) compared to women with a CST I (median 0.1215 ng/mL, p=0.002) and women with a CST III (median 0.1321 ng/mL, p=0.0069) (Figure 3.25.A). In women who experienced cervical shortening there was a higher median concentration of C1q compared to women who did not shorten in the CST I (median 0.3253 vs 0.08260 ng/mL, p=0.0095) and CST III groups (median 1.008 vs 0.0923 ng/mL, p=0.0006) (Figure 3.25.B). Women who had a CST I *L. crispatus* composition and delivered preterm also had higher median concentration of C1q than women who delivered at term (p=0.0027) (Figure 3.25.C).

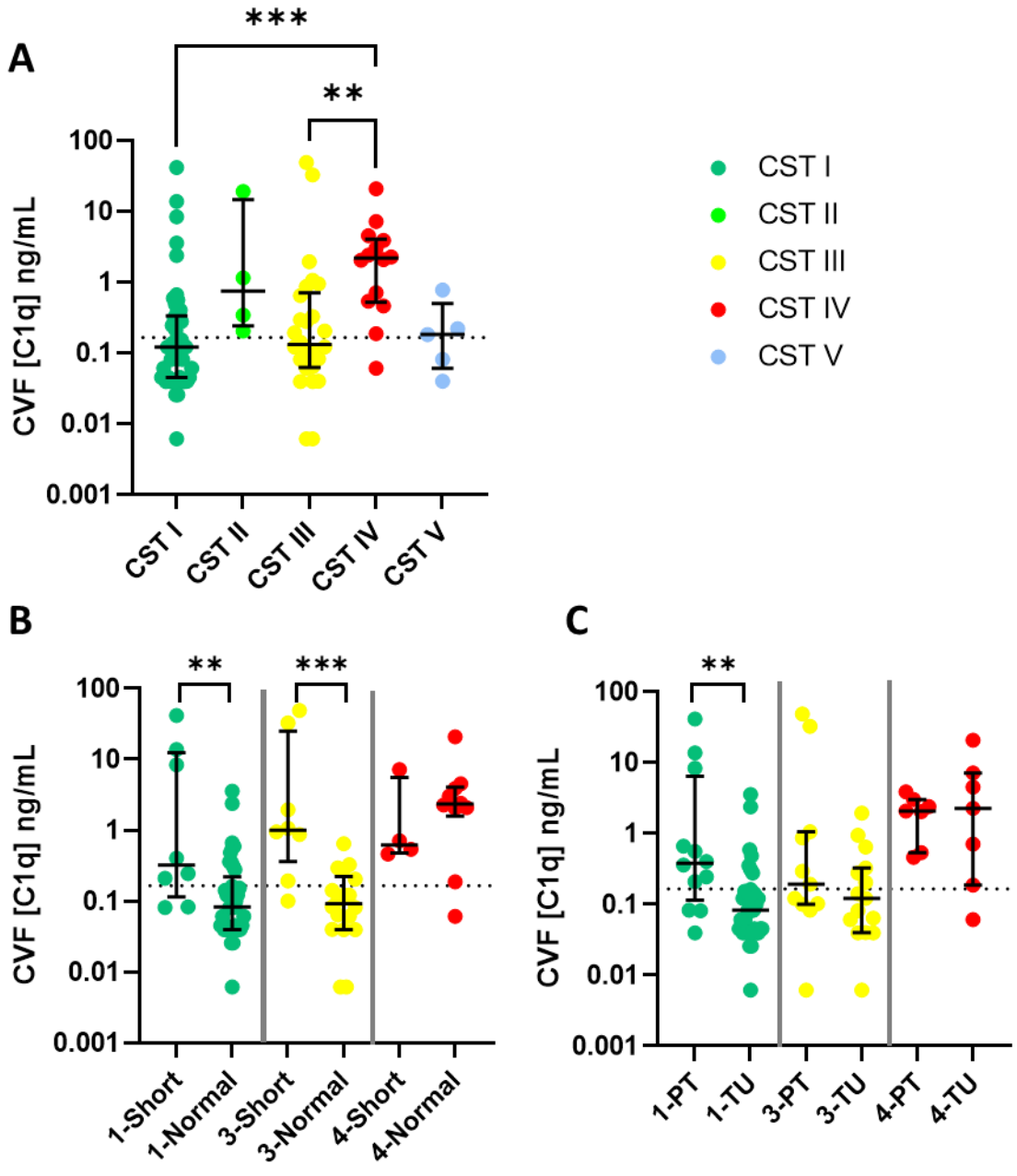


Figure 3.25. Cervicovaginal fluid concentration of C1q according to CST vaginal composition. A) Concentration of C1q according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of C1q was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. ** p<0.01; ***p<0.001. Data is presented as median values with interquartile range.

The C2 concentration did not vary across CST groups (p=0.3558) (Figure 3.26.A). However, both cervical shortening and preterm birth were associated with higher median C2 concentrations in certain CST groups. In cases of cervical shortening, women with a CST I *L. crispatus* dominance had a higher median concentration of C2 (median 24.84 ng/mL) than women with the same microbial composition who did not shorten (median 5.942 ng/mL, p<0.0001) (Figure 3.26.B). In women who delivered preterm, there was also a higher median C2 concentration compared to women who delivered at term in the groups CST I (median 20.48 vs 6.837 ng/mL, p=0.1184) and CST III (median 34.39 vs 7.395, p=0.0066) (Figure 3.26.C).

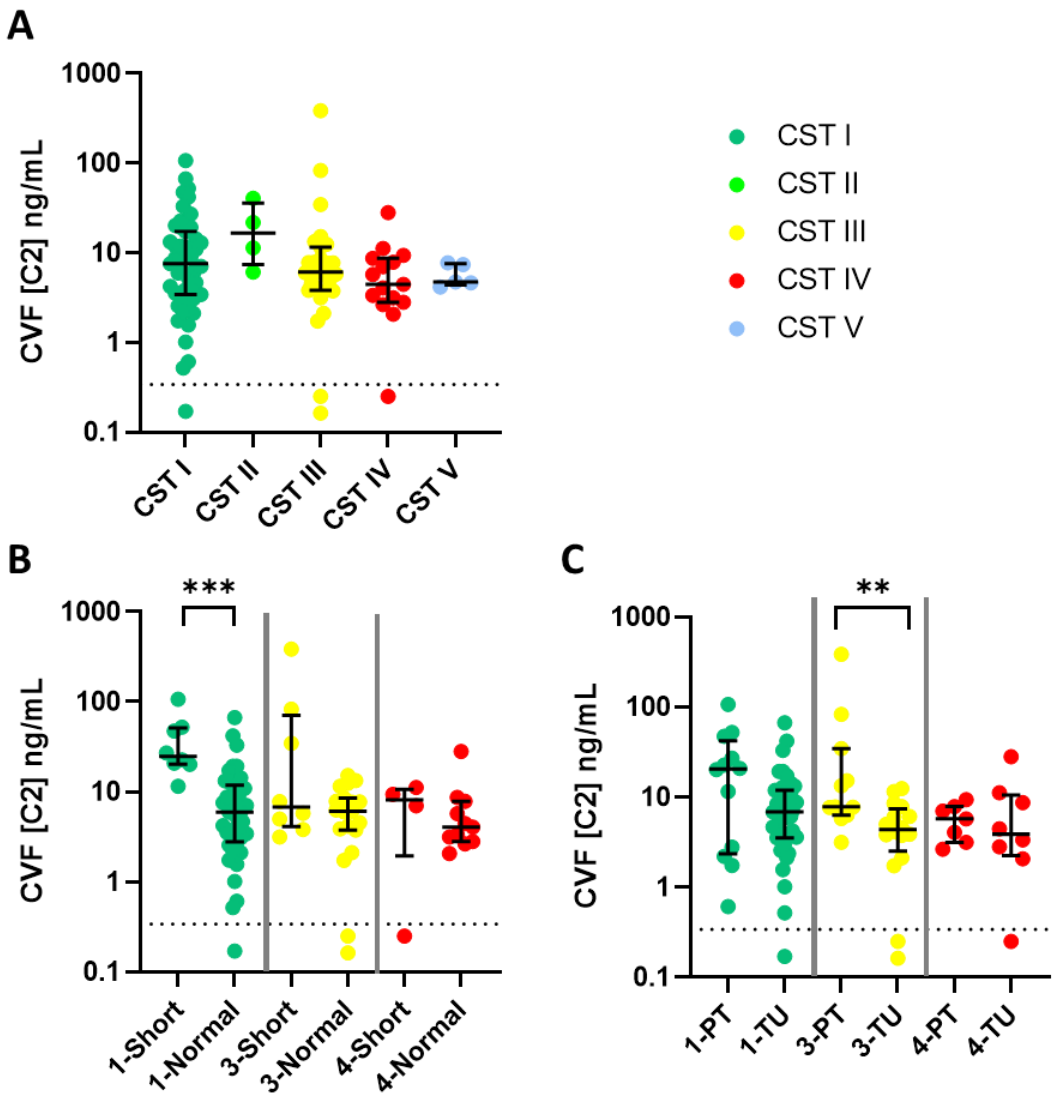


Figure 3.26. Cervicovaginal fluid concentration of C2 according to CST vaginal composition. A) Concentration of C2 according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of C2 was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. ** p<0.01; ***p<0.001. Data is presented as median values with interquartile range.

The highest median concentration of C4 was observed in women with a CST IV vaginal composition (median 68.30 ng/ml), which was significantly increased compared to women with a CST I (median 4.288 ng/mL, p=0.0001) but not CST III (median 11.91 ng/mL, p=0.0505) (Figure 3.27.A).

Based on cervical shortening, there was a higher median C4 concentration in CVF of women with cervical shortening, compared to woman who maintained a normal cervical length in women with a CST I (102.5 vs 3.029 ng/mL, p=0.0002) and CST III (median 43.60 vs 9.144 ng/mL, p=0.0112) (Figure 3.27.B). Women who delivered preterm also had higher C4 median in CST I compared to woman who delivered at term (median 60.09 vs 2.207 ng/mL, p<0.0001) (Figure 3.27.B).

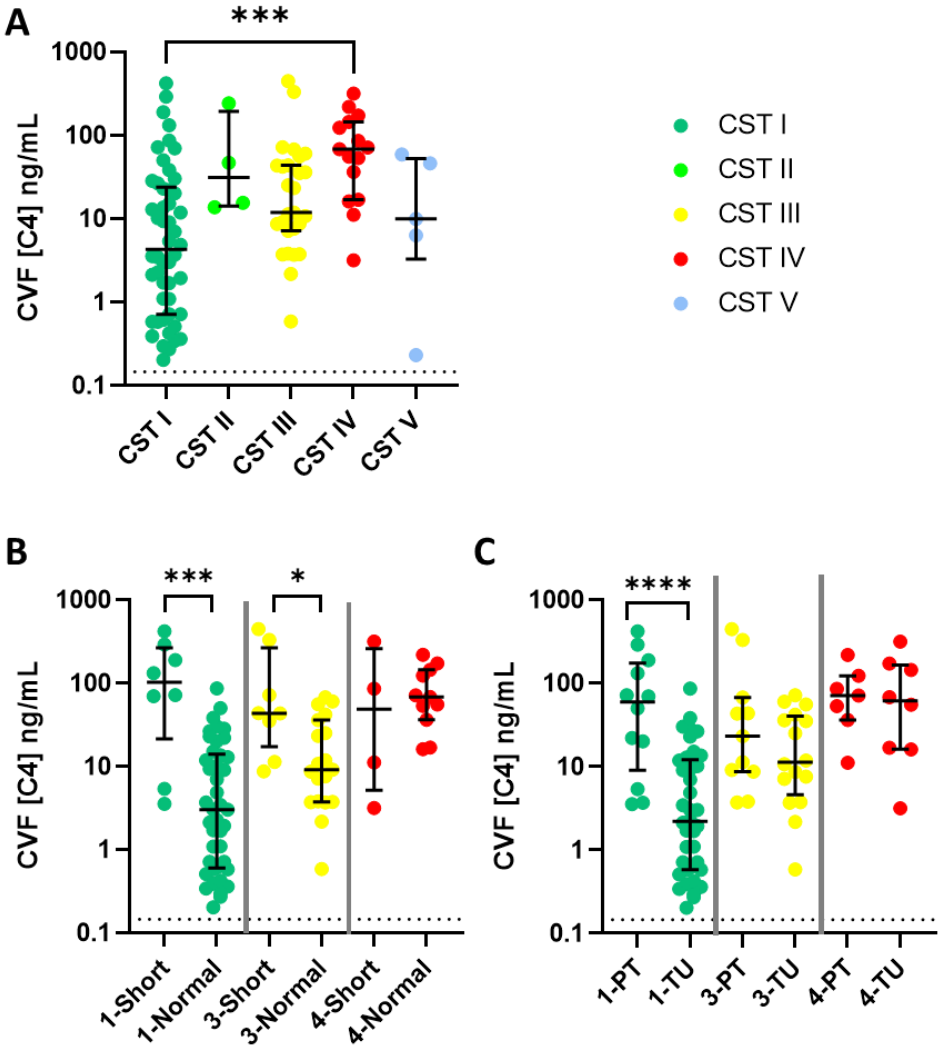


Figure 3.27. Cervicovaginal fluid concentration of C4 according to CST vaginal composition. A) Concentration of C4 according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of C4 was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. * p<0.05; *** p<0.001; ****p<0.0001. Data is presented as median values with interquartile range.

Finally, the defragmentation of C4 can be observed in the release of subproduct C4b. No differences were observed between CST IV and CST I groups (median 1.212 vs 0.407 ng/mL, p=0.0926). However, the median concentration of C4b was significantly increased in women with a CST IV microbial composition compared to CST III (p=0.0088) (Figure 3.28 A). Only women with a CST I *L. crispatus* dominance had higher median C4b in women who experienced cervical shortening (median 11.350 ng/mL) compared to women who did not shorten (0.2433 ng/mL) (p=0.0281) (Figure 3.28.B). No major differences were observed based on CST and outcome (Figure 3.28.C).

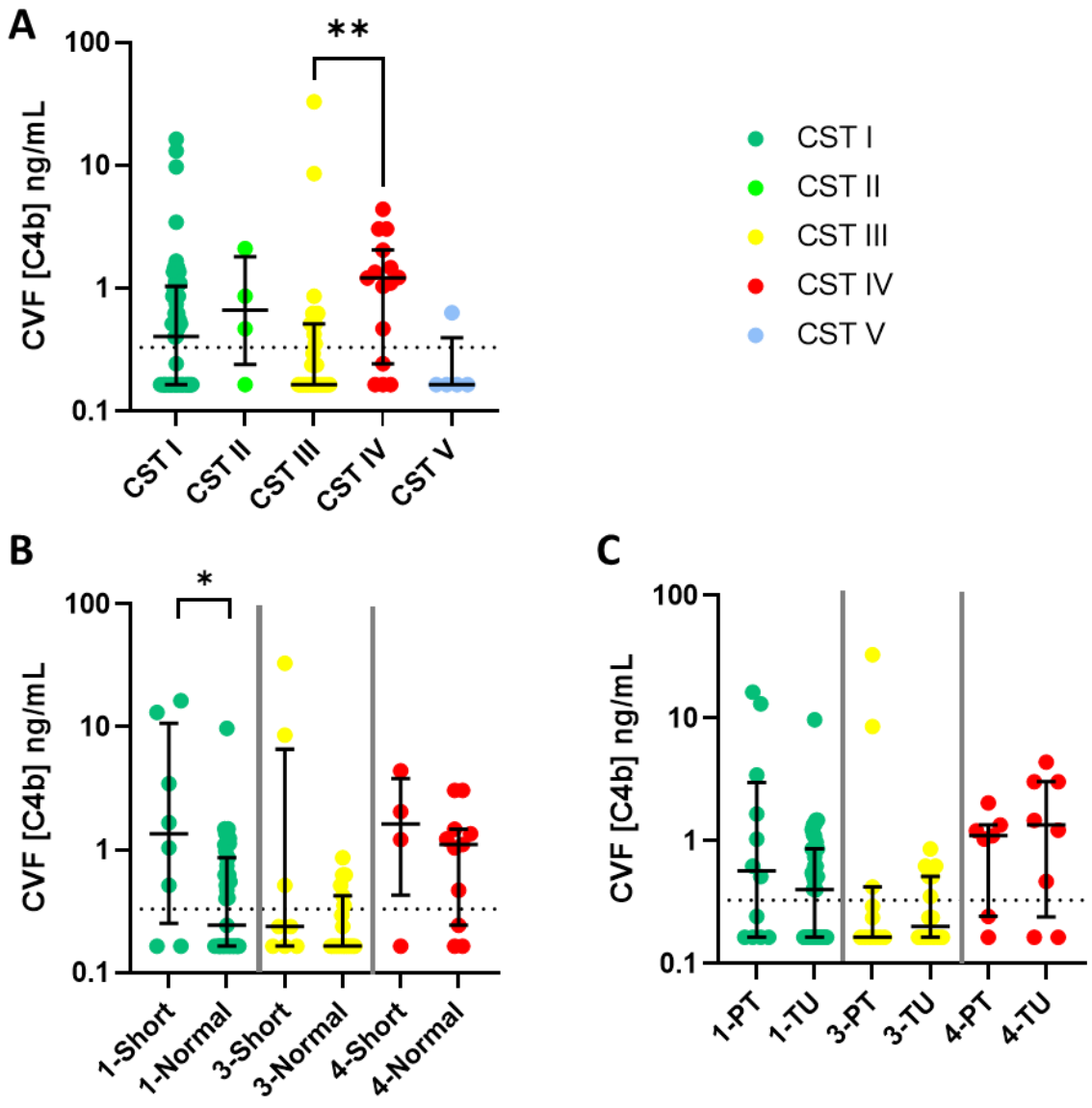


Figure 3.28. Cervicovaginal fluid concentration of C4b according to CST vaginal composition. A) Concentration of C4b according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of C4b was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. * p<0.05; ** p<0.01. Data is presented as median values with interquartile range.

The lectin pathway

With reference to the lectin pathway, the concentration of MBL did not differ significantly between women with different CST classifications (Figure 3.29.A). However, women with a CST I vaginal composition had higher concentrations of MBL when experiencing cervical shortening, compared to women who maintained their cervical length (median 1.093 vs 0.1672 ng/mL, p=0.0037) (Figure 3.29.B). When assessing pregnancy outcome, a trend was observed of higher MBL median concentrations in women who delivered preterm compared to term for each CST (I, III, and IV) but this did not reach statistical significance (p=0.0504, p=0.4433, p=0.2200, respectively) (Figure 3.29. C).

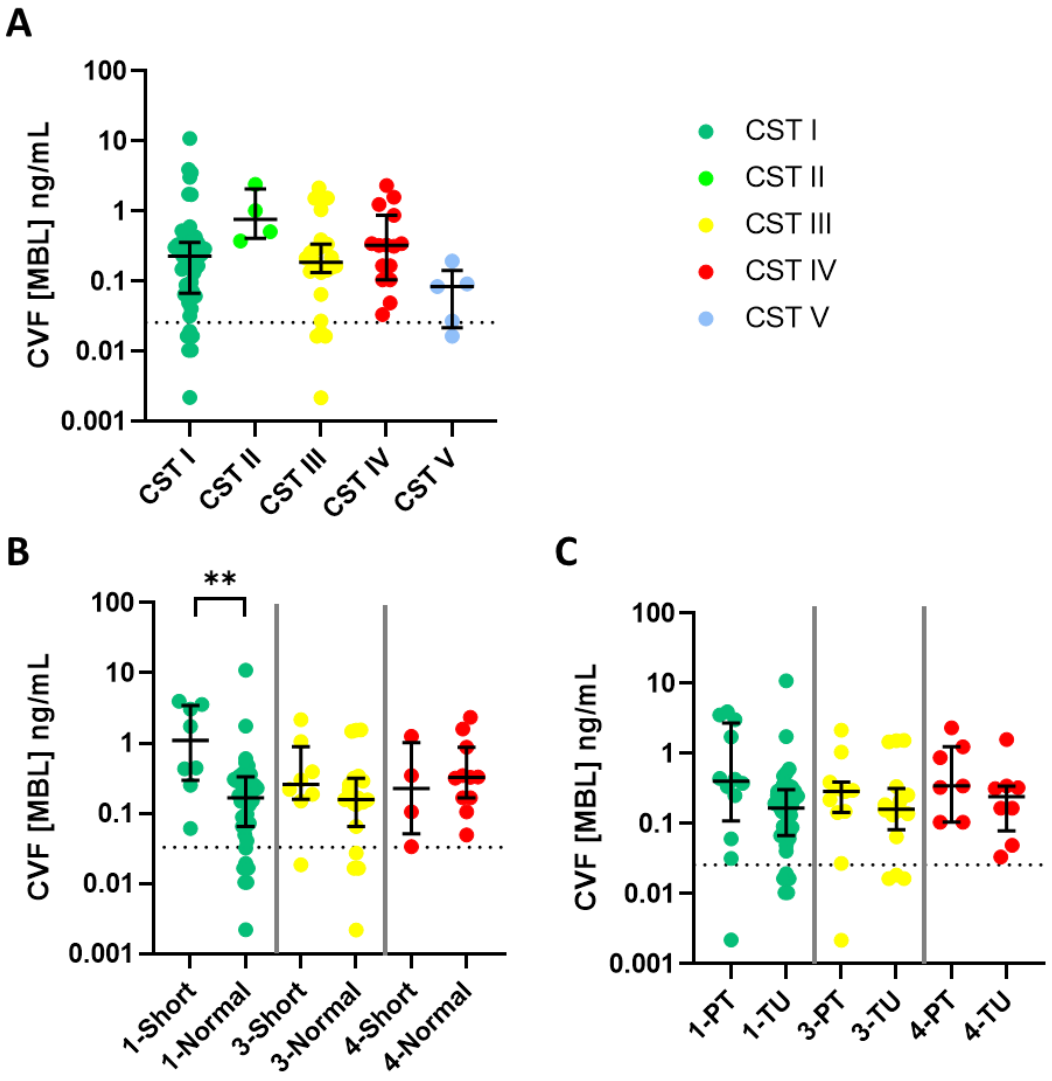


Figure 3.29. Cervicovaginal fluid concentration of MBL according to CST vaginal composition. A) Concentration of MBL according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of MBL was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. ** p<0.01. Data is presented as median values with interquartile range.

The alternative pathway

The concentration of Factor B was higher in women with a CST IV composition (median 0.3872 ng/mL) compared to women with a CST I (median 0.1100, p<0.0001) and a CST III (median 0.01618, p=0.0420) (Figure 3.30.A). No significant differences were observed in women who underwent cervical shortening (Figure 3.30. B) or who delivered preterm (Figure 3.30. C) within each CST category.

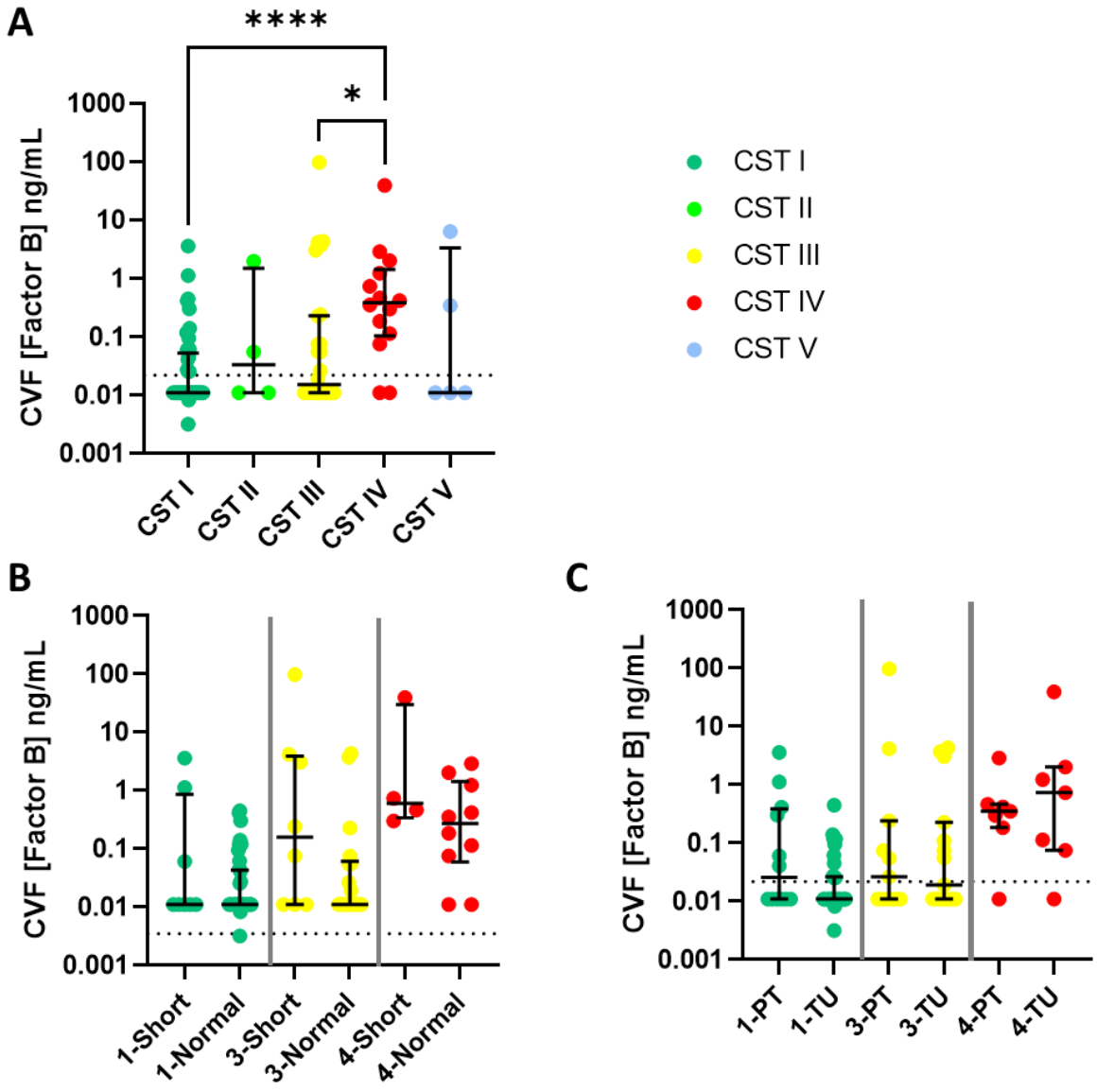


Figure 3.30. Cervicovaginal fluid concentration of Factor B according to CST vaginal composition. A) Concentration of Factor B according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of Factor B was also compared in women who shorten and woman who maintained a normal cervical and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. * p<0.05; ****p<0.0001. Data is presented as median values with interquartile range.

Factor D concentration was higher in women with a CST IV composition (median 0.2377 ng/mL) compared to women with CST I (median 0.01350 ng/mL) and CST III (median 0.05859 ng/mL) compositions (p<0.0001, p=0.0082, respectively) (Figure 3.31.A). No significant differences were seen according to cervical shortening (Figure 3.31. B), but as shown in Figure 3.31.C, women with a CST I vaginal composition that delivered preterm had a higher concentration of Factor D (median 0.03841 ng/mL) than women who delivered at term (median 0.1350 ng/mL) (p=0.033).

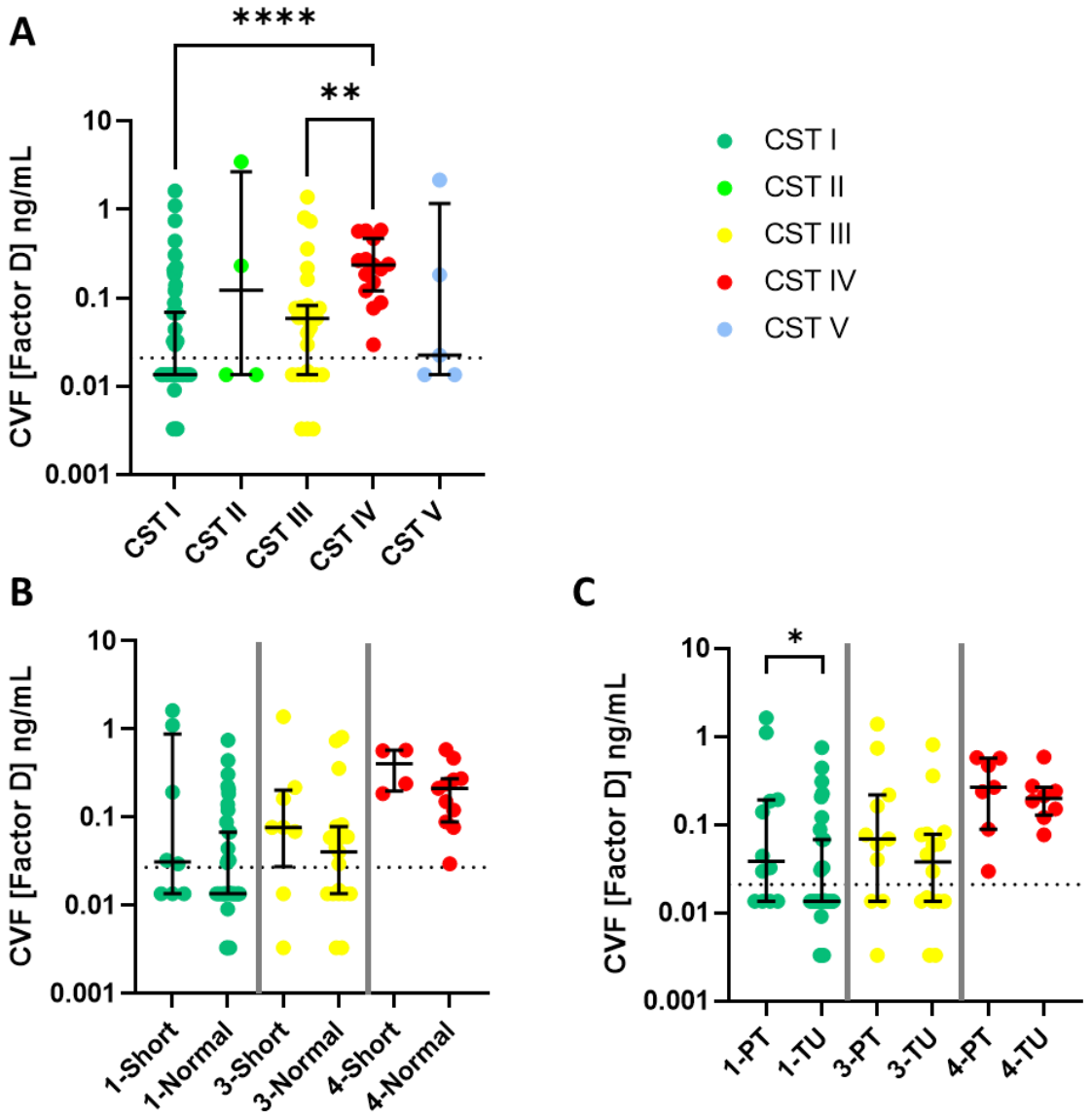


Figure 3.31. Cervicovaginal fluid concentration of Factor D according to CST vaginal composition. A) Concentration of Factor D according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of Factor D was also compared in women who shorten and woman who maintained a normal cervical and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15).. Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. * p<0.05; ** p<0.01; ****p<0.0001. Data is presented as median values with interquartile range.

Convergence on C3

The target of C3 convertases is the protein C3. The median concentration of C3 was higher in women with a CST IV microbial composition (0.8706 ng/mL) compared to women with a CST I (median 0.1100 ng/mL p=0.0001) or a CST III (median 0.1100 ng/mL, p=0.0002) (Figure 3.32.A). When assessing differences by cervical shortening, no significant differences were seen compared to women who maintained a normal length within each CST category (Figure 3.32. B).

Women who were classed as CST I had higher concentrations of C3 if they delivered preterm compared to term (p=0.0073). However, there were no statistically significant differences within CST III (p=0.7842) or IV (p=0.6126) (Figure 3.32.C.).

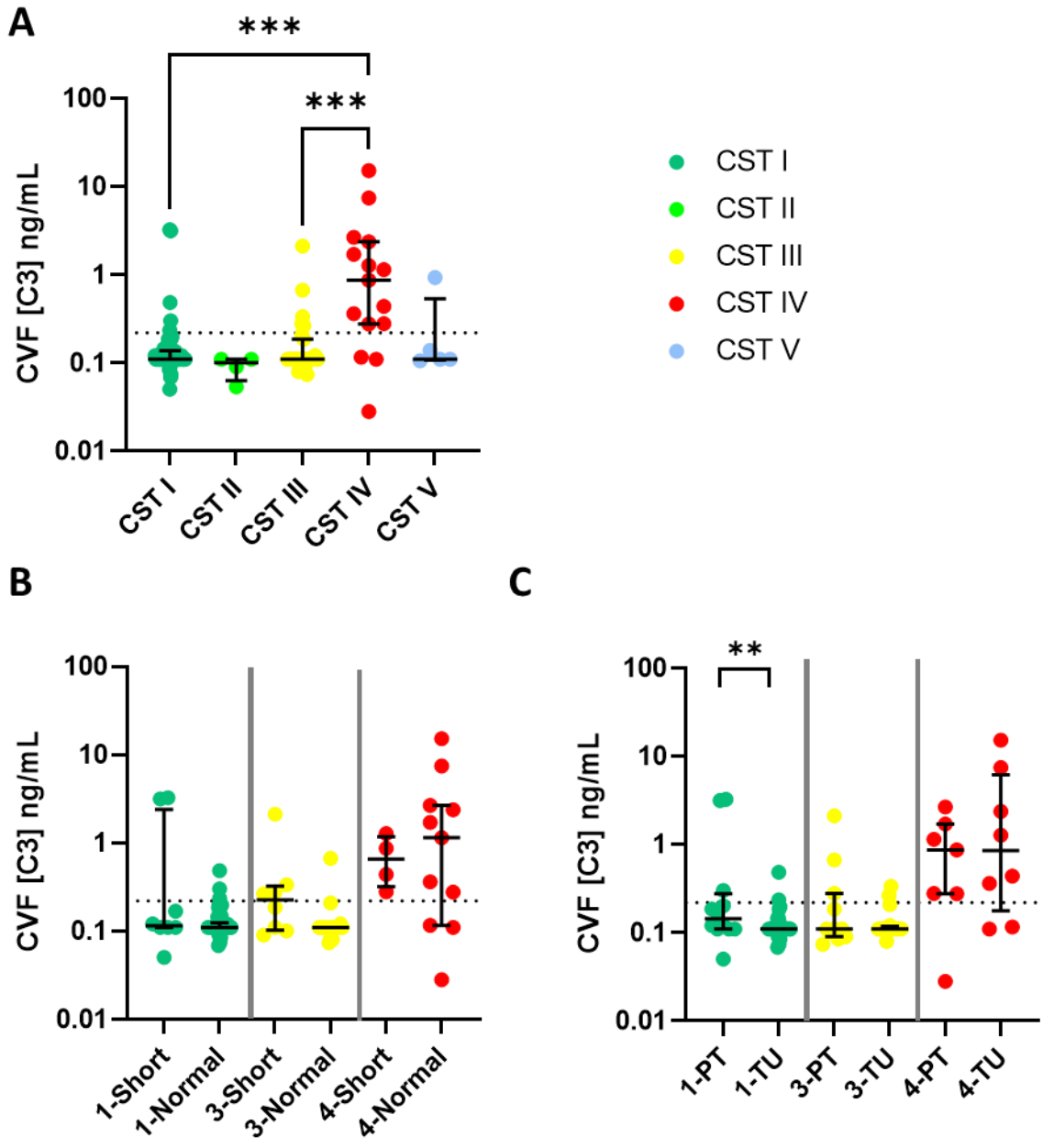


Figure 3.32. Cervicovaginal fluid concentration of C3 according to CST vaginal composition. A) Concentration of C3 according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of C3 was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. ** p<0.01; ***p<0.001; ****p<0.0001. Data is presented as median values with interquartile range.

The action of C3 convertases fragment C3 into C3a (chemoattractant) and C3b (opsonin potentiating opsonophagocytosis). There were no differences in the concentration of C3a between women with different CST composition (p=0.115) (Figure 3.33. A). In contrast, women with a CST I (p=0.0002) and CST III (p=0.0949) vaginal microbial composition that experienced cervical shortening had higher concentrations of C3a (Figure 3.33.B). In addition, the median concentration of C3a was higher in women who delivered preterm compared to at term in women dominated with CST I (median 864.2 vs 313.1 pg/mL, p=0.0007) and III (median 885.9 vs249.3 pg/mL, p=0.0501) (Figure 3. 33.C).

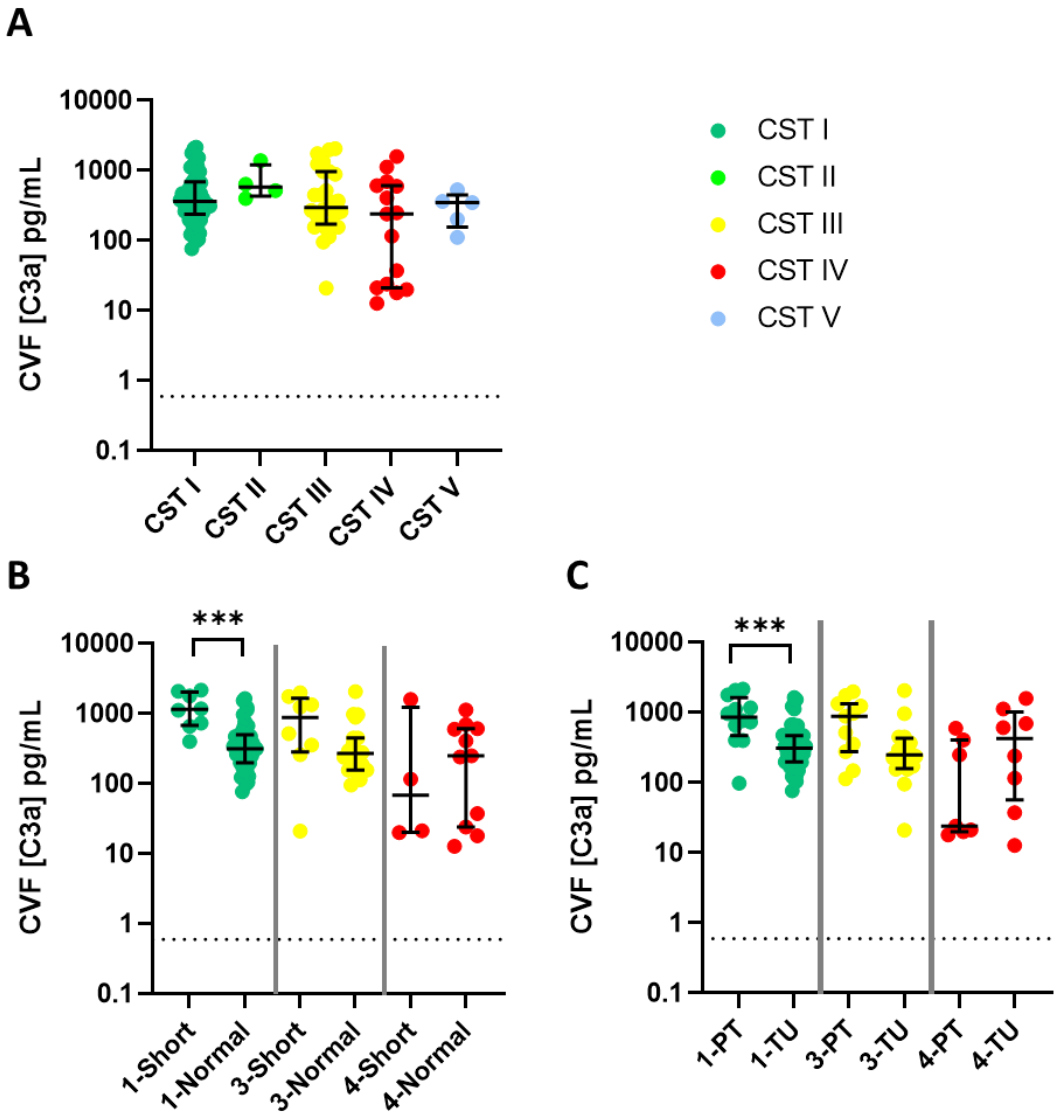


Figure 3.33. Cervicovaginal fluid concentration of C3a according to CST vaginal composition. A) Concentration of C3a according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of C3a was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. *** p<0.001. Data is presented as median values with interquartile range.

Higher concentration of C3b were observed in women with a CST IV (median 56.75 ng/mL) composition compared to women with a CST I (median 2.388 ng/mL, p<0.0001) or CST III (median 5.603 ng/mL, p=0.0039) vaginal microbial composition, as shown in Figure 3.34. A. In women who experienced cervical shortening at some during their pregnancy, a higher median concentration of C3b was detected in women with a CST III composition compared to women with a normal cervical length (p=0.0046), but not in the CST I group (p=0.0622) or CST IV (p=0.2799) (Figure 3.34 B). The CST I group exhibited higher concentrations of C3b when women delivered preterm, compared to term (p=0.027). A similar trend was observed for women with a CST III and IV, although this did not reach statistical significance (p=0.2032, p=0.2810, respectively).

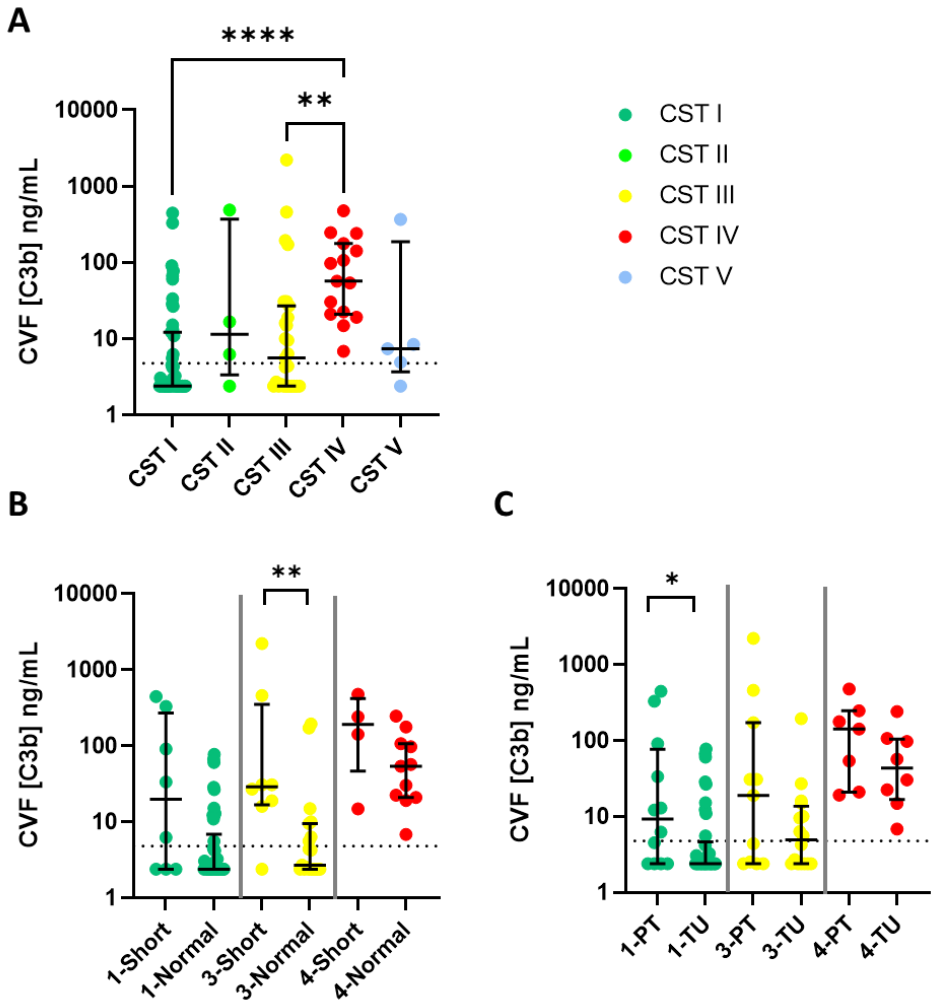


Figure 3.34. Cervicovaginal fluid concentration of C3b according to CST vaginal composition. A) Concentration of C3b according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of C3b was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. * p<0.05; ** p<0.01; ****p<0.0001. Data is presented as median values with interquartile range.

The next common step of the complement system is the activation of C5 by C5 convertases as it defragments into C5a and C5b. No significant differences (p=0.0727) were observed in the concentration of C5 across the different CST groups, but a slight increase in the median C5 was observed in women with CST IV (median 1.299 ng/mL, vs <1ng/ml in CST I and III) (Figure 3.35. A). Despite not statistically significant, all women who had a short cervix had higher median concentration of C5 compared to women with a normal cervical length with a CST I (1.465 vs 0.4 ng/mL, p=0.1021) CST III (1.386 vs 0.9258 ng/mL, p=0.3078) and CST IV (2.104 vs 1.299 ng/mL, p=0.6374) (Figure 3.35.B). Women who delivered preterm also had higher median concentration of C5 (0.9258 vs 0.40, p=0.2374) (Figure 3.35.C).

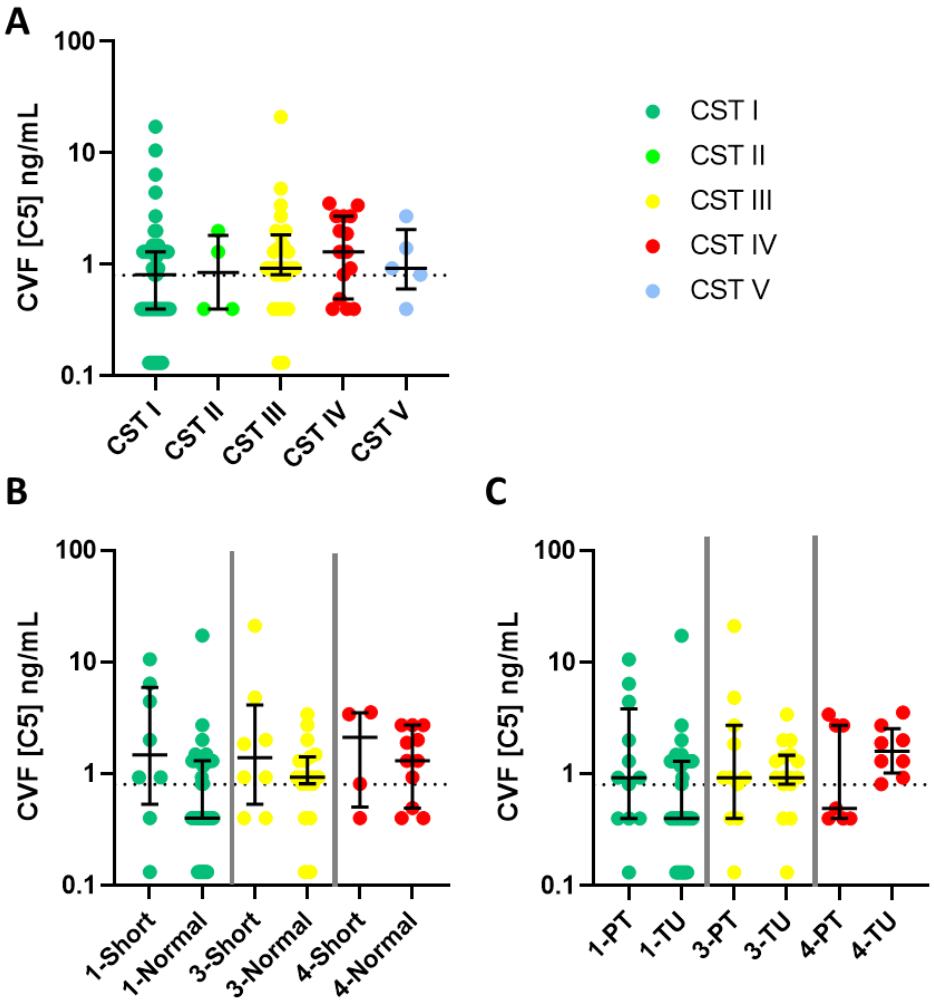


Figure 3.35. Cervicovaginal fluid concentration of C5 according to CST vaginal composition. A) Concentration of C5 according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of C5 was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. Data is presented as median values with interquartile range.

The concentration of C5a did not vary depending on the CST microbial composition (p=0.0782) (Figure 3.36.A). However, women with a short cervix classified as CST I had a significantly higher median concentration of C5a (60.63 pg/mL) compared to women who maintained a normal cervical length (19.10 pg/mL) (p=0.0006). The median was also higher in CST III (median 23.94 VS 9.724 pg/mL), but this did not reach statistical significance (p=0.0733). (Figure 3.36 B)

C5a median concentration was also higher in women who delivered preterm compared to term in women that had a CST III vaginal dominance (median 30.89 vs 7.453, pg/mL, p=0.0124) (Figure 3.36.C). No significant differences were seen by outcome in women with CST I (p=0.2733) and IV (p=0.6370).

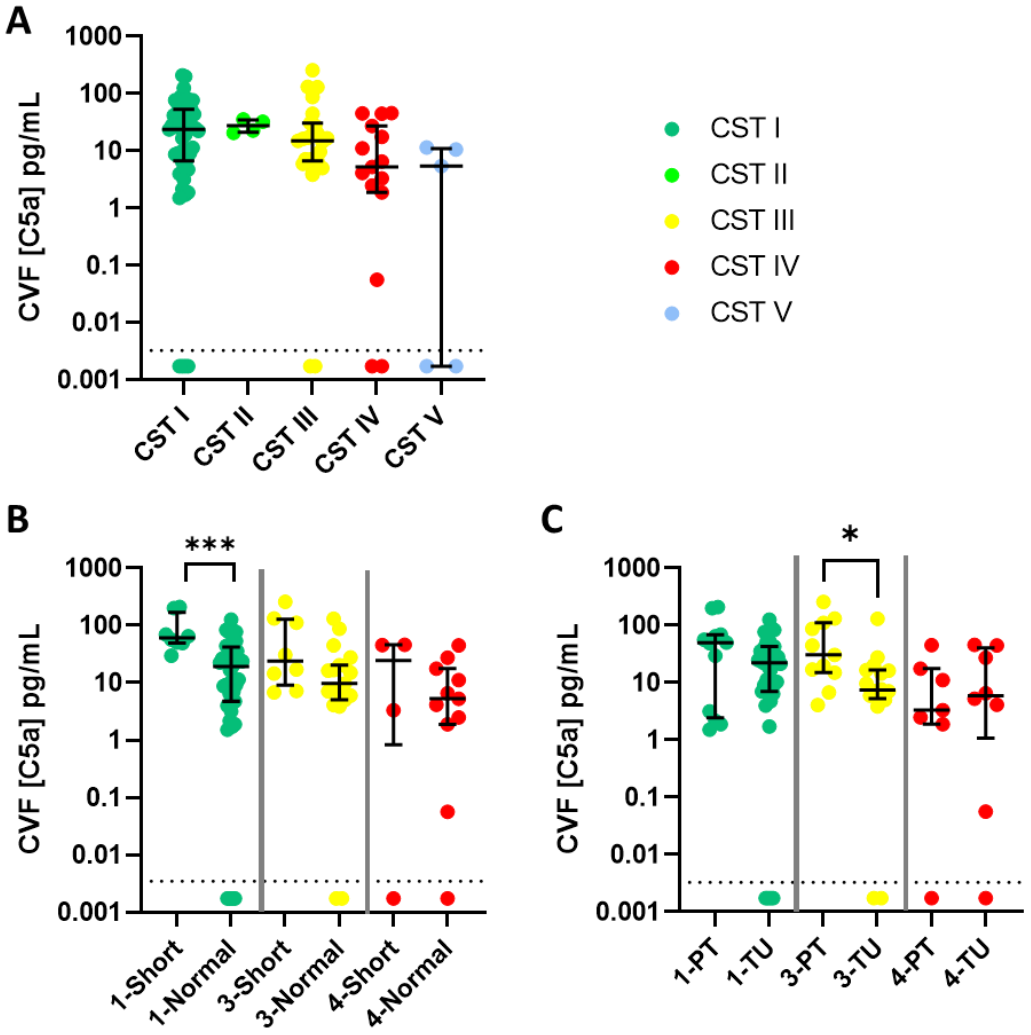


Figure 3.36. Cervicovaginal fluid concentration of C5a according to CST vaginal composition. A) Concentration of C5a according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of C5a was also compared in women who shorten and woman who maintained a normal cervical and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. * p<0.05; *** p<0.001. Data is presented as median values with interquartile range.

Complement regulators

Factor H concentration was 3-times higher in women with a CST IV vaginal composition, compared to women with a CST I (median 39.02 vs 11.83 ng/mL, p=0.0086). Women with a CST III vaginal composition also had a higher median concentration of Factor H than CST I, but this did not reach statistical significance (median 30.58 vs 11.83, p=0.0579) (Figure 3.37.A). Women who experienced cervical shortening had a higher concentration of Factor H than women that did not shorten when they were classified as CST I (p<0.0001) or CST III (p=0.0044), with no differences seen in CST IV (Figure 3.37.B). By pregnancy outcome, women who delivered preterm had a higher median concentration of Factor H in CST I (42.59 vs 8.422 ng/mL) CST III (44.17 vs 26.94 ng/mL) and IV (49.75 vs 25.68 ng/mL), however this was only statistically significant in CST I (p=0.0017, p=0.2310, p=0.2319, respectively) (Figure 3.37.C).

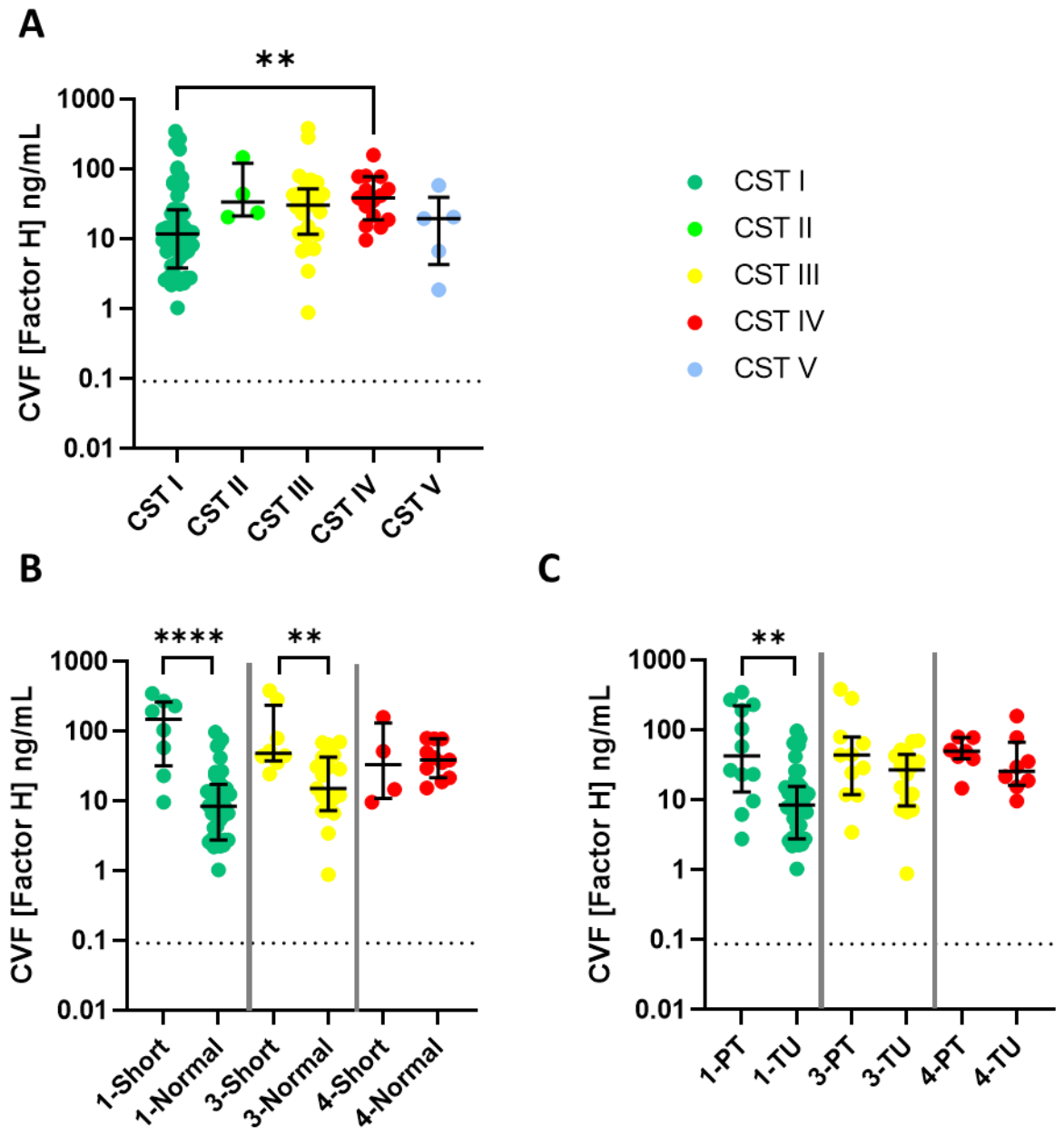


Figure 3.37. Cervicovaginal fluid concentration of Factor H according to CST vaginal composition. A) Concentration of Factor H according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of Factor H was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. ** $p<0.01$; ****$p<0.0001$. Data is presented as median values with interquartile range.

The concentration of Factor I was increased in women with a CST IV (median 2.185 ng/mL) vaginal composition compared to women with a CST I (median 0.0999 ng/mL, p<0.0001) and CST III (median 0.0900, p=0.0021) (Figure 3.38.A). Also, for women with a CST III and underwent cervical shortening, a higher median Factor I (1.332 ng/mL) concentration was seen compared to women with the same CST composition that did not shorten (0.09 ng/mL, p=0.0136). Although no significant, same pattern was observed for women with CST I (median 0.2417 vs 0.09 ng/mL) and CST IV (3.132 vs 2.185 ng/mL) (Figure 3.38.B). No differences in median concentrations of Factor I were observed according to pregnancy outcome within each CST category (Figure 3.38.C).

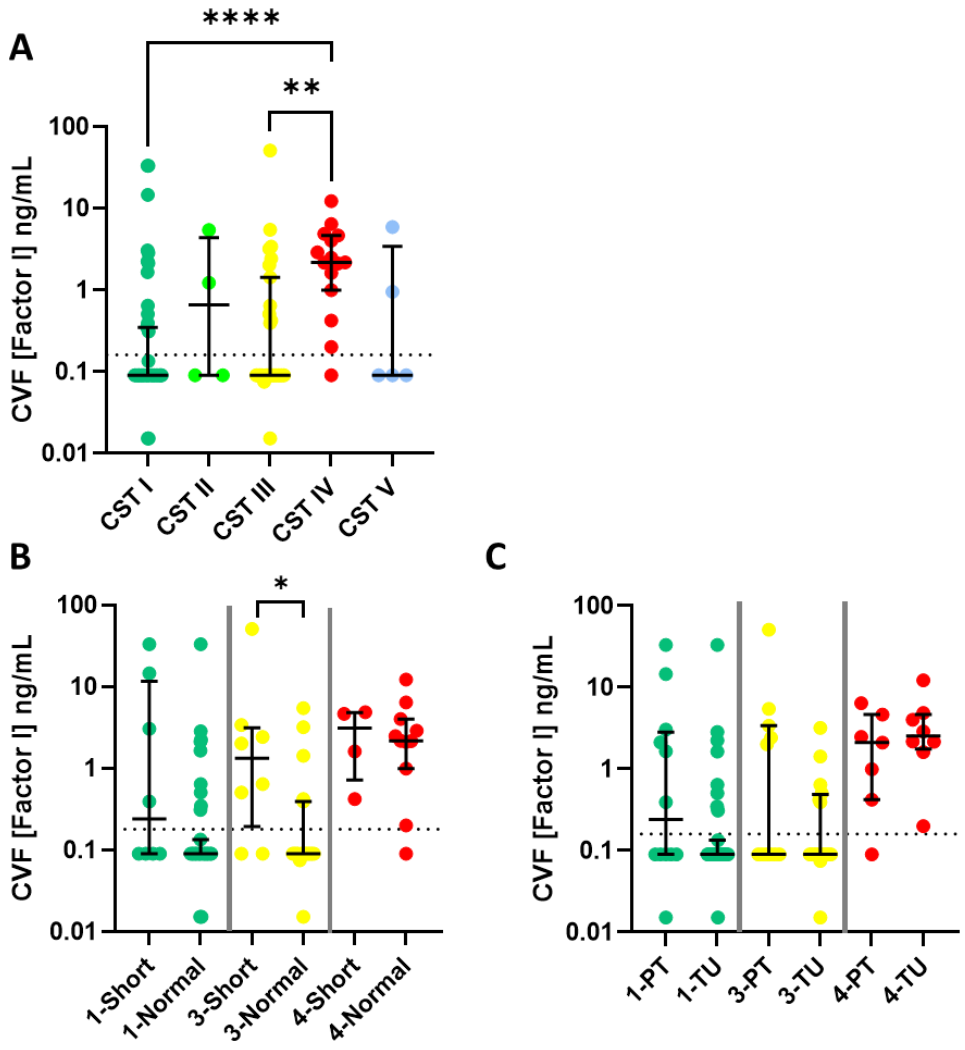


Figure 3.38. Cervicovaginal fluid concentration of Factor I according to CST vaginal composition. A) Concentration of Factor I according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of Factor I was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. * p<0.05; **p<0.01; ****p<0.0001. Data is presented as median values with interquartile range.

Matrix metalloproteinases profile

Matrix metalloproteinases degrade extracellular matrix, so MMP-2, -8 and -9 concentrations were evaluated in the context of microbial composition and pregnancy outcomes (Figure 3.39, Figure 3.40, Figure 3.41., respectively).

MMP-2 had a clear bimodal distribution in all groups. There were no statistical differences in the median concentration of MMP-2 based on CST classification (p=0.1716) (Figure 3.39.A). However, women with a CST I vaginal microbial composition had higher median concentrations of MMP-2 (3463pg/mL) compared to woman with the same vaginal microbial composition who did not shorten (54pg/mL) (p=0.0003) (Figure 3.39 A), as well as a higher median in women who delivered preterm (2996pg/mL) compared to term (54pg/mL) (p=0.0075) (Figure 3.39.C). Although not statistically significant, women with a CST III composition also had higher median concentrations in the shortening group (median 1728 vs 2657 pg/mL, p=0.2657) and in women who delivered preterm (median 1466 vs 256.9pg/mL, p=0.1209).

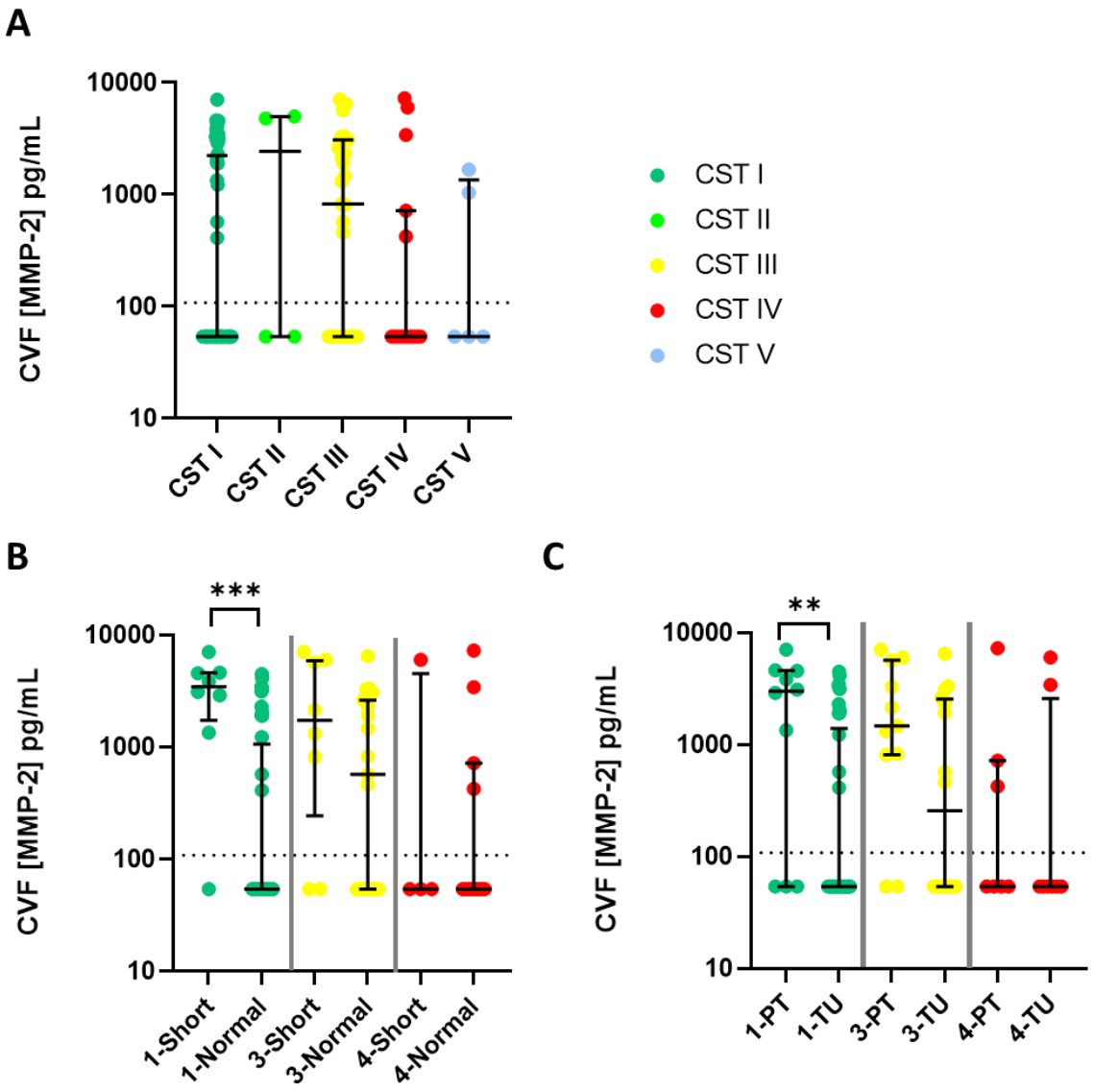


Figure 3.39. Cervicovaginal fluid concentration of MMP-2 according to CST vaginal composition. A) Concentration of MMP-2 according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of MMP-2 was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. ** $p<0.01$; ***$p<0.001$. Data is presented as median values with interquartile range.

No differences were observed in the medians of MMP-8 between groups with different CSTs composition (p=0.2341) (Figure 3.40 A). But as seen for MMP-2, the median concentration of MMP-8 was also higher in women with a CST I (median 31610 vs 3807pg/mL, p=0.0028) and CST III (median 12992 vs 7494 pg/mL, p=0.3887) who underwent cervical shortening (Figure 3.40 B) and in women who delivered preterm (CST I median 23278 vs 3807 pg/mL, p=0.0322; CST III median 15142 vs 6753 pg/mL, p=0.2310) (Figure 3.40 C).

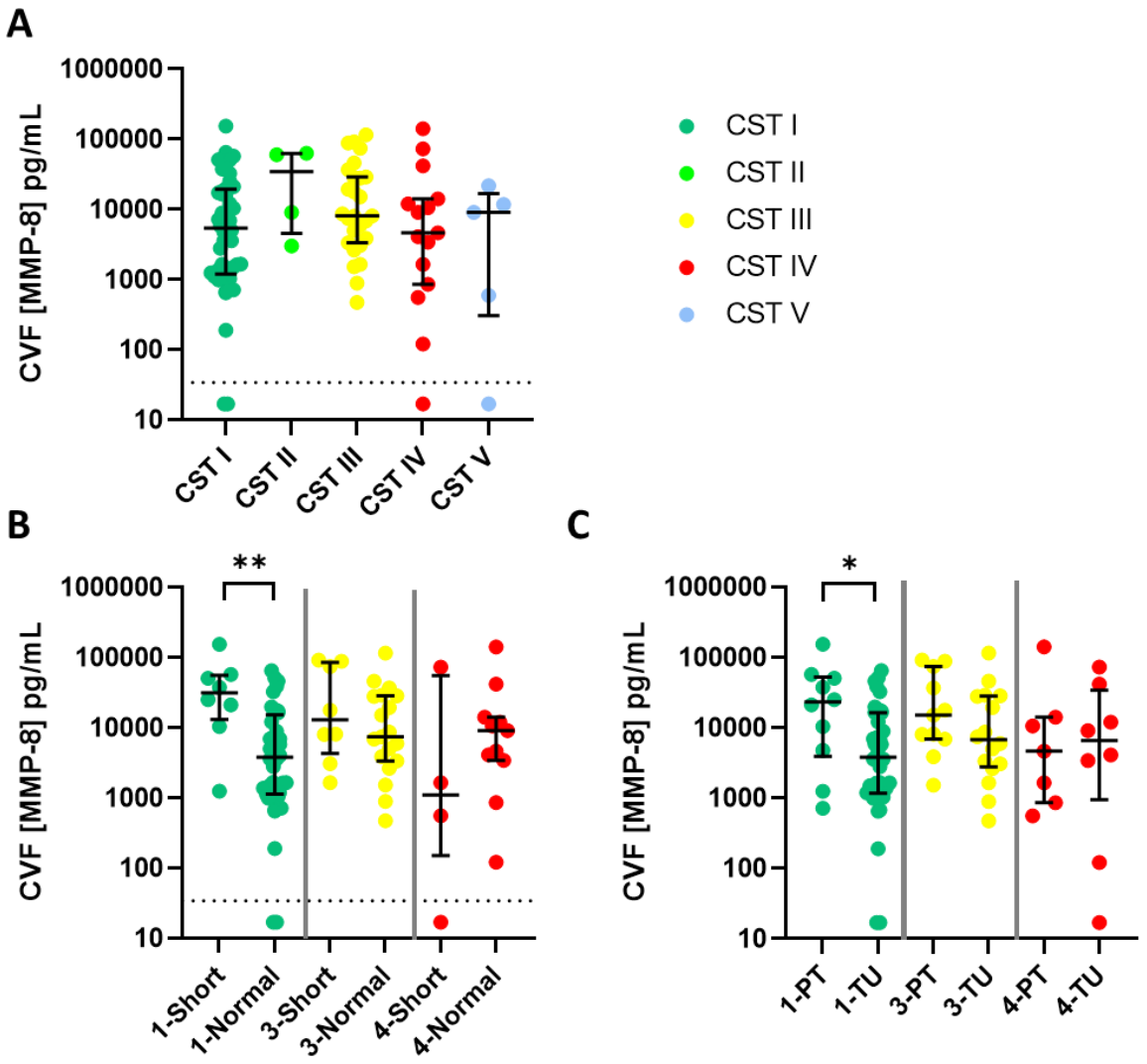


Figure 3.40. Cervicovaginal fluid concentration of MMP-8 according to CST vaginal composition. A) Concentration of MMP-8 according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of MMP-8 was also compared in women who shorten and woman who maintained a normal cervical length and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. *p<0.05; **p<0.01. Data is presented as median values with interquartile range.

As observed for the other MMPs, there were no differences in the medians of MMP-9 between groups with different CSTs composition (p=0.0783) (Figure 3.41 A). Based on pregnancy outcomes, the median concentration of MMP-9 was also higher, up to 7-times, in women with a CST I who underwent cervical shortening compared to women who maintained a normal cervical length (median 103068 vs 14117pg/mL, p=0.0018) (Figure 3.41. B) and in women who delivered preterm compared to term (median 99260 vs 14117pg/mL, p=0.0078) (Figure 3.40 C).

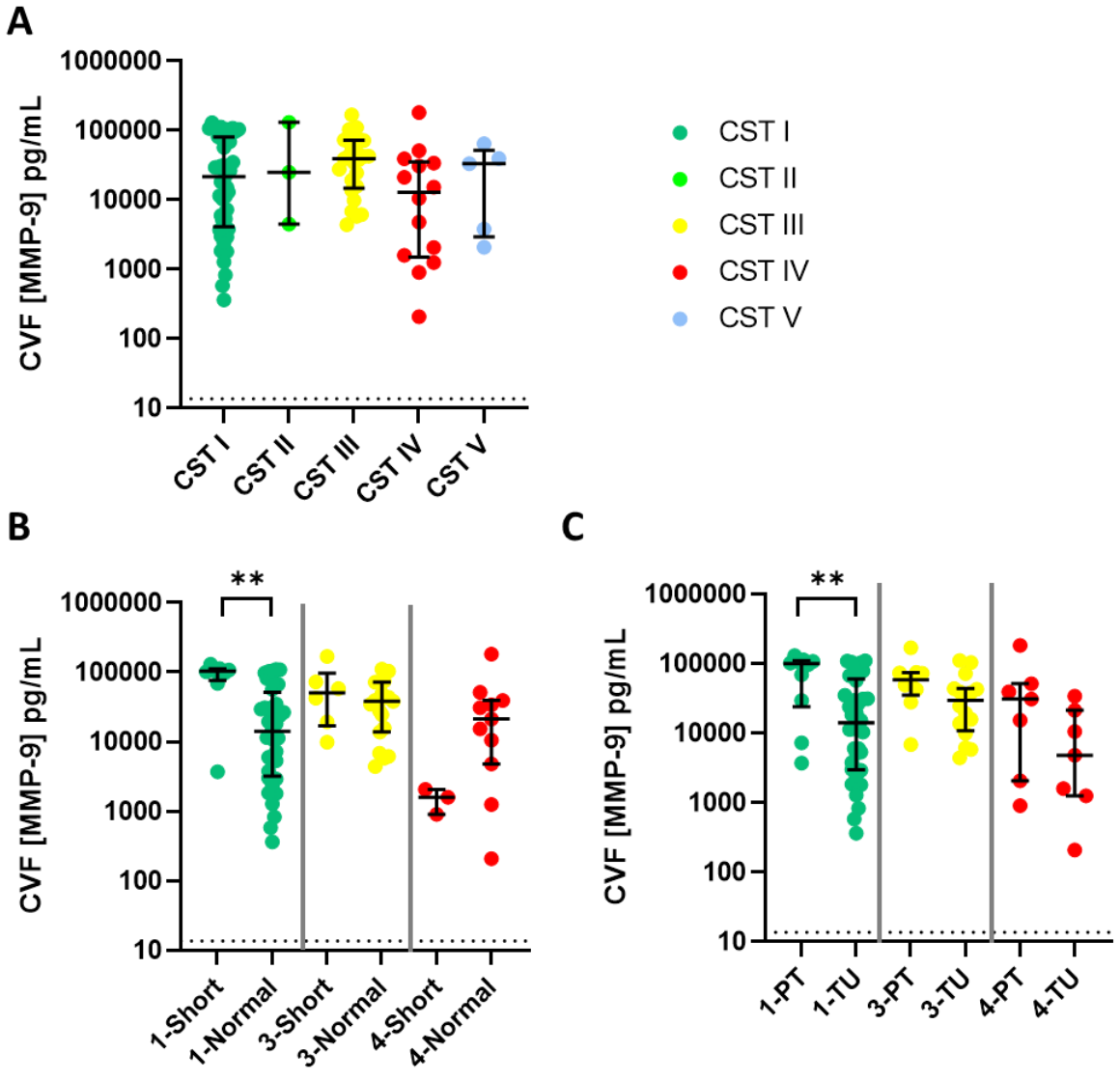


Figure 3.41 Cervicovaginal fluid concentration of MMP-9 according to CST vaginal composition. A) Concentration of MMP-9 according to CST vaginal composition (n=102 samples, n=51 women). Statistical analysis was assessed between CST I, CST III and CST IV using Kruskal-Wallis test with Dunn's multiple comparisons test. **B)** Concentration of MMP-9 was also compared in women who shorten and woman who maintained a normal cervical and **C)** based on pregnancy outcome (PT, preterm; TU, term) in those samples classed as abundant in CST I (*L. crispatus*) (n=51), CST III (*L. iners*) (n=27) or CST IV (diverse) (n=15). Statistical analysis between pairs of CSTs was assessed using a Mann-Whitney two tailed-test. ** p<0.01. Data is presented as median values with interquartile range.

3.7. Discussion

Consistent with previous studies, the data presented in this chapter indicates that women who experienced cervical shortening and those who deliver preterm are more likely to have a high-risk microbial composition. Both cervical shortening and preterm birth were associated with a strong local inflammatory response measured by cervicovaginal fluid cytokines and matrix metalloproteinases. Moreover, the data also highlight an important role for the complement system in both cervical shortening and preterm birth in both women with high and low-risk microbial compositions.

Cervical shortening and preterm birth are associated with an adverse vaginal microbial composition

The physiological process of term birth involves cervical shortening and cervical remodelling, together with contractions, after the 37th week of gestation. However, the finding of cervical shortening early in pregnancy and in the absence of contractions is a pathological process and a risk factor for preterm birth. Women who experience cervical shortening during early pregnancy have higher chances of delivering preterm (Wang et al., 2021).

In this chapter I have shown that in women who experienced cervical shortening and women who delivered preterm there is a higher proportion of *L. iners* (CST III) and heterogenous anaerobic bacteria (CST IV), whereas women who have uncomplicated term pregnancies tend to have a higher proportion of *L. crispatus* vaginal dominance (CST I). Despite not reaching statistical significance, these results are consistent with the general and widely accepted findings that *L. crispatus* is associated with pregnancies that deliver at term, whereas CST III and CST IV are associated with a higher risk of delivering preterm (Table 1.2., including (Fettweis et al., 2019; Stout et al., 2017; Tabatabaei et al., 2019)) and cervical shortening (Chan et al., 2022; Gerson et al., 2020; Kindinger et al., 2017).

Generally, *Lactobacillus* species dominance in the vagina is characteristic of optimal health. *Lactobacillus* species are thought to promote vaginal health through the maintenance of a low pH and production of antimicrobial compounds such as bacteriocins (Aroutcheva et al., 2001; Petrova et al., 2015). However, it is evident that not all *Lactobacillus* species behave the same way. *L. crispatus* is widely recognised to provide the most protection against adverse health outcomes and has been shown to maintain cervical epithelial integrity (Anton et al., 2018) and produce antimicrobial compounds against *Candida* (Wang et al., 2017) and *G. vaginalis* (Amabebe et al., 2022a). *L. crispatus* has also the ability to produce D-lactate, that contributes to the maintenance of a low pH but also inhibits collagen degradation via inhibition of EMMPRIN (extracellular matrix metalloproteinase inducer), an enzyme involved in the activation of MMP-9 (Witkin et al., 2013). In contrast, *L. iners* is

often observed in women who transition toward microbial communities enriched for BV associated taxa (Petrova et al., 2017, 2015; Zheng et al., 2021). Consistent with these observations in our cohort, there was a higher proportion of women who delivered at term with a CST I *L. crispatus* dominance. Furthermore, all observed transitions of CSTs occurred in women who at timepoint B had CST III *L. iners* vaginal dominance. In 2 women, a transition from CST IV towards CST III was observed; and 3 women transitioned from CST I or V towards CST III. Overwhelmingly, CST I dominance at Timepoint A was associated with vaginal microbiome stability with only 1/20 women transitioning to CST III at Timepoint B. Similar transitioning dynamics were observed in non-pregnant healthy black women (n=316 swab samples from n=88 women), were *L. iners* dominance, but no *L. crispatus* dominant communities were more likely to transition to high-risk microbial compositions (Munoz et al., 2021).

In the context of outcome, women who subsequently experienced uncomplicated term births tended to have higher prevalence of CST I (59% at timepoint A, 56% at timepoint B) compared to women who experience preterm birth (35% at both timepoints). Despite this not reaching statistical significance, these results are in line with those previously reported by many groups in the literature. However, in this cohort, the highest prevalence of high-risk CSTs was observed in women who delivered late preterm (>34+0 to ≤36+6) (50% CST III, 25% CST IV), whereas the majority of women who delivered early preterm (≤33+6) had CST I *L. crispatus* vaginal dominance (~50%). Work from our group (Chan et al., 2022; Kindinger et al., 2017) and others (Elovitz et al., 2019; Tabatabaei et al., 2019) has previously reported enrichment of CST III *L. iners* or CST IV in early PTB. It is possible that the differences observed between these studies is due to the relatively small numbers of patients I analysed in this cohort (17 preterm deliveries, 34 term deliveries). For example, Chan et al. worked with a similar cohort of women at high-risk of delivering preterm, with 34 preterm deliveries and 51 term uncomplicated deliveries; finding that there was a greater proportion of women with either CST III or CST IV who experienced early preterm birth (~50%, n=20) compared to late preterm birth (~25%, n=14) (Chan et al., 2022). In another larger cohort, Kindinger also found a greater proportion of *L. iners* in the early preterm birth group (12/18, 67%) compared to term (37/127, 29%) ($p<0.01$) (Kindinger et al., 2017).

It is notable that not all women in our cohort who had a high-risk microbial composition experienced a preterm delivery or cervical shortening. Similarly, some women with CST I vaginal dominance did experience cervical shortening and preterm delivery. While this indicates that there might be other non-microbial contributors driving these events it is also possible that the vaginal microbial composition is not fully representative of microbial colonisation of the upper reproductive tract. It has been proposed that microbiota continuum exists along the female reproductive tract (Chen et al., 2017), however Liu et al., in the context of recurrent spontaneous abortion, found distinct microbiota compositions between the lower and the upper reproductive tract (Liu et al., 2022). Furthermore,

there is evidence of pathobionts in fetal membranes in some women with *L. crispatus* vaginal dominance (Beleil et al., unpublished). It is also well described that even when the same species are detected in the vagina and the uterine genital tract, the biomass of these species differs greatly (Mitchell et al., 2015). This may be highly relevant to the context of preterm birth risk. Recently, Goodfellow et al. reported no major association between sPTB with vaginal microbiota composition, but a positive relationship with increased vaginal bacterial load (8.64 vs 7.89, p=0.047) (Goodfellow et al., 2021). Similarly, Freitas et al. also reported a higher total bacterial load via qPCR in preterm vs term (8.07 vs 7.78, p= 0.049) (Freitas et al., 2018). These findings indicate that in addition to cervicovaginal microbial composition, bacterial load is potentially an important mediator of host response involved in the pathophysiology of preterm birth. Moreover, they highlight the importance of carefully assessing host-response to understand more fully the underlying mechanisms driving adverse pregnancy outcomes.

Cervical shortening and preterm birth are associated with an increase of local proinflammatory cytokines

The median CVF concentration of three proinflammatory cytokines IL-1β, IL-6 and IL-8 was found to be higher in patients with cervical shortening and subsequent preterm birth. This is consistent with earlier studies reporting elevated concentrations of IL-1β (Amabebe et al., 2018), IL-6 (Chan et al., 2022; Jun et al., 2000; Park et al., 2018) and IL-8 (K. Ashford et al., 2018) in women with preterm birth and cervical shortening. However, other studies have failed to observe changes in cytokine levels in CVF associated with cervical shortening (Chandiramani et al., 2012), and some have even reported decreasing proinflammatory cytokines in women with intraamniotic infection (Simhan et al., 2003b) or who delivered preterm (Kalinka et al., 2005). These inconsistencies likely represent differences in the underlying aetiologies driving preterm birth across study cohorts. In early studies that failed to identify associations between proinflammatory CVF cytokines and preterm birth, no assessment of the vaginal microbiome or infection status of the patient was undertaken. This highlights the importance of capturing insight of both host response and microbial composition. To this extent, research from the MacIntyre and Takats laboratories has recently showed the utility of using rapid metabolic profiling of CVF to predict both immunological status and microbiota composition to monitor microbiota:host response during pregnancy as a means to identify women at risk of preterm birth (Pruski et al., 2021). However, this method does not provide a detailed assessment of key upstream adaptive and innate immune responses that may play critical roles in initiating preterm birth risk pathways.

Cervical shortening and preterm birth are associated with an increase of complement proteins in CVF

To date the understanding of the contribution of the complement system to the local cervicovaginal milieu in the context of microbiota host response and preterm birth risk is limited. In this cohort, women who subsequently experienced sPTB were found to have higher levels of key complement pathway proteins C1q, C4, MBL, C3b and Factor H as early as the first trimester of pregnancy (sampled between 12-16 weeks). Levels of C1q, C4, C3a, C3b and Factor H were also higher during mid-pregnancy (20-24 weeks of gestation) in these women. All complement proteins assessed except C3 and C5 were upregulated in women who experienced cervical shortening, suggesting a link between pathological cervical shorting and activation of the complement cascade.

The role of the complement cascade has mainly been studied in the periphery (serum and plasma) in uncomplicated pregnancy (He et al., 2020; Richani et al., 2005), preeclampsia (Denny et al., 2013; Haeger et al., 1992; Lynch et al., 2008b) and more recently preterm birth (Dunn et al., 2017; Lynch et al., 2016, 2011, 2008a; Segura-Cervantes et al., 2015; Soto et al., 2005; Vaisbuch et al., 2010). One small study has also examined peripheral complement proteins in the context of cervical shortening (Park et al., 2019). A summary of these findings was presented in the Introduction (Table 1.4.). Most of these studies have focused only on a few complement proteins, which fails to capture the complexity of the complement cascade. For example, Vaisbuch et al. reported a higher Bb concentration in plasma in women who delivered preterm, compared to woman who delivered at term (Vaisbuch et al., 2010). Lynch et al. also reported Factor B, Factor H and ficolin-3 in plasma as the highest-ranking proteins for the risk of preterm birth (Lynch et al., 2016). Soto et al. found higher C5a and C3a concentration in plasma of women with intraamniotic infection (IAI) compared to women without IAI (Soto et al., 2005). Another potential issue with some of these studies is the lack of appropriate controls. For example, Park et al. found that in women that delivered before 32 weeks of gestation there was a higher plasma concentration of C3a and C5a (Park et al., 2019). However, the comparison between outcome groups used 32 weeks of gestation as a threshold; women who delivered ≥32 weeks had a mean 37.3 ±2.0 gestational age at delivery, indicating that some of the women from this group actually delivered preterm.

The contribution of the complement system at local environments such as maternal and fetal tissues (Lappas et al., 2012; Stabile et al., 1988), amniotic fluid (Kim et al., 2018; Segura-Cervantes et al., 2015; Stabile et al., 1988; Vaisbuch et al., 2010) and cervicovaginal fluid is poorly described. This is particularly the case for the complement cascade in the cervicovaginal interface during pregnancy. Using immunoblotting techniques, Livson et al. found activation of C3 in CVF samples from both

pregnancy and labour detecting an increase of fragments of subproducts of C3 (Livson et al., 2021) and activation of C4 and Factor B (Livson et al., 2022). Since the intensity of fragments was found to be highest during pregnancy, it was suggested that labour might block complement activation (Livson et al., 2022, 2021). Hong also found higher concentrations of C3a and C5a in CVF samples from women with IAI compared to women without IAI, as well as higher concentration in early preterm births, compared to late preterm (Hong et al., 2020), however no term or uncomplicated cases were included in this study.

To provide a deeper insight into the role of the complement system in the cervicovaginal niche the work described in this chapter extended to the analysis of 14 different complement proteins covering key nodes and junctions of the complement system, including the different activation pathways: classical (C1q, C2, C4, C4b), lectin (MBL) and alternative (Factor B, Factor D); the targets and subproducts of C3 and C5 convertases (C3, C3a, C3b, C5 and C5a) and complement inhibitors (Factor H and Factor I). The majority of the complement proteins assessed were elevated in women who experienced cervical shortening or delivered preterm. This included key members of the classical pathway, which is activated via binding of IgG complexes to C1q. The concentration of C1q was elevated in both women who shorten and women who delivered preterm. The concentration of C4 was also elevated in both groups, however this was not reflected on the concentration of C4b. Usually, in cases of complement activation, there is a decrease in former proteins and an increase of subproducts indicating fragmentation. However, despite a distinctive pattern between both pregnancy outcomes, there is no clear pattern of reduction and consumption between these two proteins. This can suggest there is both consumption, since C4b subproduct is detected; together with local production, since C4 concentration is not reduced. With reference to the lectin pathway, MBL, an opsonin that binds to the surfaces of pathogens, was increased in women who shorten and women who delivered preterm. Chan et al. found that the concentration of MBL was also significantly increased between early and mid-pregnancy in women who delivered preterm, however this was not observed in women with term uncomplicated or term intervention pregnancies (Chan et al., 2022).

The other pathway that leads to the activation of the complement system is the alternative pathway. Factor B and Factor D are implicated in this pathway through binding and fragmenting sequentially to C3b to form the C3 convertase. Factor D, but not factor B, was significantly increased in women that experienced cervical shortening, but patient to patient variability was apparent in both groups. The most studied fragment from the alternative pathway is the fragment Bb, which has been shown to be increased in plasma in cases of pregnancy complications such as preeclampsia (Lynch et al., 2008b) and preterm birth (Lynch et al., 2008a; Vaisbuch et al., 2010). Fragment Bb was not included in our panel, but as Factor D targets the defragmentation of Factor B into Bb, low detection ranges of Factor

B could indicate that this fragment has already been fragmented and consumed. Indeed, Factor D is present and detected in high concentrations in women who shorten, demonstrating its presence and availability to perform the fragmentation.

All the three pathways (classical, lectin and alternative) converge on the activation of C3 convertases. Activation of C3 convertases will lead to the defragmentation of component C3 into C3a and C3b. The concentration of C3 was increased in women who experienced cervical shortening, and both C3a and C3b were increased in women who experienced cervical shortening and that delivered preterm. Lynch et al. found that plasma C3a was predictive of adverse pregnancy outcomes, including spontaneous preterm birth and PPROM (Lynch et al., 2011), together with Park et al. (increased in women who delivered before 32 weeks of gestation (Park et al., 2019)) and Soto et al. (increased in women with intraamniotic infection, IAI (Soto et al., 2005)). C3a concentrations were also increased in women with IAI in amniotic fluid (Soto et al., 2009) and in CVF (Hong et al., 2020). Consistent with these findings, Chan et al. previously reported that the concentration of C3b in CVF increased from early to mid-pregnancy in women who delivered preterm, but not in women experience healthy term pregnancy (Chan et al., 2022). The release of components C3a and C3b is a hallmark of complement activation, and both proteins have important roles in the immune response. The former, is a potent neutrophil chemoattractant; and C3b is another opsonin.

The downstream complement proteins analysed in this study included C5 and C5a. The median concentration of C5a was increased in women who experienced cervical shortening. Concentrations of C5a were also higher in women who delivered preterm, but this did not reach statistical significance, likely due to a lack of power. In plasma, C5a was reported to be higher in women with IAI (Soto et al., 2005) and preeclampsia (Haeger et al., 1992). In CVF, Hong also detected increased C5a in women with IAI; and Chan et al. also found higher levels of C5 and C5a in CVF of women who experienced cervical shortening; as well as an increase in C5a across pregnancy in women who delivered preterm (Chan et al., 2022). *In vitro* work with placental tissue has demonstrated that C5a stimulates the production of cytokines including IL-6, IL-8, as well as COX2, PGE2 and MMP-9 (Lappas et al., 2012). C5a is also a potent neutrophil chemoattractant. Therefore, free high levels of C5a in CVF could impact the host immune response.

Finally, the complement regulators Factor H and Factor I were also assessed in this chapter. The former was increased in both adverse pregnancy outcomes (cervical shortening and preterm birth). Factor H was reported by Lynch et al. to be increased in plasma in women experiencing PTB (Lynch et al., 2016).

Knowledge of the complement cascade is mainly extrapolated from autoimmune diseases and studies performed on peripheral blood. Indeed, the CVF is enriched in protein contents from the plasma

transudate, that gains access to the vaginal interface from the capillaries reaching the interstitial tissue and passing through the epithelium into the wall of the vagina (Giraldi and Levin, 2006; Graziottin and Gambini, 2015). The main origin of complement components is the liver, produced by hepatocytes, but other tissues and cell types can synthetise complement components, including leukocytes (Lubbers et al., 2017) and endothelial and epithelial cells (Morgan and Gasque, 1997). For instance, epithelial cells from luteal endometrium can produce C3, likely in a progesterone-hormonal regulated dependant manner (Hasty et al., 1994). Also, Bulla showed MBL production from vaginal epithelial cells (Bulla et al., 2010). Lubbers reviewed the contribution of leukocytes to the production of complement proteins, including mast cells, polymorphonuclear cells and macrophages (Lubbers et al., 2017). Neutrophils have been particularly studied in animal models (Ward and Hill, 1970) and human peripheral neutrophils (Botto et al., 1992). Therefore, it is likely that in the complex local microenvironment complement proteins could originate not only from blood transudating to the CVF, but also from cervical and vaginal epithelial cells, and local immune cells such as neutrophils. While this requires further investigation, our results clearly indicate local activation of the complement cascade is a key feature of cervical shortening and preterm birth.

Cervical shortening and preterm birth are associated with an increase in CVF matrix metalloproteinases

Matrix metalloproteinases are collagenases able to degrade collagen fibers. In this chapter I have shown that the concentration of three different MMPs (MMP-2, MMP-8 and MMP-9) in CVF is increased in women who underwent cervical shortening and in women who delivered preterm. All MMPs are degrading enzymes and have different targets and ability to degrade collagenous and non-collagenous components. MMP-2 is a gelatinase, that exercises its function via binding to the membrane-bound cell surface receptor. MMP-8, is a collagenase, also known as neutrophil collagenase, that can degrade type I collagen. MMP-8 is stored in the granules of PMN like neutrophils in the secondary and tertiary granules (Table 1.5.); and the release is dependent on ROS or proteases. Finally, MMP-9, also known as gelatinase B, can also be stored on PMN granules or released from other leukocytes such as macrophages (Nguyen et al., 2023).

The process of labour, both for term and preterm parturition, requires the transition of the myometrium to a contractile state as well as cervical remodelling. Cervical remodelling is a process that requires conformational changes in the ECM. Stygar et al. reported increased expression of MMP-2 and MMP-8 in cervical biopsies from women closer to labour compared to non-pregnant tissue (Stygar et al., 2002). In the periphery, it has been reported that women who delivered preterm (<34

weeks) had higher serum concentrations of MMP-9 and TIMP-4, and that imbalances between MMPs and TIMPs ratio (particularly MMP-9:TIMP-1) are associated with preterm birth (Tency et al., 2012). At the cervicovaginal interface, Becher et al. detected higher levels of MMP-8 and MMP-9 in the cervical mucus plug from preterm pregnancies compared to term (Becher et al., 2010). Furthermore, MMP-9 levels and MMP-9:TIMP-1 ratio were also higher in CVF samples from women at high-risk of delivering preterm (history of previous sPTB compared to normal risk) (Davis et al., 2020). Women who experienced cervical shortening and women who subsequently delivered preterm also had higher MMP-8 and MMP-9 concentrations in CVF, respectively (Gerson et al., 2022, 2020).

Therefore, our results of increased MMP levels (MMP-2, -8, -9) in CVF align with what is currently known in the literature. It is possible that different mechanisms trigger cervical remodelling and release of MMPs in women that delivered term and preterm, even between different aetiologies of preterm deliveries. This would explain the overall higher levels of MMPs in CVF, but specially in women who experienced cervical shortening and in women who ultimately delivered preterm, where MMP-9 concentrations for example were up to 3 to 5 times higher, depending on the timepoint. The progesterone withdrawal can trigger the induction and release of MMPs from endocervical epithelial cells and cervical fibroblast, and would be the potential source for term labour (Amabebe et al., 2022b). Whereas in preterm labour cases, cervical remodelling could be triggered by robust proinflammatory mechanisms. For example, *in vitro* work has also showed that C5a can stimulate the production of cytokines, as well as COX2, PGE2 and MMP-9 (Lappas et al., 2012). Therefore, since higher concentration of complement proteins, IL-6 and IL-8 have been detected, and there is evidence of leukocyte infiltration in this environment (Bokström et al., 1997; Gomez-Lopez et al., 2014; Osman et al., 2003; Young et al., 2002), including neutrophils and macrophages that are sources of these proteases, in preterm labour, when there is a higher influx of leukocyte and inflammatory mediators, the combination of cytokines and leukocytes could exacerbate the release of MMPs.

Finally, it is important to mention that the bimodal distribution observed in MMP-2 concentrations can, at least partially, be attributed to the dilution factor of CVF samples (1:5) to analyse 3 different MMPs simultaneously. It is obvious that there is a significant decrease of MMP-2 in certain patients, and that the percentage of detection was associated with pregnancy outcome, but a less diluted sample could provide a more accurate quantification.

Microbial-host immune responses and pregnancy outcome

This study has also addressed the associations between the local inflammatory immune response and the microbial composition, according to pregnancy outcome. Overall, women with an adverse vaginal microbial composition had increased concentrations of proinflammatory mediators. For example, women enriched in *L. iners* CST III had a higher median concentration of IL-1β compared to women with CST I. There is a large amount of evidence supporting a strong local proinflammatory cytokine production with certain microbial compositions in non-pregnant (Anahtar et al., 2015) and pregnant women. For instance, Kindinger was the first to report that women with vaginal dysbiosis during pregnancy have higher levels of proinflammatory cytokines, including IL-1β, G-CSF and TNF-α (Kindinger et al., 2016), similar to the higher concentration of IL-1β in women with a CST III vaginal composition in this cohort. More recently, Chan et al also found higher levels of IL-8 and IL-1β in women who had a CST III *L. iners* dominance or CST IV vaginal composition.

However, recent research studies are also integrating associations between the vaginal microbial composition, the inflammatory response and preterm birth (Chan et al., 2022; Elovitz et al., 2019; Fettweis et al., 2019; Florova et al., 2021; Kindinger et al., 2016). Chan et al. found that despite having the same vaginal microbial composition (CST III or CST IV), women who ultimately delivered preterm had higher levels of cytokines IL-8, IL-6 and IL-1β, compared to women who delivered at term (Chan et al., 2022). In our case, it was striking to see the same differences between pregnancy outcome in women who had a CST I vaginal microbial composition, suggesting that a dysregulated inflammatory response may be the key driver for adverse pregnancy outcomes.

When assessing complement proteins in CVF in response to the local cervicovaginal bacteria, the most consistent pattern observed was the increased concentration of complement proteins in women with CST IV, compared to a significant decrease in women with a CST I composition enriched in *L. crispatus*, suggesting the *L. crispatus* may be protective due to its inability to activate the complement system. Previously it has been mentioned how Hong et al. found higher levels of C3a and C5a in women with intraamniotic infection compared to women without infection (Hong et al., 2020). Livson also demonstrated that certain bacteria (*L. jensennii, G. vaginalis* and *Atopobium*) correlated with C4 activation (Livson et al., 2022); and our group found higher levels of MBL, C5 and C3b in women with a CST IV compared to CST I (Chan et al., 2022). Here I have shown that bacteria typically classified as CST IV activate the complement system, via detection of higher levels of C1q, C4, Factor D, C3, C3b, Factor H and Factor I. This implies that a dysregulated complement system occurs in a proportion of women with a high-risk microbial composition.

Animal studies have also supported the role of complement in microbial-driven preterm birth. For example, in a PTB murine model (via LPS vaginal administration), Gonzalez et al. detected C3 deposition in the cervix and evidence of cervical remodelling (degradation of collagen and increased MMP-9). Moreover, in a knockout pregnant mice model deficient in the C5a receptor, after LPS administration, no signs of cervical remodelling or preterm birth were observed (Gonzalez et al., 2011).

The median concentration of C1q was higher in women with a CST IV vaginal composition compared to women with a CST I. This indicates availability and potential of activation via the classical pathway since C1q has the potential to bind to Ig-complexes. However, to better understand this pathway, it is necessary to follow the cascade downstream at the subproducts level, like C4 and C4b proteins. Surprisingly, higher levels of C4 were observed in women with a CST IV vaginal composition. In cases of activation, we would have expected a reduction and consumption of levels of C4, accompanied with an increase on the subproducts; but interestingly, levels of C4b were also higher in women with a CST IV microbial composition. Higher levels of subproducts, in this case C4b, is an indicative of activation; the fact that we detect higher concentration of both C4 and C4b can indicate a continuous production and consumption of these proteins. No differences were observed in the concentration of MBL across different CSTs, but there are other participants in the MBL pathway like ficolins (Matsushita, 2013), MASPs (Wallis, 2007) and collectins (Lubbers et al., 2017) that could be mediating the activation of this pathway. Finally, the alternative pathway requires of Factor B and Factor D, both of which were detected in higher levels in women with a CST IV vaginal composition.

The three different pathways converge on the activation of C3 and C5 convertases. There was a similar trend as seen in C4/C4b concentrations in the levels of C3, which were significantly higher in women with a CST IV composition. At the same time higher levels of C3b were also detected in women with CST IV vaginal composition. Interestingly, no differences were seen in C3a, the other subproduct of C3. C3a, as an anaphylatoxin, could be potentially less stable when released, which may partially explain why the concentration of C3a was not associated with any CST. Release of C3a and C3b in the local environment has direct effect on the inflammatory response, since the former recruits phagocytes like neutrophils, and C3b is implicated in the opsonization of pathogens. Finally, higher levels of Factor H and Factor I were higher in women with a CST IV vaginal composition, potentially demonstrating an attempt to regulate the dysregulated complement system.

When assessing complement proteins integrated with both microbial composition and pregnancy outcome, women classed as CST III had higher concentration of C1q, C4, C3b and Factor H in women who underwent cervical shortening, compared to woman with normal cervical length with the same

cervicovaginal microbial composition. In addition, the median concentrations of C2, C4 and C5a were increased in women classed as CST III who delivered preterm, compared to their counterparts who delivered at term with the same microbial composition. Chan et al. 2022 also found higher concentration of C3b and C5 in women who experienced cervical shortening and were classed as CST III; and higher concentration of MBL, C3b, and C5 in women with an adverse vaginal microbial composition (L. deplete, CST III or CST IV) (Chan et al., 2022). This suggests a dysregulated complement activation in microbial-driven cervical shortening and preterm birth.

What was unexpected was to find the same pattern of increased complement proteins in women with CST I dominance who experienced cervical shortening and preterm birth. Most of the analytes assessed were increased in cases of cervical shortening (C1q, C2, C4, C4b, MBL, C3a, C3b, C5, Factor H and Factor I) and in women who delivered preterm (C1q, C2, Factor D, C3, C3a, C3b, C5a, Factor H). There are a few explanations that may clarify this finding. The first is based on the classification of the vaginal microbial composition using CSTs. The VALENCIA classification method classifies vaginal microbial communities based on composition, where the matches are based on similarity, not being a clustering algorithm (France et al., 2020). In other words, samples will be classified into different CSTs based on the high relative abundance of certain species. Therefore, it may fail to represent samples with lower relative abundance of other species also present. This phenomenon must be considered in our cohort, whereby in 3/4 of the women classed as CST I with a high inflammatory response, it may account for the findings observed. In 2/4, the assignation score to CST I in these samples was lower than 0.99, where in addition to the dominating *L. crispatus* focal specie, there was also other *Lactobacillus* species present (*L. jensenii* or *L. gasseri*). Interestingly, one particular woman also classed as CST I *L. crispatus* dominance also contained *Gardnerella vaginalis*. Therefore, a possible explanation is that it is not *L. crispatus* that drives the inflammatory immune response, but rather the other species present even if they are present in a lower proportion. This highlights again the fact that combining relative abundance data with actual bacterial load using quantitative methodologies can provide a better understanding of specific thresholds of bacteria triggering an inflammatory response.

Another explanation is that not all microbial-driven preterm births are triggered by bacteria. The microbiota includes a whole range of microorganisms, from bacteria to other microorganisms like archaea, fungi and viruses (Methé et al., 2012). The majority of studies, including the one presented here, usually explore the bacterial composition of the vaginal milieu, but this does not include associations with other microbiota members that could be relevant too to microbial-host interactions in the cervicovaginal milieu. Two other members that may be relevant and are quite unexplored at the vaginal milieu are the mycobiome (fungal organisms) and the virome (viruses). The contribution to health and disease of the fungal component has been underrepresented (Bradford and Ravel,

2017), but characterisation in reproductive-age women has showed that *Candida* colonisation might be higher than expected in asymptomatic women (65% vs the basic consideration of 20%) (Drell et al., 2013). One study found similar colonisation rates in women with lactobacillus-dominant and non-dominant signatures, although *L. iners* appeared to be more likely to cohabit with *Candida* spp. (Tortelli et al., 2020). Bacteria produces a range of metabolites and small molecules that can affect the metabolism and morphology and *Candida* spp, and *Candida* itself can alter the host cytokine production too (Bradford and Ravel, 2017), as well as activate the complement cascade (Harpf et al., 2020). Therefore, fungal communities may play a role in vaginal health and disease too.

On the other hand, viral infections can also have an effect on pregnancy outcomes (Mor et al., 2017). Cardenas et al proposed the "double-hit hypothesis" of preterm labour to address the effect of polymicrobial infections. Using a murine model infected with murine herpes virus (MHV68), a subsequent inoculation with LPS resulted in preterm birth and fetal death, whereas inoculation of MHV68 or LPS alone did not have any effect on pregnancy. This suggests that even a relatively benign and common viral infection can predispose for a later bacterial infection (Cardenas et al., 2011). More recently, Wylie et al. evaluated the vaginal virome in pregnancy (Wylie et al., 2018). Using high-throughput sequencing they were able to identify six families of eukaryotic DNA viruses. The majority of pregnant women carried at least 1 virus in the vagina (80%, n=60). There was also evident patient to patient virome variability, with the median being 2 viruses per individual, and 7 viruses the highest recorded. As observed from microbiome studies, no single specific virus or group of viruses *per se* was associated with preterm birth, however, viral richness was associated with preterm birth, the risk being higher if both bacterial and viral diversity was higher (Wylie et al., 2018). Furthermore, viruses and viral particles can activate and interact with the complement system. Viral infections can activate all the three pathways, via direct binding of C1q to viral surfaces or recognising Ig-viral complexes (classical pathway), binding of MBL to carbohydrates expressed on viral surfaces (lectin), or lack/decreasing Factor H after viral infection (alternative pathway). Activation of the complement system in cases of viral infection can result on virus opsonisation and neutralisation with opsonins (C1q, C3b, C4b) targeting the surface of the virions (Blue et al., 2004).

Therefore, other causes such as adverse bacteria present in lower proportions, viral infections, or even sterile inflammation could potentially be driving some of the inflammatory effects observed in the typical healthy *L. crispatus* dominant population.

MMPs are involved in cervical shortening and preterm birth, however not in association with the vaginal microbial composition

In our cohort, none of MMPs levels assessed varied depending on the microbial composition. However, others have found that cervicovaginal concentration of MMP-8 was distinctive in cases of BV in both non-pregnant (Beghini et al., 2015) and pregnant women (Diaz-Cueto et al., 2006). Cervical shortening and preterm birth were associated with increased MMPs on its own. Moreover, the concentration was higher in women with a CST I microbial composition that experienced cervical shortening and preterm birth. This would suggest that MMPs release is not a direct effect of a microbial-induced event, but from another contributor. It is likely that there are different MMPs origins, and that in cases of sterile inflammation, cervical and vaginal epithelial cells contribute to the release of MMPs.

Strengths and limitations

One of the strengths of this study is the use of Luminex Multiplex Immunoassays. This technology has allowed us to investigate a wide panel of mediators from the local cervicovaginal milieu. In this chapter I have shown the feasibility of a large and comprehensive assessment of CVF samples using six different panels, covering cytokines (4 analytes), complement proteins (14 analytes) and MMPs (3 analytes). Given the nature of human studies, where usually there is precious limited sample available, Luminex Multiplex Immunoassays were ideal for this project since they require a low volume compared to other techniques (e.g., ELISAs), where it would have been impossible to cover the full amount of analytes of interest. Moreover, the immunoassay technique is very robust and reproducible, showing a relatively low intra-plate and inter-plate variation, which contributes to consistency and accuracy of results. It is important to mention that some of the analytes analysed were out of range for a noticeable set of samples. This was further investigated and demonstrated that the detection ranges were significantly different depending on pregnancy outcome, indicating that the differences observed between groups are legitimate, and indeed women with favourable outcomes (e.g., term pregnancies) entailed a decreased inflammatory response.

Another strength of this study is the novel data presented about the contribution of the complement system at the cervicovaginal milieu. A wide range of complement analytes were analysed covering the different pathways in which the complement system can be activated (classical, lectin and alternative pathways), as well as the downstream complement proteins from the C3 and C5 convertases action, together with complement inhibitors. This has conferred a more detailed overview about the potential

role of the complement system in response to the local cervicovaginal microbial composition and in women who undergo cervical shortening and in women who delivered preterm.

The cohort included in this study was highly phenotyped. The very detailed metadata for every study participant has contributed to a better understanding of the immunophenotyping of different pathophysiological events, including cervical shortening. As a retrospective study, it was possible to include two very well-defined clinical populations. The PT group included only women who spontaneously delivered preterm. The term uncomplicated served as a strong control comparator, being representative of a healthy term pregnancy without interventions (e.g., cerclage that could have potentially biased the "normal" trajectory of a healthy term pregnancy).

Our findings of proinflammatory cytokines, MMPs and the high-risk microbial composition proportions are consistent with the current perspective in microbial-driven cervical shortening and preterm birth. However, some limitations need to be taken into account too. Despite our findings aligning with the existing literature, there was not enough statistical power to address the CST composition with pregnancy outcome. A larger cohort would have allowed us to assess longitudinal trajectories across pregnancy and achieve a greater stratification of patients, such as assessing preterm birth categories (early vs late preterm), or microbial composition classification into sub-CSTs classifications, to potentially reveal more details between specific communities. For example, it is well known that CST IV is characterised by a high diversity and low abundance of lactobacilli species. However, CST IV, based on the VALENCIA classification method, could be further subclassified into CST IV-A (high relative abundance of BVAB1, with moderate *Gardnerella vaginalis* abundance), CST IV-B (low relative abundance of BVAB1 and high *G. vaginalis* relative abundance) and CST IV-C (low abundance of *G. vaginalis*, BVAB1 and *Lactobacillus* spp.) (France et al., 2020). However, this does not exclude the clinical significance of the associations presented.

Finally, the novelty of exploring the complement system in CVF highlights the complexity inherent to this component of the immune response. It remains unclear the exact patterns of activation, as well as the clinical significance of finding both consumption and increased proteins concentrations.

Future work

This chapter has revealed new microbial-host immune response interactions and a potential role of the complement system in cervical shortening, and preterm birth. With a larger cohort, it will be possible to separate patients based on risk factors and reasons for referral to the preterm birth surveillance clinics. Stratifying patients based on history of adverse pregnancy outcomes (e.g.,

previous PTB) from patients with history of cervical treatment, could help us understand and define specific microbial-driven or sterile-inflammation mechanisms.

After revealing the implication of the complement system in cervical shortening, future work could focus on a more detailed description of these events. For example, to explore the effect that interventions may have on the immune response at the complement system level. One of the main interventions for cervical shortening is insertion of a cervical cerclage; since previous data suggests that cervical cerclage may impact the local immune response (Chan et al., 2022), future work could evaluate the local immune response pre- and post-cerclage insertion.

Finally, *in vitro* experiments co-culturing bacterial isolates with cervical/vaginal epithelial cells and immune cells are needed to ascertain the contribution of different cell types to the production of immune mediators.

3.8. Conclusion

Chapter 3 is an integration of metagenomic and immunological approaches that demonstrate associations between cervicovaginal bacterial communities and cervicovaginal inflammation in cervical shortening and spontaneous preterm birth. A dysregulated inflammatory response, involving proinflammatory cytokines, complement proteins and matrix metalloproteinases, appears to be implicated in cervical shortening and preterm birth (Figure 4.42.). However, the extent to which the immune response modulates the risk and specific microbial-host interactions are unclear. There is evidence that women with a CST IV vaginal composition are more likely to exhibit increased complement proteins. However, women enriched with *L. crispatus* classed as CST I also exhibited inflammatory responses in cases of cervical shortening and preterm birth. This highlights the multi-aetiological immune responses and the extreme variability among patients. In pure microbial-driven aetiologies, where pathogenic bacteria is involved, it is possible to suggest that immune cells such as neutrophils may be present and recruited into sites of infection, via sensing chemoattractants like IL-8, C3a and C5a, enhancing a stronger inflammatory response via degranulation and opsonisation of pathogens.

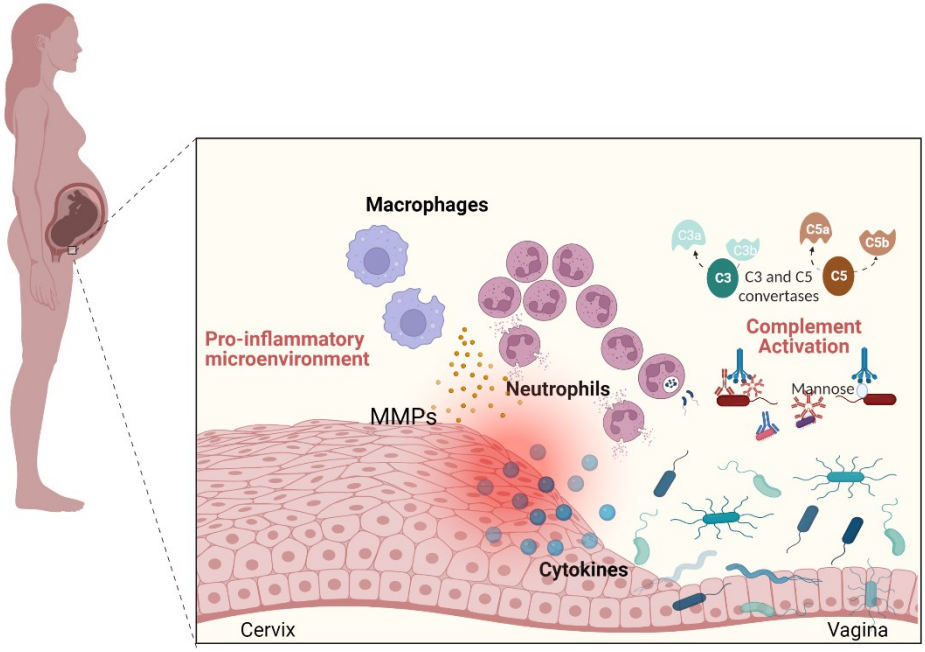


Figure 4.42. Local cervicovaginal microbial-host immune responses: a summary. There is a strong proinflammatory local immune response (increased cytokines, complement proteins and matrix metalloproteinases) in women with an adverse vaginal microbial composition and in women who underwent cervical shortening and who subsequently delivered preterm. Image created with Biorender. Adapted from Gimeno-Molina et al., 2022b.

4. THE ROLE OF NEUTROPHILS IN MICROBIAL-DRIVEN PRETERM BIRTH

4.1. Chapter Summary

Introduction

Neutrophils have long been implicated in physiological and pathophysiological processes of labour, both in the peripheral blood and at the maternal-fetal interface. Both cervical shortening and preterm birth are associated with adverse vaginal microbial composition, but it is unclear how the immune response, in particular the immune cells at the cervicovaginal interface can modulate the risk. The potential role of neutrophils has not been established in the context of human microbial driven preterm birth.

Hypothesis

- The immunophenotype of cervical neutrophils can influence the local immune milieu in response to a high-risk microbial signature, cervical shortening and preterm labour

Aims

- To optimise methodologies for the assessment of peripheral and cervical neutrophils
- To characterise the immunophenotype of cervical neutrophils and compare with matched peripheral blood neutrophils
- To correlate the activation status and functional capacity of cervical and peripheral neutrophils with pregnancy outcomes
- To compare the immunophenotype of neutrophils with vaginal microbial composition
- To correlate the presence and activation status of cervical and peripheral neutrophils with the presence of local immune mediators

Methods

Pregnant women at high-risk of delivering preterm (n=98) were included in this study and provided matched peripheral blood, cytobrush and vaginal swabs at least at one of the following timepoints: timepoint A: 12-16 weeks; timepoint B: 20-24 weeks; timepoint C: 30-34 weeks. CVF samples were assessed for microbial composition surveying the V1-V2 region of the 16S rRNA by Illumina MiSeq. Supernatant was used for measuring cytokines, complement proteins and matrix metalloproteinases

with Luminex Multiplex immunoassays. Peripheral blood and cytobrush samples were analysed with Aurora Cytek™ flow cytometry to assess the immunophenotype of neutrophils.

Results

Cervical neutrophils exhibited a different immunophenotype compared to peripheral neutrophils, with reduced expression of CD11b and CD62L markers ($p<0.0001$) and increased expression of CD63, CD66b, CD88 ($p<0.0001$) and CD55 ($p<0.001$) as determined by mean fluorescence intensity (MFI) by flow cytometry. Cervical neutrophils were more often detected in women with an adverse microbial composition. In women with a CST IV vaginal composition, there was a higher cervical MFI expression of CD63 and CD66b compared to women with a CST I ($p<0.01$, $p<0.05$, respectively). Cervical shortening ($p=0.0409$), but not preterm birth ($p=0.5108$), was associated with more frequently detected cervical neutrophils. Peripheral neutrophils from women who experienced cervical shortening exhibited a reduced CD62L MFI expression ($p=0.009$); whereas CD10 was increased ($p=0.0088$) and CD35 decreased ($p=0.0017$) in cervical neutrophils from women with a short cervix. Women who delivered preterm had a decreased expression of CD16 in peripheral neutrophils ($p=0.0171$), and cervical neutrophils appeared to have a higher expression of CD63 ($p=0.0880$) and CD66b ($p=0.2803$). Finally, a higher proportion of cervical neutrophils was associated with higher CVF concentrations of inflammatory mediators such as IL-1β ($p=0.0497$), IL-8 ($p=0.0386$), C3a ($p=0.0072$), C3b ($p=0.0013$) and MMP-8 ($p=0.0362$).

Conclusion

Cervical neutrophils exhibit a phenotype consistent with increased activation compared to peripheral neutrophils. The increased proportion of neutrophils observed in association with an adverse microbial composition, cervical shortening, and the concentration of immune mediators, supports the role of neutrophils in the mechanisms of microbial-driven inflammation at the cervicovaginal interface.

4.2. Introduction

Pregnancy is characterised by neutrophilia (Aghaeepour et al., 2017; Luppi et al., 2002; Plum et al., 1978; Pramanik et al., 2007; von Dadelszen et al., 1999). Peripheral neutrophils are also more activated, based on higher levels of reactive oxygen species (ROS), higher expression of CD11b and increased phagocytic activity (Pramanik et al., 2007; von Dadelszen et al., 1999). A change in surface antigen expression impacts on effector functions of the cells (Elghetany and Lacombe, 2004). There has been some effort to understand the role of peripheral neutrophils the context of pregnancy and preterm birth (Gervasi et al., 2001; Yuan et al., 2009; Zhang et al., 2017). For example, Gervasi et al. found a higher MFI expression of CD11b and CD66b and higher ratio of oxidative burst in granulocytes from women in preterm labour compared to peripheral granulocytes from healthy term pregnancies (Gervasi et al., 2001).

Peripheral blood neutrophil concentrations have been explored as a predictor of pregnancy complications using the neutrophil-to-lymphocyte ratio (NLR) in cases of chorioamnionitis (Ridout et al., 2023), PPROM and threaten preterm labour (Lakshmi and Sravani, 2021; Ozel et al., 2020) and spontaneous preterm birth (Vakili et al., 2021).

Neutrophils in pregnancy have been most explored in the context of chorioamnionitis. The diagnosis of chorioamnionitis refers to the diffuse infiltration of neutrophils into the chorioamniotic membranes based on placental histology reports. Chorioamnionitis represents the presence of intraamniotic infection, although it can occur in the absence of infection too, induced by danger signals, known as sterile intra-amniotic inflammation (Kim et al., 2015). Chorioamnionitis is reported in 40% of preterm birth cases, but it is more common with lower gestational ages at delivery (up to 94% in women who deliver between 21-24 weeks) (Andrews et al., 2006; Tita and Andrews, 2010).

The amniotic fluid is rich in leukocytes, including neutrophils (Gomez-Lopez et al., 2018). Amniotic fluid neutrophils have been suggested to play a key role in cases of microbial invasion of the amniotic fluid (MIAC) since they are able to perform phagocytosis (Gomez-Lopez et al., 2017). Gomez-Lopez et al. demonstrated *ex vivo* that neutrophils retrieved from the amniotic fluid can phagocytose different species of bacteria, including *Streptococcus agalactiae*, *Ureaplasma urealyticum*, *Gardnerella vaginalis* and *Escherichia coli* (Gomez-Lopez et al., 2017).The same group also suggested the possibility that amniotic fluid neutrophils can perform NETosis after detecting a different morphology and a net/mess structure surrounding neutrophils and bacteria in amniotic fluid smears (Galaz et al., 2020). More recently, they also assessed amniotic fluid neutrophils by RNA-seq from women who delivered at term (n=2) and preterm (n=4), revealing a greater transcriptional activity in women who delivered preterm (Gomez-Lopez et al., 2021).

Moreover, there is *in vitro* evidence demonstrating the migration ability of neutrophils following a chemotactic gradient (Tong et al., 2019). Others have also shown that peripheral neutrophils isolated from women with PPROM or with preterm labour have a higher migration capacity than neutrophils from women not in labour (Takeda et al., 2017). Exposure of neutrophils to conditioned media from LPS stimulated fetal membranes lead to a higher activation of neutrophils in terms of cytokine release, ROS production, degranulation and NETs (Tong et al., 2019).

Most of the evidence implicating non-circulating neutrophils in processes related to labour is based on immunohistochemistry localization. Neutrophils have been detected in fetal membranes and cervix closer to labour (Osman et al., 2003), as well as in the cervical stroma of term pregnancies (Bokström et al., 1997), in the cervix of idiopathic preterm labour (Whitworth et al., 2007) and in vaginal lavages of women who delivered preterm (Yamada et al., 1998).

Despite knowing about the presence of neutrophils at the cervicovaginal interface, it was not until 2015 that the potential role of cervical leukocytes in human spontaneous preterm birth was explored. Hunter et al. used flow cytometry to characterise cytobrush samples and found that neutrophils were the most abundant cell type. Early preterm births (<34 weeks) were associated with absence of macrophages and a reduction of polymorphonuclear cells (PMN) presence (Hunter et al., 2016). It is known that early preterm births are more likely to be driven by infectious aetiologies, however there was no assessment of microbial composition to correlate with immune cell populations or activation markers.

Mohd Zaki et al. also evaluated cervical leukocytes with a different approach using RNA-seq on a FACS sorted (fluorescence-activated cell sorting) population of cervical neutrophils (n=10). They managed to correlate the transcriptome of cervical neutrophils with the vaginal microbial composition and found that some clusters of genes implicated in neutrophil activation and degranulation were negatively correlated with *G. vaginalis* abundance but positively correlated with *L. iners* abundance (Mohd Zaki et al., 2022).

These studies support the growing interest in the key role that neutrophils might play in pregnancy and parturition. However, the potential role of neutrophils at the cervicovaginal interface has not been established in the context of human microbial-driven preterm birth. After demonstrating the presence of a proinflammatory milieu at the cervicovaginal interface in Chapter 3, Chapter 4 presents data describing the immunophenotype of peripheral and cervical neutrophils, their potential role in influencing the local proinflammatory milieu and its association with cervical shortening and preterm birth.

4.3. Study design

Pregnant women at high-risk of delivering preterm were recruited form Preterm Birth Prevention Clinics at Queen Charlotte's and St Mary's Hospital as part of the VMET 2 and FLIP-1 research studies. Women were followed up across their pregnancy and asked to provide samples during the routine visits at timepoint A (12-16 weeks of gestation), timepoint B (20-24 weeks of gestation), timepoint C (30-34 weeks of gestation). At each timepoint, women were asked to provide matched peripheral blood, cytobrush and a high-vaginal swab (Figure 4.1.). Peripheral neutrophils (isolated from blood as described in section 2.4.1.-Method 2) and cervical neutrophils (section 2.4.2.) were stained with monoclonal antibodies (Table 2.1.) and analysed by flow cytometry using the Aurora Cytek™ (section 2.5.). The high-vaginal swab served for both DNA extraction (section 2.6.1.) and 16S rRNA sequencing (section 2.6.3.); as well as for Luminex Multiplex Immunoassays using the supernatant (section 2.7.). Cytokines IL-1β, IL-6, IL-8 (section 2.7.2.2.), complement proteins (C1q, C2, C4, C4b, MBL, Factor B, Factor D, C3, C3a, C3b, C5, C5a, Factor H and Factor I) (section 2.2.7.3.) and MMPs (-2, -8 and -9) (section 2.7.4.) were measured in CVF.

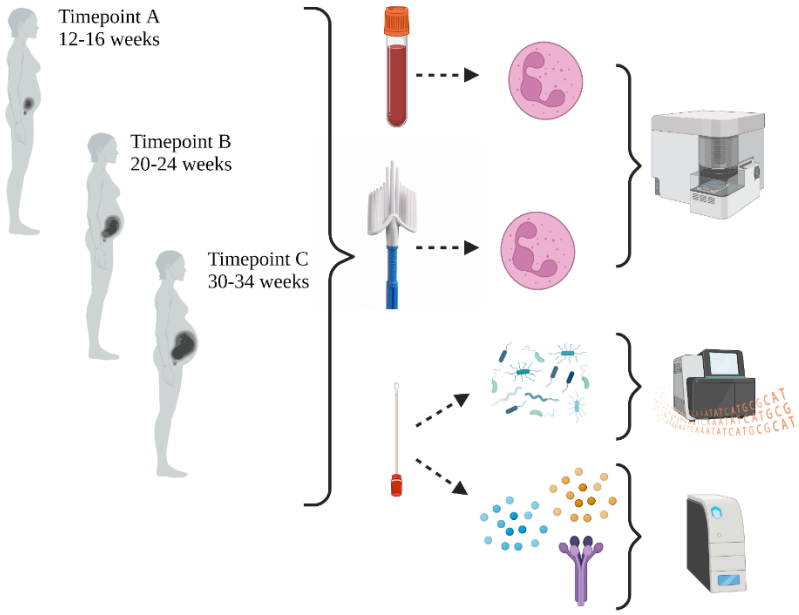


Figure 4.1. The role of neutrophils: the study design. Women at high risk of preterm birth were recruited from preterm birth prevention clinics and different samples were taken at three different timepoints: A (12-16 weeks), B (20-24 weeks) and C (30-34 weeks). Neutrophils from peripheral blood and cytobrush samples were assessed by flow cytometry with the Aurora Cytek™. CVF samples were assessed for vaginal microbial composition, surveying the V1-V2 region of the 16S rRNA gene on an Illumina MiSeq platform; as well as evaluating cytokines (IL-1β, IL-6, IL-8), complement proteins (C2, C4b, C5, C5a, Factor D, MBL, Factor I, C3, C3b, C4, Factor H, C1q, Factor B, C3a) and matrix metalloproteinases (MMP-2, MMP-8, MMP-9) with Luminex Multiplex Immunoassays.

4.4. Statistical analysis

GraphPad Prism version 9 software was used for statistical analysis. Comparisons between two groups required a t-test or Mann-Whitney test, depending on the distribution of the data. For multiple comparisons, differences between groups were analysed using the Kruskal Wallis test with the Dunn's post hoc test. To analyse proportional differences, the Fisher's exact or Chi square test were used. To assess differences between matched samples (matched peripheral vs cervical neutrophils) a Wilcoxon signed rank test was used. Finally, statistical significance was only accepted with p-values below 0.05.

4.5. Protocol optimisation

4.5.1. Sample collection and general procedures

Initially, isolation of peripheral blood neutrophils was performed by layering blood over Ficoll-Paque (Method 1- Section 2.4.1.), however, during the optimisation phase the protocol was amended to adopt the double gradient of Histopaque-1119 and Histopaque-1077 (Method 2, Section 2.4.1.) since a) there was no difference in the retrieval of the population of interest (Figure 4.2.), b) it did not affect the viability of the cells (N=6) and c) it involved a shorter protocol. This was considered to be beneficial for working with short-lived cells like neutrophils and to maximise the parallel sample collection and processing of cytobrush samples.

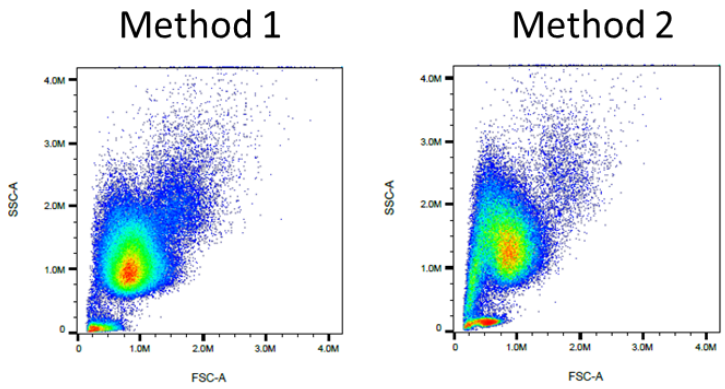
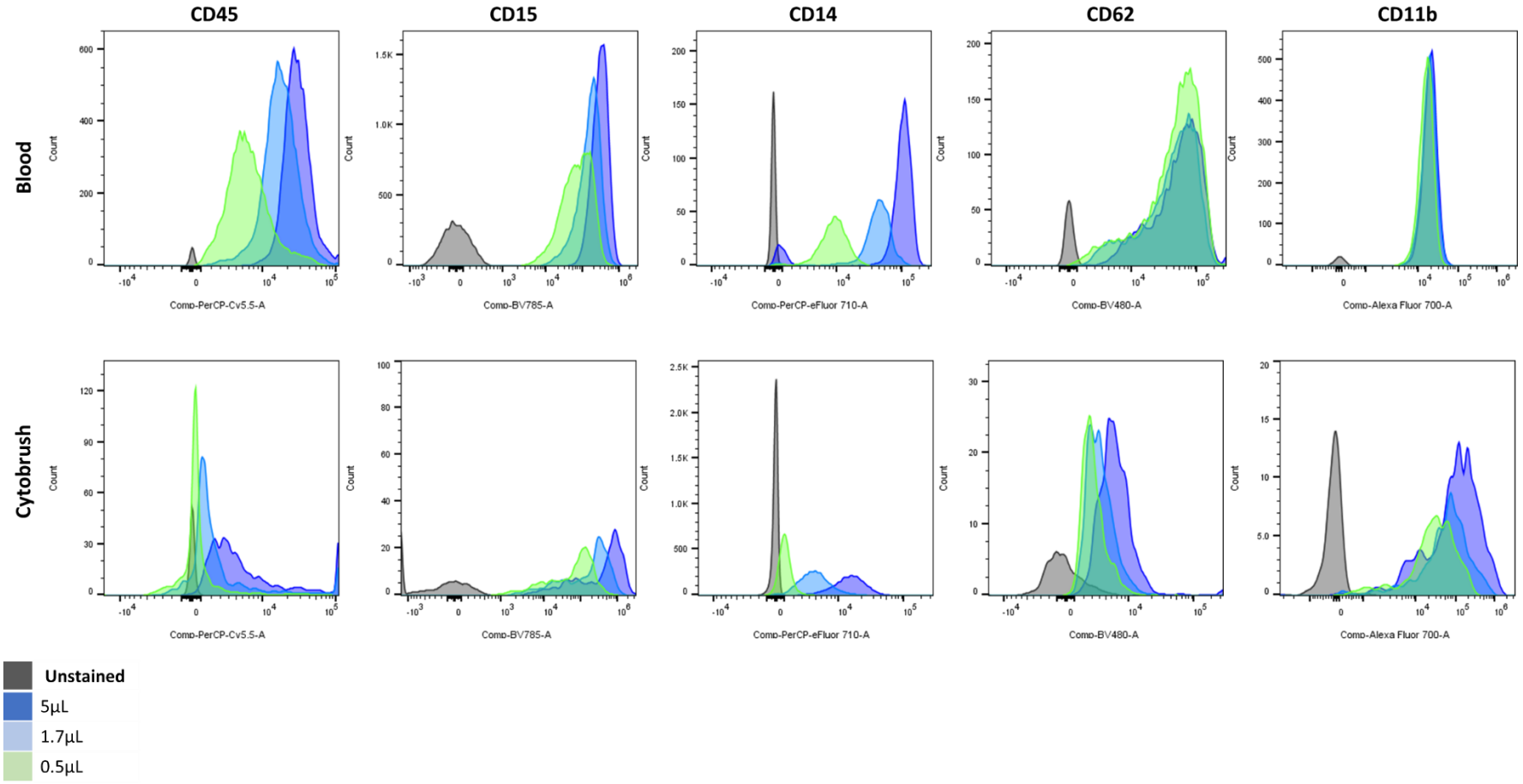


Figure 4.2. Comparison between two methods of isolation of neutrophils from peripheral blood. A) Method 1 consisted of layering whole blood over Ficoll-Paque. **B)** Method 2 consisted of layering whole blood over a double gradient of Histopaque-1119 and Histopaque-1077. (See Chapter 2.4.1. for details on methodology).

4.5.2. Antibody titration

The multicolour flow cytometry panel (Table 2.1.) to immunophenotype neutrophils by flow cytometry included a total of 13 monoclonal antibodies, and a viability staining solution, making a total of 14 different fluorochromes. Antibody titration can improve the accuracy of staining as well as minimise non-specific binding. All antibodies were titrated for both peripheral blood (n=3) and cytobrush samples (n=3). Three different volumes were assayed per test: 5µL (suggested per manufacturer), 1.7µL and 0.5 µL per 100µL cell suspension. Histogram plots of unstained and single colour stains are shown in Figure 4.3. Channel fluorescence is indicated after gating on the singlets and granulocytes population, except to establish CD14 volume, that was performed on a PBMC fraction, since CD14 is expressed on monocytes but not on granulocytes. A volume of 0.5 µL was selected to assess all antibodies, except for CD45, where a volume of 1.7 µL showed a better differentiation between negative and positive peaks on the cytobrush samples.


CD45
CD15
CD14
CD62
CD11b
Blood
Cytobrush
Count
Comp-PerCP-Cy5.5-A
Comp-BV785-A
Comp-PerCP-eFluor 710-A
Comp-BV480-A
Comp-Alexa Fluor 700-A
Unstained
5µL
1.7µL
0.5µL

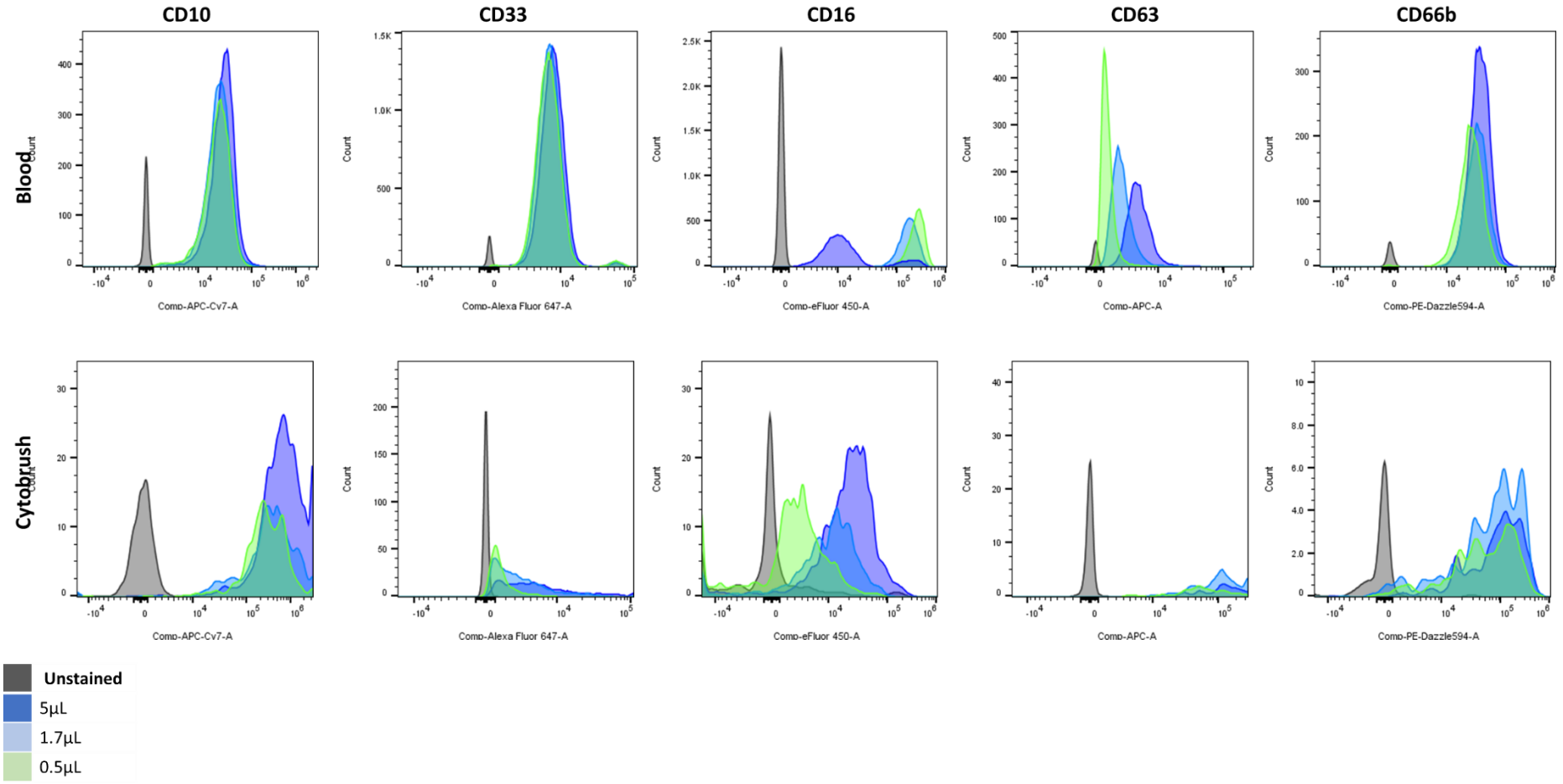


Figure 4.3. Titrating Antibodies for Flow Cytometry. Histogram overlay of unstained and different volumes tested for titration of antibody: volume per test of 5µL (suggested per manufacturer), 1.7µL and 0.5 µL for peripheral blood (top row) and cytobrush samples (bottom row), assayed for markers of identification (CD45, CD15, CD14) and markers of activation (CD11b, CD62L, CD10, CD33, CD16, CD63, CD66b, CD88). Colour legend from darker to lighter reflects more volume to less volume (See colour legend). Histogram overlay after gating on singlets and granulocyte population, for all markers, except CD14 (gating on singlets and monocytes in PBMC fraction).

4.5.3. Acquisition timings and fixation

To maximise the collection of samples during the patients visits in the clinic, and to recruit from two different hospitals, the effect of sample fixation over the viability was assessed. After antibody staining, samples were resuspended in 0.25% PFA, 0.5% PFA and 2% PFA. Samples were acquired on the Aurora® immediately after staining (Timepoint 1), between 2-3 hours after staining (Timepoint 2) and overnight with next day acquisition (Timepoint 3). No significant differences were observed between Timepoint 1 and Timepoint 2, whereas results from Timepoint 3 decreased the retrieval of live cells (Figure 4.4). Also, a higher percentage of fixative (2%) did not improve the viability and affected the distribution on the SSC-A, therefore it was decided to use the lowest percentage: 0.25% PFA.

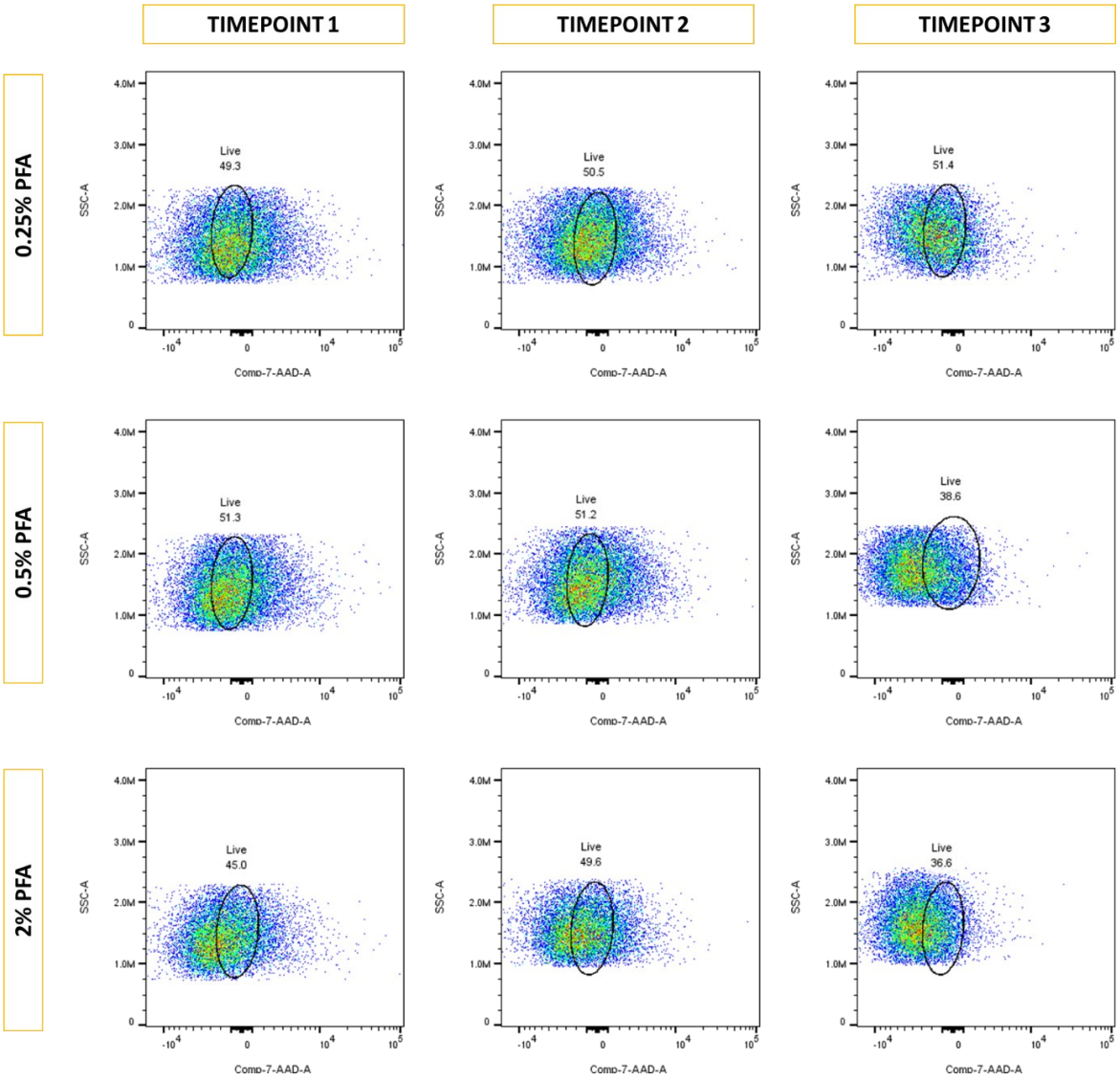


Figure 4.4. Fixation and timing of acquisition test. Pseudocolor plots indicating percentage of viability of samples resuspended in different fixative concentration (0.25%, 0.5% and 2% PFA in PBS), that were acquired at three different timepoints: straight after antibody incubation (timepoint 1), end of the day acquisition (timepoint 2), next day acquisition (timepoint 3).

The timing of acquisition and their effects on the expression of markers of activation were also assessed. Overall, no major differences were seen between timepoints, except for CD63 MFI that was elevated on timepoint 3 and CD16 that was slightly reduced (Figure 4.5.). Given the data shown in Figure 4.4. and 4.5., it was decided that acquisition could be variable within the day of collection (up to 6 hours) but not acquired the next day (greater than 16 hours post samples collection). This component of optimisation demonstrated it was possible to increase the numbers of women recruited in a session whilst not adversely effecting results. Also, this also meant samples could be collected from St Mary's Hospital, and analysed at Queen Charlotte's and Chelsea Hospital, since the Aurora ® instrument is located in Hammersmith Campus. For samples collected in St Mary's hospital, samples were processed onsite and transported for acquisition in Hammersmith location after 0.25% fixation.

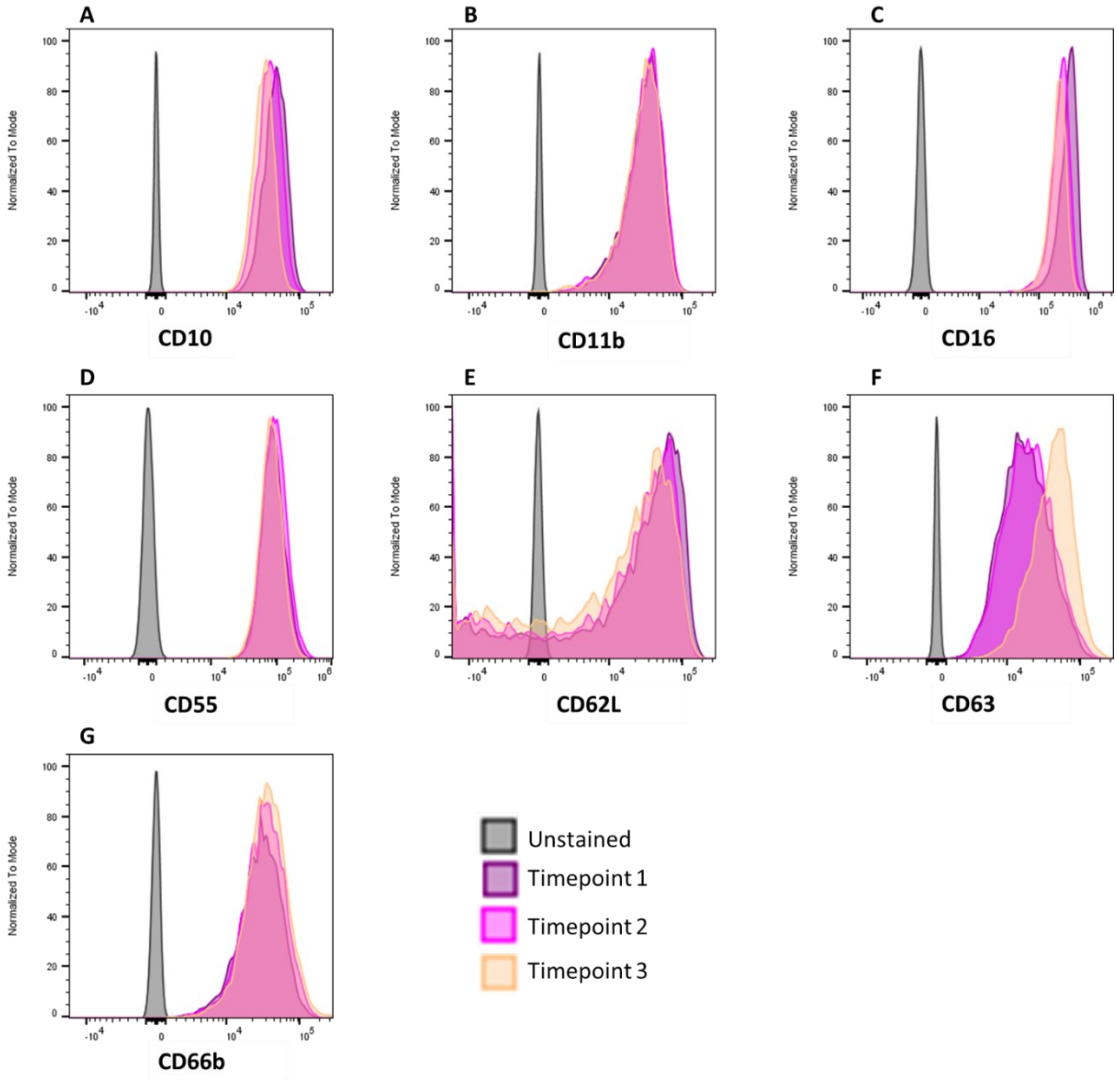


Figure 4.5. Timing of acquisition effect on markers of activation. Histogram overlay (normalized to the mode) of activation markers **A**) CD10, **B)** CD11b **C)** CD16, **D)** CD55, **E)** CD62L, **F)** CD63, and **G)** CD66b, after samples were resuspended in 0.25%, PFA in PBS and acquired at three different timepoints: straight after antibody incubation (timepoint 1), end of the day acquisition (timepoint 2), next day acquisition (timepoint 3). Samples were previously gated on singlets, live population, CD45+, CD15+ and CD14-.

4.5.4. Microscopy

Direct visualization of cytobrush samples with brightfield microscopy (Figure 4.6.A) revealed the cellular complexity within these samples, since cellular structures observed were identified by morphology as epithelial cells, immune cells and bacteria. Samples stained with Giemsa solution (Figure 4.6.B) verified the detection of neutrophils since the typical morphology of lobulated multinuclear cells were observed. Further confirmation of neutrophils collected by cytobrush was performed using cell surface markers detected by immunofluorescence using the confocal microscope. The same antibodies as used by flow cytometry were used: anti-CD15 (Figure 4.6.C), anti-CD16 (Figure 4.6.D) and anti-CD63 (Figure 4.6.E).

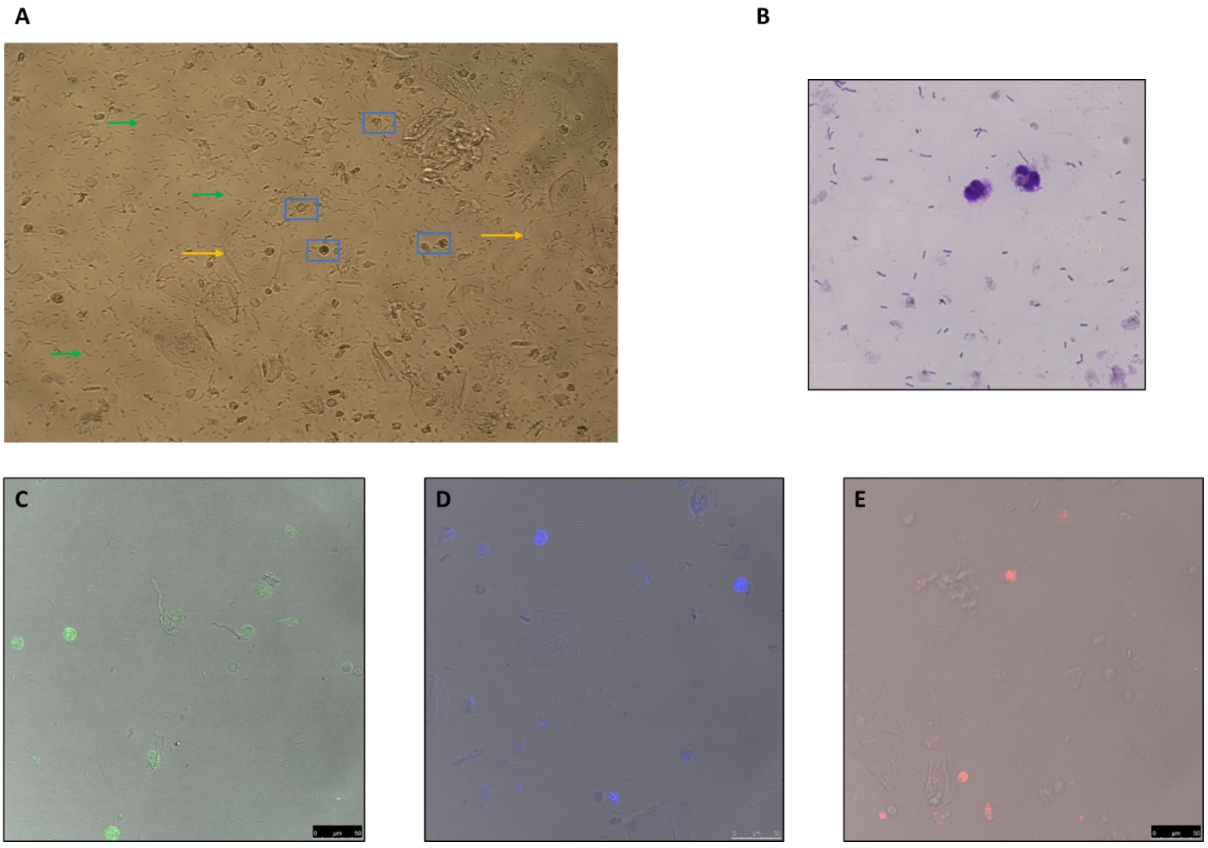


Figure 4.6. Imaging of cytobrush samples. A) Brightfield microscopy image of cytobrush samples. Orange arrows depicts epithelial cells. Blue squares depict immune cells. Green arrows showing bacteria. **B)** Giemsa staining reveals typical morphology of neutrophils with multilobulated nuclei. Confocal microscopy reveals neutrophils from cytobrush samples stained with **C)** anti-CD15, **D)** anti-CD16 and **E)** anti-CD63.

4.5.5. Gating strategy for flow cytometry

The same gating strategy was used to identify both peripheral and cervical neutrophils. Figure 4.7. and Figure 4.8. shows respectively the sequential procedures. Firstly, singlets were selected to exclude doublets by gating in the FSC-A (area) vs FSC-H (height). Live cells were gated on the viability dye-negative population. Next, immune cells were selected based on CD45+ marker. Finally, neutrophils are selected on the CD15+, CD14-/low populations. MFI of different markers was assessed within the neutrophil gate.

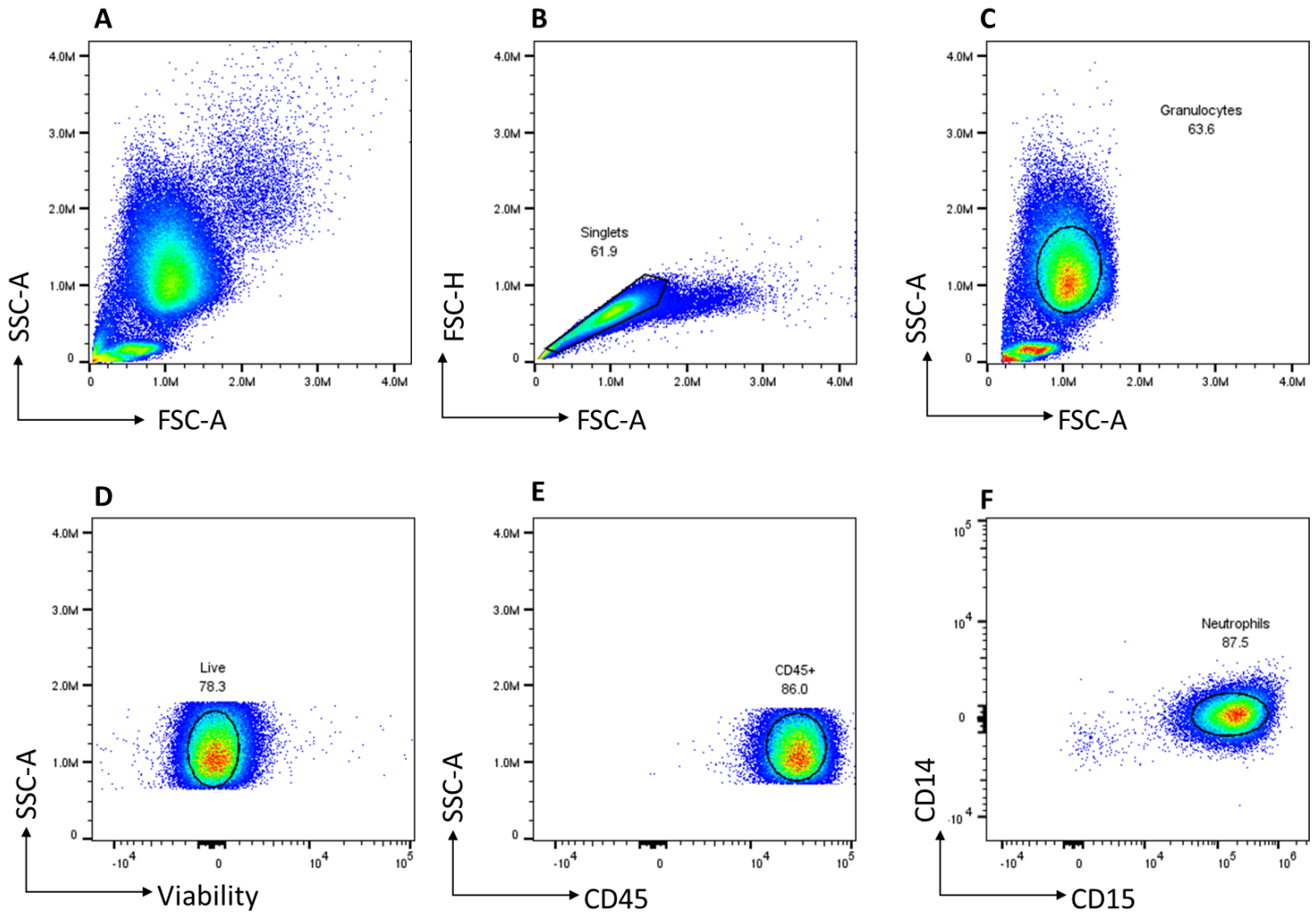


Figure 4.7. Gating strategy for flow cytometric analysis of peripheral blood samples. Gating was performed on the following strategy. A) Cells are visualised on the FSC-A, SSC-A. B) Cell doublets were excluded by setting gates in the FSC-A (area) vs FSC-H (height). C) Granulocyte population was detected on the FSC-A, SSC-A. D) Live cells are gated on the viability dye-negative population. E) Immune cells are selected in the $CD45^{+}$ gate. F) Neutrophils are detected in the $CD15^{+}$ $CD14^{-/low}$ population.

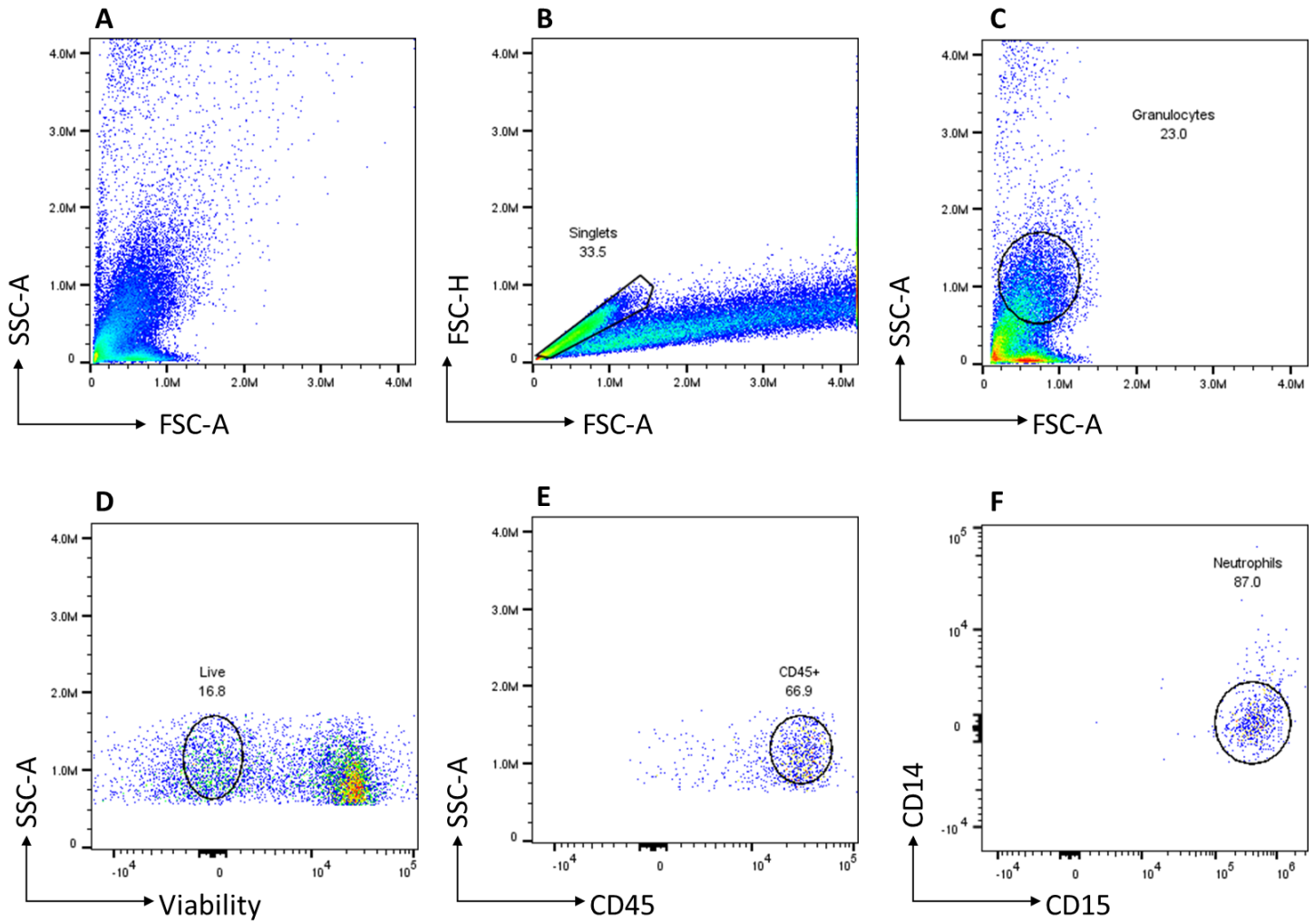


Figure 4.8. Gating strategy for flow cytometric analysis of cytobrush samples. Gating was performed on the following strategy. A) Cells are visualised on the FSC-A, SSC-A. B) Cell doublets were excluded by setting gates in the FSC-A (area) vs FSC-H (height). C) Granulocyte population was detected on the FSC-A, SSC-A. D) Live cells are gated on the viability dye-negative population. E) Immune cells are selected in the $CD45^{+}$ gate. F) Neutrophils are detected in the $CD15^{+}$ $CD14^{-/low}$ population.

4.6. Results

4.6.1. Study population characteristics

A total of 98 women were included (Figure 4.9.), providing a total of 147 samples. All matched peripheral and cytobrush samples were included for the assessment of the immunophenotype of neutrophils regardless of outcome. Out of the 98 women, three were lost to followed-up (transferred cared, moved country). From all the women who delivered before 37 weeks of gestations, two had a miscarriage, three had iatrogenic preterm deliveries (one for pre-eclampsia, one for pre-eclampsia and gestational diabetes, one due to fetal complications), one was classified as other (medical termination of pregnancy) and 10 were classified as spontaneous preterm birth (sPTB). In the term cohort (delivery after 37 weeks of gestation), 28 women had a term uncomplicated delivery (TU) and did not receive any type of intervention (neither progesterone and/or cerclage) at any point during their pregnancy. 50 women delivered at term following intervention; 8 women were part of the FLIP study (Vaginal *Lactobacillus* Supplementation in women at High Risk of Preterm Birth). Their samples were taken before administration of the vaginal probiotic, but based on outcome were included in the intervention group. For the correlation of the immunophenotype of neutrophils with pregnancy outcome, only the women with a spontaneous preterm delivery (PT), term uncomplicated (TU) or term with intervention (TI) were considered. Their clinical characteristics are fully described in Table 4.1.

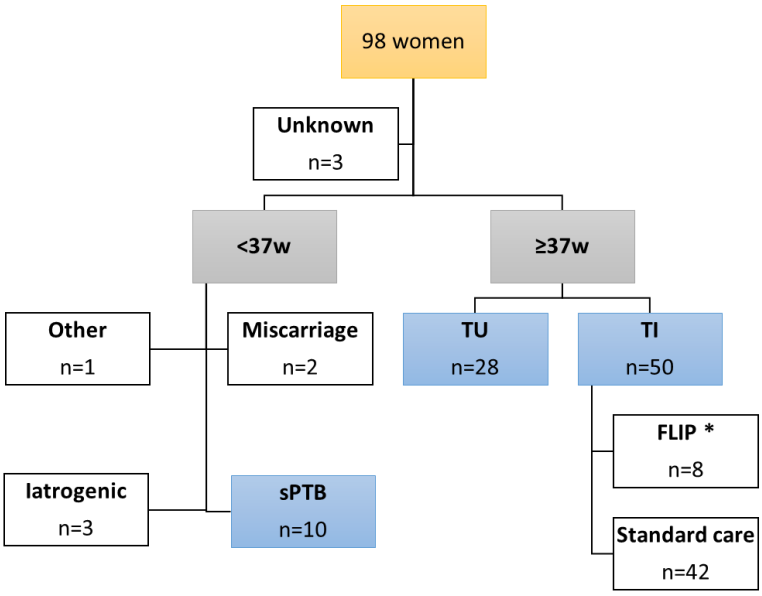


Figure 4.9. Overview of the total population included in the study of the immunophenotype of peripheral and cervical neutrophils in pregnancy. To establish the immunophenotype, 98 women with matched peripheral and cervical samples were included in the study regardless of outcome. To determine the associations between the immunophenotype of neutrophils and pregnancy outcome, only women with a spontaneous preterm delivery (sPTB), a term uncomplicated delivery (TU) or a term with intervention (TI) (highlighted in blue) were included in the analysis. *Samples from women part of the FLIP study were collected before administration of the vaginal probiotic.

The clinical characteristics of the patients included in the study are fully detailed in table 4.1. Ten women had a spontaneous preterm delivery (PT, 10/88, 11%). Half of them had an early preterm delivery, and the other half a late preterm delivery. The term uncomplicated group (TU) included 28 women (31%). The majority of women (50/88, 56%) had a term delivery after receiving an intervention. The most common intervention was cervical cerclage (n=33); and 10/50 women received progesterone only. The majority of women who delivered preterm (8/10) also had a cervical cerclage. The reason for cervical cerclage and the material used is also recorded on Table 4.1. The mean gestational age at delivery was statistically significant between groups (32+3 for PT, 39+4 for TU and 39+0 for TI, $p<0.0001$).

The difference between the age mean in the preterm and term group was statistically but not clinically significant (30 vs 35, respectively, $p=0.0029$). No differences were observed in the median BMI between groups (0.5720). The majority of women from all groups were white (>50%); and the proportion of ethnicity did not differ between the groups ($p=0.3866$).

Table 4.1. Study cohort demographics and clinical description

	PT	TU	TI	p-value
N (%)	10 (11.36%)	28 (31.82%)	50 (56.82%)	50 (56.82%)
Gestation at delivery				One-way Anova[a] ****p<0.0001
Mean	32+3	39+4	39+0	PT-TU
Range	24+0-36+5	37+0-41+4	37+1-41+6	PT-TI
Early PT ≤33+6 N (%)	5 (50%)	n.a.	n.a.	
Late PT ≥34 N (%)	5 (50%)	n.a.	n.a.	
Age				One-way Anova[a]
Mean	30.3	33.56	35.24	PT-TI p=0.0029
Range	24-39	25-42	28-43	
BMI				Kruskal-Wallis test[b]
Median	24.05	24.4	25	p=0.5720
Range	20-36.6	18.10-33.0	17-45	
Ethnicity				Chi-square[c]
White	6 (60%)	19 (67.86%)	25 (50%)	p=0.3866
Black	0 (0.0%)	3 (10.71%)	9 (18%)	
Other	4 (40%)	6 (21.43%)	17 (34%)	
Interventions				
Cervical cerclage	8	n.a.	33	
Pre-pregnancy		n.a.	1	
History indicated (Material)	5 (3 nylon, 2 mersilene)	n.a.	13 (12 nylon, 1 unk)	
USS indicated (Material)	3 (2 nylon, 1 mersilene)	n.a.	19 (16 nylon, 1 mersilene, 2 unk)	
Progesterone only	0	n.a.	10	
FLIP	1	n.a.	7	
Gravida				Kruskal-Wallis test [b]
Median	3	2	3	TU-TI * p=0.0242
Range	1-7	1-8	1-9	
Parity				Kruskall-Wallis test[b]
Median	0.5	0	1	p=0.5007
Range	0-3	0-5	0-5	

a) One-Way Anova with Tukey's multiple comparison test. b) Dunn's multiple comparisons test. c) Approximate chi-square calculations since conditions (expected values should be greater than 1.0 and at least 20% of the expected values greater than 5) have not been met. Ethnicity differences were also assessed combining groups and calculating Fisher's exact tests. No significantly differences were observed.

n.a. not applicable. unk: unknown

Table 4.1. Cont.

Gestation at sampling				Kruskal-Wallis test[b]
Timepoint A (n)	3	22	27	
Median	14+3	14+5	14+2	p=0.5122
Range	13+6-16+1	12+6-16+4	11+1-17+6	
Timepoint B	7	16	23	
Median	22+6	23+2	22+6	p=0.8060
Range	20+0-24+2	19+1-24+2	20+4-25+5	
Timepoint C (n)	2	12	20	p=0.4246
Median	30+6	30+5	31+4	
Range	30+4-31+2	20+6-33+6	29+6-33+6	

a) One-Way Anova with Tukey's multiple comparison test. b) Dunn's multiple comparisons test. c) Approximate chi-square calculations since conditions (expected values should be greater than 1.0 and at least 20% of the expected values greater than 5) have not been met. Ethnicity differences were also assessed combining groups and calculating Fisher's exact tests. No significantly differences were observed.

n.a. not applicable. unk: unknown

Clinical characteristics based upon risk factors and reasons for referral to the preterm birth surveillance clinic were also recorded (Table 4.2.). In the preterm cohort, the most common risk factor was a history of previous mid trimester loss (MTL). In the term uncomplicated pregnancy group, the majority of women were seen due to a history of cervical treatment (15/28). The most common risk factor for the term intervention group was also a history of cervical treatment (15/50), followed by incidental finding of a short cervix (7/50) and a history of preterm birth and PPROM in a previous pregnancy (5/50). As shown in Table 4.2., there is a wide variety of risk factors within each group.

Table 4.2. Risk factor for referral to preterm birth surveillance clinic

	PT	**TU**	**TI**
N (%)	10 (11.36%)	28 (31.82%)	50 (56.82%)
Uterine anomaly		3	1
Uterine anomaly + PTB+PPROM		1	
Uterine anomaly + incidental short cervix			1
Cx treatment	1	15	14
Cx treatment + incidental short cervix			1
Cx treatment+ PTB + PPROM			2
Cx treatment + MTL+PPROM			1
Cx treatment+ MTL+ incidental short cervix			1
Cx treatment+ Hx cerclage	1		2
Cx treatment+ PPROM			1
PTB		3	
PTB + incidental finding short cervix			1
PTB+MTL+PPROM+Cerclage			1
PTB+MTL			2
PTB+PPROM	3		5
PTB+PPROM+Hx cerclage	1		1
MTL	4	2	
MTL+ Cerclage			2
MTL+PPROM		1	3
MTL+PPROM+Cerclage			1
Hx cerclage			2
Incidental finding of short cervix			7
Fully dilated C-section		3	1

Hx: history. History of PTB (spontaneous preterm birth), PPROM (premature prelabour rupture of membranes), MTL (mid-trimester loss). Cx treatment: cervical treatment. C-section: caesarean section.

Finally, all the women from the cohort were also grouped based on the detection of cervical shortening (cervical length ≤25mm) during pregnancy. 32 women (32/98, 32.65%) underwent cervical shortening at some point during pregnancy (Table 4.3.). The rest of the women (66/98, 67.35%) maintained a normal cervical length throughout pregnancy. The median length of the shortest cervical length was significantly shorter in women who experienced cervical shortening (19mm), compared to women that did not shorten (median 33mm) ($p<0.0001$). The median gestational age at which cervical shortening occurred was 21+2. The median latency period from shortening until delivery was 113 days (16+2), but it ranged from 5 to 160 days.

The most common intervention in women who experienced cervical shortening was cervical cerclage (29/32, 90%). The majority of the cerclages were ultrasound indicated (22/29, 76%). In contrast, the reason for cervical cerclage in women with a normal cervical length was history indicated (15/15).

The majority of women who underwent cervical shortening delivered at term following intervention (22/32, 68.75%). The preterm birth rates were higher in women who experienced cervical shortening (8/32, 25%) compared to women who did not develop a short cervix (3/66, 4.55%) ($p<0.0001$).

Table 4.3. Clinical characteristics of the study population according to the detection of cervical shortening

	Cervical shortening	**Normal cervical length**	**p-value**
N (%)	32 (32.65%)	66 (67.35%)	
Shortest cervical length			Mann-Whitney
Median	19	33	****
Range	(4-25)	(26-47)	p<0.0001
Gestation at shortening			
Median	21+2	n.a	
Range	14+4-32+3	n.a	
Latency until delivery			
Median	16+2 (113days)	n.a	
Range	5days-160 days	n.a	
Cerclage insertion	29/32	15/66	Unpaired t-test
Mean gestational age	20+0	15+4	p<0.0001
Range	(11+5-25+3)	(12+2-19+6)	****
Pre-pregnancy	1	0	
History indicated, n (material)	6 (3 nylon, 3 mersilene)	15 (13 nylon, 1 mersilene, 1unk)	
USS indicated, n (material)	22 (18 nylon, 2 mersilene, 1 unk)	0	
Outcome			
PT	8 (25%)	3 (4.55%)	Chi-square
TU	0 (0.0%)	28 (42.42%)	****
TI	22 (68.75%)	28 (42.42%)	p<0.0001
Other	3[a] (9.4%)	6[b] (9.1%)	
Samples			Unpaired t-test
Timepoint A			
Mean	14+2	14+3	p=0.5767
Range	11+1-16+6	12+4-17+6	
Timepoint B			
Mean	22+4	23+0	p=0.4361
Range	20+0-25+3	21+0-24+2	
Timepoint C			p=0.503
Mean	31+3	31+0	
Range	30+2-31+6	30+2-32+1	

a) Reason for other: 1 miscarriage, 2 unknown; b) reason for other: 1 miscarriage, 2 preeclampsia, 1 breech, 1 medical termination of pregnancy, 1 unknown.

4.6.2. Immunophenotype of peripheral and cervical neutrophils

The immunophenotype of peripheral and cervical neutrophils were characterised in 77 pairs of matched samples, assessing the MFI of different markers in the live neutrophil population (CD45+, CD15+, CD14-/low). First, markers related to neutrophil maturation were evaluated. The expression of CD10 was similar between cervical and peripheral neutrophils (Figure 4.10.A). The MFI of CD33 was higher in peripheral neutrophils compared to cervical neutrophils (Figure 4.10.B) (p=0.0274).

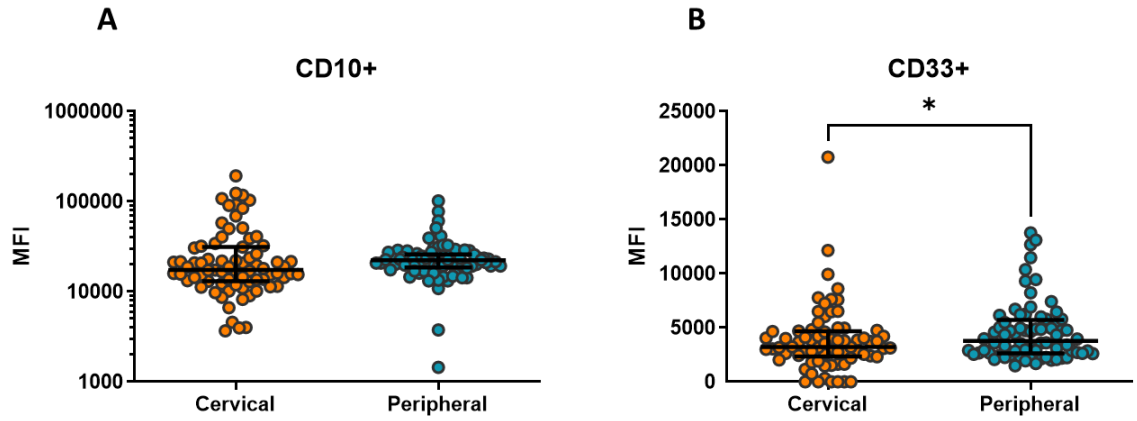


Figure 4.10. Median fluorescence intensity of markers implicated in neutrophil maturation and immune regulation in cervical and peripheral granulocytes. A) MFI of CD10. **B)** MFI of CD33. Statistical differences of MFI expression between cervical and peripheral neutrophils (n=77 matched pairs) were evaluated using a Wilcoxon matched-pairs signed rank test two-tailed. *p<0.05. Data is presented as median values with interquartile range.

Markers implicated in neutrophil migration were also examined. The expression of CD11b, an integrin, was reduced in cervical neutrophils (median MFI 9323) compared to peripheral neutrophils (median MFI 19201) ($p<0.0001$) (Figure 4.11. A). The expression of CD62L, a selectin, was also reduced in cervical neutrophils compared to matched peripheral neutrophils (median MFI 2080, compared to median MFI 22143, respectively; $p<0.0001$) (Figure 4.11.B).

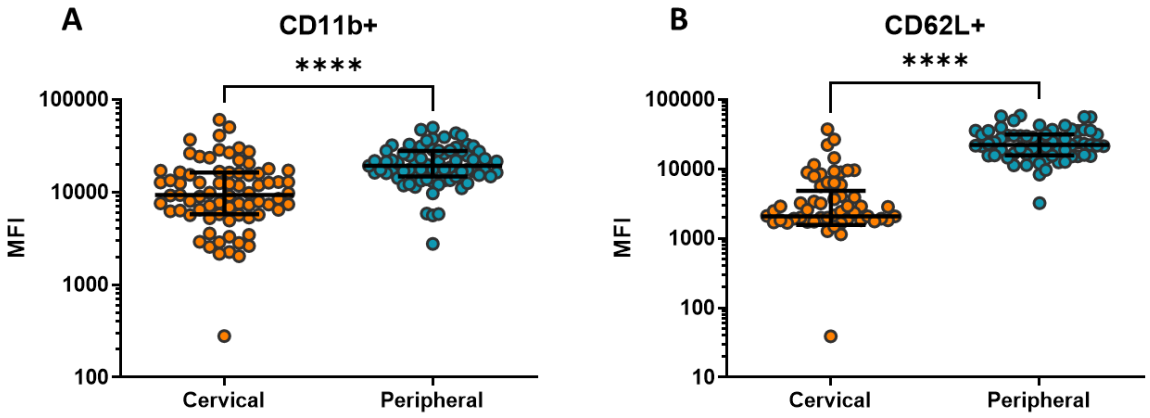


Figure 4.11. Median fluorescence intensity of markers implicated in neutrophil migration. A) MFI of CD11b. **B)** MFI of CD62L. Statistical differences of MFI expression between cervical and peripheral neutrophils (n=77 matched pairs) were evaluated using a Wilcoxon matched-pairs signed rank test two-tailed. ****$p<0.0001$. Data is presented as median values with interquartile range.

The following section will cover markers implicated in microbial recognition and effector functions of neutrophils, including CD16, CD63 and CD66b. CD16 is also known as the FCγ receptor III (FcγRIII) and participates in signal transduction. The MFI of CD16 was more than 5 times higher in peripheral neutrophils than in cervical neutrophils ($p<0.0001$) (Figure 4.12.A). CD63 is a marker of degranulation and its expression was up to 3 times higher in cervical neutrophils compared to peripheral neutrophils ($p<.0.0001$) (Figure 4.12.B). Finally, CD66b is a marker of granulocyte activation involved in many roles (degranulation, ROS production and phagocytosis). CD66b expression was also increased in cervical neutrophils compared to peripheral neutrophils ($p<0.0001$) (Figure 4.12.C).

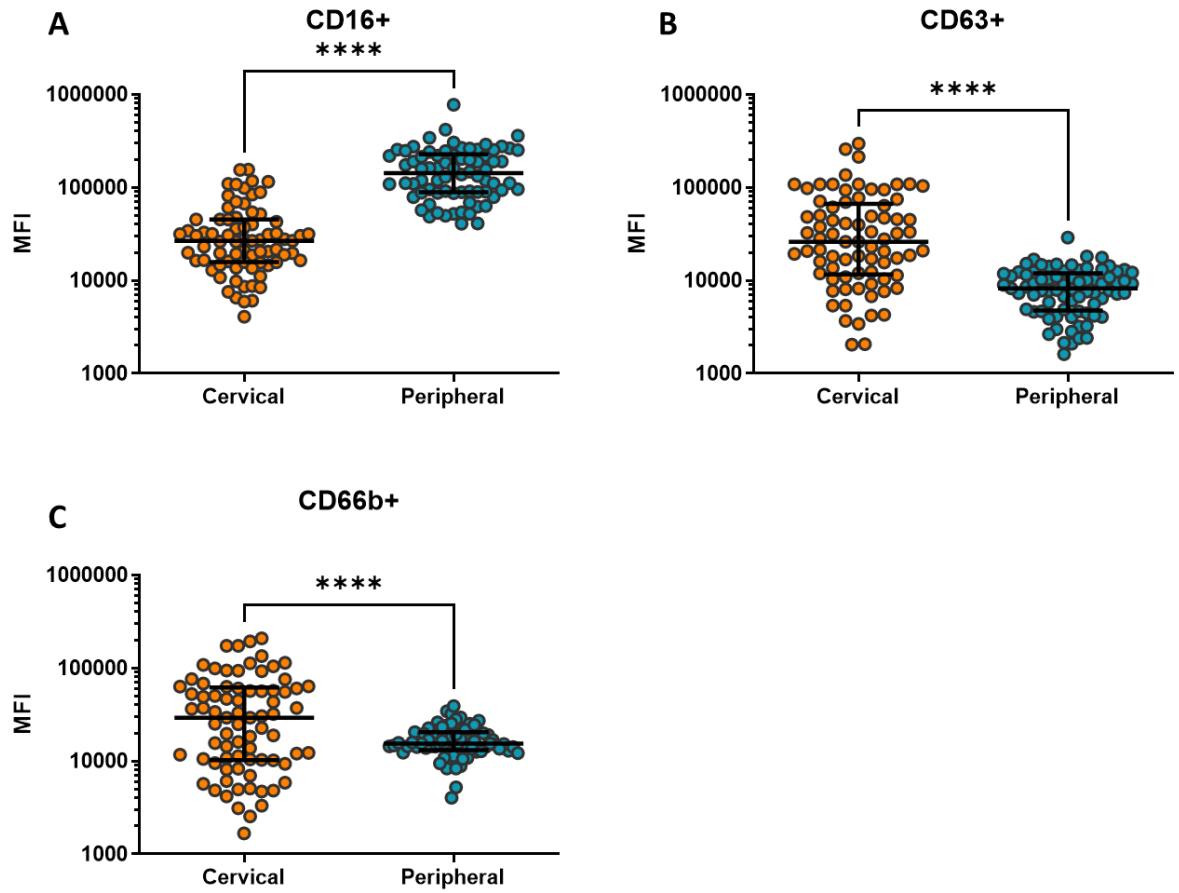


Figure 4.12. Median fluorescence intensity of markers implicated in neutrophil activation. A) MFI of CD16. **B)** MFI of CD63. **C)** MFI of CD66b. Statistical differences of MFI expression between cervical and peripheral neutrophils (n=77 matched pairs) were evaluated using a Wilcoxon matched-pairs signed rank test two-tailed. ****p<0.0001. Data is presented as median values with interquartile range.

The MFI expression of CD35, CD55 and CD88, markers implicated in the complement system, were also examined. CD35 is also known as complement receptor 1 (CR1); this immune adherence receptor can bind C3b and C4b. A significantly lower MFI was detected on cervical neutrophils (median 8945), compared to peripheral neutrophils (median 23735) ($p<0.0001$) (Figure 4.13.A). CD55 is also known as the complement decay accelerating factor (DAF) and is an inhibitor of the complement cascade. The MFI expression of CD55 was higher on cervical neutrophils (median MFI 79364) compared to peripheral neutrophils (median MFI 71678) ($p=0.0007$) (Figure 4.13.B). CD88 is the C5a receptor and the MFI on cervical neutrophils was almost 3 times higher than in peripheral neutrophils ($p<0.0001$) (Figure 4.13.C).

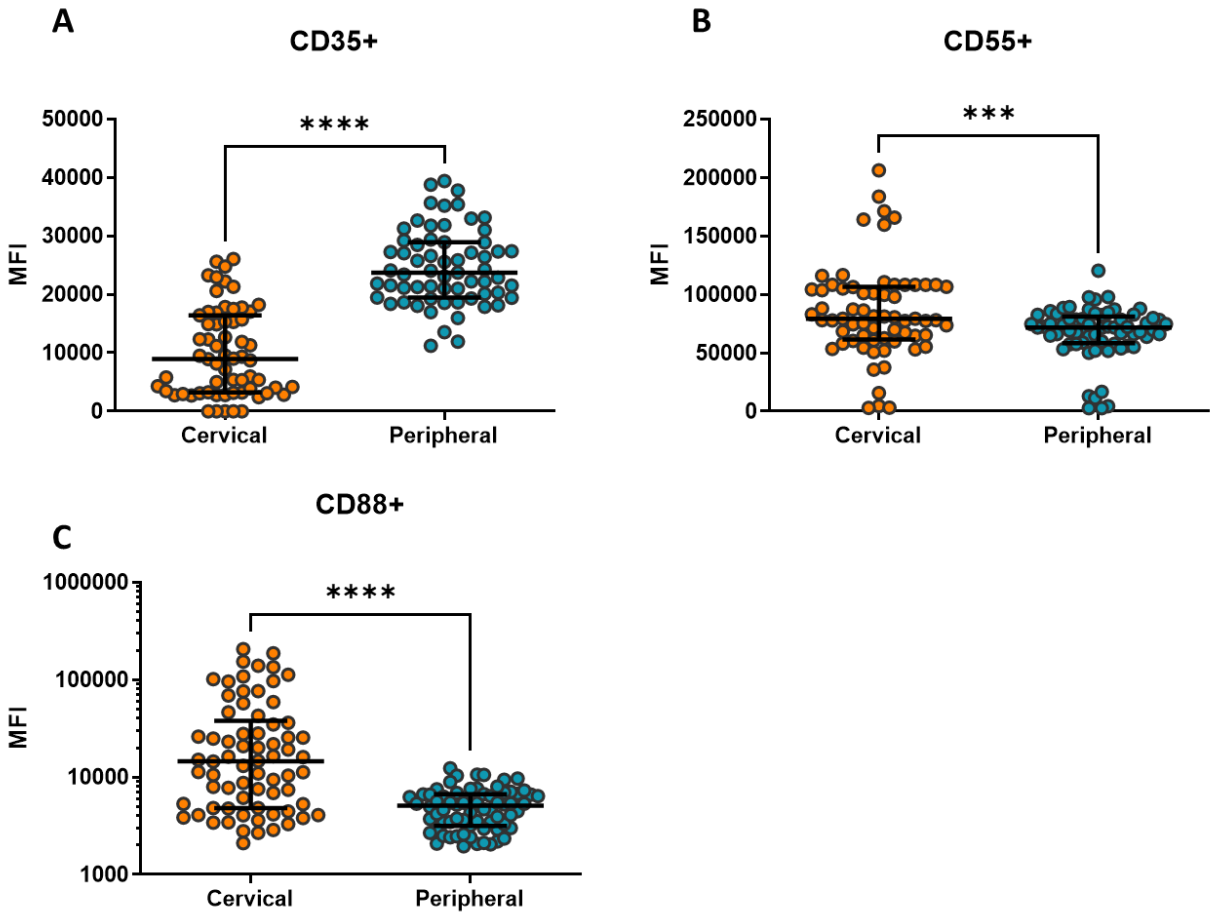


Figure 4.13. Median fluorescence intensity of markers implicated in the complement system. A) MFI of CD35. **B)** MFI of CD55. **C)** MFI of CD88. Statistical differences of MFI expression between cervical and peripheral neutrophils (n=77 matched pairs) were evaluated using a Wilcoxon matched-pairs signed rank test two-tailed. ***p<0.001; ****p<0.0001. Data is presented as median values with interquartile range.

4.6.3. Detection and immunophenotype of neutrophils according to the vaginal microbial composition

In this study cohort, cervical neutrophils were not always detected in cytobrush samples. From a total of 141 samples, cervical neutrophils were detected in 109/141 (77%), and undetectable in 22% of the samples (32/141). However, live neutrophils were only detected in 83/141 samples (58%). The detection of neutrophils relating to microbial composition, cervical shortening and pregnancy outcome was explored.

Neutrophil detection according to the vaginal microbial composition

Women were divided in two groups based on the local cervicovaginal microbial composition: women with a low-risk microbial signature (classified as CST I, CST II and CST V) and women with a high-risk microbial signature (dominance of CST III or CST IV). The percentage of detection of neutrophils was slightly higher in women with a high-risk microbial composition (81%) compared to women with a low-risk vaginal microbial composition (74%) (Figure 4.14. A), however this did this not reach statistical significance (p=0.2454). No differences were observed in the percentage of detection of live neutrophils in women with a low- or a high-risk microbial composition (55% vs 60%, p=0.3731) (Figure 4.14.B.). However, when live neutrophils were detected, women with a high-risk microbial composition had a higher proportion of live neutrophils (>50% live neutrophils) than women with a low-risk microbial composition (82% vs 18%, p=0.075) (Figure 4.14.C).

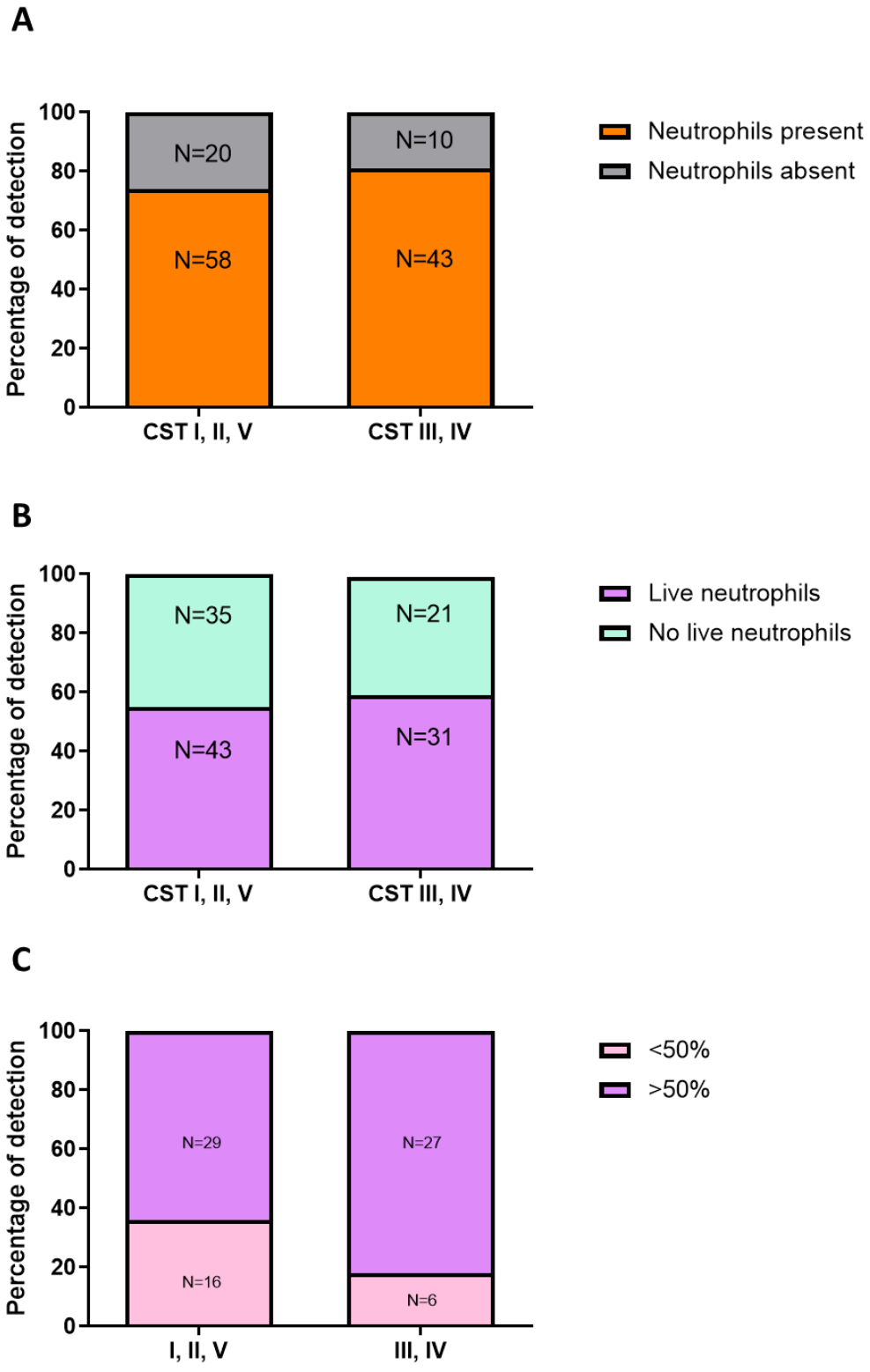


Figure 4.14. Detection of cervical neutrophils based on a low-risk and high-risk vaginal microbial composition. Percentage of detection of **A)** neutrophils and **B)** live neutrophils in women with a low-risk vaginal microbial composition (CST I, CST II or CST V) compared to women with a high-risk vaginal microbial composition (CST III or CST IV). **C)** Detection of live neutrophils in a low proportion (<50%) and in a high proportion (>50%). Statistical differences between the proportions of both groups were assessed with Fisher's exact test.

Since there was a trend towards a higher proportion of women with high-risk microbial signature with neutrophils present, to further understand the potential association between the detection of neutrophils and the local cervicovaginal composition, differences were also assessed across the 5 different Community State Types: CST I (*L. crispatus* dominated), CST II (*L. gasseri* dominated), CST III (*L. iners* dominated) CST V (*L. jensenii* dominated) and CST IV (low abundance of lactobacilli, heterogeneous anaerobic bacteria) (Figure 4.15.). Comparisons were made between CSTs I, III, IV due to their varying differences in mediating different immune responses as established in Chapter 3. There was a higher proportion of women with a CST III *L. iners* vaginal dominance with neutrophils present (94%) compared to women with a CST I *L. crispatus* dominance (74%) (p=0.0247). The same trends were observed in women with live neutrophils present, but this did not reach statistical significance (p=0.1804) (Figure 4.15.B). When live cervical neutrophils were present, they were present in higher proportions (>50% live neutrophils) in women with a CST III and a CST IV (Figure 4.15.C).

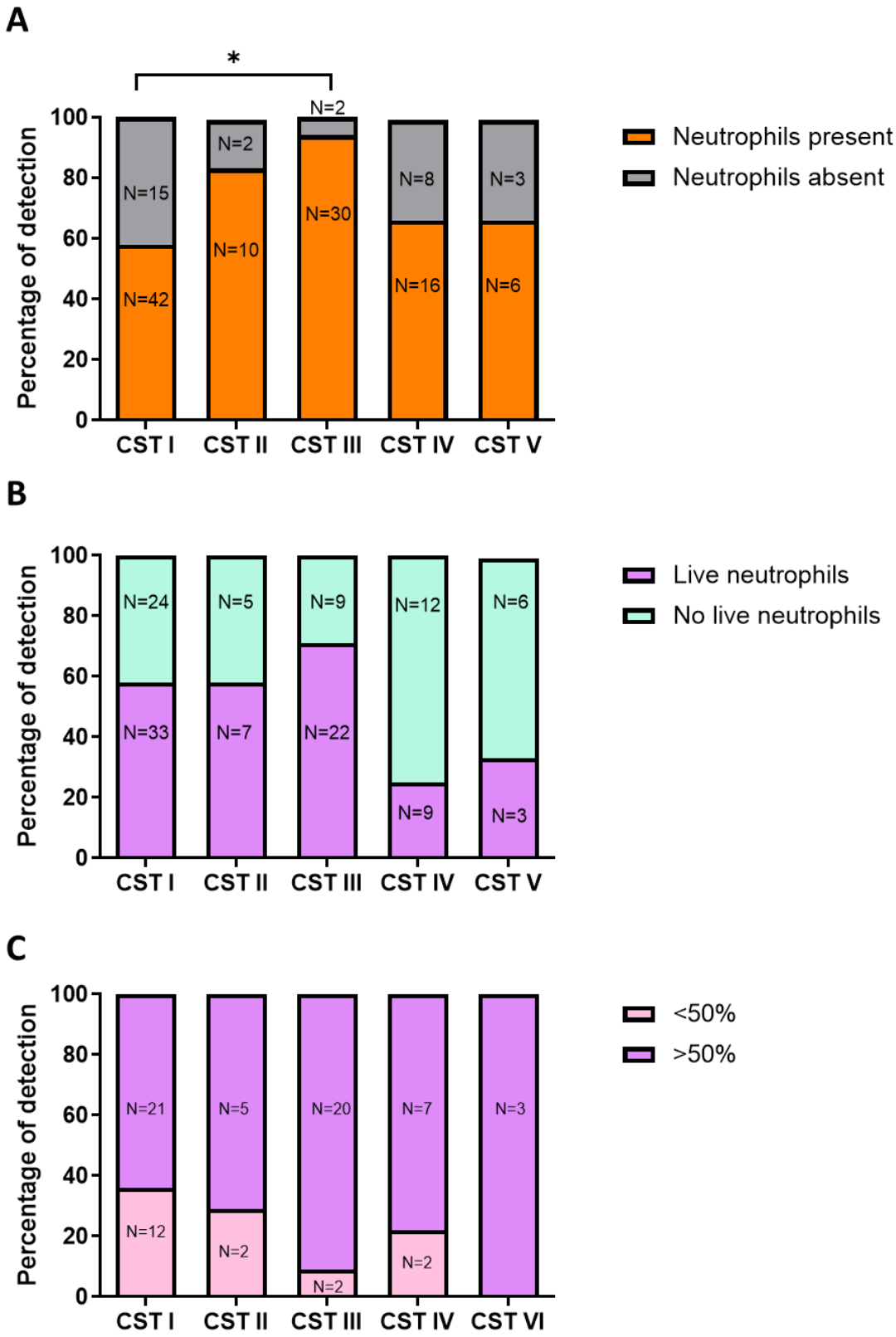


Figure 4.15. Detection of cervical neutrophils based on CSTs vaginal microbial classifications. Percentage of detection of **A)** neutrophils and **B)** live neutrophils in women with a CST I (*L. crispatus* dominance), CST II (*L. gasseri* dominance), CST III (*L. iners* dominance), CST IV (reduced lactobacilli) and CST V (*L. jensenii*). **C)** Proportion of live neutrophils present (<50%, >50%). Statistical differences between the proportions of both groups were assessed with Fisher's exact test. *p<0.05.

Immunophenotype of peripheral and cervical neutrophils in low-risk and high-risk vaginal microbial compositions

The immunophenotype of both peripheral and cervical neutrophils was assessed and comparisons made in women who had a low-risk (CST I, CST II, CST V) or a high-risk (CST III, CST IV) vaginal microbial composition.

The MFI of CD10 was significantly increased in peripheral neutrophils from women with a high-risk microbial signature, compared to women with a low -risk microbial signature (p=0.0396) (Figure 4.16.A). The immunophenotype of peripheral neutrophils did not vary between women with a low-risk and a high-risk microbial composition for the markers CD33 (Figure 4.16.B), CD11b (Figure 4.16.C) or CD62L (Figure 4.16.D).

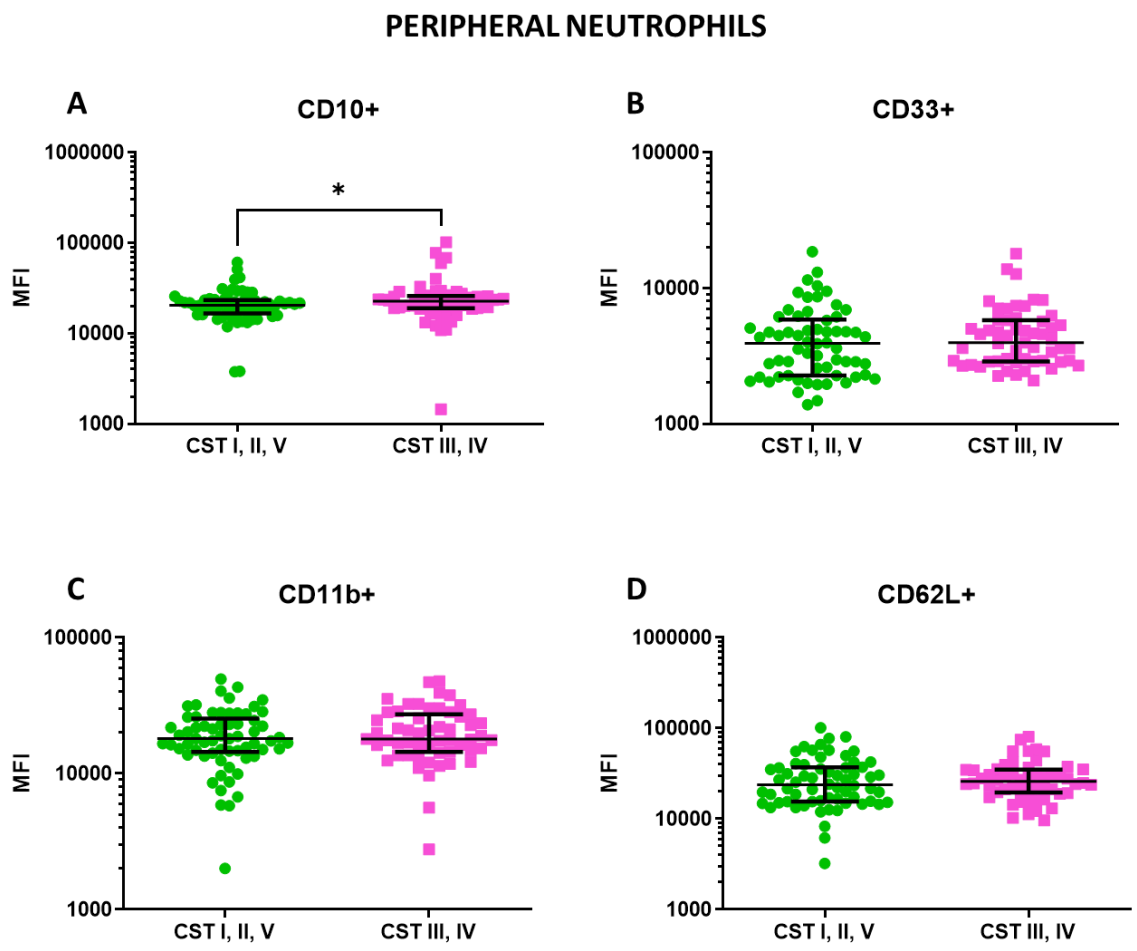


Figure 4.16. Median fluorescence intensity of markers implicated in neutrophil maturation, immune regulation and migration in peripheral neutrophils in low-risk and high-risk microbial vaginal compositions. **A)** MFI of CD10. **B)** MFI of CD33. **C)** MFI of CD11b. **D)** MFI of CD62L. Statistical differences of MFI expression between a low-risk microbial signature (CST I, CST II, CST V) (n=68) and a high-risk microbial signature (CST III, CST IV) (n=53) were evaluated using a Mann Whitney one-tailed test. *p<0.05. Data is presented as median values with interquartile range.

When assessing markers of microbial recognition (CD16) and neutrophil activation (CD63, CD66b) in the periphery, no differences were observed between the median MFI expression based on the microbial composition (Figure 4.17).

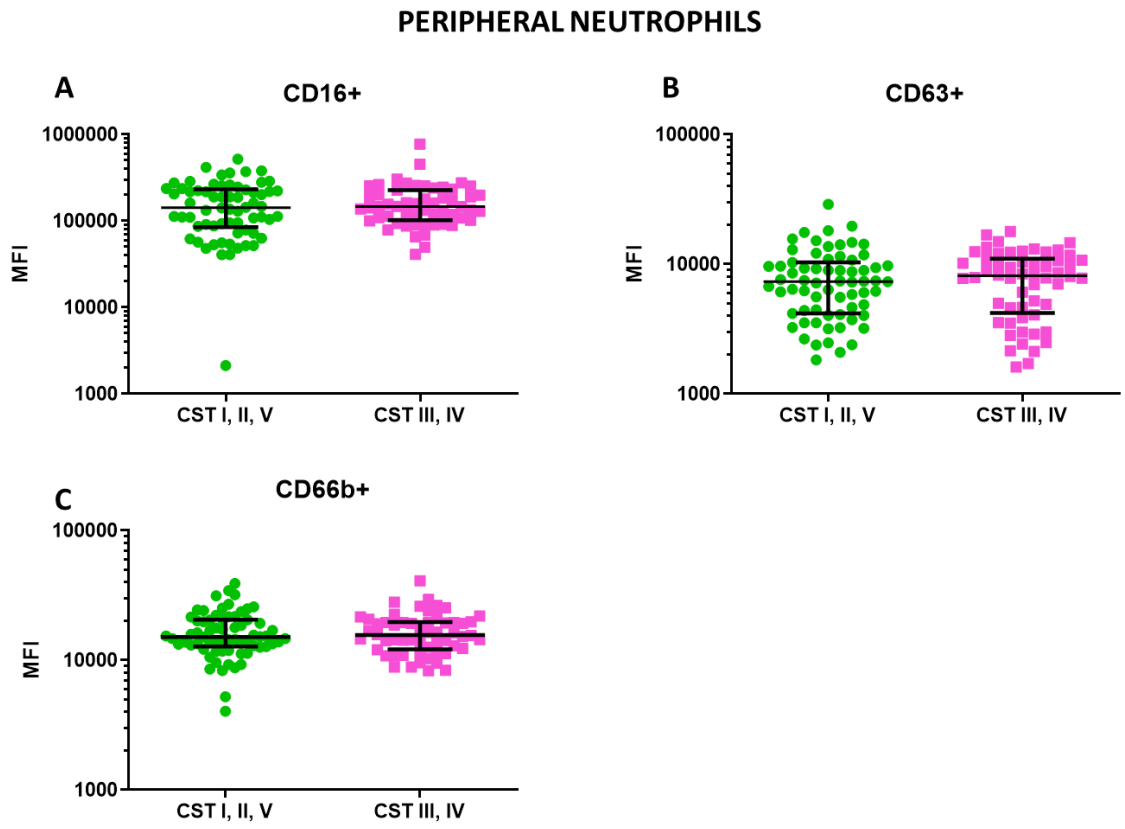


Figure 4.17. Median fluorescence intensity of markers implicated in neutrophil activation in peripheral neutrophils in low-risk and high-risk microbial vaginal compositions. A) MFI of CD16. **B)** MFI of CD63. **C)** MFI of CD66b. Statistical differences of MFI expression between a low-risk microbial signature (CST I, CST II, CST V) (n=68) and a high-risk microbial signature (CST III, CST IV) (n=53) were evaluated using a Mann Whitney one-tailed test. Data is presented as median values with interquartile range.

In terms of complement activation (Figure 4.18), no differences were found for the median MFI of CD35 or CD88, however the MFI of complement regulator CD55 was reduced in women with a CST III or CST IV cervicovaginal composition, compared to women with a low-risk microbial signature (p=0.0025) (Figure 4.18.B).

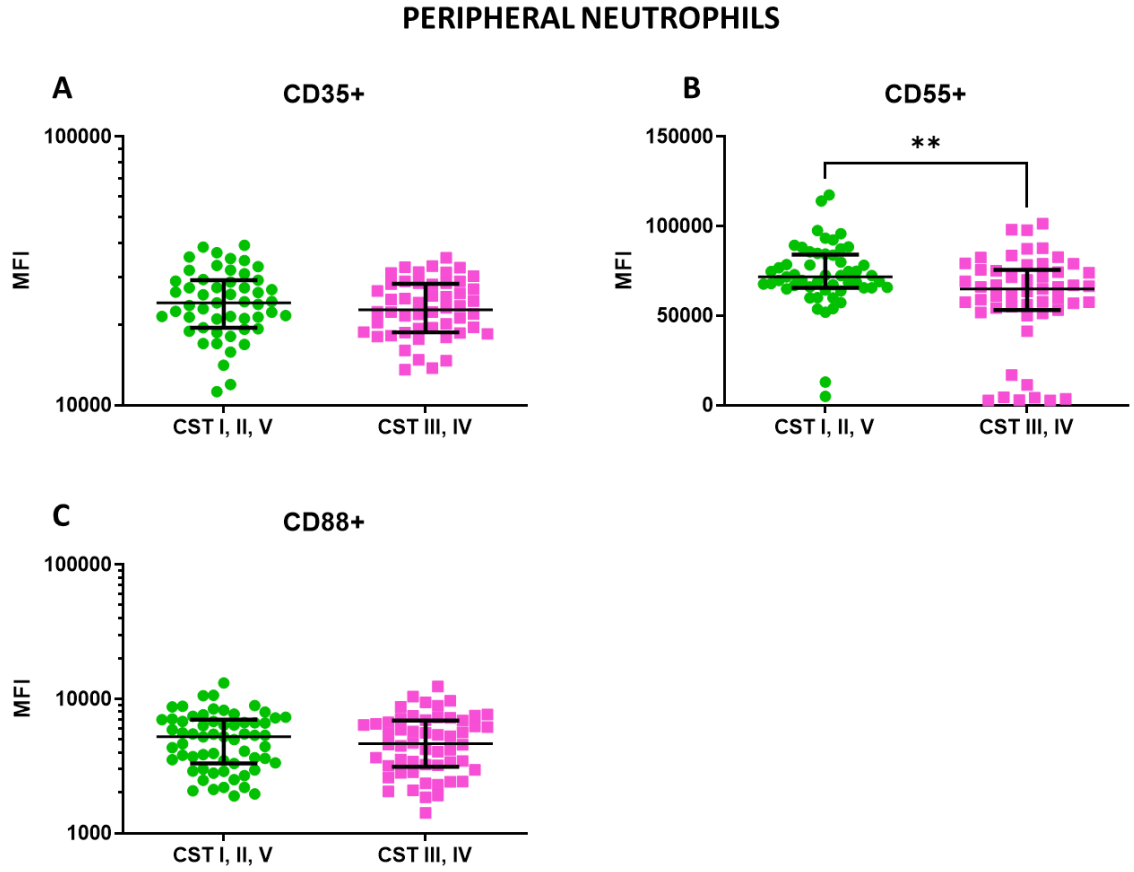


Figure 4.18. Median fluorescence intensity of markers implicated in complement activation in peripheral neutrophils in low-risk and high-risk microbial vaginal compositions. A) MFI of CD35. **B)** MFI of CD55. **C)** MFI of CD66b. Statistical differences of MFI expression between a low-risk microbial signature (CST I, CST II, CST V) (n=68) and a high-risk microbial signature (CST III, CST IV) (n=53) were evaluated using a Mann Whitney one-tailed test. **p<0.01. Data is presented as median values with interquartile range.

As seen in the periphery, the immunophenotype of cervical neutrophils including the following markers CD10, CD33, CD11b and CD62L did not significantly change based on the microbial composition (Figure 5.19).

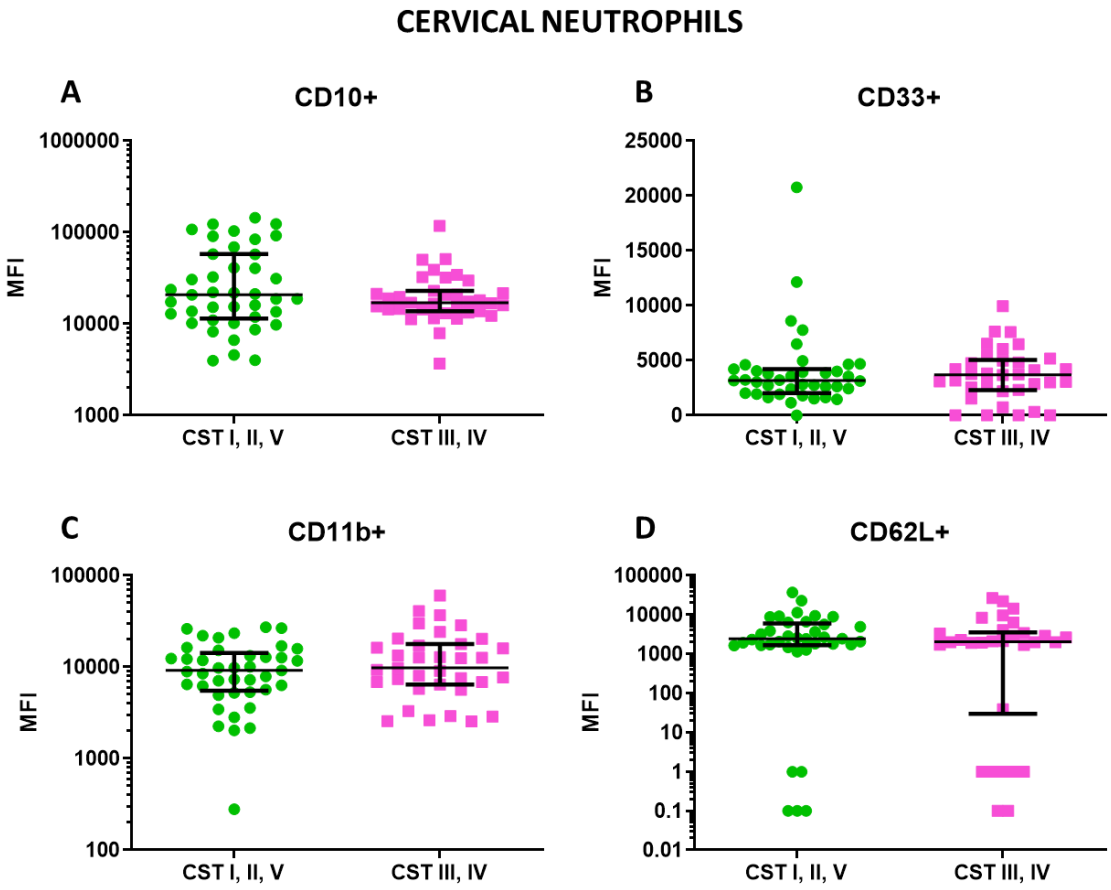


Figure 4.19. Median fluorescence intensity of markers implicated in neutrophil maturation, immune regulation and migration in cervical neutrophils in low-risk and high-risk microbial vaginal compositions. A) MFI of CD10. **B)** MFI of CD33. **C)** MFI of CD11b. **D)** MFI of CD62L. Statistical differences of MFI expression between a low-risk microbial signature (CST I, CST II, CST V) (n=43) and a high-risk microbial signature (CST III, CST IV) (n=31) were evaluated using a Mann Whitney one-tailed test. Data is presented as median values with interquartile range.

In contrast to peripheral neutrophils, cervical neutrophils had a non-significant but higher MFI of CD63 (28265 vs 19331, p=0.0596) (Figure 4.20.B) and CD66b (MFI 46516 vs 14395, p=0.0093) (Figure 4.20.C) in women with a high-risk microbial composition (Figure 4.20).

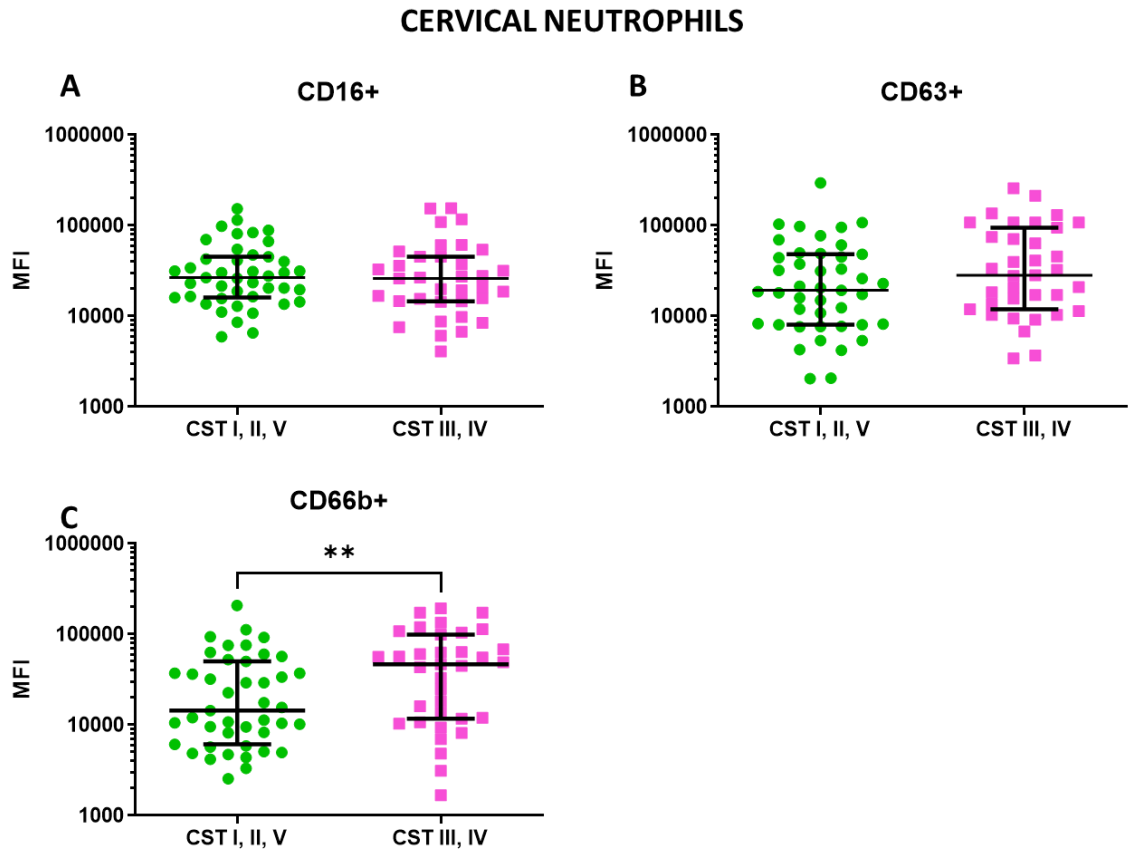


Figure 4.20. Median fluorescence intensity of markers implicated in neutrophil activation in cervical neutrophils in low-risk and high-risk microbial vaginal compositions. A) MFI of CD16. **B)** MFI of CD63. **C)** MFI of CD66b. Statistical differences of MFI expression between a low-risk microbial signature (CST I, CST II, CST V) (n=43) and a high-risk microbial signature (CST III, CST IV) (n=31) were evaluated using a Mann Whitney one-tailed test. **p<0.01. Data is presented as median values with interquartile range.

Finally, markers of complement activation were assessed in cervical neutrophils. No statistically significant differences were observed in the MFI of CD35 (C3b receptor) (Figure 4.21.A), CD55 (p=0.4988) (Figure 4.21.B) or CD88 (C5a receptor) between the low and high-risk CSTs. However, the median CD88 MFI was lower in women with a CST III or IV high-risk microbial composition (p=0.0526) (Figure 4.21.C).

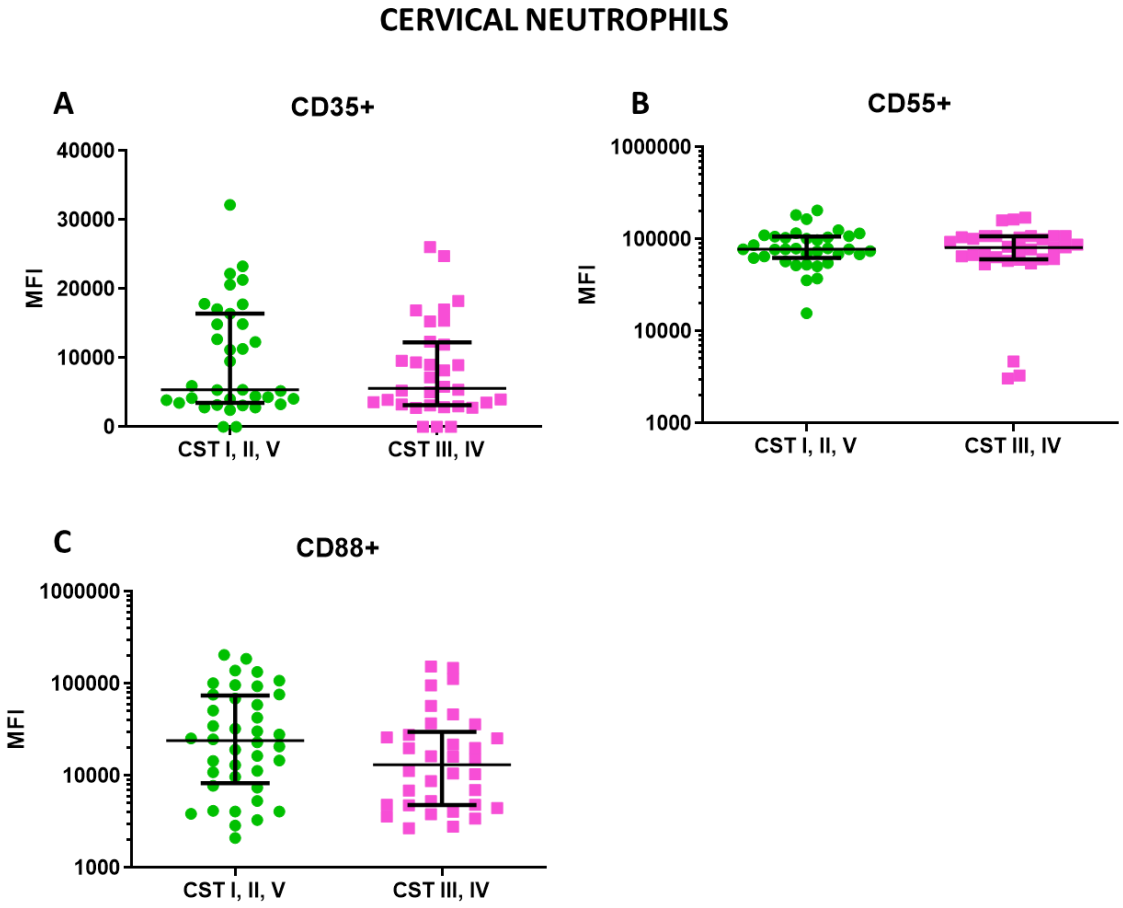


Figure 4.21. Median fluorescence intensity of markers implicated in complement activation in cervical neutrophils in low-risk and high-risk microbial vaginal compositions. A) MFI of CD35. **B)** MFI of CD55. **C)** MFI of CD66b. Statistical differences of MFI expression between a low-risk microbial signature (CST I, CST II, CST V) (n=43) and a high-risk microbial signature (CST III, CST IV) (n=31) were evaluated using a Mann Whitney one-tailed test. Data is presented as median values with interquartile range.

Immunophenotype of peripheral and cervical neutrophils based on CST classifications

Since we detected interesting associations (CD10 and CD55 in peripheral neutrophils; CD66b in cervical neutrophils) and trends (CD63 and CD88 in cervical neutrophils) of the MFI of markers between low-risk and high-risk microbial signatures, this was further assessed based on individual CST groups.

The MFI expression of CD10, CD33, CD11b and CD62L (Figure 4.22) on peripheral neutrophils did not significantly change according to each CST classification in isolation.

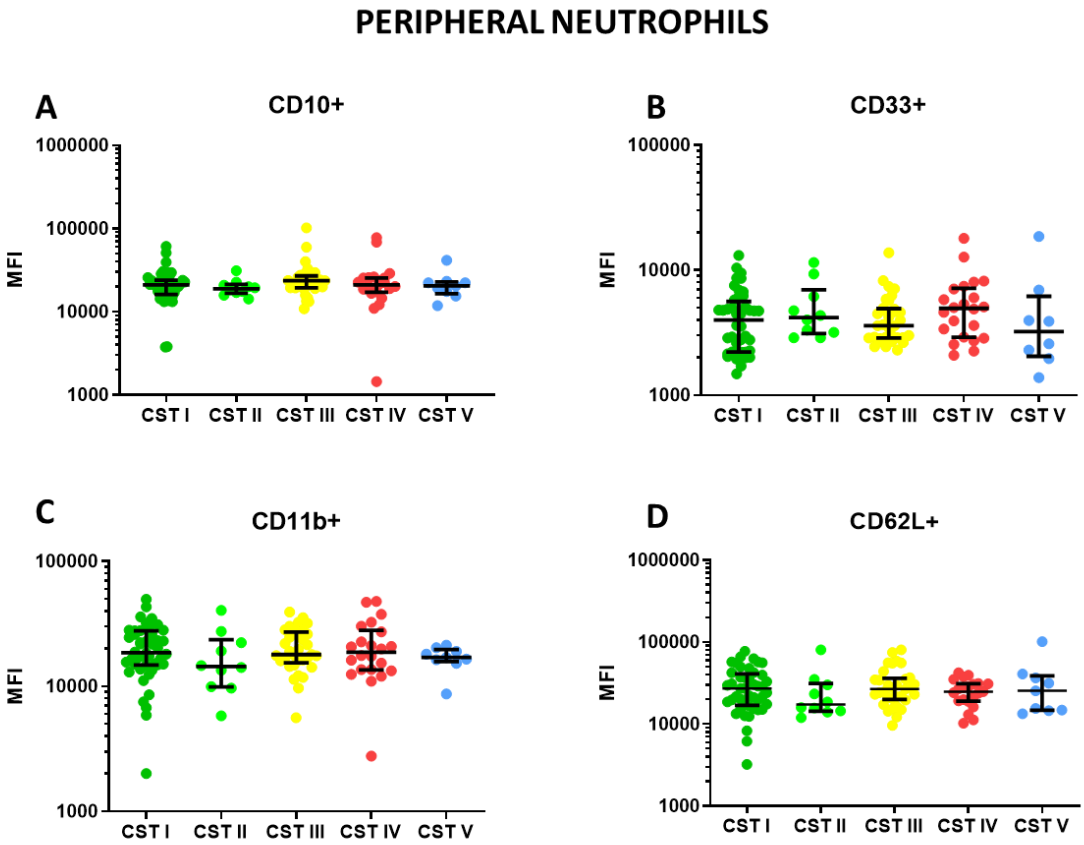


Figure 4.22. Median fluorescence intensity of markers implicated in neutrophil maturation, immune regulation and migration in peripheral neutrophils based on CSTs classifications. A) MFI of CD10. **B)** MFI of CD33. **C)** MFI of CD11b. **D)** MFI of CD62L. Statistical differences of MFI expression between groups (CST I, n=47; CST III, n=33; and CST IV, n=22) were evaluated using a Kruskal-Wallis test with Dunn's multiple comparison post hoc test. Data is presented as median values with interquartile range.

In addition, no differences were seen in the MFI of the markers of activation CD16, CD63 or CD66b (Figure 4.23.A, B, C) according to each of the CST groups.

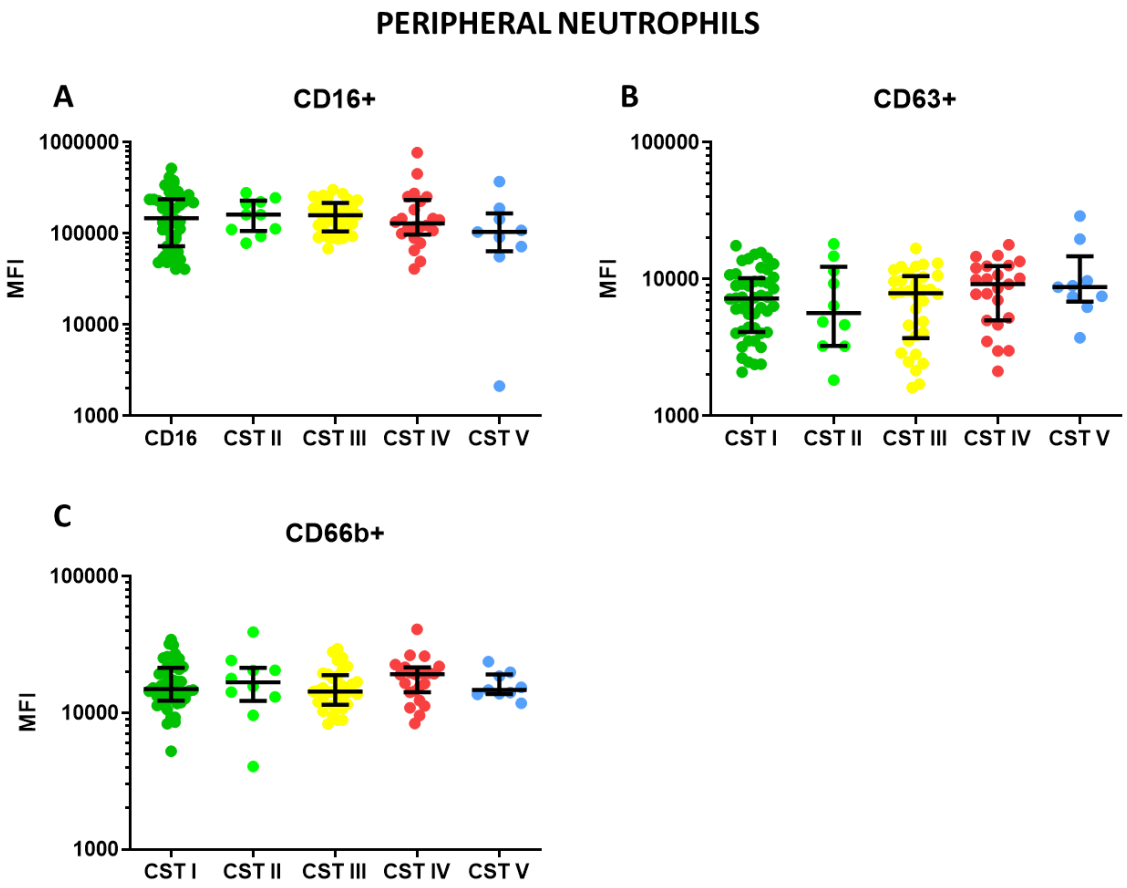


Figure 4.23. Median fluorescence intensity of markers implicated in neutrophil activation in peripheral neutrophils based on CSTs classifications. A) MFI of CD16. **B)** MFI of CD63. **C)** MFI of CD66b. Statistical differences of MFI expression between groups (CST I, n=47; CST III, n=33; and CST IV, n=22) were evaluated using a Kruskal-Wallis test with Dunn's multiple comparison post hoc test. Data is presented as median values with interquartile range.

Markers related to the complement system were also assessed according to the local vaginal microbial composition. Peripheral neutrophils had a higher CD35 MFI (C3b receptor) in women with a CST IV composition compared to women with a CST III (p=0.0151) (Figure 4.24.A). The MFI expression of CD55 in peripheral neutrophils changed between women with different CST classification (p=0.0434), but significance was lost after applying the Dunn's multiple comparisons post hoc test (Figure 4.24.B). We can see a decrease of the median MFI of CD55 in women with a CST IV, potentially being the contributor for the significantly decreased CD55 observed in the periphery in women with a high-risk microbial composition (CST III- and CST IV). Finally, no differences were observed in the MFI of CD88 in peripheral neutrophils by CST (Figure 4.24.C).

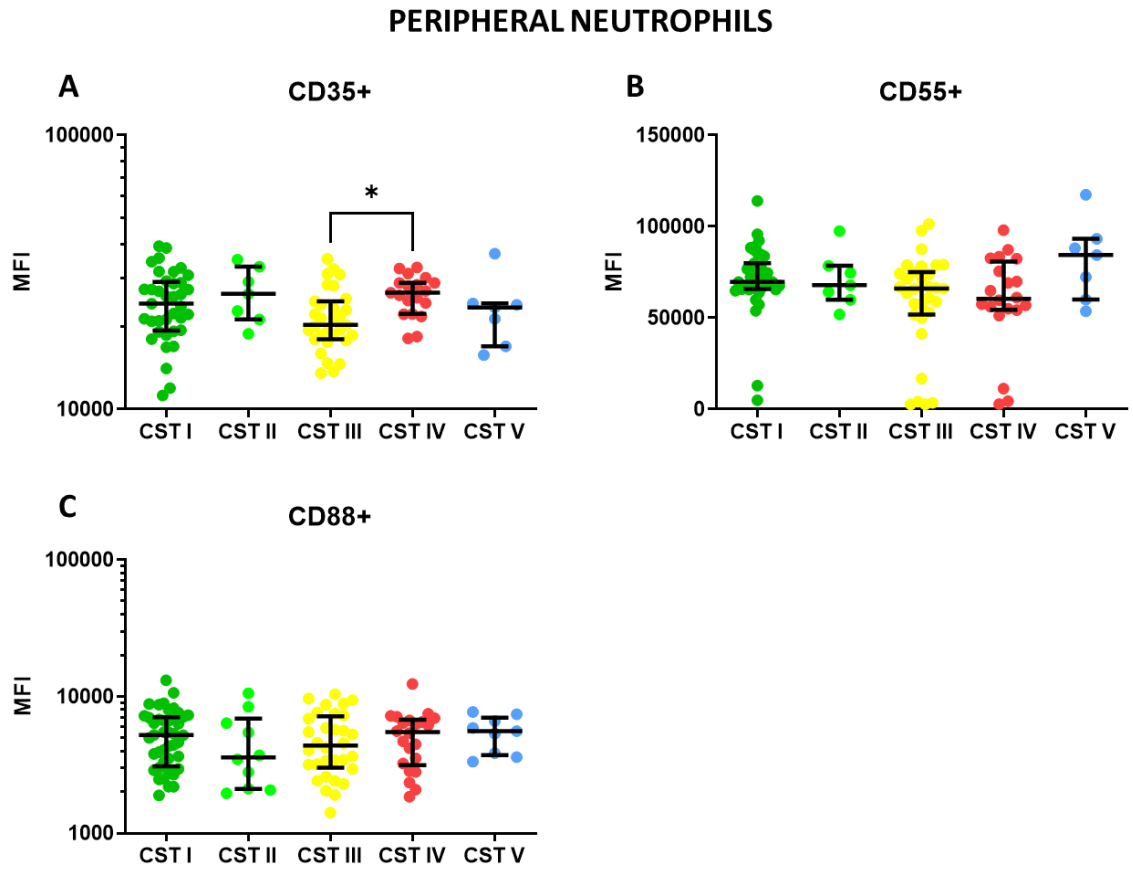


Figure 4.24. Median fluorescence intensity of markers implicated in complement activation in peripheral neutrophils based on CSTs classifications. A) MFI of CD35. **B)** MFI of CD55. **C)** MFI of CD66b. Statistical differences of MFI expression between groups (CST I, n=47; CST III, n=33; and CST IV, n=22) were evaluated using a Kruskal-Wallis test with Dunn's multiple comparison post hoc test. *p<0.05. Data is presented as median values with interquartile range.

The MFI of cervical neutrophils was also assessed based on the vaginal CST classifications. There were no differences in the MFI of CD10, CD33, CD11b and CD62L based on CST subclassifications (Figure 4.25.).

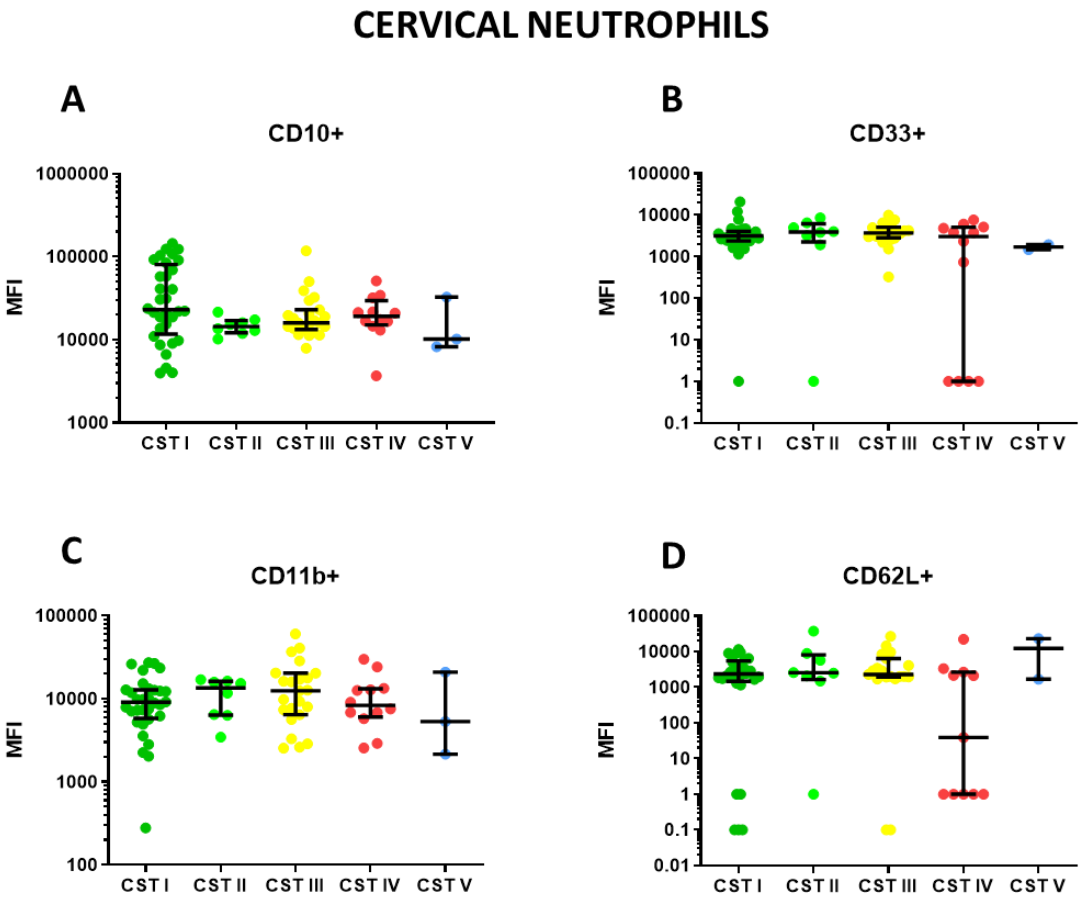


Figure 4.25. Median fluorescence intensity of markers implicated in neutrophil maturation, immune regulation and migration in cervical neutrophils based on CSTs classifications. A) MFI of CD10. **B)** MFI of CD33. **C)** MFI of CD11b. **D)** MFI of CD62L. Statistical differences of MFI expression between groups (CST I, n=33; CST III, n=22; and CST IV, n=9) were evaluated using a Kruskal-Wallis test with Dunn's multiple comparison post hoc test. Data is presented as median values with interquartile range.

In terms of markers of activation, CD16 (FcγRIII) MFI expression significantly decreased in women with a CST IV composition compared to women with a CST I (p=0.0441) and CST III (p=0.0101) (Figure 4.26.A). Additionally, there was a higher CD63 MFI in women with a CST IV microbial composition compared to women with a CST I (p=0.0012) and women with a CST III (p=0.0044) (Figure 4.26.B). Furthermore, CD66b MFI was significantly increased by 5-times in women with a CST IV composition compared to women with a CST I (p=0.0049) (Figure 4.26.C).

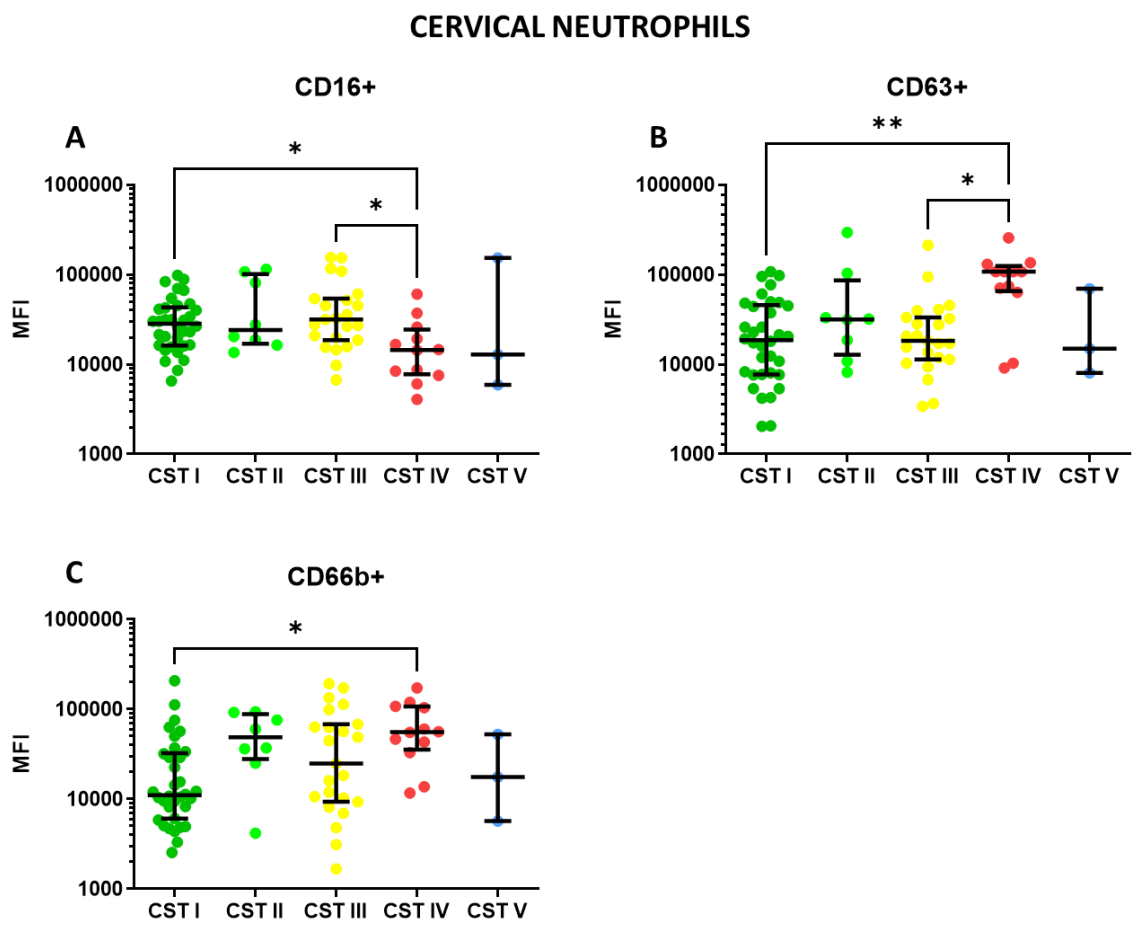


Figure 4.26. Median fluorescence intensity of markers implicated in neutrophil activation in cervical neutrophils based on CSTs classifications. A) MFI of CD16. **B)** MFI of CD63. **C)** MFI of CD66b. Statistical differences of MFI expression between groups (CST I, n=33; CST III, n=22; and CST IV, n=9) were evaluated using a Kruskal-Wallis test with Dunn's multiple comparison post hoc test. *p<0.05; **p<0.01. Data is presented as median values with interquartile range.

Finally, markers implicated in complement system activation were assessed in cervical neutrophils based on CST classifications. No differences were observed in the median MFI across different vaginal microbial compositions for CD35 or CD55 (Figure 4.27.A, B). The expression of marker CD88 was significantly different depending on the CSTs classification (p=0.0376), being the median of CD88 higher in cervical neutrophil from women with a CST I compared to CST IV (p=0.0597) (Figure 4.27. C).

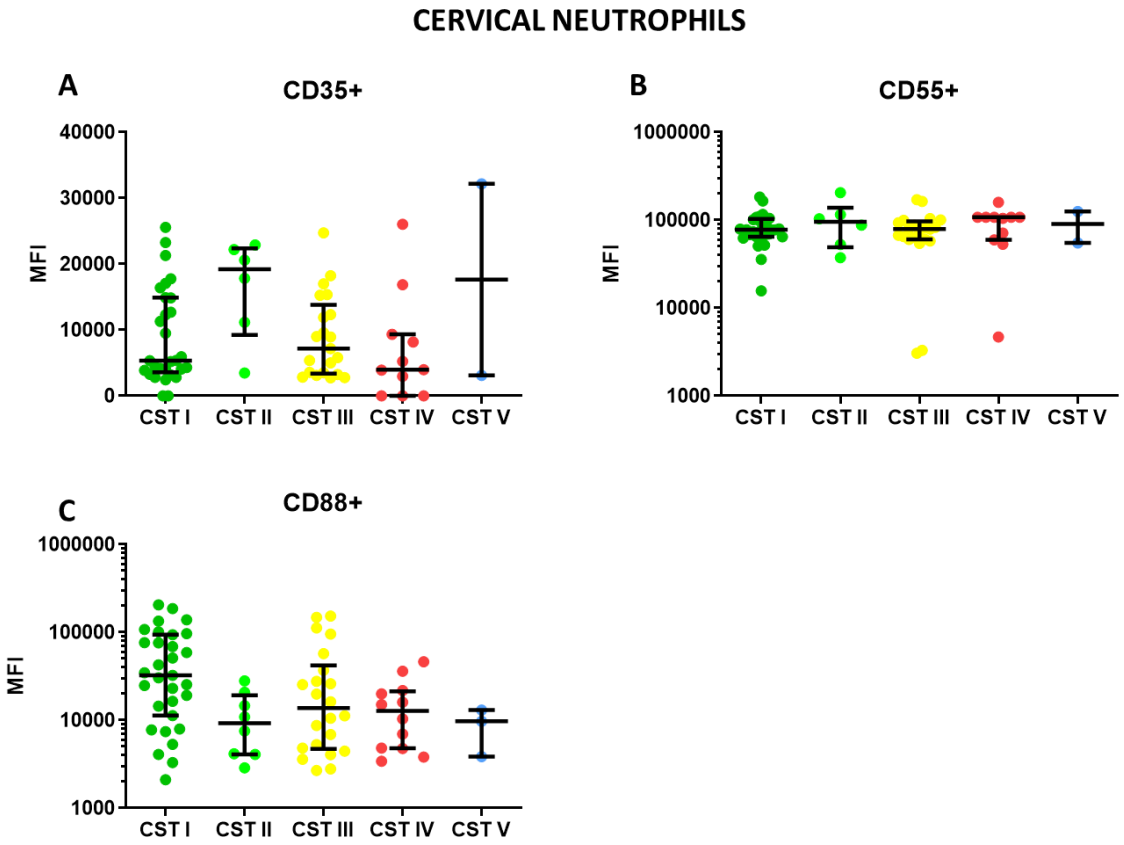


Figure 4.27. Median fluorescence intensity of markers implicated in complement activation in cervical neutrophils based on CSTs classifications. A) MFI of CD35. **B)** MFI of CD55. **C)** MFI of CD66b. Statistical differences of MFI expression between groups (CST I, n=33; CST III, n=22; and CST IV, n=9) were evaluated using a Kruskal-Wallis test with Dunn's multiple comparison post hoc test. Data is presented as median values with interquartile range.

4.6.4. Detection and immunophenotype of neutrophils in women who undergo cervical shortening

Detection of cervical neutrophils in women who undergo cervical shortening

As previously mentioned, not all samples had cervical neutrophils present. The presence or absence of cervical neutrophils was evaluated in women who experienced cervical shortening and compared with women who maintained their cervical length during pregnancy.

When assessing the total percentage of neutrophil detected (both live and dead), there was a higher proportion of neutrophils in women who develop a short cervix (80%), but this was not statistically significant (p=0.3193) (Figure 4.28.A). However, live neutrophils were more often detected in women who experienced cervical shortening at some point during their pregnancy (71%) compared to women who maintained a normal cervical length throughout pregnancy (54%) (p=0.0409) (Figure 4.28.B). Focusing on the subset of live neutrophils, there was a higher proportion of samples with a large proportion of live neutrophils present in women who experienced cervical shortening (86% samples with more than 50% live neutrophils), compared to women with a normal cervical length (74%); however, this was not statistically significant (p=0.1486) (Figure 4.28.C).

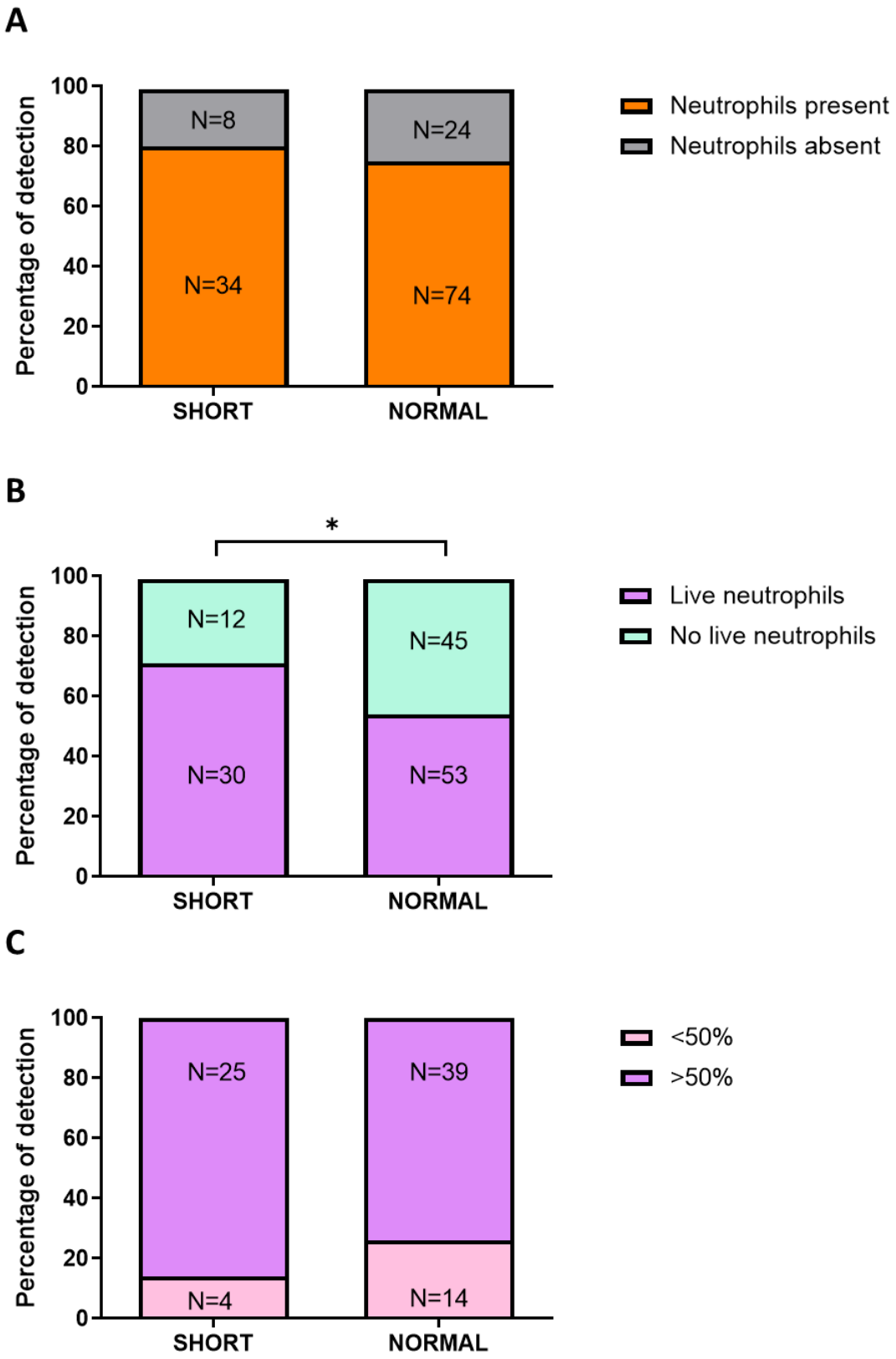


Figure 4.28. Detection of cervical neutrophils in women who undergo cervical shortening. Percentage of detection of **A)** neutrophils and **B)** live neutrophils in samples from women who experienced cervical shortening (≤25mm) (n=42) at some stage during their pregnancy, compared to women who had a normal cervical length (>25mm) throughout pregnancy (n=98). Statistical differences between the proportions of both groups were assessed with Fisher's exact test. *p<0.05.

Immunophenotype of peripheral neutrophil in women who undergo cervical shortening

Since there was a higher percentage of live neutrophils present in women who experience cervical shortening, it was hypothesised that the immunophenotype of neutrophils would be altered in women who experienced cervical shortening. The next section presents the MFI expression in neutrophils from women who had cervical shortening compared to women who maintained a normal cervical length during their pregnancy.

The MFI of peripheral neutrophils markers CD10, CD33 and CD11b did not change between women with short or normal cervical length. However, the CD62L MFI was significantly decreased in women who experienced cervical shortening at some point during their pregnancy (≤25mm) compared to women who maintained a normal cervical length throughout pregnancy (>25mm) (p=0.0090) (Figure 4.29).

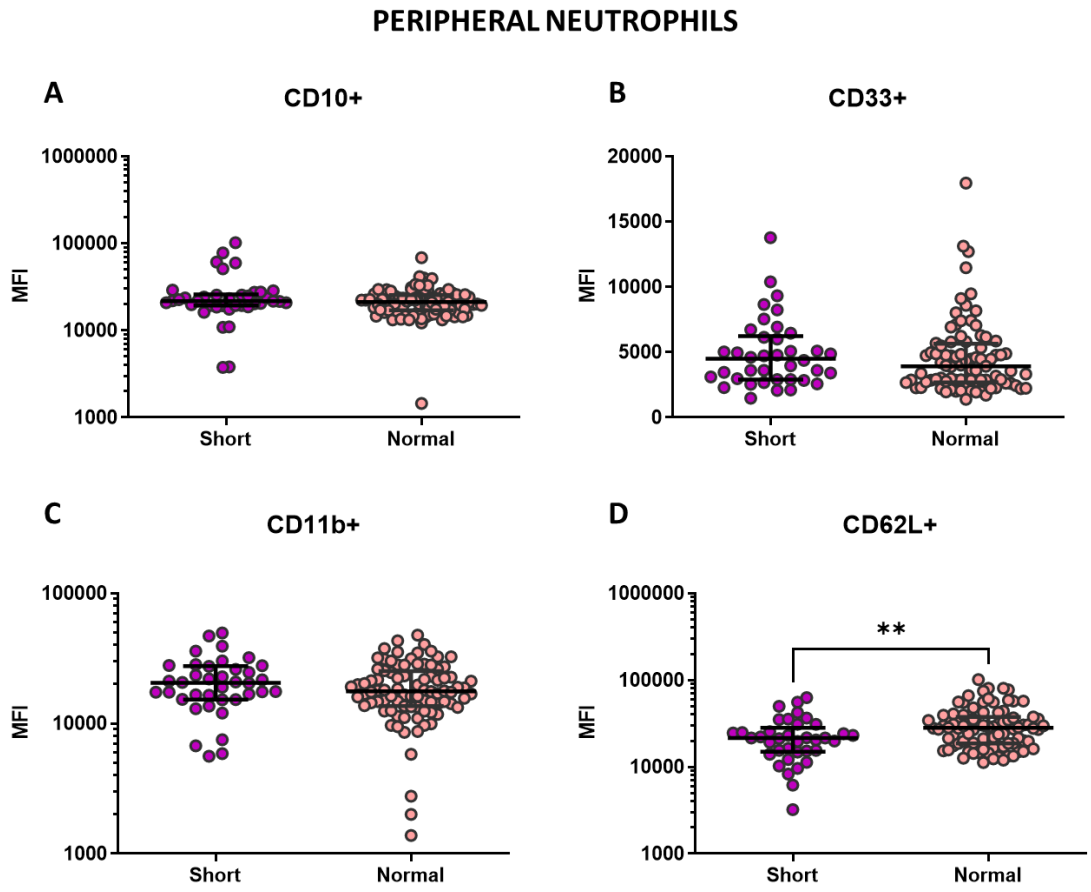


Figure 4.29. Median fluorescence intensity of markers implicated in neutrophil maturation, immune regulation and migration in peripheral neutrophils in women who undergo cervical shortening. A) MFI of CD10. **B)** MFI of CD33. **C)** MFI of CD11b. **D)** MFI of CD62L. Statistical differences of MFI expression of different markers between samples from women who undergo cervical shortening (≤25mm) (n=38) and from women who maintain a normal cervical length (>25mm) (n=89) were evaluated using a Mann Whitney two-tailed test. **p<0.01. Data is presented as median values with interquartile range.

Compared to women with a normal cervical length, in women who underwent cervical shortening, there were no differences in the MFI of CD16 (p=0.7205), CD63 (p=0.0779) or CD66b (p=0.2567) in peripheral neutrophils (Figure 4.30).

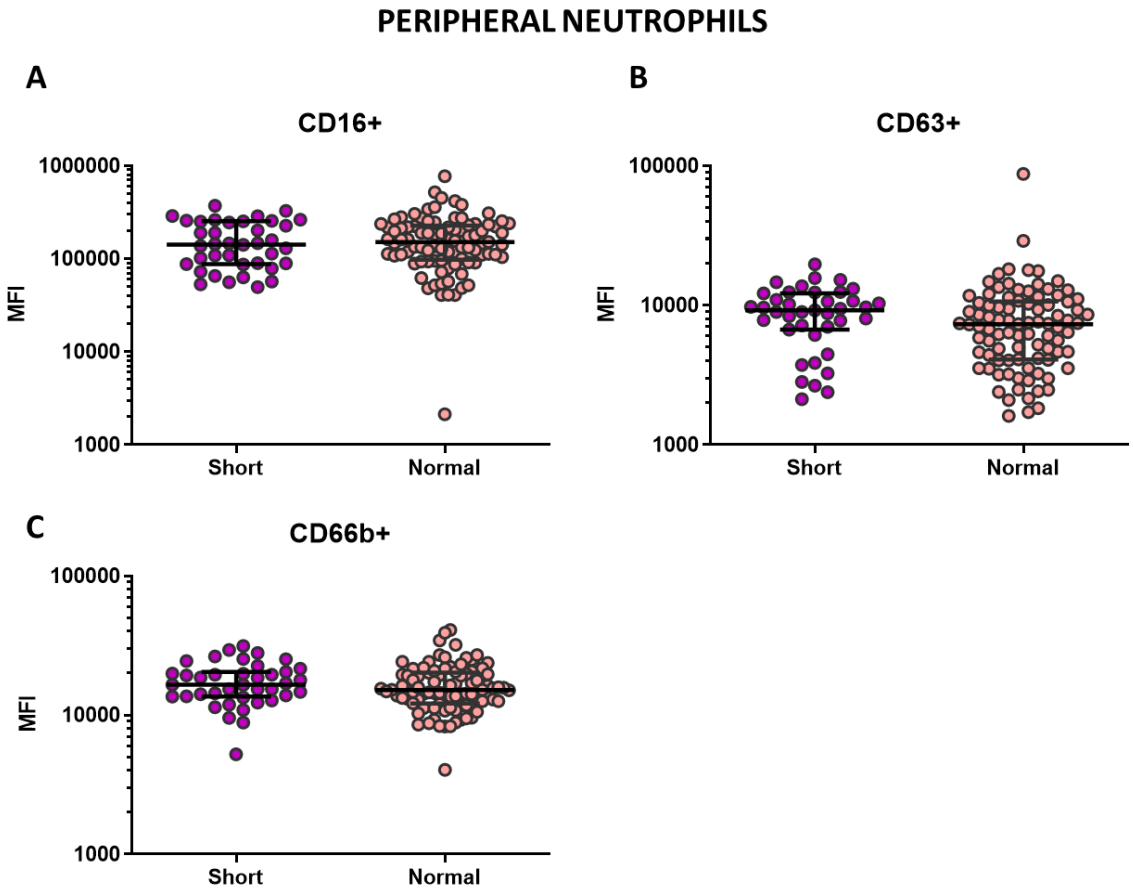


Figure 4.30. Median fluorescence intensity of markers implicated in neutrophil activation in peripheral neutrophils in women who undergo cervical shortening. A) MFI of CD16. **B)** MFI of CD63. **C)** MFI of CD66b. Statistical differences of MFI expression of different markers between samples from women who undergo cervical shortening (≤25mm) (n=38) and from women who maintain a normal cervical length (>25mm) (n=89) were evaluated using a Mann Whitney two-tailed test. Data is presented as median values with interquartile range.

Finally, peripheral neutrophils did not exhibit a different immunophenotype in markers implicated in complement activation between short and normal cervical length groups, including CD35 (p=0.5204) and CD55 (p=0.3637) (Figure 4.31.A, B). There was a trend of higher MFI in CD88 marker within women who experienced cervical shortening, however this was not statistically significant(p=0.0516) (Figure 4.31.C).

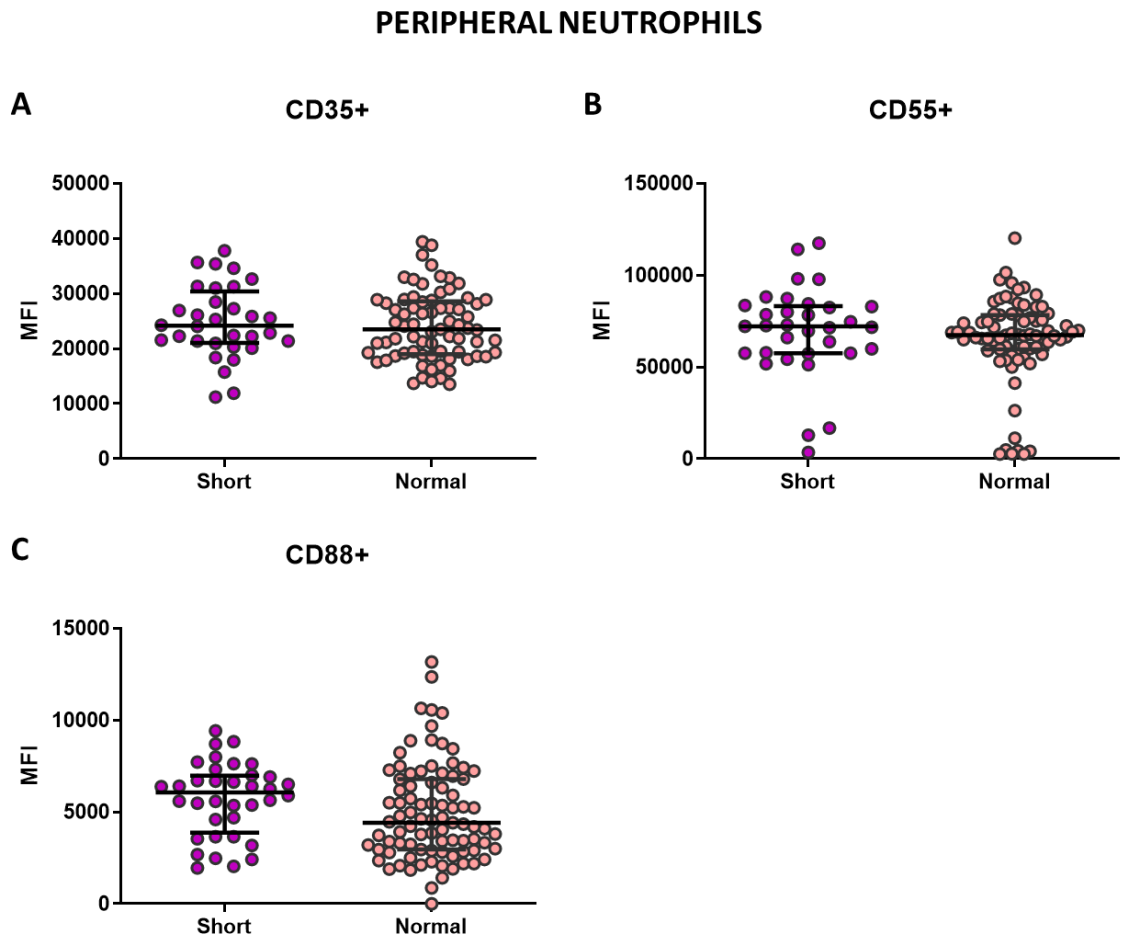


Figure 4.31. Median fluorescence intensity of markers implicated in complement activation in peripheral neutrophils in women who undergo cervical shortening. A) MFI of CD35. **B)** MFI of CD55. **C)** MFI of CD88. Statistical differences of MFI expression of different markers between samples from women who undergo cervical shortening (≤25mm) (n=36) and from women who maintain a normal cervical length (>25mm) (n=87) were evaluated using a Mann Whitney two-tailed test. Data is presented as median values with interquartile range.

Immunophenotype of cervical neutrophils in women who undergo cervical shortening

The immunophenotype of cervical neutrophils were also assessed in women who developed cervical shortening. From the markers implicated in maturation, immune regulation and migration, only CD10 had a higher expression in cervical neutrophils from women who developed a short cervix at some point during their pregnancy (p=0.0088) (Figure 4.32.).

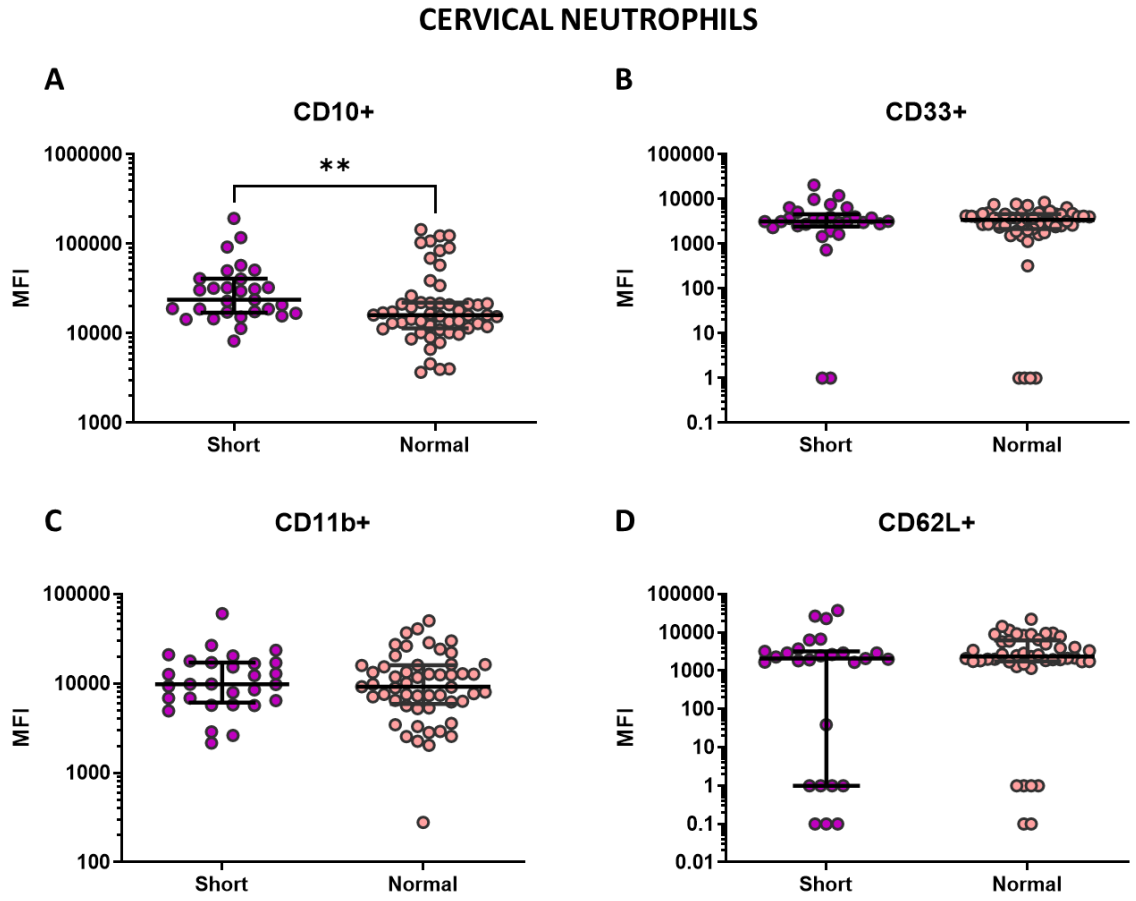


Figure 4.32. Median fluorescence intensity of markers implicated in neutrophil maturation, immune regulation and migration in cervical neutrophils in women who undergo cervical shortening. A) MFI of CD10. **B)** MFI of CD33. **C)** MFI of CD11b. **D)** MFI of CD62L. Statistical differences of MFI expression of different markers between samples from women who undergo cervical shortening (≤25mm) (n=30) and from women who maintain a normal cervical length (>25mm) (n=53) were evaluated using a Mann Whitney two-tailed test. **p<0.01. Data is presented as median values with interquartile range.

No differences were observed between groups based on the MFI expression of CD16, CD63 and CD66b of cervical neutrophils (Figure 4.33.).

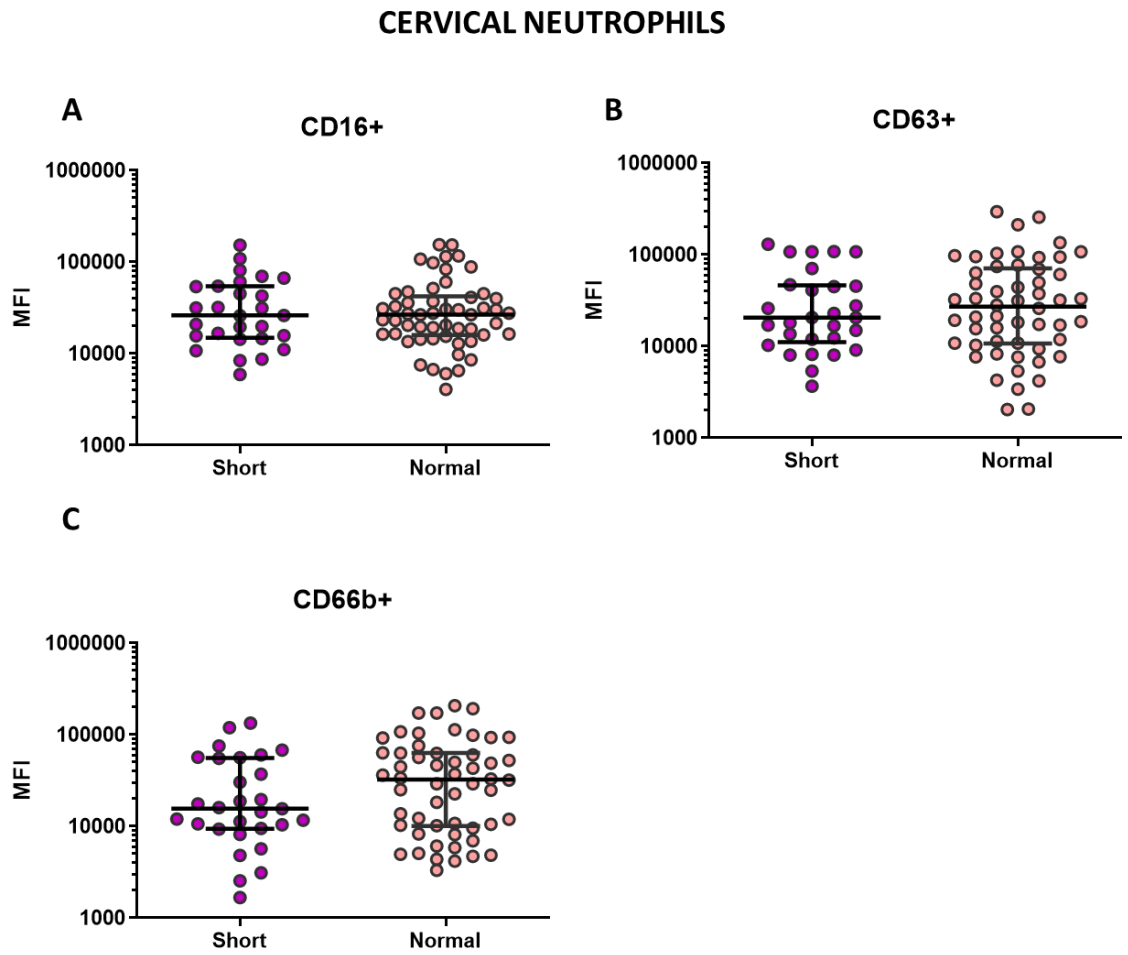


Figure 4.33. Median fluorescence intensity of markers implicated in neutrophil activation in cervical neutrophils in women who undergo cervical shortening. A) MFI of CD16. **B)** MFI of CD63. **C)** MFI of CD66b. Statistical differences of MFI expression of different markers between samples from women who undergo cervical shortening (≤25mm) (n=30) and from women who maintain a normal cervical length (>25mm) (n=53) were evaluated using a Mann Whitney two-tailed test. Data is presented as median values with interquartile range.

Finally, markers implicated in the complement system were assessed in cervical neutrophils. In women who experienced cervical shortening there was a significantly decreased MFI of CD35 compared to women who did not shorten (p=0.0017) (Figure 4.34. A). No differences were observed for CD55 (p=0.3619) (Figure 4.34.B) or CD88 (p=0.0671) (Figure 4.34.C).

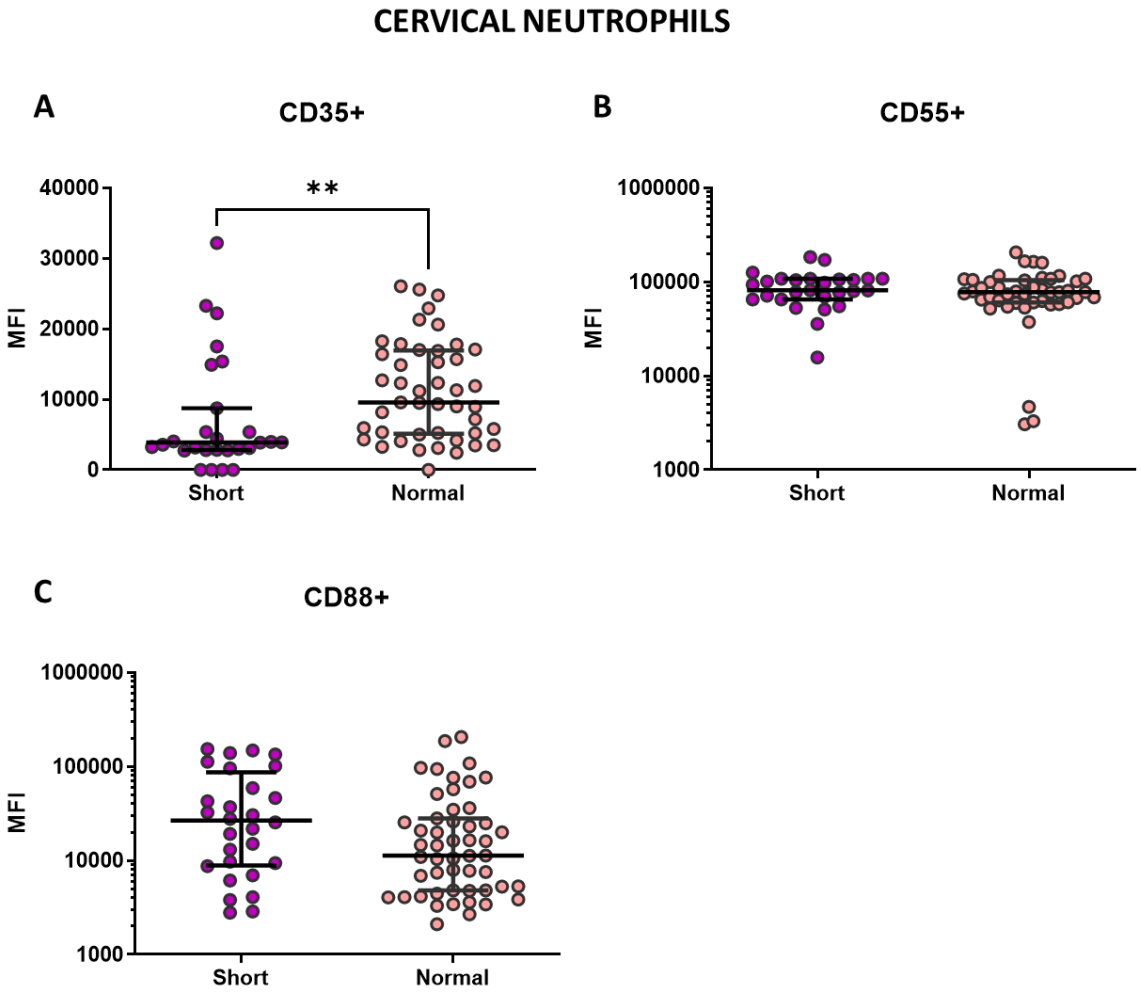


Figure 4.34. Median fluorescence intensity of markers implicated in complement activation in cervical neutrophils in women who undergo cervical shortening. A) MFI of CD35. **B)** MFI of CD55. **C)** MFI of CD88. Statistical differences of MFI expression of different markers between samples from women who undergo cervical shortening (≤25mm) (n=29) and from women who maintained a normal cervical length (>25mm) (n=53) were evaluated using a Mann Whitney two-tailed test. **p<0.01. Data is presented as median values with interquartile range.

4.6.5. Detection and immunophenotype of neutrophils in women who deliver preterm

Detection of cervical neutrophils in women who deliver preterm

The final outcome to address was the detection of cervical neutrophils depending on pregnancy: women who delivered preterm (PT), term uncomplicated pregnancy (TU) and term with intervention (TI). The percentage of cervical neutrophils present did not vary between groups (Figure 4.35.A). However, women who delivered preterm tended to have more live neutrophils present (75%, compared to 57% in TU and 56% in TI) (Figure 4.35.B). However, this did not reach statistical significance (p=0.4567). There was a high proportion of live neutrophils present (>50% live cervical neutrophil) regardless of pregnancy outcome (Figure 4.35.C).

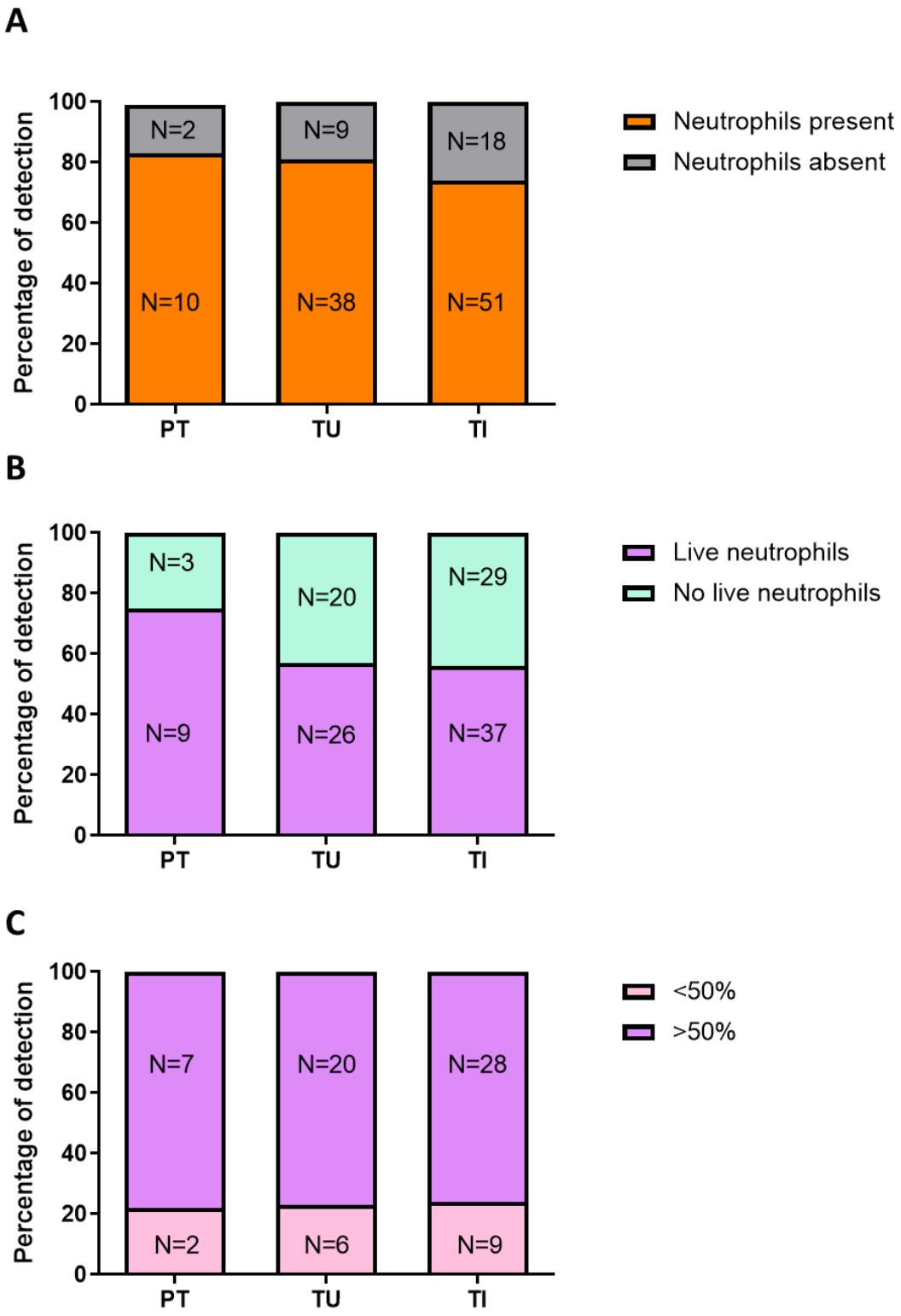


Figure 4.35. Detection of cervical neutrophils in women who ultimately deliver preterm. Percentage of detection of **A)** neutrophils and **B)** live neutrophils in women who deliver preterm compared to women with term uncomplicated and term intervention pregnancies. **C)** Proportion of live neutrophils in low amount (<50% live neutrophils) and high amount (>50%). Statistical differences between the proportions of groups were assessed with Chi-square test.

Immunophenotype of peripheral neutrophil in women who deliver preterm

The median fluorescence intensity of activation markers for both peripheral and cervical neutrophils were compared between women depending on pregnancy outcome.

In peripheral neutrophils, expression of markers implicated in neutrophil maturation (CD10), immune regulation (CD33) and migration (CD11b, CD62L) were not altered with respect to pregnancy outcome (Figure 4.36.).

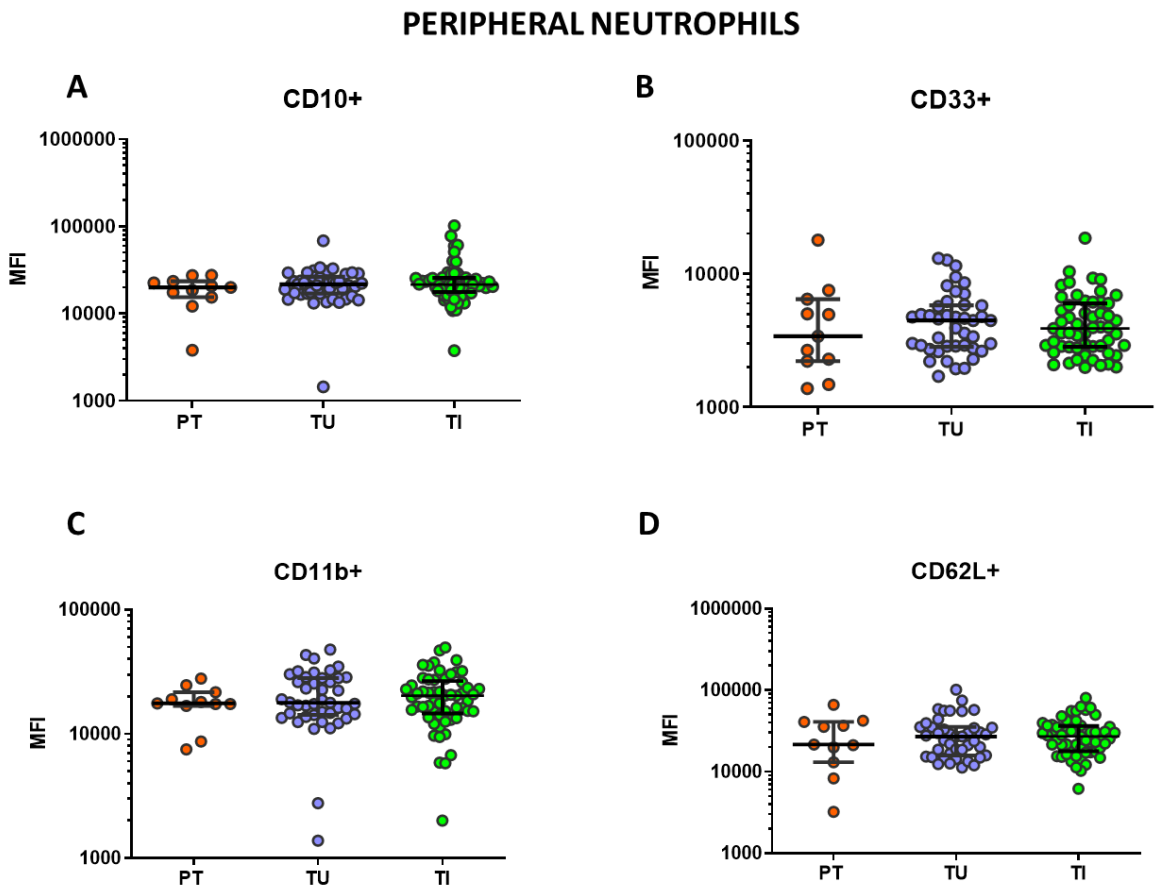


Figure 4.36. Median fluorescence intensity of markers implicated in neutrophil maturation, immune regulation and migration in peripheral neutrophils based on pregnancy outcome. A) MFI of CD10. **B)** MFI of CD33. **C)** MFI of CD11b. **D)** MFI of CD62L. Statistical differences of MFI expression of different markers were assessed between samples from women who delivered preterm (PT, n=61), term uncomplicated (TU, n=43) and term with intervention (TI, n=65) using a Kruskal Wallis test with Dunn's multiple comparison post hoc test. Data is presented as median values with interquartile range.

When assessing markers implicated in microbial recognition (CD16) and neutrophil activation (CD63, CD66b) in peripheral neutrophils, median MFI of CD16 was lower in women who delivered preterm (Figure 4.37.A). In fact, significantly decreased CD16 MFI was observed between women who delivered preterm compared with women who delivered both term uncomplicated (p=0.0421) and term with intervention (p=0.0050). No differences were observed in the MFI of CD63 and CD66b by pregnancy outcome (Figure 4.37. B, C).

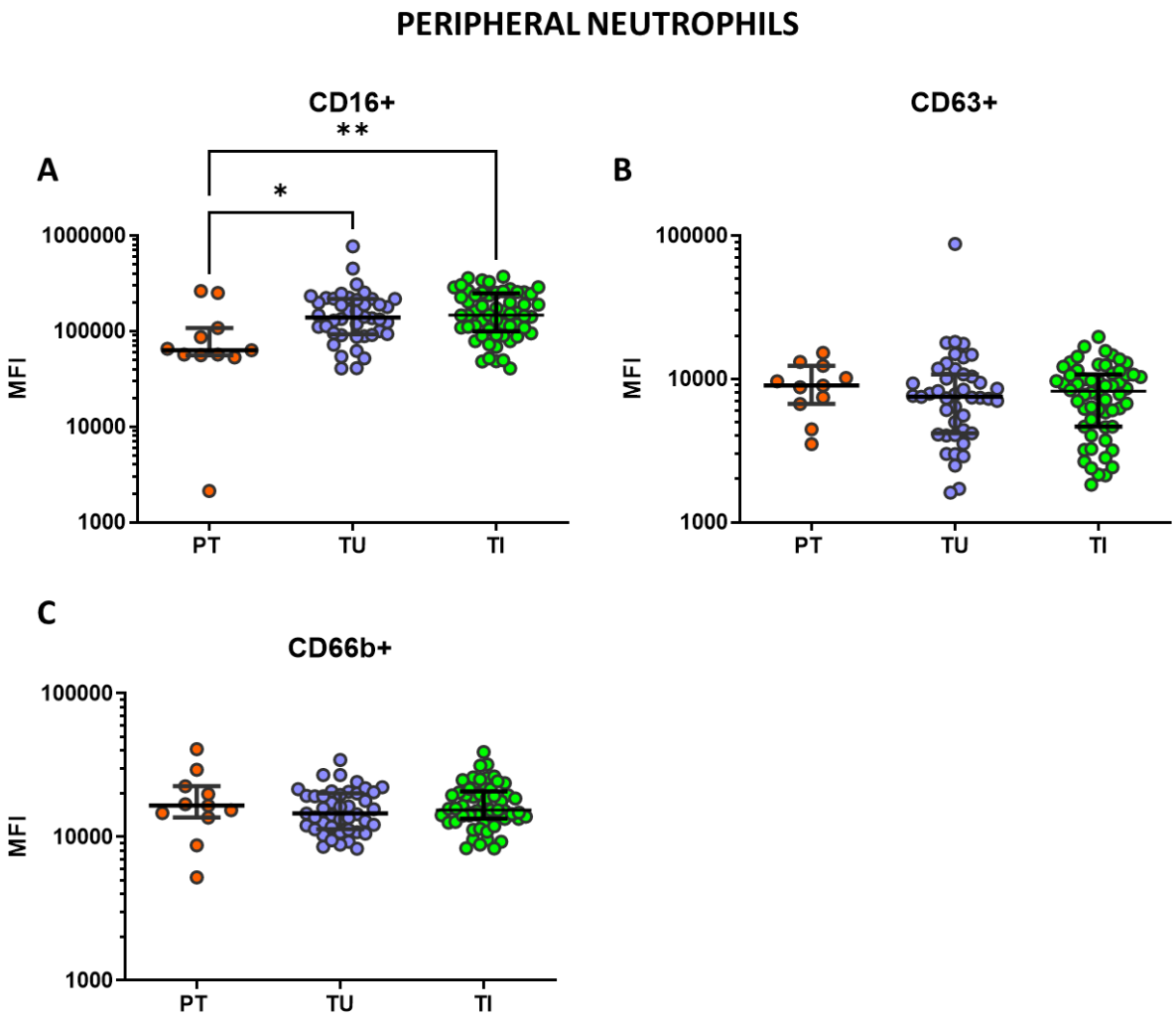


Figure 4.37. Median fluorescence intensity of markers implicated in neutrophil activation in peripheral neutrophils based on pregnancy outcome. A) MFI of CD16. **B)** MFI of CD63. **C)** MFI of CD66b. Statistical differences of MFI expression of different markers were assessed between samples from women who delivered preterm (PT, n=11), term uncomplicated (TU, n=43) and term with intervention (TI, n=65) using a Kruskal Wallis test with Dunn's multiple comparison post hoc test. *p<0.05; **p<0.01. Data is presented as median values with interquartile range.

Finally, the median fluorescence intensity of markers implicated in complement activation CD35, CD55 and CD88 remained unchanged between pregnancy outcome groups in peripheral neutrophils (Figure 4.38.).

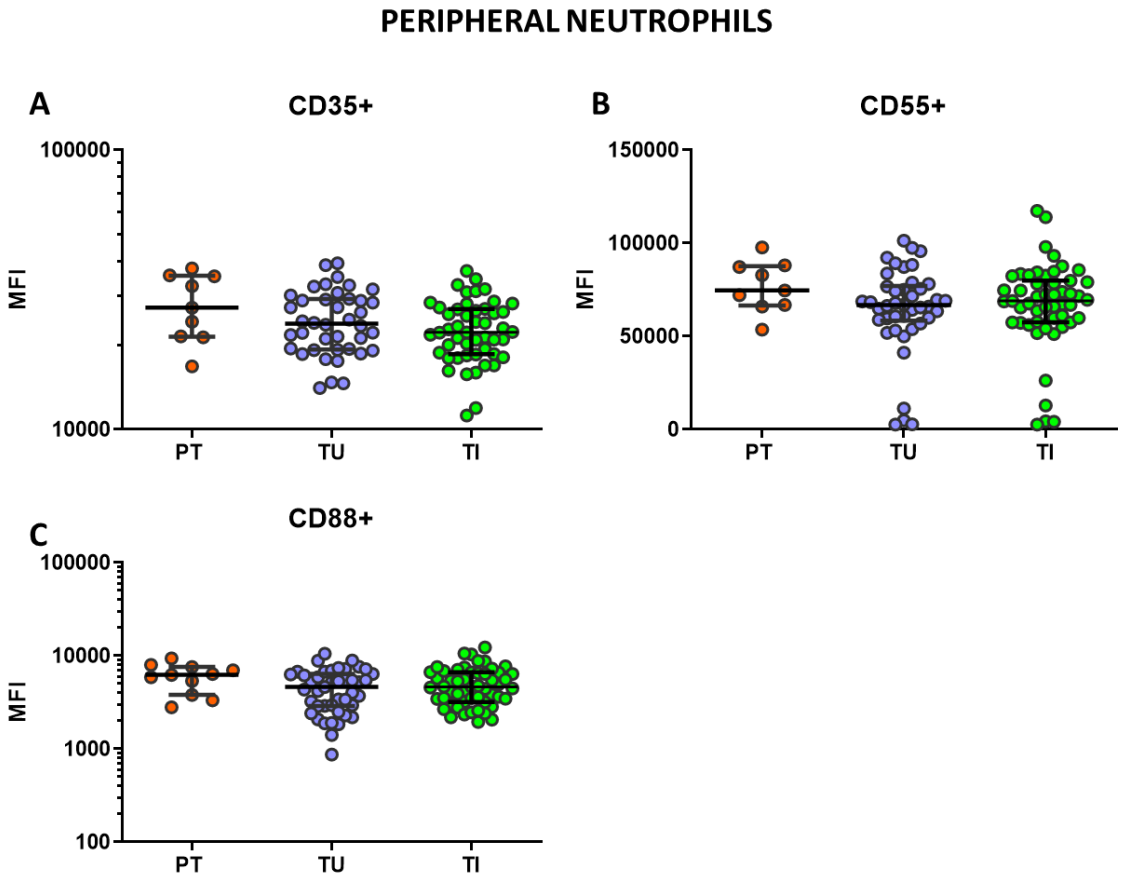


Figure 4.38. Median fluorescence intensity of markers implicated in complement activation in peripheral neutrophils based on pregnancy outcome. A) MFI of CD35. **B)** MFI of CD55. **C)** MFI of CD88. Statistical differences of MFI expression of different markers were assessed between samples from women who delivered preterm (PT, n=11), term uncomplicated (TU, n=43) and term with intervention (TI, n=65) using a Kruskal Wallis test with Dunn's multiple comparison post hoc test. Data is presented as median values with interquartile range.

Immunophenotype of cervical neutrophils in women who deliver preterm

Finally, the median fluorescence expression of the different markers in cervical neutrophils were also assessed by pregnancy outcome. Like peripheral neutrophils, the MFI of markers CD10, CD33, CD11b and CD62L on cervical neutrophils did not differ between women with preterm, term uncomplicated or term with intervention pregnancies (Figure 4.39.).

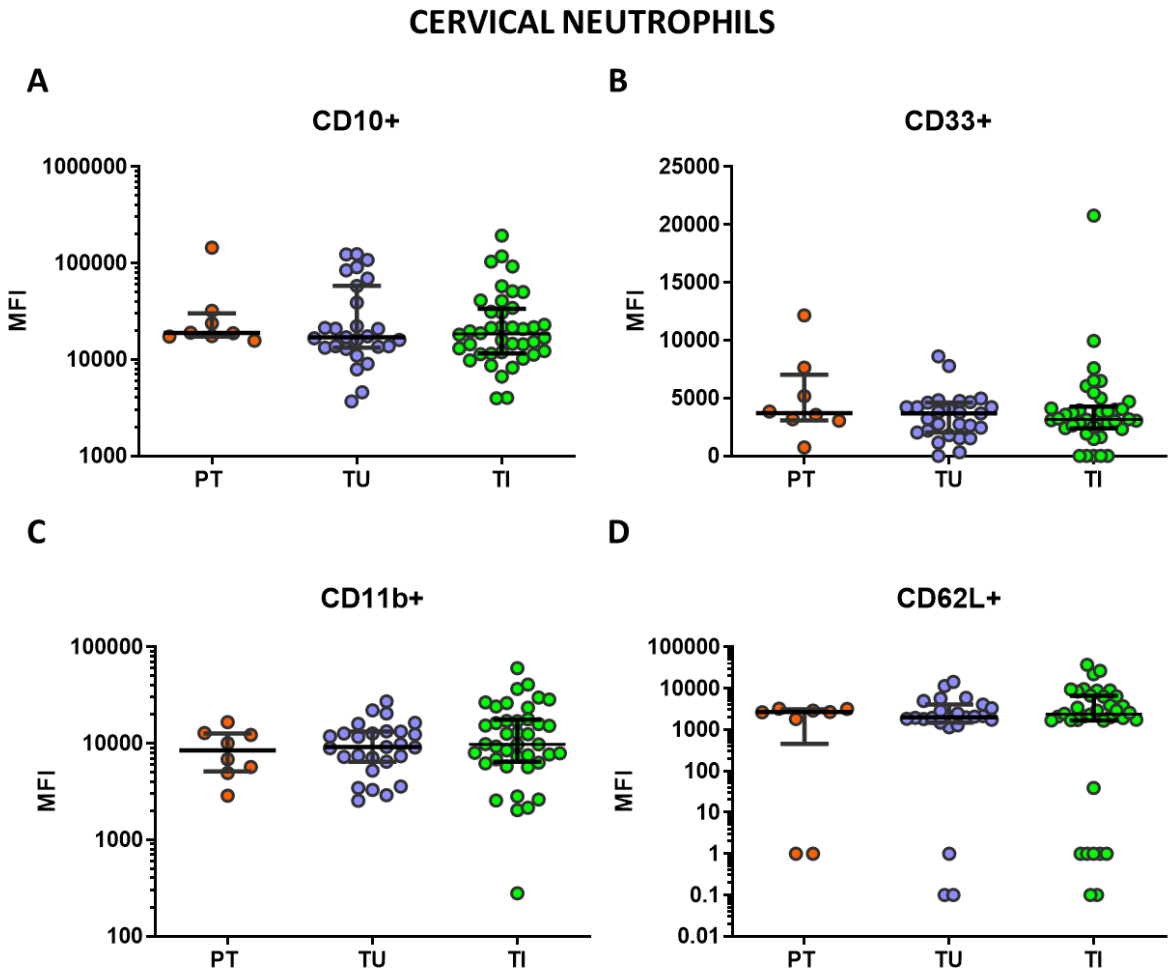


Figure 4.39. Median fluorescence intensity of markers implicated in neutrophil maturation, immune regulation and migration in cervical neutrophils based on pregnancy outcome. A) MFI of CD10. **B)** MFI of CD33. **C)** MFI of CD11b. **D)** MFI of CD62L. Statistical differences of MFI expression of different markers were assessed between samples from women who delivered preterm (PT, n=9), term uncomplicated (TU, n=26) and term with intervention (TI, n=37) using a Kruskal Wallis test with Dunn's multiple comparison post hoc test. Data is presented as median values with interquartile range.

In terms of markers of microbial recognition and activation in cervical neutrophils, no statistical differences were observed in expression of CD16, CD63 or CD66b between women who delivered preterm and women who delivered at term (Figure 4.40.). A trend of a higher cervical MFI expression of CD63 and CD66b appeared in women who delivered preterm compared to women who delivered at term without intervention, but this did not reach statistical significance.

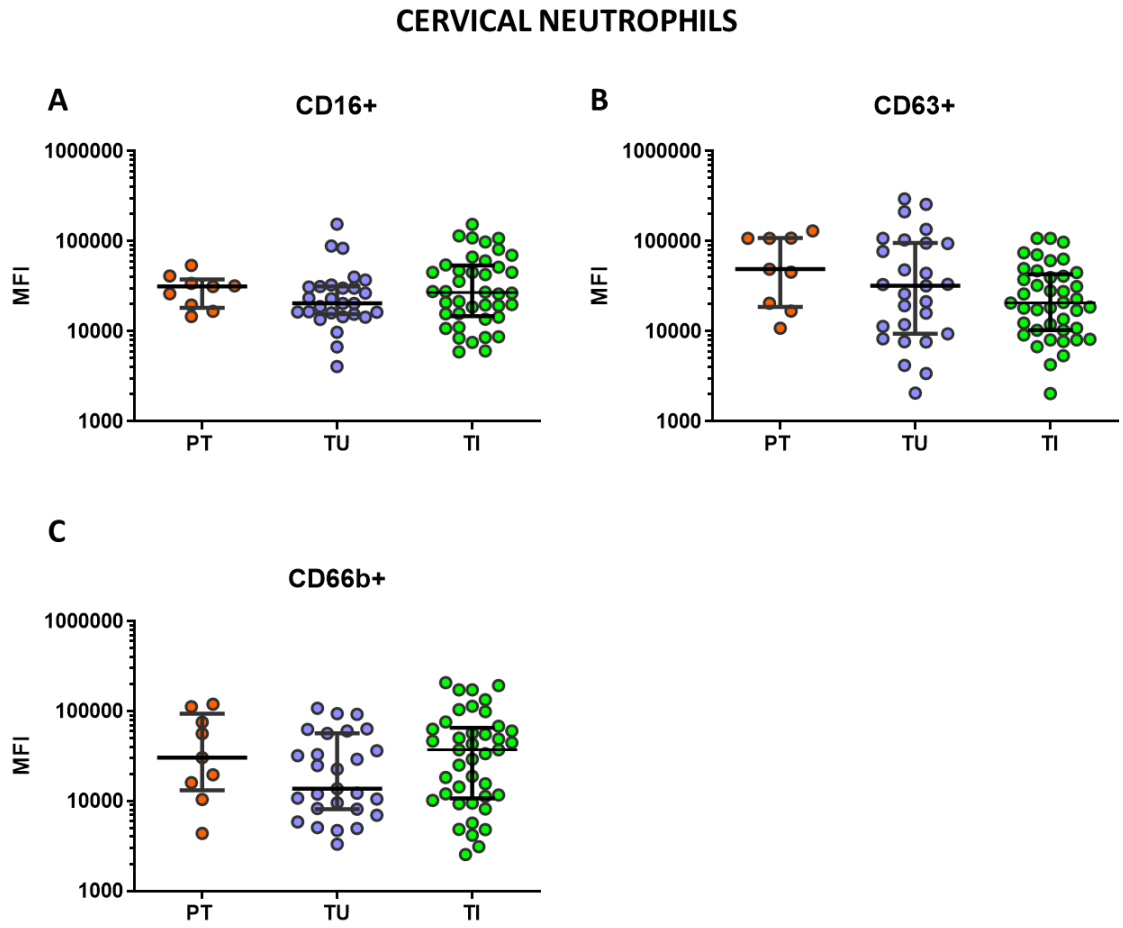


Figure 4.40. Median fluorescence intensity of markers implicated in neutrophil activation in cervical neutrophils based on pregnancy outcome. A) MFI of CD16. **B)** MFI of CD63. **C)** MFI of CD66b. Statistical differences of MFI expression of different markers were assessed between samples from women who delivered preterm (PT, n=9), term uncomplicated (TU, n=26) and term with intervention (TI, n=37) using a Kruskal Wallis test with Dunn's multiple comparison post hoc test. Data is presented as median values with interquartile range.

Finally, no statistical differences were observed either in markers mediating complement activation CD35, CD55 or CD88 in cervical neutrophils (Figure 4.41.).

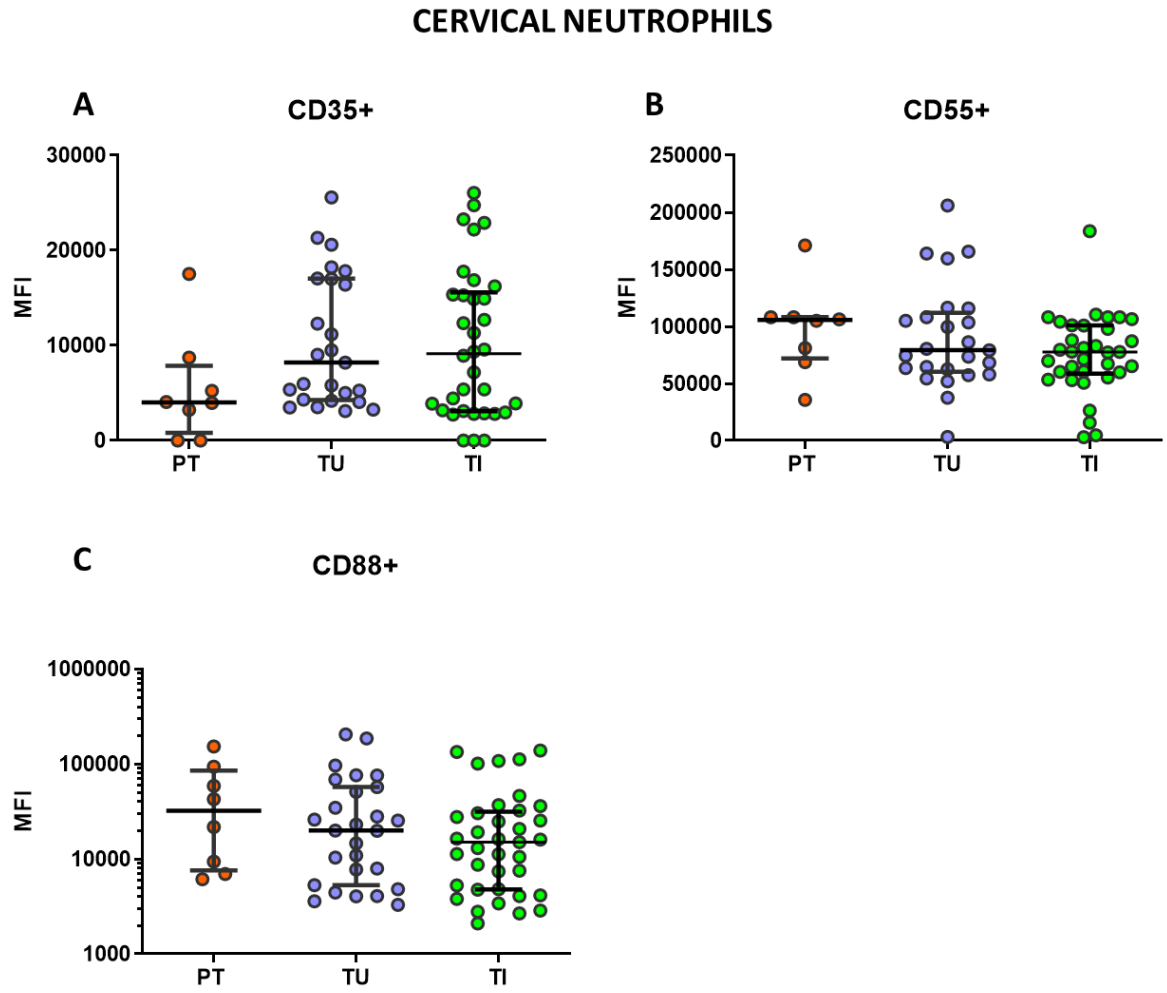


Figure 4.41. Median fluorescence intensity of markers implicated in complement activation in cervical neutrophils based on pregnancy outcome. A) MFI of CD35. **B)** MFI of CD55. **C)** MFI of CD88. Statistical differences of MFI expression of different markers was assessed between samples from women who delivered preterm (PT, n=9), term uncomplicated (TU, n=26) and term with intervention (TI, n=37) using a Kruskal Wallis test with Dunn's multiple comparison post hoc test. Data is presented as median values with interquartile range.

4.6.6. The presence of cervical neutrophils and the local immune response

Finally, following the hypothesis that neutrophils are mediators of the inflammatory response, the detection of cervical neutrophils was correlated with the microbial composition and the local inflammatory response (Table 4.4.). For illustrative purposes, data from IL-1β, IL-8, C3b and MMP-9 is also graphically represented as median with interquartile range (Figure 4.42). As showed in the Table 4.4. and Figure 4.42., the concentration of certain analytes was significantly increased in the presence of cervical neutrophils, together with a high-risk microbial composition. However, even in the absence of cervical neutrophils, the concentration was still elevated for some mediators (e. g., IL-1β).

Table 4.4. Concentration of analytes based on presence and absence of cervical neutrophils and microbial composition.

ANALYTE [Concentration] (units)	NEUTROPHILS ABSENT			NEUTROPHILS PRESENT			High-risk absent vs present	Overall KW
	Low-risk signature (CST I, II, V)	High-risk signature (CST III, IV)	p-value	Low-risk signature (CST I, II, V)	High-risk signature (CST III, IV)	p-value	p-value	p-value
IL-1β (pg/mL)	22.25 (9.982-85.61)	59.66 (24-51-615.8)	0.1966	36.28 (17.88-104.3)	100.3 (24.13-4441.1)	0.0124	>0.9999	**0.0011**
IL-6 (pg/mL)	0.8659 (0.8500-1.992)	0.8500 (0.5368-2.954)	>0.9999	0.8500 (0.8500-2.060)	0.8500 (0.7763-3.898)	>0.9999	>0.9999	0.7435
IL-8 (pg/mL)	774.3 (224.4-2163)	683.6 (85.16-2358)	>0.9999	1216 (574.6-2310)	1668 (675.5-7048)	0.6887	0.2018	**0.0388**
C1q (ng/mL)	0.2751 (0.0813-0.7579)	1.213 (0.0471-3.632)	>0.9999	0.2090 (0.0948-0.8241)	0.8282 (0.1762-2.789)	0.0232	>0.9999	**0.0206**
C2 (ng/mL)	10.11 (2.262-18.78)	6.915 (0.3664-14.46)	>0.9999	11.71 (5.529-36.16)	13.10 (5.316-28.26)	>0.9999	0.5091	0.1682
C4 (ng/mL)	6.379 (1.605-40.90)	46.65 (4.072-182.3)	0.6824	16.67 (4.055-56.15)	53.39 (21.50-162.3)	0.0056	>0.9999	**0.0008**
C4b (ng/mL)	0.3473 (0.1999-1.193)	0.7093 (0.1650-5.383)	>0.9999	0.6358 (0.3122-1.022)	0.9534 (0.4896-3.257)	0.1637	>0.9999	0.0760
MBL (ng/mL)	0.2713 (0.0628-0.5426)	0.2138 (0.028-0.7388)	>0.9999	0.3478 (0.117-0.8289)	0.3061 (0.137-0.7787)	>0.9999	>0.9999	0.4375
Factor B (ng/mL)	0.02828 (0.011-4.873)	2.666 (0.0166-12.13)	>0.9999	0.01100 (0.0110-0.424)	0.1849 (0.011-3.405)	>0.9999	0.0912	0.0815
Factor D (ng/mL)	0.08605 (0.0143-0.178)	0.2651 (0.0135-0.981)	>0.9999	0.05365 (0.0135-0.297)	0.1618 (0.0839-0.349)	0.0093	>0.9999	**0.0104**
C3 (ng/mL)	0.1100 (0.11-0.8190)	0.7916 (0.11-1.643)	>0.9999	0.1100 (0.11-0.11)	0.1146 (0.11-0.7805)	0.0941	>0.9999	**0.0148**
C3a (pg/mL)	405.9 (232.1-556.8)	564.2 (114.9-858.0)	>0.9999	530.1 (301.8-871.5)	694.9 (377.3-1220)	0.7554	0.6564	**0.0420**

Table 4.4. Cont.

ANALYTE [Concentration] (units)	NEUTROPHILS ABSENT			NEUTROPHILS PRESENT			High-risk absent vs present	Overall KW
	Low-risk signature (CST I, II, V)	**High-risk signature (CST III, IV)**	**p-value**	**Low-risk signature (CST I, II, V)**	**High-risk signature (CST III, IV)**	**p-value**	**p-value**	**p-value**
C3b (ng/mL)	5.156 (3.025-14.11)	35.81 (2.388-320.5)	0.7342	9.246 (3.748-24.33)	46.13 (11.88-74.83)	0.0011	>0.9999	**0.0002**
C5 (ng/mL)	0.8762 (0.400-1.661)	0.8247 (0.400-0.9193)	>0.9999	0.8247 (0.400-1.238)	1.203 (0.400-2.892)	0.1291	0.4800	0.0916
C5a (pg/mL)	18.3 (4.338-33.70)	1.720 (0.4517-21.12)	>0.9999	16.80 (7.781-54.27)	24.60 (5.513-43.06)	>0.9999	0.1463	0.1188
Factor H (ng/mL)	13.07 (6.986-34.55)	19.37 (6.784-78.49)	>0.9999	26.64 (11.63-53.95)	48.86 (19.80-107.8)	0.1780	0.3099	**0.0029**
Factor I (ng/mL)	0.2137 (0.09-1.165)	1.817 (0.09-7.953)	0.5439	0.2643 (0.09-1.168)	1.141 (0.5305-3.804)	0.0067	>0.9999	**0.0027**
MMP-2 (pg/mL)	54.00 (54-1622)	1607 (54-5345)	0.6429	54.00 (54-2956)	1675 (54-5445)	0.0553	>0.9999	**0.0076**
MMP-8 (pg/mL)	3630 (1497-7448)	15250 (3583-66464)	0.3692	8750 (2125-26687)	17981 (5907-77645)	0.0736	>0.9999	0.**0034**
MMP-9 (pg/mL)	14765 (4307-25081)	31700 (11821-97171)	0.6324	25363 (8694-61703)	60472 (16732-138819)	0.0322	>0.9999	**0.0041**

Data presented as median (IQR). Statistical analysis was performed with Kruskal-Wallis test with Dunn's multiple comparisons test. Overall Kruskal-Wallis test p-value and relevant pair comparisons are showed in the table. Statistical significance is indicated in orange shadowing.

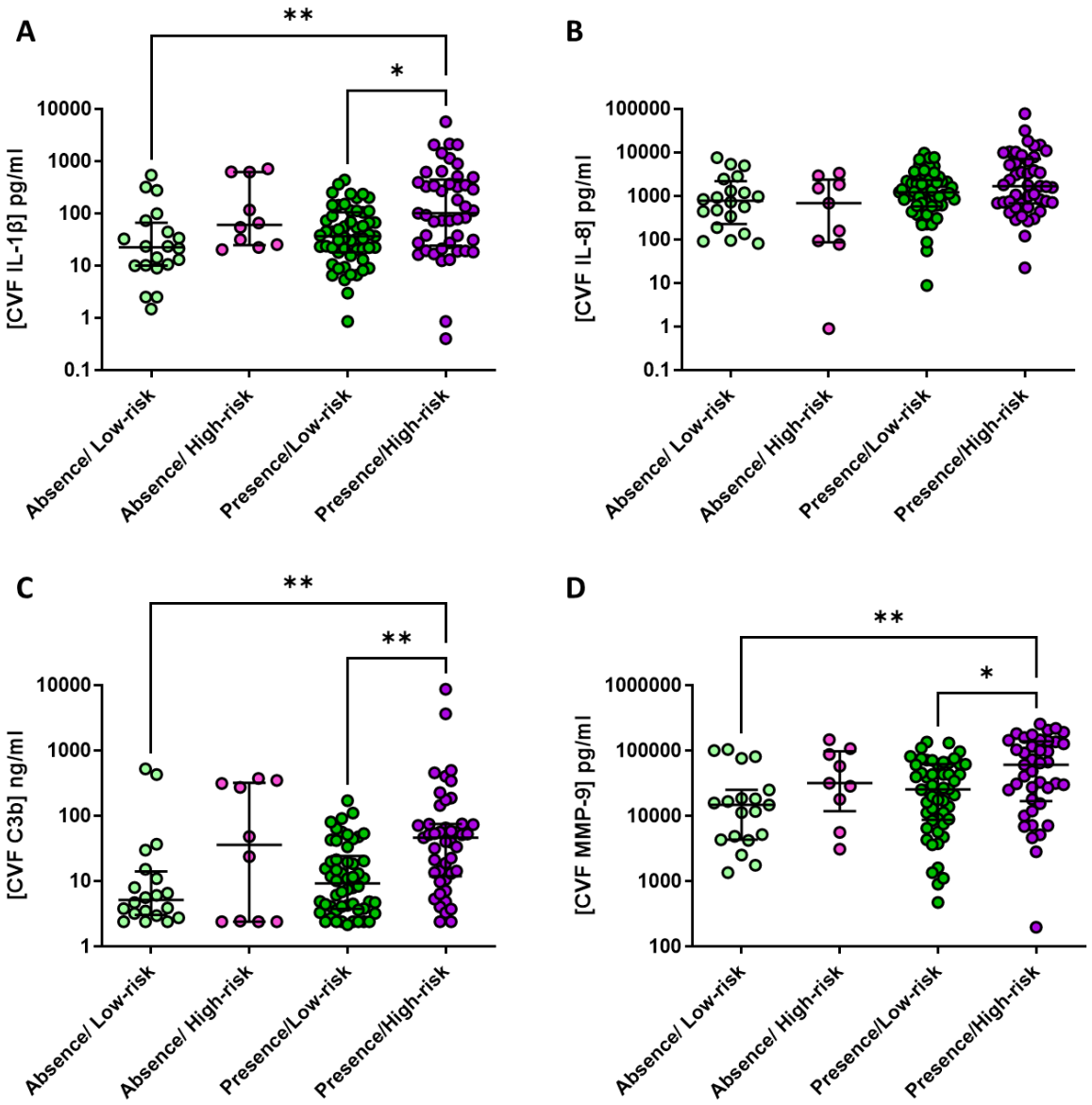


Figure 4.42. Concentration of analytes based on presence and absence of cervical neutrophils and microbial composition. CVF concentration of **A)** IL-1β, **B)** IL-8, **C)** C3b, **D)** MMP-9 based on presence and absence of cervical neutrophils and microbial composition: low-risk (CST I, II, V), high risk (CST III, IV). *p<0.05; **p<0.01. Data is presented as median values with interquartile range.

Since the presence of cervical neutrophils was associated with a stronger inflammatory response, next it was investigated if the proportion of neutrophils present can also modulate the inflammatory response. The percentage of live neutrophils detected was divided into two categories: low percentage of neutrophils (less than 50% neutrophils) and high percentage of neutrophils (>50% neutrophils). The cervicovaginal fluid concentrations of cytokines, complement proteins and matrix metalloproteinases were compared between both groups.

Detection of cervical neutrophils and cervicovaginal cytokines

Firstly, the proportion of neutrophils present were compared with three proinflammatory cytokines: IL-1β, IL-6 and IL-8. When there were more neutrophils present, the concentration of proinflammatory IL-1β was significantly higher in the cervicovaginal fluid (p=0.0497) (Figure 4.43.A). No differences were observed in the concentration of IL-6 based on the proportion of neutrophils (Figure 4.43.B). However, women with a higher proportion of neutrophils present had a significantly higher concentration of IL-8 in CVF compared to women with less neutrophils (p=0.0386) (Figure 4.43.C).

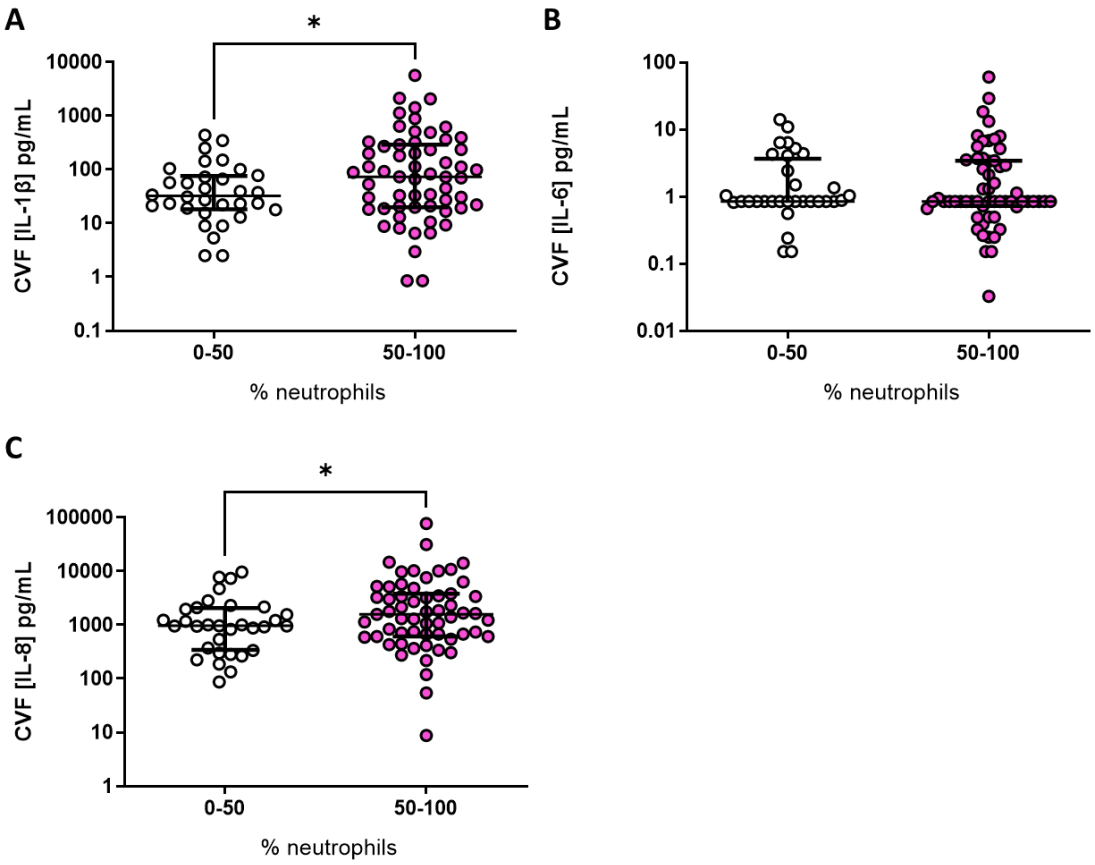


Figure 4.43. Cervical neutrophils presence in association with cervicovaginal cytokines. The concentration of **A)** IL-1β, **B)** IL-6 and **C)** IL-8 in CVF was compared between women who had greater number of neutrophils (>50% neutrophils, n=59) and lower number of neutrophils (<50% neutrophils, n=32) using a Mann-Whitney one-tailed test. *p<0.05. Data is presented as median values with interquartile range.

Detection of cervical neutrophils and cervicovaginal complement proteins

Next, the percentage of neutrophils and cervicovaginal complement proteins were examined.

The median concentration of C1q (complement protein required for complement activation on the classical pathway via binding to immune complexes) was higher in women who had a greater proportion of neutrophils present, compared to women with less neutrophils (p=0.0005) (Figure 4.44.A). No differences were observed in the concentration of C2 (figure 4.44.B). The concentration of C4 significantly decreased in women with less neutrophils present (p=0.0003) (Figure 4.44.C), but this reduction of C4 was not reflected by an increase in the concentration of C4b since there was no difference in C4b concentration between neutrophil percentages (Figure 4.44.D).

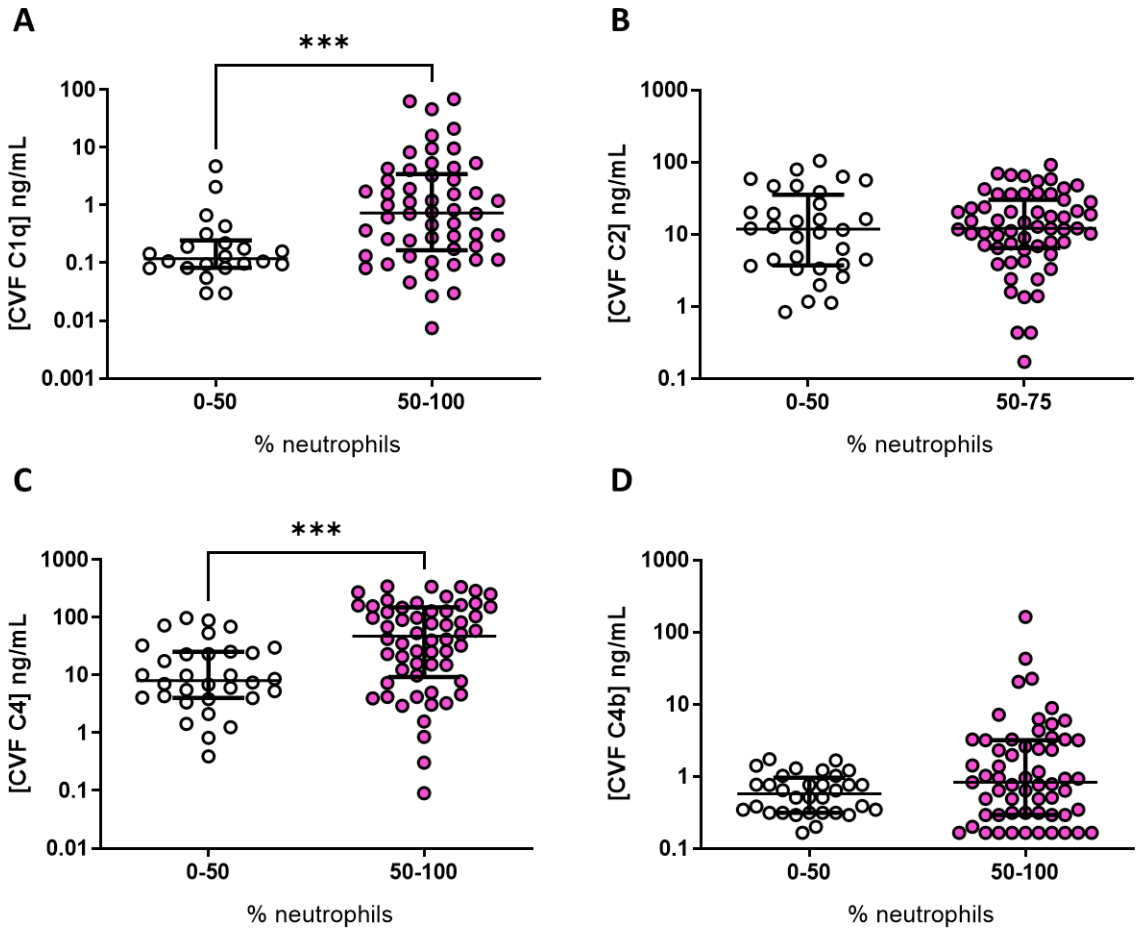


Figure 4.44. Cervical neutrophils presence in association with cervicovaginal complement proteins from the classical pathway. The concentration of **A)** C1q, **B)** C2, **C)** C4 and **D)** C4b In CVF was compared between women who had greater number of neutrophils (>50% neutrophils, n=59) and lower number of neutrophils (<50% neutrophils, n=32) using a Mann-Whitney two-tailed test. ***p<0.001. Data is presented as median values with interquartile range.

In the lectin pathway, the percentage of neutrophils present did not affect the cervicovaginal fluid concentration of MBL (p=0.0676) (Figure 4.45).

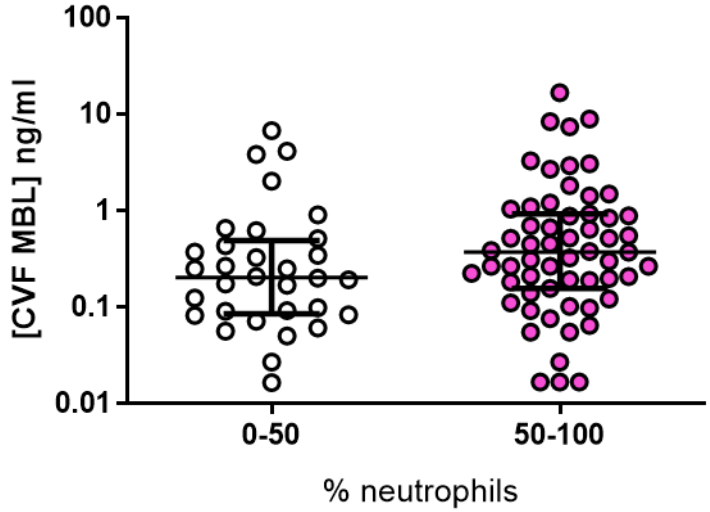


Figure 4.45. Cervical neutrophils presence in association with cervicovaginal complement proteins from the lectin pathway. The concentration of MBL in CVF was compared between women who had greater number of neutrophils (>50% neutrophils, n=59) and lower number of neutrophils (<50% neutrophils, n=32) using a Mann-Whitney two-tailed test. Data is presented as median values with interquartile range.

In the alternative pathway, no differences were observed in the concentration of Factor B within women with different percentages of cervical neutrophils (Figure 4.46.A). However, women who had a greater proportion of neutrophils present had a significant higher median of Factor D in the CVF (p=0.0033) (Figure 4.46.B).

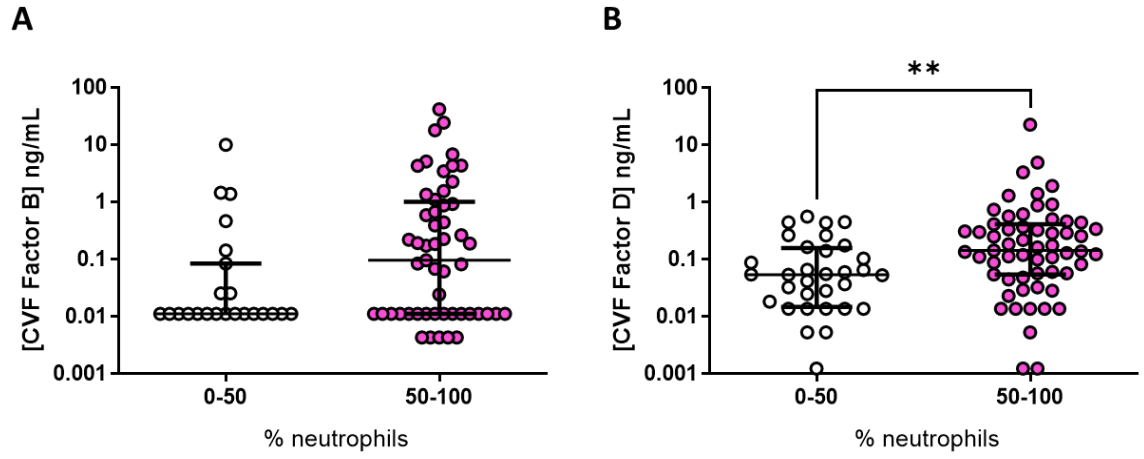


Figure 4.46. Cervical neutrophils presence in association with cervicovaginal complement proteins from the alternative pathway. The concentration of **A)** Factor B and **B)** Factor D in CVF was compared between women who had greater number of neutrophils (>50% neutrophils, n=59) and lower number of neutrophils (<50% neutrophils, n=32) using a Mann-Whitney two-tailed test. **p<0.01. Data is presented as median values with interquartile range.

The target of C3 convertases is the protein C3, which is defragmented into C3a and C3b upon activation. The median concentration of C3 (p=0.0149), C3a (p=0.0072) and C3b (p=0.0013) were higher in association with the higher proportions of neutrophils detected (Figure 4.47.A, B, C). However, no differences were observed in downstream proteins C5 and C5a (Figure 4.47.D, E).

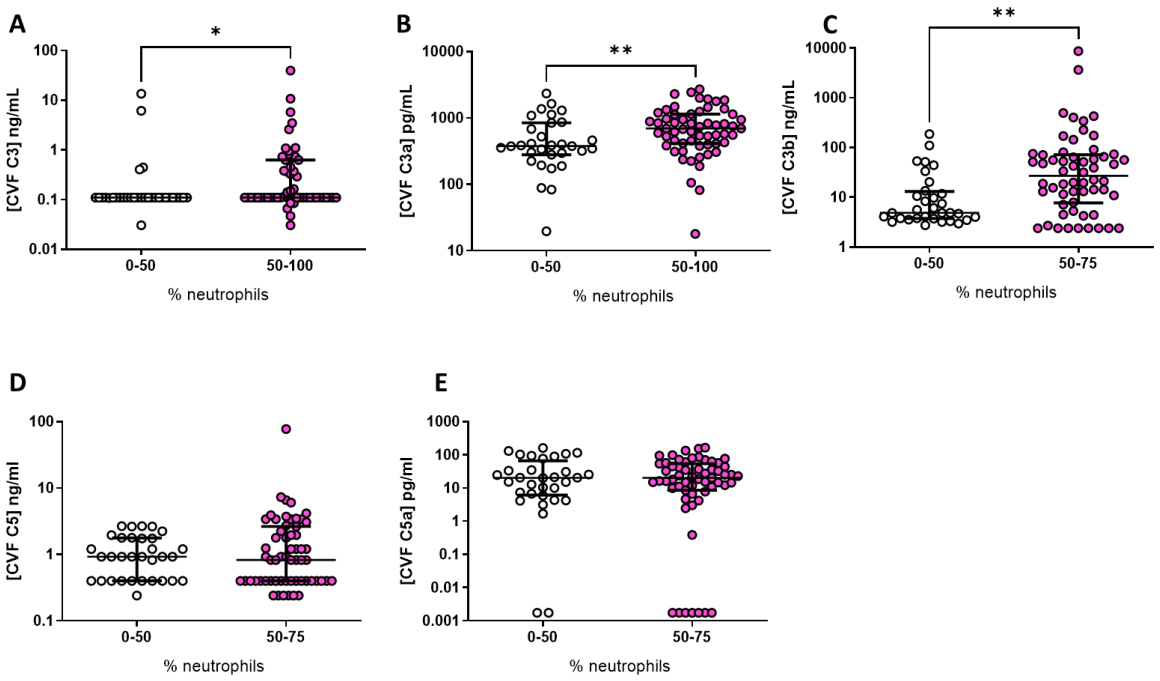


Figure 4.47. Cervical neutrophils presence in association with cervicovaginal complement proteins from the convergence of pathways in C3 and C5 convertases. The concentration of **A)** C3, **B)** C3a, **C)** C3b, **D)** C5 and **E)** C5a in CVF was compared between women who had greater number of neutrophils (>50% neutrophils, n=59) and lower number of neutrophils (<50% neutrophils, n=32) using a Mann-Whitney two-tailed test. *p<0.05; **p<0.01. Data is presented as median values with interquartile range.

Both complement regulators were significantly increased in women who had more neutrophils compared to women with less cervical neutrophils (Factor H, p=0.0241; Factor I, p=0.0009) (Figure 4.48).

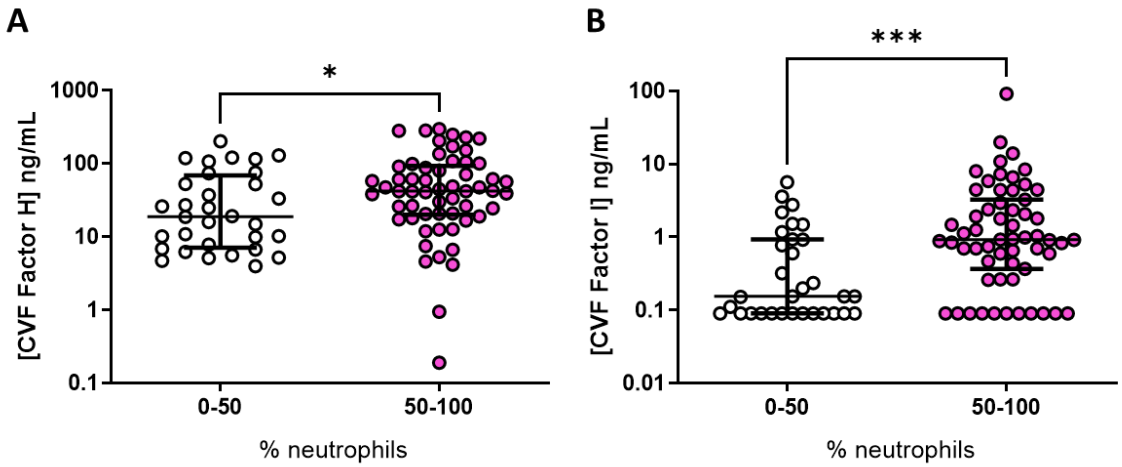


Figure 4.48. Cervical neutrophils presence in association with cervicovaginal complement regulators. The concentration of **A)** Factor H and **B)** Factor I in CVF was compared between women who had greater number of neutrophils (>50% neutrophils, n=59) and lower number of neutrophils (<50% neutrophils, n=32) using a Mann-Whitney two-tailed test. *p<0.05; ***P<0.001. Data is presented as median values with interquartile range.

Detection of cervical neutrophils and cervicovaginal matrix metalloproteinases

Finally, cervical neutrophil detection and the concentration of MMPs in CVF was also examined. The concentration of MMP-2 had a bimodal distribution in both groups of women, but women with a higher presence of neutrophils had an increased MMP-2 concentration in CVF (p=0.0125) (Figure 4.49.A), as well as higher median concentration of MMP-8, compared to women with less neutrophils (p=0.0362) (Figure 4.49.B). No difference was observed in the concentration of MMP-9 (p=0.0944) (Figure 4.49.C).

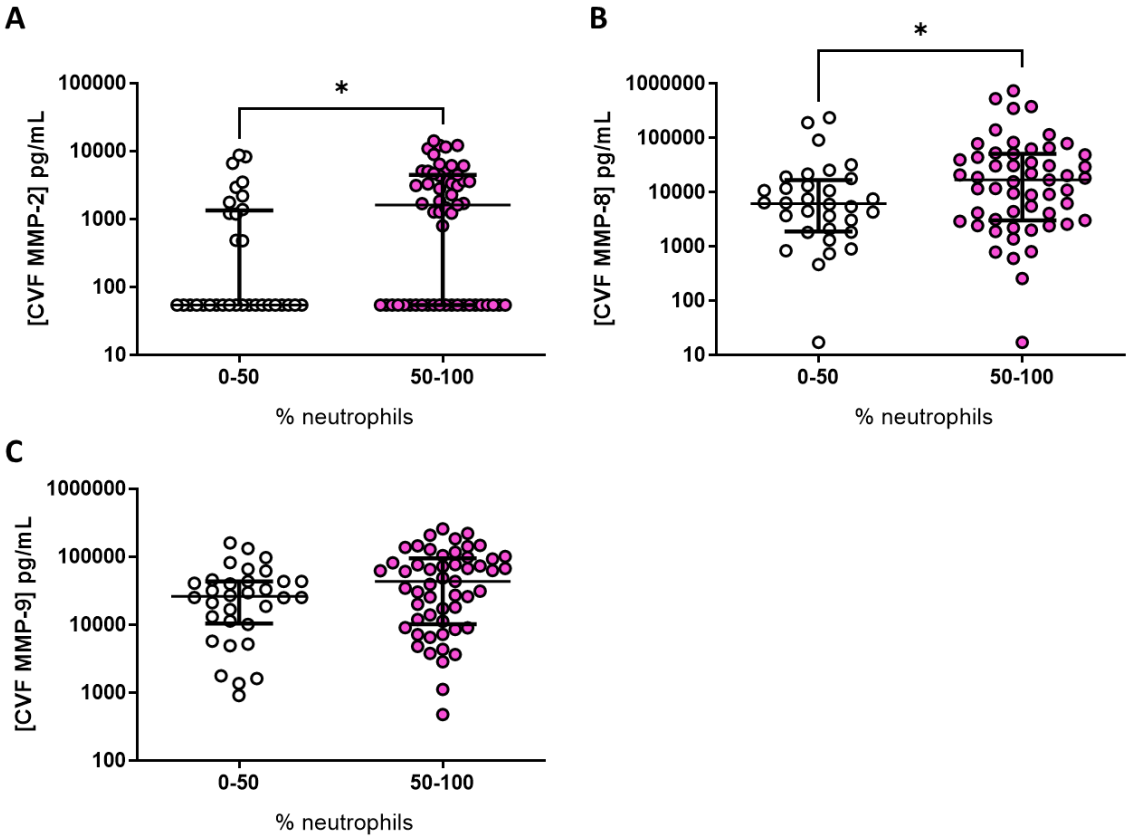


Figure 4.49. Cervical neutrophils presence in association with cervicovaginal matrix metalloproteinases. The concentration of **A)** MMP-2, **B)** MMP-8 and **C)** MMP-9 in CVF was compared between women who had greater number of neutrophils (>50% neutrophils, n=59) and lower number of neutrophils (<50% neutrophils, n=32) using a Mann-Whitney two-tailed test. *p<0.05. Data is presented as median values with interquartile range.

4.7. Discussion

In Chapter 4 the potential for neutrophils to play a role as mediators of microbial-driven inflammation, cervical shortening, and preterm birth has been explored. In summary, the data confirms that cervical neutrophils exhibit a phenotype reflecting greater activation than peripheral neutrophils. Neutrophils are not always present in the cervicovaginal interface but are more often detected in the presence of an adverse microbial composition, and in women who undergo cervical shortening. In addition, the presence of higher proportions of cervical neutrophils is associated with higher concentrations of cytokines, complement and MMPs.

Cervical and peripheral neutrophils exhibit a different immunophenotype

Until this study, the immunophenotype of cervical neutrophils in pregnancy was relatively unexplored. Hunter et al. showed via histogram plots that cervical neutrophils express CD16, CD66b, CD11b and CD11c (Hunter et al., 2016). However, no comparisons in the expression of these markers were made.

In this study, the immunophenotype of matched cervical and peripheral neutrophils was evaluated. The immunophenotype differed between peripheral and cervical neutrophils and can impact the role that this cell plays on different locations. CD33 is a protein expressed on the surface of hematopoietic cells that mediates cell-cell adhesion and also acts as a marker of immature neutrophils and immune regulation (Elghetany, 2002; Ssemaganda et al., 2014). The expression of CD33 was higher in peripheral neutrophils compared to cervical neutrophils, suggesting that cervical neutrophils could have an immunosuppressive role (Mao et al., 2018).

Integrins and selectins are adhesion molecules that mediate neutrophil attachment and migration and tend to shed from the neutrophil surface after migration and cellular activation (Lee et al., 2007; Rebuck et al., 1995). In our panel CD11b (integrin) and CD62L (L-selectin) were assessed, and expression of both markers was significantly reduced in cervical neutrophils. These findings align with the shedding of integrins after tethering and rolling of neutrophils to migrate into the sites of infection.

Focusing on markers of microbial recognition, the MFI of CD16, the FcγRIII, was assessed. Neutrophils express different Fcγ receptors, which allows them to interact with IgG antibodies, facilitating the phagocytosis of IgG-opsonised pathogens (Wang and Jönsson, 2019). A reduced expression of CD16 was observed in cervical neutrophils; this could indicate internalisation of the receptor due to binding of IgG-complexes for opsonisation of pathogens. CD63 and CD66b markers are highly related to effector functions of neutrophils. The former is a marker contained within azurophilic granules; and the latter is involved in a variety of functions, including respiratory burst, adhesion, degranulation and

phagocytosis (Elghetany, 2002; Källquist et al., 2008). Both markers were highly expressed in cervical neutrophils (CD66b expression in cervical neutrophils was double than peripheral MFI; and CD63 was triple than peripheral MFI), suggesting that neutrophils in the cervical milieu are highly activated and have degranulated. A decrease in CD16 has been reported on the surface of apoptotic neutrophils (Moulding et al., 1999), together with changes and reduction of other cell surface markers such as CD63, CD66b and CD88. None of these markers were decreased in cervical neutrophils, but they were increased, suggesting that cervical neutrophils are not apoptotic.

There is a close relationship between neutrophils and complement system activation. The anaphylatoxins C3a and C5a recruit phagocytes into sites of infection and inflammation for clearance of pathogens via expression of complement receptors and regulators. To better understand the interrelationship between peripheral and cervical neutrophils with the complement system, the expression of CD35, CD55 and CD88 was assessed. CD35 and CD88 are both complement receptors. CD35 is the complement receptor 1 (CR1) that can bind to C3b and C4b; and CD88 is the C5a receptor. Cervical neutrophils exhibited a reduced expression of CD35 compared to peripheral neutrophils, whereas CD88 that was more expressed in the cervix. This could suggest that CD35 is bound to C3b-opsonin complexes and that there is a potential internalisation, while CD88 is more expressed in order to sense and follow a C5a driven chemoattractant gradient, since in chapter 3 I showed how these complement proteins are present in the CVF in high concentrations. CD55 has a different role in the complement system, it is known as Decay Accelerating Factor (DAF) which is a membrane-bound complement inhibitor. The fact that CD55 is also more expressed on cervical neutrophils could be indicative of an attempt to compensate for the dysregulated release of complement proteins in the cervicovaginal environment.

Amsalem and colleagues also found different MFI expression of certain markers between peripheral blood and human decidua neutrophils. Decidual neutrophils expressed higher CD66b and decreased CD181 and CD182 (IL-8 receptors) (Amsalem et al., 2014). These results align with our findings of an increased CD66b expression and decreased expression of receptors in local neutrophils compared to periphery, suggesting a more active phenotype and internalisation of receptors after binding ligands *in situ*.

Cervical neutrophils are present with a different immunophenotype in women with an adverse vaginal microbial composition

Cervical neutrophils were more often detected in the presence of an adverse microbial composition, specifically in the presence of *L. iners* (CST III). Cervical neutrophils have also been detected increased in non-pregnant women with BV sampled with cytobrush (Farr et al., 2020) and by cervicovaginal lavages (Gardner et al., 2020). Therefore, it was hypothesised that the differences observed between cervical and peripheral neutrophils could be related to the composition of the cervicovaginal microbiome. The immunophenotype of cervical neutrophils was influenced by CSTs, in contrast to peripheral neutrophils. Women with CST IV vaginal dominance had a significantly higher MFI expression of CD63 and CD66b in cervical but not peripheral neutrophils. These data suggests that cervical neutrophils are more activated in the presence of pathogenic bacteria typically classed in CST IV. Cervical neutrophils also exhibited a reduced expression of CD16 in association with CST IV. However, Beghini et al. observed that CD16 expression was elevated in neutrophils from vaginal lavages from non-pregnant women with BV (Beghini et al., 2013). The typical bacteria causing BV clusters in CST IV (*G. vaginalis, Mobilincus, Mycoplasma, Atopobium, Prevotella*). In Beghini's study, BV was assessed by Amsel criteria and Nugent score, a useful clinical diagnostic for BV, however, accurate identification of species cannot be provided, whereas 16S rRNA sequencing allows to identify bacteria at species level. By classifying the vaginal microbiota composition in CSTs, and being CST IV a diverse group with several species observed, our study is limited too to some extent too to understand the response of cervical neutrophils to different bacterial species. This can be overcome with future work with larger sample sizes for a better stratification and evaluation of responses to specific bacterial species.

In this study I have showed how the immunophenotype of cervical neutrophils is altered depending on the vaginal microbial composition. Zhang et al. reviewed how the microbiota can regulate neutrophil production from the bone marrow priming and modulating neutrophil function in response to microbes and microbial subproducts (Zhang and Frenette, 2019). In this context, Mohd Zaki et al. also investigated the role of cervical neutrophils in a small subset of samples by RNA-seq (n=10). They found that clusters of genes related to neutrophil activation and degranulation were negatively correlated with *G. vaginalis* and positively correlated with *L. iner*s (Mohd Zaki et al., 2022). In our cohort (n=131 samples), cervical neutrophils were more often detected in women dominated with *L. iners* ; and the highest expression of CD63 and CD66b was associated with CST IV, which includes *G. vaginalis* among other members. Our data implies that the vaginal microbial composition influences the immunophenotype of cervical neutrophils.

Cervical shortening is associated with the presence of cervical neutrophils

Detection of cervical neutrophils was more likely in women who developed cervical shortening compared to women who did not shorten. Neutrophils have previously been located in the cervix by immunohistochemistry, together with proinflammatory cytokines including IL-1β, IL-6 and IL-8 (Young et al., 2002). In chapter 3 I described how all of these cytokines were significantly increased in CVF of women who underwent cervical shortening. IL-8 is a strong neutrophil chemoattractant and could be recruiting neutrophils into the cervix. Neutrophils are a source of proteases and collagenases, including neutrophil elastase, which has been detected in CVF of women with cervical shortening (Choi et al., 2018; Hezelgrave et al., 2020), therefore neutrophils should be on site to release it. Neutrophils are also a source of other pro-labour mediators such as COX-2 (Maloney et al., 1998), prostaglandins (St-Onge et al., 2007) and MMPs (Kelly, 2002; Rath et al., 1998).

The neutrophil immunophenotype differed between women who had cervical shortening and women who maintained a normal cervical length. In the periphery, neutrophils showed a reduced expression of CD62L. In the cervix, CD35 expression was reduced, and CD10 expression increased. Perhaps cervical neutrophils internalise the CD35 complement receptor, consistent with the increase in complement system activation seen in women who underwent cervical shortening (data presented in Chapter 3).

No differences were observed in the CD63, CD66b expression in peripheral nor cervical neutrophils. This was surprising as cervical shortening has been associated with adverse microbial composition, and data from cervical neutrophil immunophenotype based on CST composition demonstrated differences in women with a CST IV composition. The heterogeneity of cervical shortening aetiologies could be reflective of these findings, since women with previous history of cervical treatment are more likely to shorten due to cervical insufficiency and perhaps in cases of sterile/mechanical shortening, neutrophils behave differently or do not play a role. Also, in this study women who underwent cervical shortening were included, but I did not correct for the timing of the shortening, therefore there is also a heterogeneity of samples (mixture of before shortening/ at the time of shortening/ after shortening) that might not be reflective enough of specific immunophenotypes associated with cervical shortening. If samples were collected before the event of cervical shortening happening, cervical neutrophils might not be activated yet, whereas at the time of shortening or post shortening are more likely to already have been activated, or at least degranulated to release mediators such as matrix metalloproteinases and other collagenases.

Associations between cervical neutrophils and pregnancy outcome

Despite no significant associations between detection of cervical neutrophils and pregnancy outcome, there was a trend suggesting a higher proportion of women with neutrophils ultimately delivered preterm. Previously it has been reported that there are higher concentrations of PMN in vaginal lavages from women in preterm labour compared to uncomplicated term pregnancies (Yamada et al., 1998). This study did not specify the nature of those PMN, but potentially the majority were neutrophils, since they observed granules in electron micrographs and also assessed granulocyte elastase, which was higher in preterm cases. The time of sampling could be the reason for the lack of statistical difference in our study, as this is a retrospective assessment of neutrophil detection during pregnancy, so the latency between collection and delivery was highly variable. In contrast, in Yamada's study samples were collected at the time of admission because of preterm labour. Therefore, neutrophil counts might change closer to the event of labour. Another study also reported presence of vaginal neutrophils (>5 neutrophils per optic field in gram stains vaginal smears) in association with elevated vaginal pH and sPTB (Simhan et al., 2003a).

The immunophenotype of granulocytes in pregnancy, even more in preterm birth, is quite unexplored. Zhang et al. found that peripheral granulocytes (CD45+CD15+) increased during pregnancy and between women in preterm labour vs women in no labour, and that term labour is associated with increased MFI expression of CD11b, CD55 and CD192. However, no associations between neutrophils and preterm birth were reported (Zhang et al., 2017). Yuan et al. also explored peripheral blood neutrophils immunophenotype between preterm and term patients that were in labour or not in labour. Neutrophils from patients in labour had higher MFI of CD11a, CD11b and CD62L (Yuan et al., 2009). Gervasi found that peripheral neutrophils from women in preterm labour had higher expression of CD11b, CD15 and CD66b. They did not find differences in the other markers assessed (CD14, CD16, CD18, CD49d, CD64, HLA-DR) (Gervasi et al., 2001). In our cohort, only CD16 expression in peripheral neutrophils was associated with pregnancy outcome. Peripheral neutrophils from women who delivered preterm had a decreased MFI expression compared to both women who delivered at term with or without intervention (Figure 4.36.). A potential explanation for the differences observed between our study and Gervasi study is that they had a larger cohort (55 women who delivered preterm and 50 who delivered at term). But more importantly, again the time of sampling is quite crucial to understand these differences. Our study is a retrospective assessment of neutrophil detection during pregnancy; whereas in Gervasi's cohort, despite no differences observed between gestational age at sampling between preterm and term group, it is inferred that cases were sampled at the time of preterm labour, and term uncomplicated controls across pregnancy. Since labour is already a proinflammatory state, this could potentiate some of the differences observed.

Also, our cohort included only women who delivered preterm, whereas in Gervasi's study not everyone included in the preterm labour subsequently delivered preterm (39/55 PTB<37 weeks, 70.9%).

The role of cervical neutrophils in pregnancy has only been assessed by Hunter and Mohd Zaki previously. Hunter et al. showed that a decrease in PMN and macrophages was associated with early PTB (Hunter et al., 2016). Despite no vaginal microbial composition included in this study, it is known that early PTB are associated with microbial-driven aetiologies. Mohd Zaki also evaluated cervical neutrophils (Mohd Zaki et al., 2022), and like ours, no differences in the detection of neutrophils was associated by birth outcome.

Murine models do not support the role of neutrophils in cervical remodelling and preterm birth but suggest the participation of macrophages instead (Gonzalez et al., 2011). However, in human cervicovaginal milieu the most abundant cell type are PMN (Hunter et al., 2016; Mohd Zaki et al., 2022), and Hunter et al. actually linked the absence of macrophages with PTB.

Cervical neutrophils are active participants of the local cervicovaginal immune response

Finally, I have shown how a higher proportion of neutrophils is associated with a stronger inflammatory response. Higher CVF concentrations of IL-1β and IL-8 were seen when cervical neutrophils were present compared to absent, and when there were higher proportions of live neutrophils within the immune cell population. IL-8 is a potent neutrophil chemoattractant that could be bringing more neutrophils into sites of inflammation. In addition to being recruited, cervical neutrophils may be contributing to the inflammatory response on its own, since *in vitro* work has shown how neutrophils can secrete cytokines such as IL-1β, IL-6 and IL-8 following LPS stimulation (Naegelen et al., 2015). Moreover, associations with complement proteins revealed an increased concentration of C3a and C3b when more live neutrophils were present. C3a is another chemoattractant and C3b is an opsonin that binds bacteria and to the C3b receptor of neutrophils to facilitate the clearance of pathogens via neutrophil driven phagocytosis.

Finally, neutrophils are known for the secretion of proteases after degranulation. MMP-8 and MMP-9 are present in secondary and tertiary neutrophilic granules. The levels of MMP-8 were higher when more neutrophils were present. Since high CD63 expression was seen in cervical neutrophils, it is likely that MMPs are released as the neutrophils degranulate.

Despite the evidence of a strong inflammatory response when the proportion of neutrophils was higher, when assessing the local inflammatory response in the presence and absence of cervical

neutrophils, many inflammatory mediators were also increased in association with high-risk microbial compositions even in the absence of cervical neutrophils. Therefore, neutrophils may not be the only modulators in the local immune response. Macrophages and vaginal and cervical epithelial cells can be active participants too. For example, extrahepatic complement production has been shown from mast cells, macrophages (Lubbers et al., 2017) and vaginal epithelial cells (Bulla et al., 2010). Macrophages are also a source of proinflammatory cytokines (Arango Duque and Descoteaux, 2014) and there is evidence of a strong inflammatory response and release of cytokines from vaginal and cervical epithelial cells incubated with bacterial supernatant (Anton et al., 2018) or co-cultures with bacteria (Anton et al., 2022) in the presence of *G. vaginalis.*

Strengths and limitations

One of the strengths of this study is that I have shown the feasibility to work with neutrophils, both peripheral and cervical, considering how delicate these cells are. The protocol was carefully designed and optimised, including antibody titration in both type of samples, microscopy techniques and timings between collection and acquisition to maximise the collection of both peripheral and cervical neutrophils, from two different London hospitals.

The Aurora Cytek ® instrument was used to analyse the immunophenotype of both peripheral and cervical neutrophils. The panel included 14 different fluorophores, and this instrument presented the advantage, as a spectral flow cytometer, to detect multiple fluorophores with minimal spillover correction required.

Another strength of this project is the large amount of cytobrush samples collected. A total of 147 samples were collected from 98 women at high-risk of delivering preterm birth. This is a considerably large number considering that cytobrush sampling is a relatively invasive technique, and the cohort are women who are at high-risk of delivering preterm and have often suffered with previous adverse pregnancy outcomes. This was achieved thanks to the patients, the continuity of care produced by the clinical research team, and the infrastructure available at the preterm birth prevention clinics and the Women's Health Research Centre (WHRC) at the Imperial College NHS Trust Hospitals from Queen Charlotte's and St Mary's hospital.

Moreover, the collection of matched peripheral and cervical samples is another strength, since I have showed that the immunophenotype of neutrophils differs between location, being cervical neutrophils more associated with an activation phenotype. Both samples allowed to better understand the role of neutrophils during pregnancy in microbial-driven mechanisms.

Finally, a combination of immunology techniques with metagenomic approaches allowed us not only to characterise neutrophils in pregnancy, but also to correlate our findings with microbial signatures and immune responses. The assessment of cervical neutrophils itself is a very novel work, since there are only two other research publications in pregnancy assessing leukocytes at the cervicovaginal interface (Hunter et al., 2016; Mohd Zaki et al., 2022), only one of them providing associations with the microbial composition (Mohd Zaki et al., 2022), and none covering their role with the local immune milieu.

One of the limitations of this project is that the multicolour flow cytometry panel was designed specifically for the study of neutrophils, as it was our population of interest. Therefore, it is not possible to evaluate the contribution of other cell types such as B cells and T cells. However, given the fact that neutrophils were selected in the CD15+ CD14- fraction, stored data can be used to assess the presence and contribution of the macrophage population (CD15-, CD14+) from cytobrush samples. It would not be possible though to assess their counterpart on the periphery (monocytes), as only the granulocyte fraction was isolated and analysed from peripheral blood.

Potential future work

One of the limitations of this study is the lack of stratification of women according to risk factor for preterm birth. Future work could evaluate if cervical neutrophils detection and immunophenotype differs between women with a history of previous cervical treatment or previous history of PTB, to reflect two different PTB aetiologies: microbial-driven and sterile/mechanical mechanisms. Also, the role of neutrophils could be evaluated in women who undergo cervical shortening in a timeline of events, comparing before going on to shorten and at the time the cervix is already short. Potentially, the effect of interventions could be evaluated too, to determine differences in the immunophenotype of neutrophils pre and post vaginal progesterone administration, pre and post cervical cerclage, and pre and post different cerclage suture materials (nylons vs mersilene). It also remains unknown the role of cervical neutrophils in other pregnancy complications such as PPROM.

Cervical neutrophils appear to play a role in mediating microbial-driven inflammatory events during pregnancy. After demonstrating by flow cytometry measuring surface markers that peripheral and cervical neutrophils exhibit a different immunophenotype in association with adverse microbial compositions and cervical shortening, future *in vitro* work could be performed to look at the effects that different bacterial species can have on the effector functions of neutrophils. Also, since markers of degranulation are highly expressed, assessing other proteases that can be released by neutrophils,

such as myeloperoxidase or neutrophil elastase, and seeing if concentration correlate with presence of neutrophils can be part of future work.

4.8. Conclusion

Cervical neutrophils exhibit a phenotype reflecting increased activation compared to peripheral neutrophils. There is higher expression of markers related to neutrophil effector functions, including CD63 and CD66b, and increased expression in association with adverse microbial compositions (Figure 4.50). Cervical neutrophils are more often detected in women who undergo cervical shortening, and in women who had a vaginal microbial composition dominated with *L. iners*. Increased cervical neutrophil proportions were associated with higher concentrations of cytokines, complement proteins and MMPS. These data support the role of cervical neutrophils in local inflammation and microbial-driven cervical shortening.

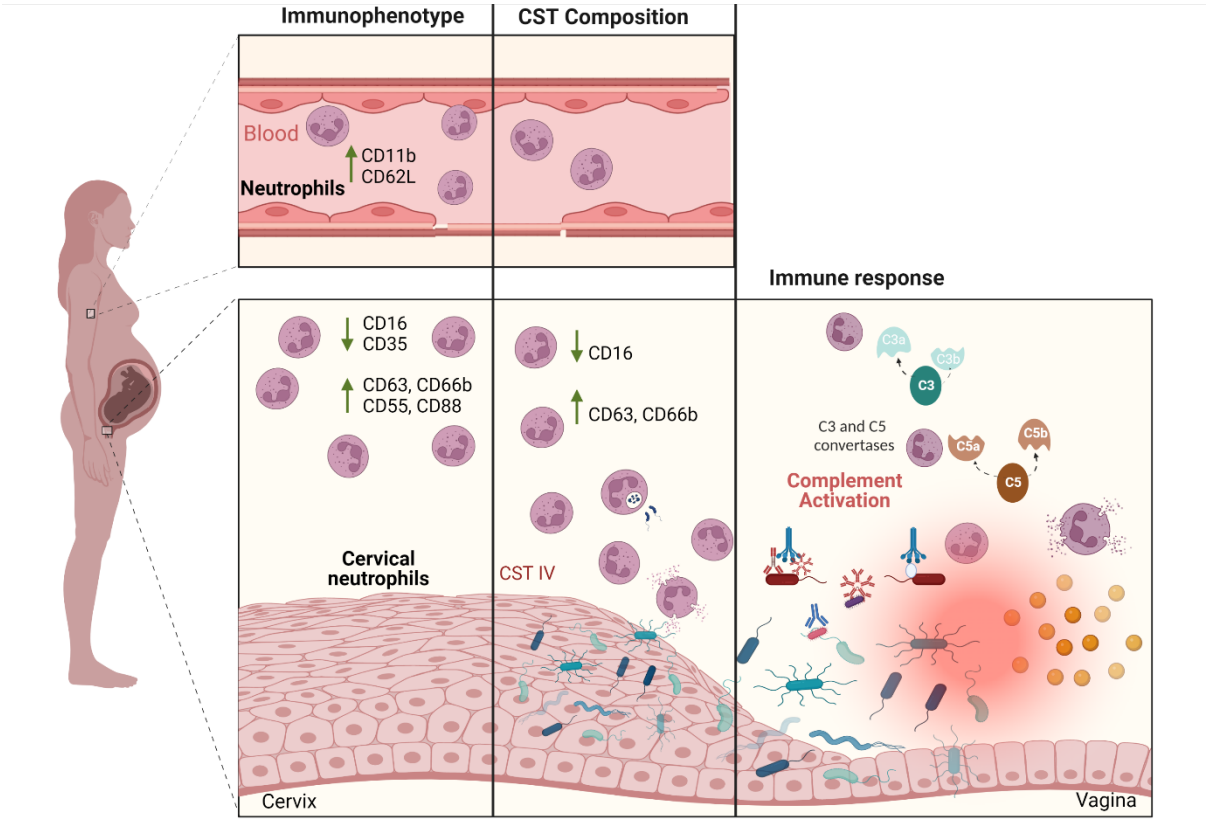


Figure 4.50. The role of neutrophils in pregnancy, term and preterm birth. Cervical neutrophils exhibit a phenotype reflecting increased activation compared to peripheral neutrophils. Peripheral neutrophils exhibited an increased expression of migration markers CD11b and CD62L. Cervical neutrophils exhibited a decreased expression of markers CD16 (FcγRIII) and CD35 (C3b receptor), likely due to internalisation of receptors after binding to ligands. Cervical neutrophils exhibited an increased expression of marker CD63 and CD66b implicated in effector functions. Complement receptors CD55 and CD88 were also increased in cervical neutrophils. The immunophenotype of peripheral neutrophils did not differ according to the CST vaginal microbial composition, however, women dominated with CST IV vaginal composition exhibited a decreased CD16 expression and increased CD63, CD66b expression in cervical neutrophils. Finally, presence of cervical neutrophils was associated with a local inflammatory immune response (cytokines, complement proteins, MMPs). Despite being recruited into sites of infection (IL-8, C3a, C5a are chemoattractants), and contribute to the local inflammatory response (cytokines, MMPs) it cannot be rules out the participation of other cell types (e.g., epithelial cells and or macrophages) to the local cervicovaginal immune response.

5. MICROBIAL-HOST INTERACTIONS AT THE CERVICOVAGINAL INTERFACE: OPSONISATION OF VAGINAL MICROBIOTA

5.1. Chapter Summary

Introduction

Inflammation and infection have long been implicated in the aetiology of sPTB. *Lactobacillus* spp. can modulate the risk of preterm birth: *Lactobacillus* dominance, particularly *L. crispatus* has been associated with a protective role, whereas *Lactobacillus* depletion and the presence of species of CST IV are consistently implicated in a higher risk of microbial-induced sPTB. However, how the host immune response mediates these mechanisms is poorly understood. This study aims to develop an understanding of the degree of recognition of cervical-vaginal bacteria by host immune factors (immunoglobulins and opsonins); and how this interaction influences the local proinflammatory response and the risk of sPTB.

Hypothesis

- There is a variation in bacterial opsonisation patterns which is dependent on cervicovaginal microbial composition
- The variation in bacterial opsonisation influences the local immune milieu and risk of preterm birth

Aims

- To optimise bacterial opsonisation protocols for the assessment of opsonisation of cervicovaginal bacteria
- To determine the percentage of opsonised cervicovaginal bacteria
- To determine if the percentage of opsonisation influences the local immune milieu
- To determine if the percentage of opsonisation is altered depending on the local microbial composition, cervical shortening and preterm birth

Methods

Forty-three women from the VMET2 Research Study were included in this study. Of the 43 women, 10 experienced spontaneous preterm birth and 33 had an uncomplicated term delivery. All women

included in the study provided CVF (two swabs) at an early (A: 12-16 weeks of gestation) and mid-pregnancy (B: 20-24 weeks of gestation) collection timepoints. CVF from one of the swabs was dedicated for 16S rRNA sequencing and Luminex multiplex immunoassays. The second matched CVF swab was used to establish the percentage of bacteria bound to immunoglobulins. Briefly, the bacterial pellet was extracted and fixed overnight. Following incubation with a nucleic acid stain and anti-human immunoglobulins (anti-IgA, anti-IgG) or anti-opsonin C3b (anti-C3b), samples were acquired by flow cytometry to assess the percentage of bacteria bound to these mediators. Pure bacterial cultures were used as a positive (detection of bacteria within flow cytometric settings) and negative control (no binding to immunoglobulins or opsonin).

Results

The most frequent CST in the overall population was CST I *L. crispatus*. No differences (in composition, diversity or richness) were observed in the vaginal microbial composition across pregnancy. The percentage of bacteria bound to IgG, IgA and C3b was stable across pregnancy and remained unchanged between timepoint A and timepoint B. Correlation analysis showed a statistically significant positive correlation between percentages of bound IgA and bound IgG ($r=0.8492$), bound IgA and bound C3b ($r=0.7014$) and bound IgG and bound C3b ($r=0.6692$) (all cases $p<0.0001$).

A higher percentage of binding to IgG was observed in women with a CST I vaginal composition (70%) compared to women with a CST IV (~20%) ($p=0.0008$), as well as percentage bound to IgA (CST I 74% vs CST IV 48.5%, $p=0.0554$). The same pattern was observed for binding to C3b (variation from 62% to 39% in women with CST I and CST IV, $p=0.0419$). There was a negative correlation between binding of bacteria and the concentration of cytokine and complement inflammatory mediators. Finally, no differences in percentage of bacterial binding to opsonins was observed in women who underwent cervical shortening. However, in association with pregnancy outcome, women who delivered preterm had lower binding of IgG ($p=0.0174$), IgA ($p=0.0283$) and C3b ($p=0.0042$) compared to women who delivered at term.

Conclusion

This chapter presents data confirming the feasibility of using flow cytometry to demonstrate the percentage of bacteria coated with opsonins from vaginal samples taken from pregnant women. The percentage of bacterial coating was stable across pregnancy. However, women who had a vaginal microbial composition enriched in *Lactobacillus* spp. had higher percentages of bacterial coating,

whereas CST IV was associated with lower bacterial recognition. This suggests that species in CST IV are more likely to be able to evade the immune response, and that opsonisation is adopted as a neutralisation strategy. In contrast to what was anticipated, less bacterial opsonisation was associated with higher concentrations of proinflammatory cytokines and complement proteins and higher rates of preterm birth.

5.2. Introduction

The cervicovaginal fluid (CVF) is a very complex biological fluid composed from secretions from the endocervix and the vagina, rich in epithelial cells, leukocytes, antibodies, and vaginal microbiota (Lacroix et al., 2020). The hallmark of vaginal health is the dominance of *Lactobacillus* spp., whereas depletion of *Lactobacillus* spp. predisposes to a higher risk of adverse pregnancy outcomes. However, not everyone with a high-risk vaginal microbial composition (CST III and/or CST IV) will ultimately deliver preterm. This is particularly relevant since correlation is not a synonym of causation, and many factors can influence the microbiome, and the specific molecular mechanisms that drive preterm birth are yet to be elucidated. Therefore, there might be individual responses and interactions between the host and the microbiota modulating physiological and pathological responses. However, these interactions are yet to be clarified in the cervicovaginal interface.

The local interactions between the microbiota and the host immune response have been studied in most detail at the intestinal mucosal interface, with a particular interest in microbiota-immunoglobulin interactions (Round and Palm, 2018; Weis and Round, 2021), which appears to be key for the gut homeostasis. Not surprisingly, there is a particular interest in the role of IgA, the most abundant immunoglobulin in the mucosal secretions. One of the major functions of IgA is the prevention of microorganism expansion, via binding to the microorganisms itself or their toxins. Secondly, it is involved in antigen-specific responses since dendritic cells can present IgA-bound complexes to T cells. Thirdly, binding to IgA can be associated with commensal species, probably due to its ability to aggregate bacteria, and in this case enhance colonisation and outcompete other strains (Weis and Round, 2021). The potential to identify IgA-coated bacteria interactions has been proved in both mouse models (e.g., identification of commensal bacteria by (Jackson et al., 2021); model of undernutrition by (Huus et al., 2020)) and human gut samples (inflammatory bowel disease, (Palm et al., 2014); asthma, (Dzidic et al., 2017)). Collado et al. also showed the potential of studying immunoglobulin-coated bacteria in a variety of samples apart from faeces, like in saliva and breast milk, using both IgA and IgG (Simón-Soro et al., 2015). These studies relied on the use of FACS to establish and purify the percentage of immunoglobulins coating bacterial cells. This purification approach, followed by 16S rRNA sequencing (Ig-Seq), allows to establish the identity of the specific microorganisms from the coated and uncoated fractions (Round and Palm, 2018).

The flow cytometry approach has been applied to CVF samples in a healthy non-pregnant cohort (Breedveld et al., 2022). Similar to the gut functionality, IgA was suggested to be implicated in vaginal homeostasis, since women with *L. crispatus* vaginal dominance showed increased IgA coating of bacteria. Interestingly, both *L. crispatus* dominant and non-dominant populations were coated with

IgA and IgG (Breedveld et al., 2022). However, what happens during physiological pregnancy, microbial-driven preterm birth, or any associations with the local immune response has never been addressed.

During pregnancy, CVF and cervical mucus are rich in immunoglobulins, especially IgA and IgG (Chan et al., 2022; Hein et al., 2005), and immunoglobulins levels can be associated with the vaginal microbial composition and pregnancy outcome. Chan et al. found that IgM and all IgG subtypes (IgG1, IgG2, IgG3, IgG4) levels were higher in women with CST III and CST IV vaginal dominance, compared to CST I. In addition, when stratifying participants by both pregnancy outcome and vaginal composition, women with the same vaginal classification (e.g., *L.* deplete, or CST III, CST IV) had higher levels of IgM and IgG2-4 when delivering preterm compared to term within the given microbial composition (Chan et al., 2022), suggesting an increased capacity to opsonise bacteria in women with high-risk microbial composition.

Opsonisation is the immunological process by which pathogens are coated and targeted by opsonins like immunoglobulins and/or complement proteins to enhance phagocytosis (Peiser, 2016). However, bacterial opsonisation at the cervicovaginal interface has not been studied in pregnancy despite the need to understand the mechanism of microbial-driven inflammation and sPTB. Therefore, this chapter aims to describe the percentage of bacterial opsonisation across gestational age, in relation to vaginal microbial composition, local immune milieu and pregnancy outcome. The percentage of opsonisation was measured by flow cytometry, using anti-human IgA and IgG antibodies, since they have long been implicated in mucosal surfaces (Chen et al., 2020), as well as anti-human C3b, a typical opsonin product of the activation of the complement cascade, that I (Chapter 3) and others (Chan et al., 2022) found higher free levels in CVF of women with a CST IV vaginal composition.

5.3. Study design

A subset of the pregnant women at high-risk of delivering preterm that were included in Chapter 3 (section 3.3.) were randomly selected for the current study presented in Chapter 5 to establish the percentage of bacterial opsonisation (bacterial coating). These women were recruited from preterm birth prevention clinics across London (Queen Charlotte's and Chelsea Hospital, St Marys Hospital, University College London Hospital, and Chelsea and Westminster). Women included in the study had provided a second high vaginal swab apart from the one already employed for bacterial DNA extraction and sequencing (Chapter 2, section 2.6.) and Luminex Multiplex Assays (Chapter 2, section 2.7.).

The preparation of samples for bacterial flow cytometry was described in Chapter 2, section 2.8 and graphically in Figure 5.1. Briefly, the bacterial pellet was extracted from a high-vaginal swab and fixed overnight in 3.6% PFA. Bacteria was washed twice in PBS and resuspended in 1mL of FACS buffer (PBS + 1% BSA). A 100µL of the resulting bacterial supernatant was stained with SYTO dye and anti-human monoclonal antibodies (Table 2.7.) for 30 minutes in the dark. Samples were washed twice with PBS and resuspended in 0.5mL of PBS. Samples were acquired on the flow cytometry (FACS Symphony, BD Biosciences).

Women included in the study were recruited between June 2018 and February 2021 as part of the VMET2 Research study. Measurement of the percentages of opsonisation was conducted during the first and second trimester of pregnancy (timepoint A: 12-16 weeks of gestation; timepoint B: 20-24 weeks of gestation).

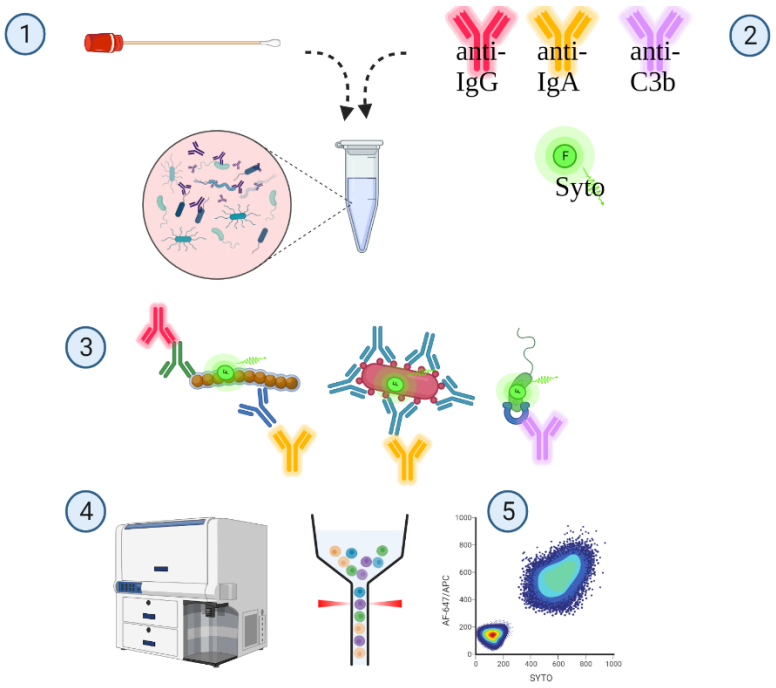


Figure 5.1. Protocol overview for evaluation of opsonised bacteria. 1) Bacterial cells were extracted from vaginal swabs and fixed overnight with 3.6% PFA. 2) Samples were stained with Syto Bacterial dye and anti-human antibodies: IgG, IgA, or C3b. 3) Samples were incubated for 30 minutes in the dark. 4) Samples were acquired by flow cytometry and 5) analysed with Flowjo to assess the percentage of double positive populations (SYTO+, IgG+; SYTO+ IgA+; SYTO+ C3b+). Image created with Biorender.com.

FlowJo was used for the analysis of the flow cytometry data to obtain the percentage of bacteria bound to immunoglobulins or C3b. The median fluorescence intensity (MFI) was calculated within the population of interest. The integrated MFI (or coating index) was calculated by multiplying the percentage of bacteria bound with the MFI.

5.4. Statistical analysis

Statistical analysis was performed using Graphpad Prism 9. Comparison between two groups were analysed with an unpaired t-test or a Mann Whitney test, depending on if the data was normally or not-normally distributed. The Kruskal Wallis test with the Dunn's post hoc test was used to analysed differences between three or more groups, for example, to compare differences according to the vaginal microbial composition where groups of interest (I, III, IV) (e.g., differences according to the vaginal microbial composition). To analyse proportional differences, the Fisher's exact test or Chi square test was used. As usual, a p-value less than 0.05 was considered to be statistically significant.

5.5. Protocol optimisation

The protocol optimisation was performed by adapting a protocol previously developed and described by Collado et al. (Simón-Soro et al., 2015). The first and crucial part of the protocol optimisation consisted in establishing the right settings on the flow cytometer, initially using a BD® LSR II Flow Cytometer. To do so, 1µm polystyrene beads were used as a reference (Figure 5.2.A), since the size of typical species commonly found in the vagina (*Lactobacillus*, (Schär-Zammaretti and Ubbink, 2003); *Gardnerella vaginalis*, (Turovskiy et al., 2011) range between 1 and 1.5µm. Bacterial cells from vaginal swabs were located within the 1µm beads gate (Figure 5.2. B), and bacterial detection was further confirmed using a fluorescent nucleic acid stain (Figure 5.2.C.). Based on the latter histogram distribution (Figure 5.2.) we were able to detect two distinct populations, revealing the necessity to draw tight gates around the population of interest (positive for Syto™ BC Green).

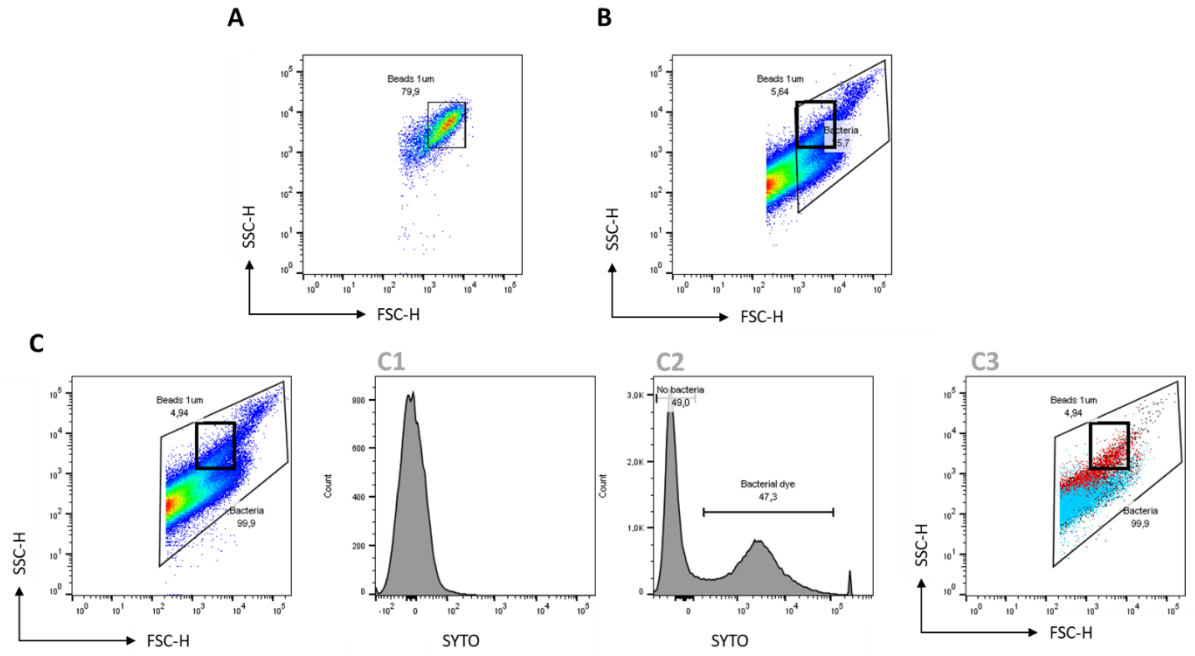


Figure 5.2. Defining flow cytometry settings for bacterial detection. A) Detection of 1µm beads. **B)** Bacterial cells extracted from vaginal are located within the established settings (gate with thick black border represents 1µm size). **C)** Bacterial detection is confirmed staining bacterial pellet with SYTO™ BC Green Fluorescent Nucleic Acid Stain. It is possible to detect a shift of the fluorescence from the unstained control (C1) on the green channel (525nm) with the stained sample (C2). Backgating on the pseudocolour plot (C3) reveals the necessity of using tight gates around the population of interest. Red (positive population), blue (negative population).

Secondly, we used pure bacterial cultures of bacteria that typically colonise the lower reproductive tract (*G. vaginalis, L. crispatus, L. iners,* provided by Lauren Roberts), as well as bacterial cells extracted from vaginal swabs (N=2) stained with two different bacterial DNA dyes from different regions of the spectra (green: SYTO™ BC and red: Cell Trace™ Far Red Cell Stain). This allowed us to prove that a) bacterial cells from liquid culture could be detected within the settings; and that b) we were indeed detecting bacteria based on the fluorescence. Figure 5.3. depicts the performance of the two different nucleic acid stains in a *L. iners* sample. Plots for *G. vaginalis*, *L. crispatus* and human vaginal swabs are available in Appendix 5.1. Since both dyes performed similarly, SYTO™ BC dye was selected for the rest of the experiments.

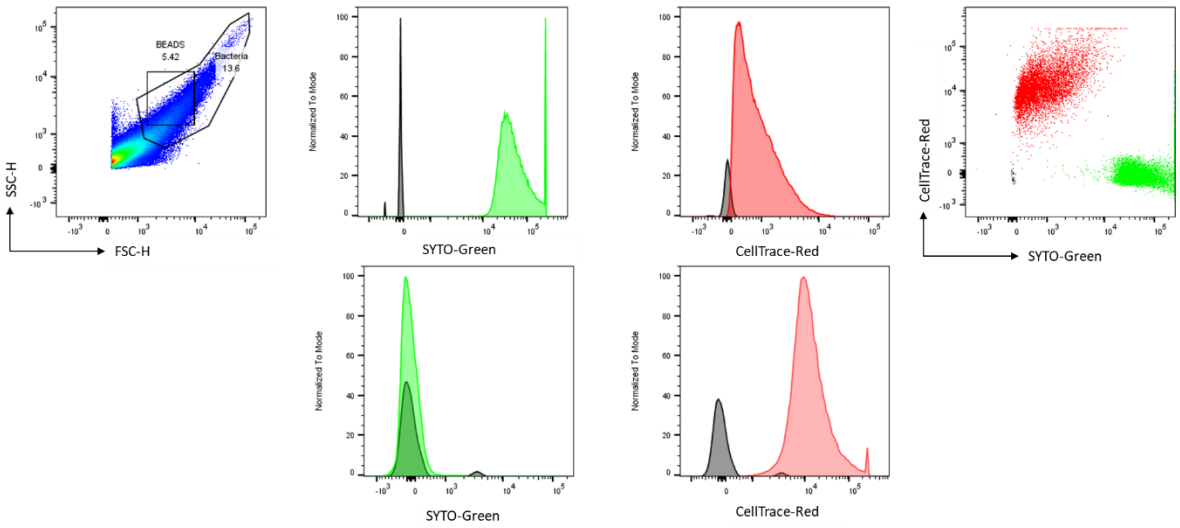


Figure 5.3. Performance of two different nucleic acid stains. A sample from pure bacterial liquid cultures of *L. iners* **(A)** was single stained with SYTO™ BC Green Fluorescent Nucleic Acid Stain **(B)** and CellTrace™ Far Red **(C)**. Histogram and pseudocolor plot overlay of unstained reference control (black) and single colour stain (red or green, respectively) **(D)**. All histograms were normalized to the mode.

Simultaneously, the staining incubation times were optimised. Nucleic acid stain with SYTO^{TM}BC was performed for 15 minutes and 30 minutes. Cells were also single stained with a representative anti-human immunoglobulin, IgG conjugated with Alexa Fluor647 and FITC (Table 5.1.) for 30 minutes, 1 hour, 2 hours. Results are presented in form of histogram overlay (Figure 5.4) of unstained control and single colour staining for Syto^{TM} BC (Figure 5.4.A), IgG-FITC (Figure 5.4.B) and IgG-Alexa Fluor 647 (Figure 5.4.C). Since the time of incubation did not affect the MFI. the time of incubation for both immunoglobulins and nucleic acid staining was standardised to 30 minutes.

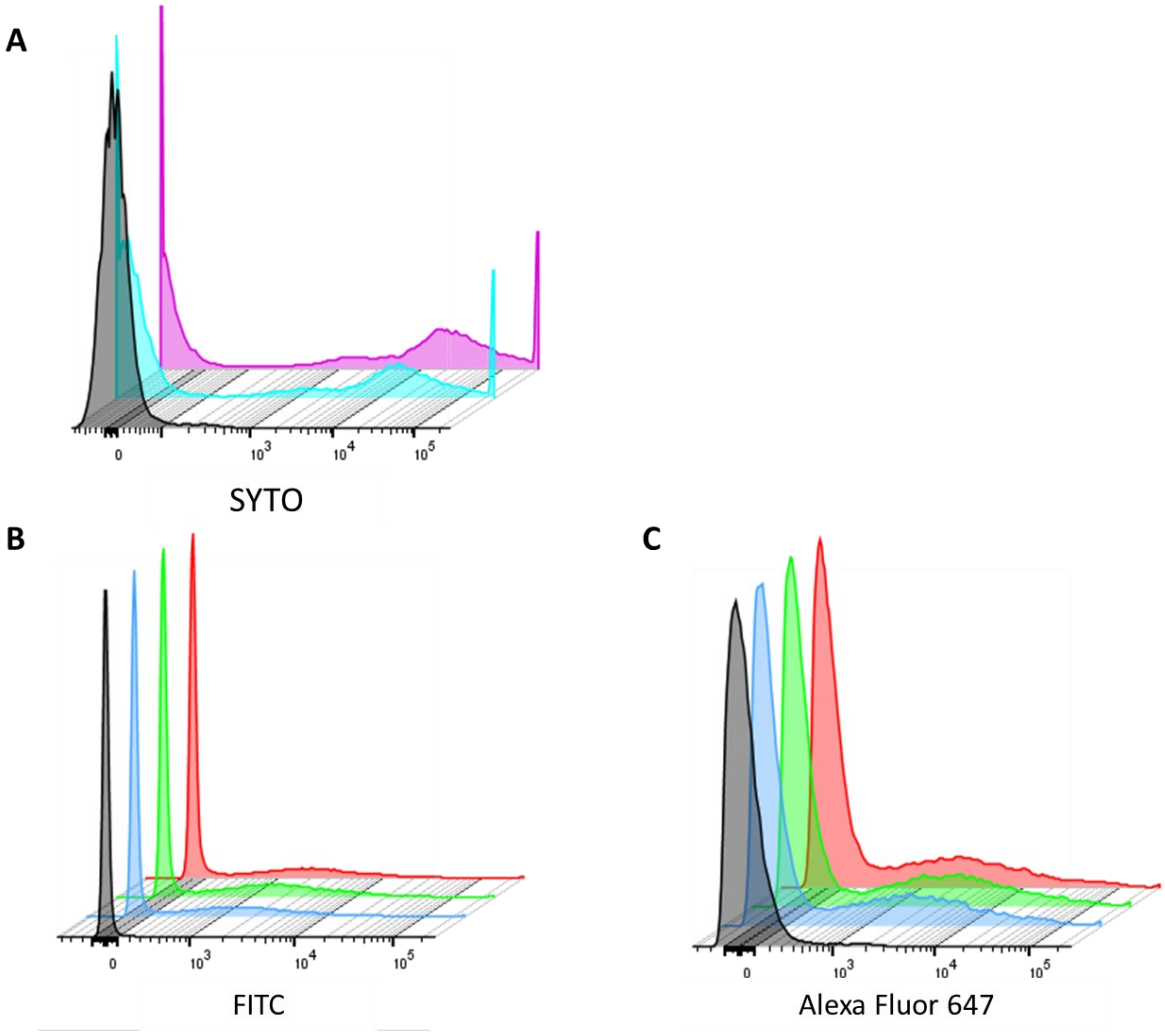


Figure 5.4. Optimisation of staining incubation periods. A) Histogram overlay of unstained (black) and single colour stain Syto^{TM}BC for 15 minutes (pink) and 30 minutes (blue). **B)** Histogram overlay of unstained (black) and single colour stain with anti-human IgG-FITC for 30 minutes (blue), 1 hour (green) and 2 hours (red). **C)** Histogram overlay of unstained (black) and single colour stain with anti-human IgG-Alexa Fluor 647 for 30 minutes (blue), 1 hour (green) and 2 hours (red).

During the optimisation of the protocol, the Flow Cytometry Facilities at Imperial College London-Hammersmith Campus changed instruments and substituted the BD® LSR II Flow Cytometer instrument for a BD FACSymphony™ A3 Cell Analyzer. Before continuing with the optimisation, the settings were re-settled and re-established in the new instrument, following the previous strategy using microparticle beads and bacterial cells extracted from vaginal swabs. Single tubes containing 1µm beads (Figure 5.5 A) and 6µm beads (Figure 5.5 B) were acquired individually to set and drag gates. Bacterial cell pellet from a vaginal swab was located within the established gates (Figure 5.5.C). Bacterial cells were also single and mix stained with Syto and IgG-Alexa Fluor 647. Single colour stains were used to adjust the laser settings (Figure 5.5.D, E). The double stain group (Figure 5.5.F) was used as a proof of concept to detect the population of interest. The BD FACSymphony™ A3 Cell Analyzer was used to acquire the data for the rest of the experiment (both optimisation and real samples acquisition).

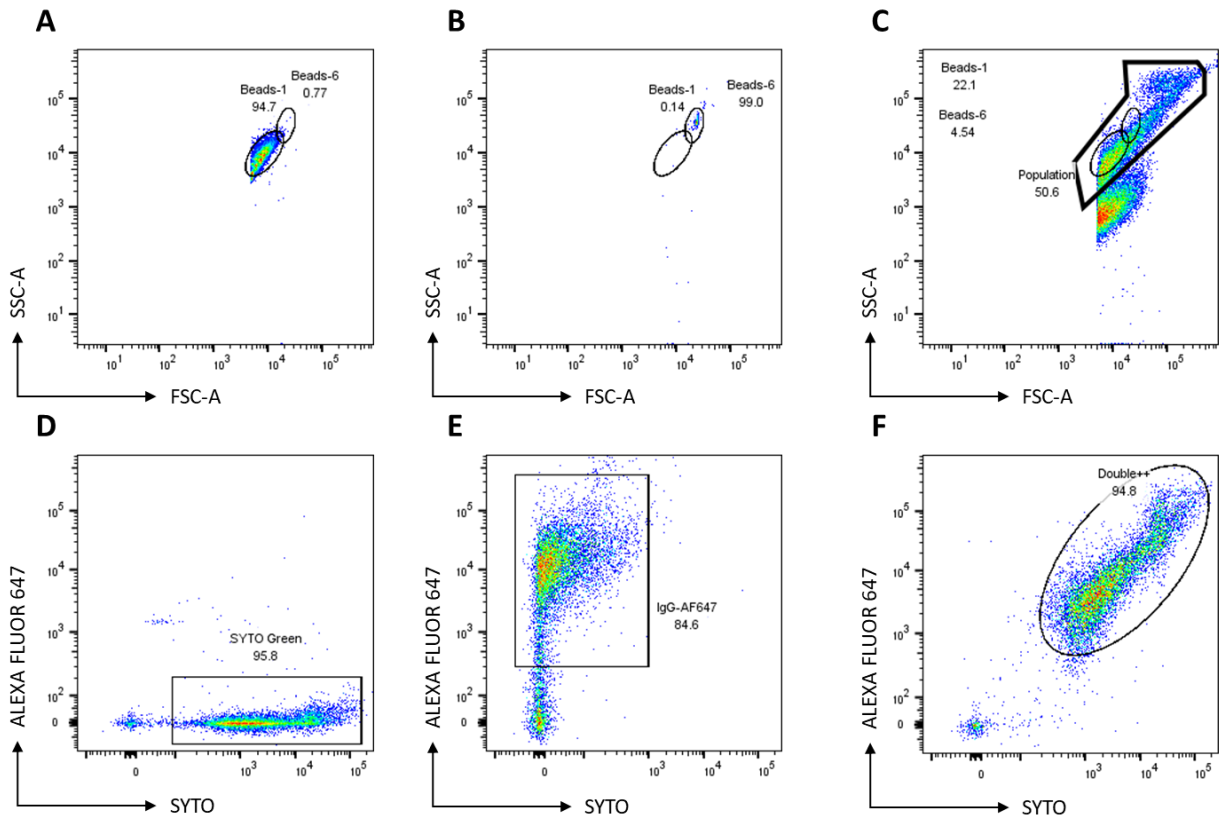


Figure 5.5. Establishment of instrument settings on a BD FACSymphony A3 Analyzer. A) FSC and SSC pseudocolor plot of 1µm beads. **B)** FSC and SSC pseudocolor plot of 6µm beads. **C)** FSC and SSC pseudocolor plot of bacterial pellet extracted from vaginal swab sample. Circular gates indicate the location of the beads. Note that the population of interest is covered within this range. **D)** Single colour stain with Syto™ Green. **E)** Single colour stain with anti-human IgG-Alexa Fluor 647. **F)** Double positive population for Syto and IgG will determine the percentage of immunoglobulin-coating bacteria.

After selecting the Syto™ BC nucleic acid stain to detect bacteria, and taking into account its wavelength of excitation/emission ranges from 485/500nm on the green channel (Appendix 5.2), the following panel was designed to include anti-human immunoglobulins (IgG, IgA) and C3b antibodies (Table 5.1) conjugated to a fluorochrome that would emit further on the spectra, choosing Alexa-Fluor 647 (Appendix 5.2.) to avoid major compensation issues. The anti-C3b antibody was conjugated in our facilities as described in Chapter 2 Section 2.8. by Dr Alexiane Decout. All antibodies (Table 5.1) were titrated to establish the optimal antibody volume (concentration) required (Figure 5.6.). After titration experiments, it was concluded that 0.5µL was the most appropriate volume to assay all antibodies, as well as nucleic acid stain.

Table 5.1. Preliminary antibody panel for opsonisation studies

Antibody	Fluorochrome	Cat #/ Company	Justification*	Volume ** [Concentration]	Isotype
Goat anti-human IgG (H+L) Secondary Antibody	FITC	A18806 Invitrogen	Same antibody as IgG in different colour	0.5 µL [0.01mg/mL]	n.a.
Goat anti-human IgG (H+L) Cross-Adsorbed Secondary Antibody	Alexa Fluor® 647	A-21445 Invitrogen	(Simón-Soro et al., 2015)	0.5 µL [0.01mg/mL]	Goat IgG Isotype Control Cat # 02-6202 (Invitrogen)
Goat anti-human IgA	Alexa Fluor® 647	109-606-011 Jackson ImmunoResearch Laboratories Inc.	(Breedveld et al., 2022)	0.5 µL [0.0075mg/mL]	Goat IgG Isotype Control Cat # 02-6202 (Invitrogen)
Complement C3b Monoclonal Antibody (10C7)	Unconjugated	MA1-70054 Invitrogen	(Sharma et al., 2020)	0.5 µL [0.05mg/mL]	Mouse IgG1 κ Isotype Control Cat # 566011 (BD Biosciences)

*Previous use in a study related to opsonisation or bacterial coating. ** After antibody titration (Figure 5.5.). Cat #: catalogue number

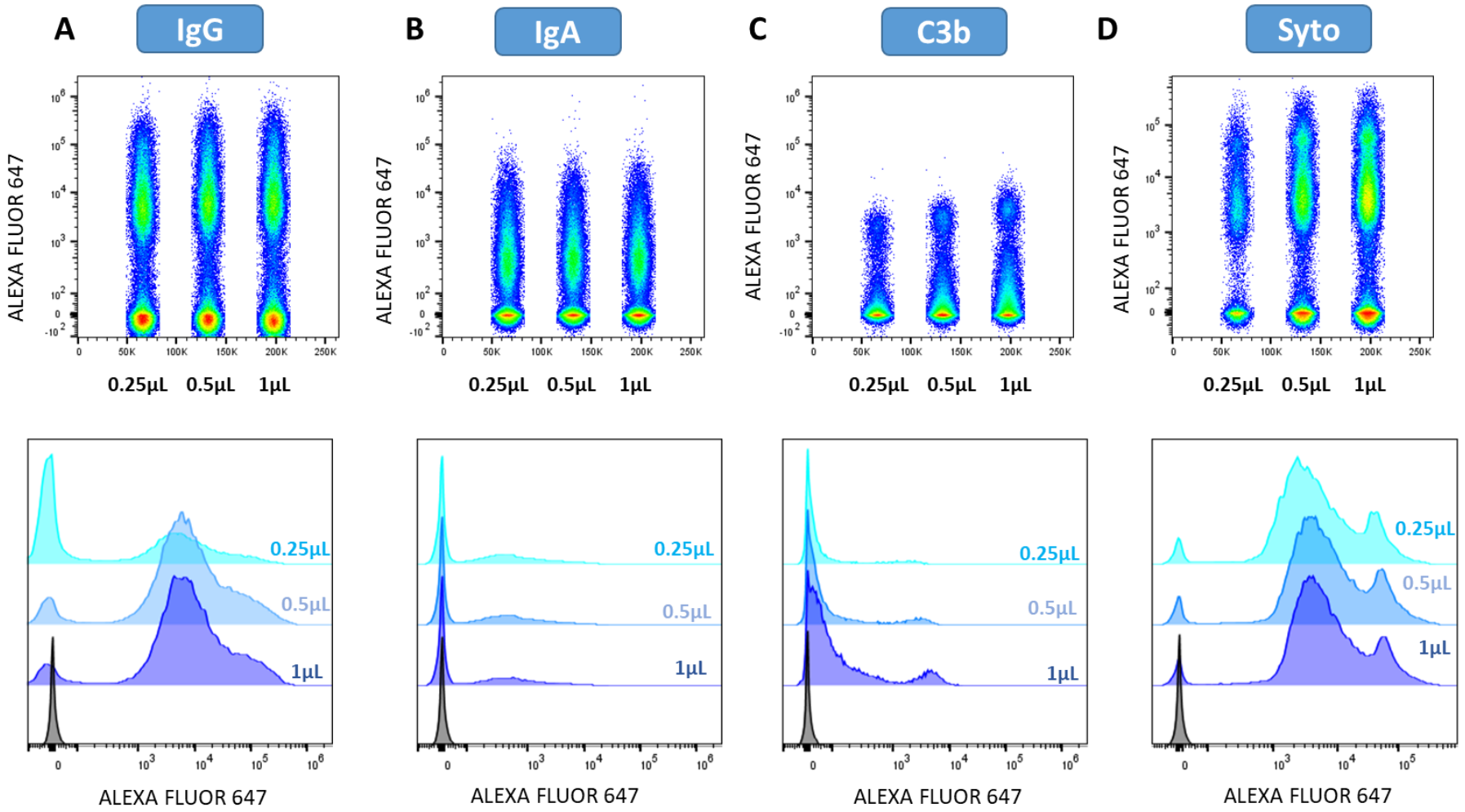


Figure 5.6. Antibody Titration. A) Different volumes of anti-human IgG-AF647 were single-colour assayed (0.25µL, 0.5µL,1µL). **B)** Different volumes of anti-human IgA-AF647 were single-colour assayed (0.5µL, 1µL,2µL). **C)** Different volumes of anti-human C3b-AF647 were single-colour assayed (0.25µL, 0.5µL,1µL). **D)** Different volumes of Syto™ BC Green were single-colour assayed (0.25µL, 0.5µL,1µL). Top Row: Pseudocolor plot of samples vs fluorescent channel of concatenated file of ungated populations. Bottom Row: histogram overlay of unstained (black) and single colour stain concentrations. All histograms were normalised to the mode.

Pure bacterial cultures were used both as a positive and negative control to assess the specificity of the antibodies. Since the titration of the nucleic acid stain was performed in cell pellet from vaginal swabs, bacterial liquid cultures of *G. vaginalis, L. crispatus* and *L. iners* (provided by Lauren Roberts)) were stained with the chosen new volume (0.5µL) of SYTO^{TM} BC Green dye. As expected, a clear discrimination between the unstained and stained positive population was observed in the three bacteria (Appendix 5.3). Secondly, pure bacterial cultures were used as negative controls since these cultures were not derived from clinical isolates and have not been coated with human immunoglobulins. Therefore, pure bacterial cultures were stained with antibodies and their respective isotype controls (Table 5.1.) under the same conditions as the vaginal swab's extractions procedure. Since all bacteria tested showed the same distribution, only data for *L. crispatus* (Figure 5.7.) is shown below, but results for *L. iners* and *G. vaginalis* are available in Appendix 5.4. and Appendix 5.5, respectively. Pure bacterial liquid cultures showed negative or negligible nonspecific binding percentages for anti-human IgG (Figure 5.7A), anti-human IgA (Figure 5.7B), goat IgG Isotype Control (Figure 5.7.C), anti-C3b (Figure 5.7D), and mouse IgG1 κ Isotype Control (Figure 5.7E).

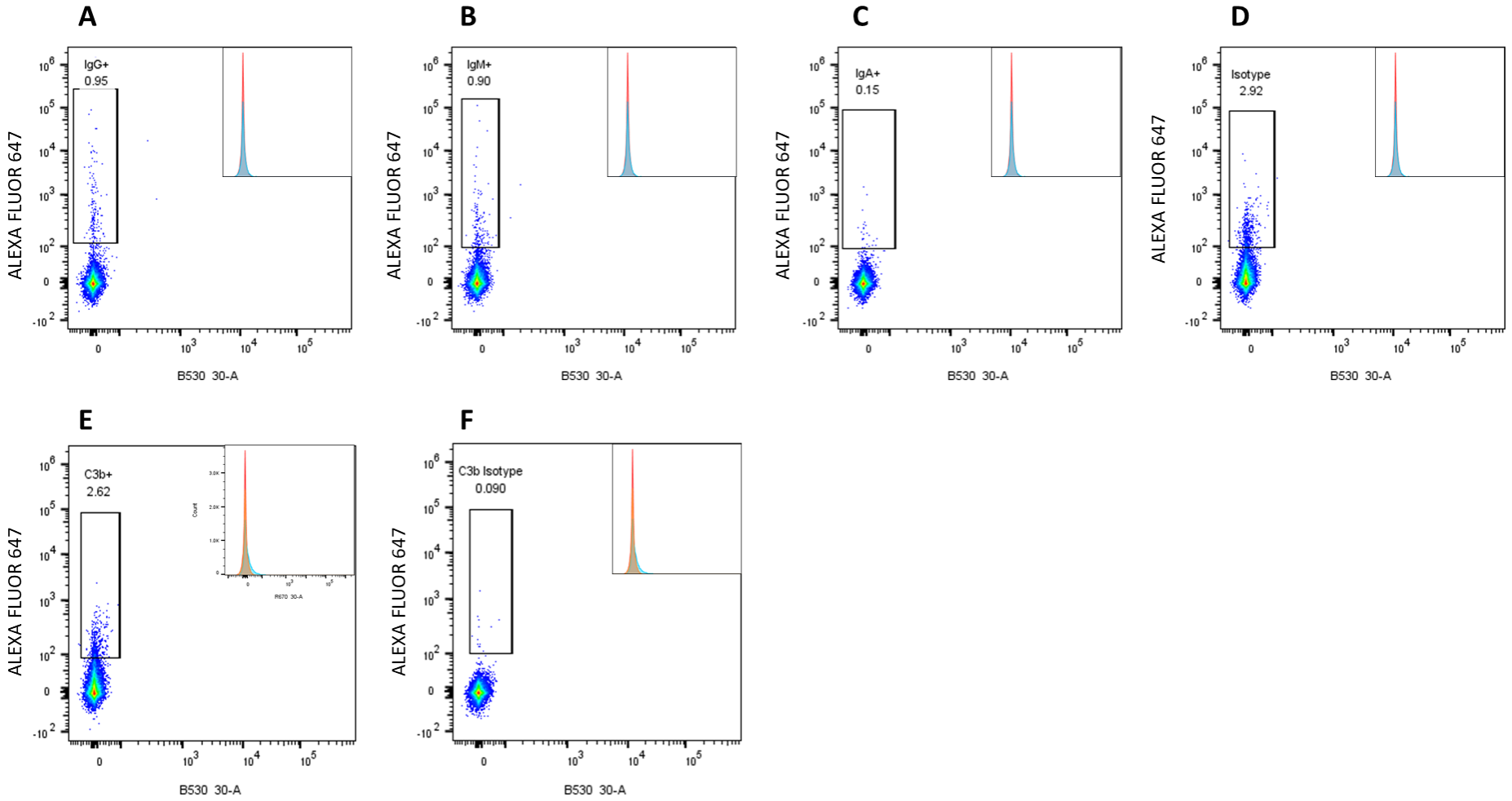


Figure 5.7. Pure bacterial cultures as controls. *L. crispatus* overnight cultures were washed three times and fixed overnight. After washing, bacterial pellet was stained with anti-human immunoglobulins and isotype controls. Gating strategy consisted in sequentially identifying the singlets (on FSC-A vs FSC-H), the bacterial population (on FSC-A vs –SSC-A) and the percentage of immunoglobulin bound by fluorescent expression of AF-647. Samples had negative or negligible binding percentages for **A)** anti-human IgG; **B)** anti-human IgA; **C)** goat IgG Isotype Control; **E)** anti-C3b; and **F)** mouse IgG1 κ Isotype Control. Data is shown as both pseudoplots and histogram overlay of unstained (red) and single colour stains for the relevant antibodies (blue) and isotype (orange).

However, in bacterial cells from vaginal swabs, the isotype control for the immunoglobulins' antibody (goat IgG Isotype Control) showed high fluorescence on the red channel, demonstrating a high unexpected non-specific binding (Figure 5.8 A). In contrast, the anti-C3b antibody showed no fluorescence/binding (Figure 5.8 B). These results indicated that C3b antibody (and its isotype) had a good quality performance but revealed the need of further optimisation of the immunoglobulins antibodies and their isotype (Table 5.2). I repeated the experiments up to n=3 vaginal swabs to assess if the non-specific binding was consistent across different samples and all of the tests showed a significant range of non-specific binding (1-70%). Different conditions were assayed (Table 5.2), including double staining with Syto + Isotype Control (to exclude non-bacterial cells or debris that could be causing the non-specific binding), pre-incubating the samples with Fc blocker, and to further dilute the antibody and the isotype. None of the conditions tested appeared to improve the performance of the isotype or reduce the non-specific binding (Table 5.2.), neither did the results show any consistency between different types of samples (e.g., pregnancy status, gestational age at sampling) to establish the reason of the non-specificity.

Therefore, it was concluded that there were several potential sources of error that could impact the reliability of the anti-immunoglobulin antibodies listed in Table 5.1.:1) selection of wrong isotype control, 2) usage of polyclonal antibodies, and 3) application not tested by manufacturer for flow cytometry for some of the antibodies.

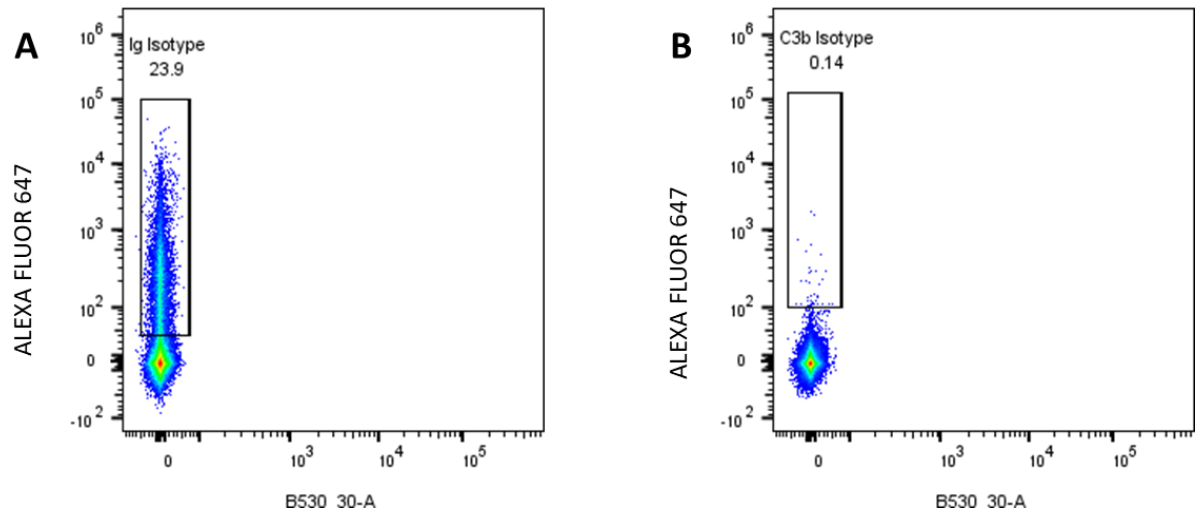


Figure 5.8. Performance of isotype control in real samples. Bacterial cells extracted from vaginal swabs were stained with isotype controls. **A)** Goat IgG Isotype Control served as an isotype control for anti-human immunoglobulins antibodies. **B)** Mouse IgG1 κ Isotype Control served as an isotype control for anti-C3b antibody.

Table 5.2. Troubleshooting for Goat IgG Isotype Control

Sample	Gestation	Conditions	Dilution 0.01mg/mL	Dilution 0.0034mg/mL	Dilution 0.0034mg/mL
Non-pregnant	NA	Single stain	22%	-	-
2V1484	24+4	Single stain	19%	-	-
Non-pregnant	NA	SYTO + Isotype	27%	-	-
Non-pregnant	NA	Dilute isotype and antibody FC blocker	25.2%	10.1%	5.91%
2V1736	20+4	Dilute isotype and antibody FC blocker	1.41%	0.53%	0.81%
2V1697	31+1	Dilute antibody and isotype	11.3%	2.77%	2.93%
2V1702	30+4	Dilute antibody and isotype	69.2%	48.6%	35.6%
2V1701	31+0	Dilute antibody and isotype	6.67%	0.99%	1.47%
2V1751	15+3	Dilute antibody and isotype	57.8%	56.2%	42.4%

Therefore, anti-human immunoglobulins polyclonal antibodies described in Table 5.1. were disregarded and a new set of anti-human immunoglobulin monoclonal antibodies conjugated with fluorochromes that emit on the red (APC) that were commercially available for flow cytometry were selected to help reduce and eliminate non-specific binding that can be associated with polyclonal antibodies (Table 5.3).

Table 5.3. Set of anti-human immunoglobulin monoclonal antibodies

Antibody	Fluorochrome	Clone	Isotype	Company	Catalogue number
Anti-human IgG	APC	IS11-3B2.2.3	Mouse IgG1κ	Miltenyi Biotec	130-119-772
Anti-human IgA	APC	IS11-8E10	Mouse IgG1κ	Miltenyi Biotec	130-113-998

After titrating the new set of antibodies (Figure 5.9.), we selected a volume of 1.5µL (0.0075mg/mL) for anti-IgA and anti-IgG staining. In this case, the performance of the isotype was as expected, and no non-specific binding was detected (Figure 5.10.). Pure bacterial liquid cultures of *G. vaginalis, L. iners* and *L. crispatus* (provided by Lauren Roberts) were used again as a control to confirm no binding of the new antibodies nor their isotypes (Figure 5.11. and Figure 5.12.).

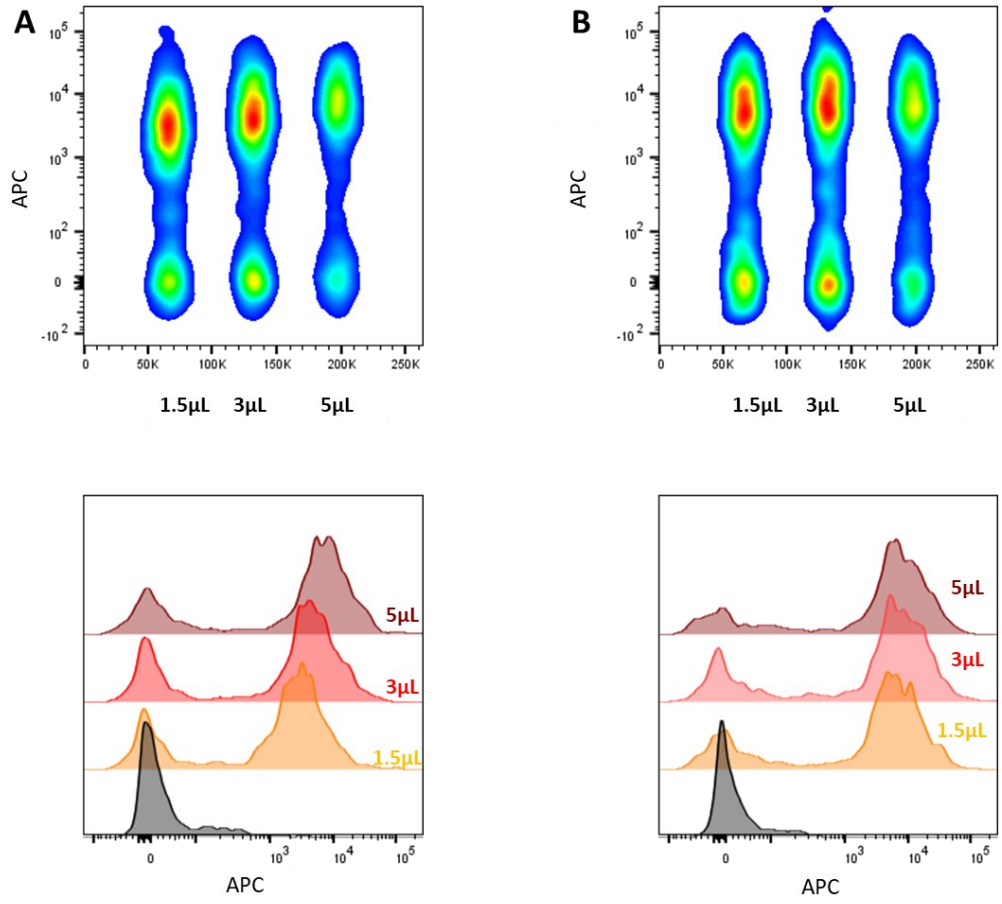


Figure 5.9. Antibody Titration APC. A) Different volumes of anti-human IgG-APC were single-colour assayed (1.5µL, 3µL, µL). **B)** Different volumes of anti-human IgA-APC were single-colour assayed (1.5µL, 3µL, 5µL). Top Row: Smooth pseudocolor plot of samples vs fluorescent channel of concatenation file of ungated populations. Bottom Row: histogram overlay of unstained (black) and dye concentrations (red tonality representative of concentration). All histograms were normalised to the mode.

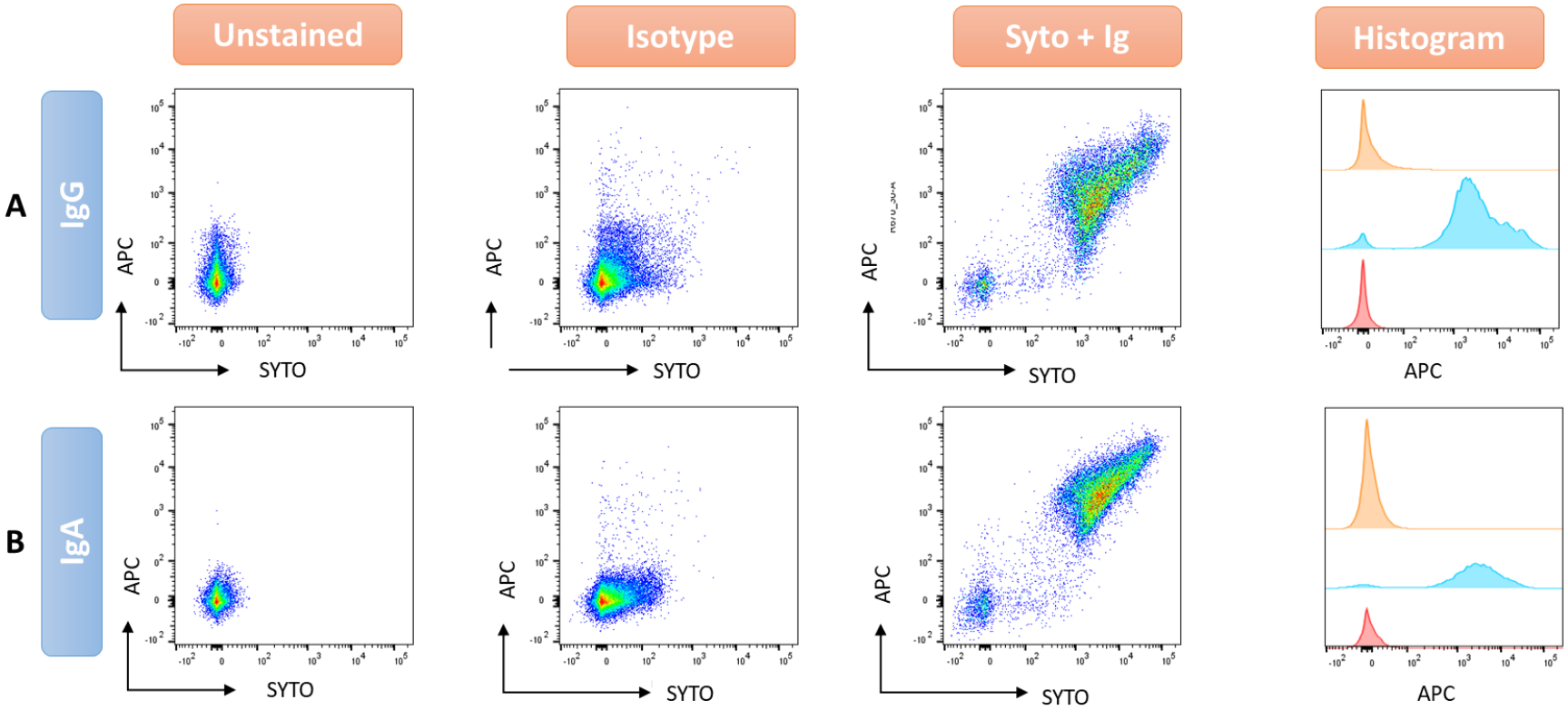


Figure 5.10. Performance of isotype in CVF samples. Bacterial cells extracted from vaginal swabs were stained with isotype controls and SYTO and anti-human immunoglobulins: **A)** IgG and **B)** IgA. First column indicates Unstained reference control pseudocolor plot. Second column represents the good performance of the isotype controls showing a pseudocolour plot similar to the unstained, with no detection of positive population. Third column represents the double staining detection of SYTO+ anti-human Ig. Finally, the fourth colour is a histogram overlay of unstained (red), single colour stain of anti-Ig (blue) and isotype control (orange).

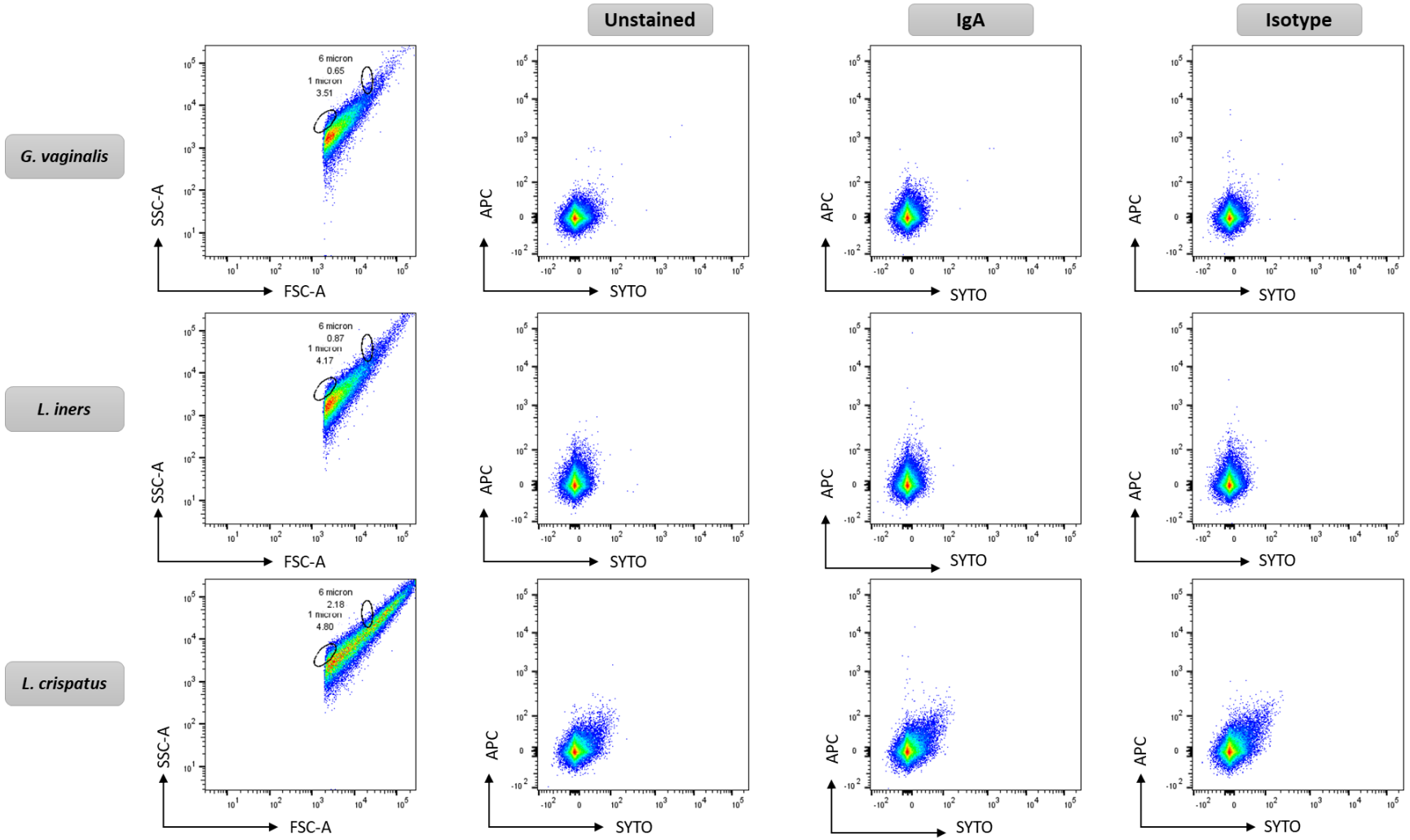


Figure 5.11. Pure bacterial cultures as control of anti-human IgA. Overnight cultures of *G. vaginalis, L. iners* and *L. crispatus* were washed three times and fixed overnight. After washing 3 times, bacterial pellet was stained with anti-human IgG-APC Gating strategy consisted in sequentially identifying the singlets (on FSC-A vs FSC-H), the bacterial population (on FSC-A vs –SSC-A). Data is shown as both pseudoplots and no differences were observed between pseudoplots of unstained and single colours of anti-IgG or Isotype.

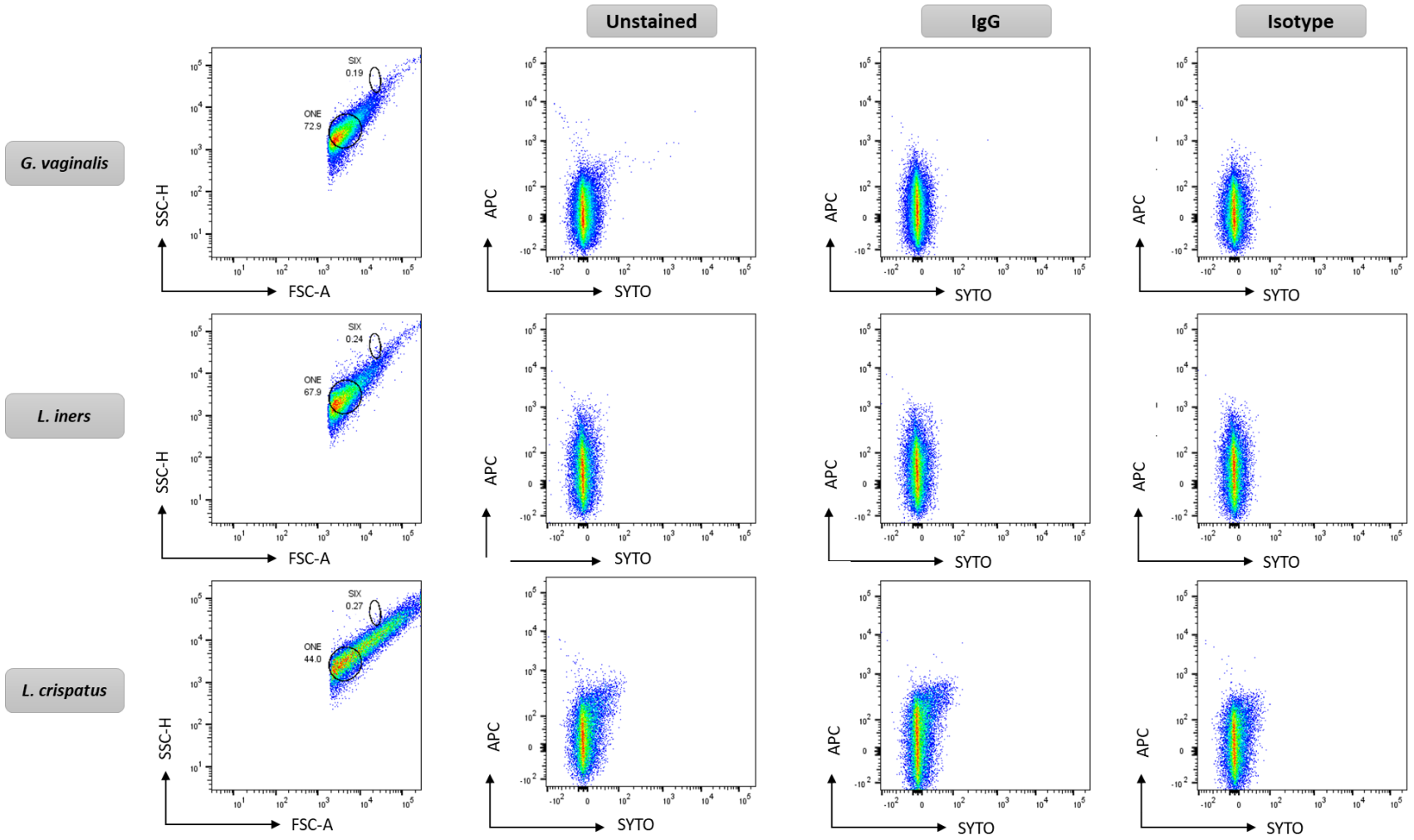


Figure 5.12. Pure bacterial cultures as control of anti-human IgG. Overnight cultures of *G. vaginalis, L. iners* and *L. crispatus* were washed three times and fixed overnight. After washing 3 times, bacterial pellet was stained with anti-human IgG-APC Gating strategy consisted in sequentially identifying the singlets (on FSC-A vs FSC-H), the bacterial population (on FSC-A vs –SSC-H). Data is shown as both pseudoplots and no differences were observed between pseudoplots of unstained and single colours of anti-IgG or Isotype.

As a final proof of detection of bacteria, the three species of bacteria used throughout the optimisation process (*L. crispatus, L. iners, G. vaginalis*)) were stained with SYTO and visualised using the confocal microscope (Figure 5.13.).

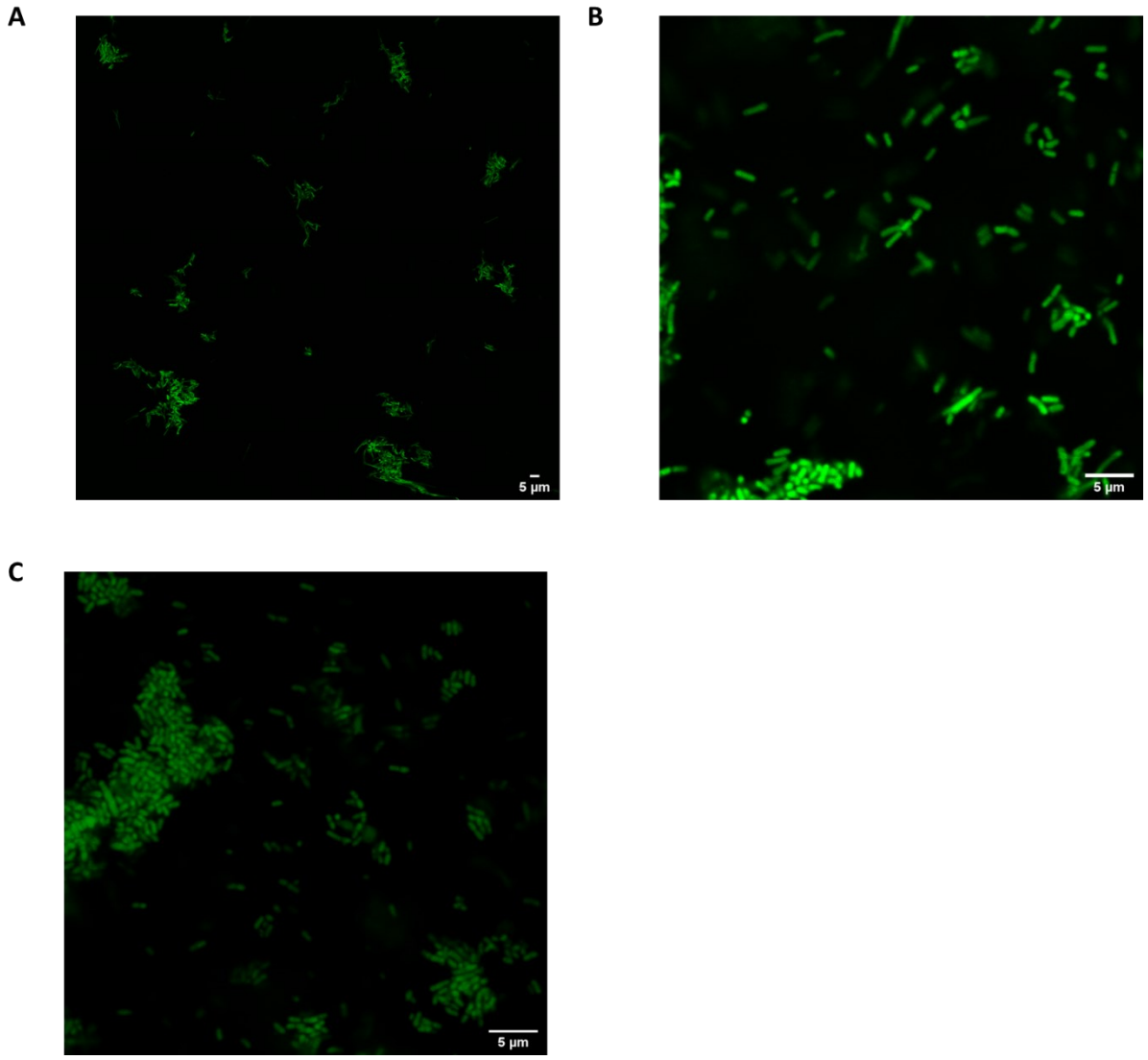


Figure 5.13. Confocal microscopy of bacteria typical from the reproductive tract stained with SYTO nucleic acid stain. Pure bacterial cultures of **A)** *L. crispatus*, **B)** *L. iners* **and C)** *G. vaginalis* were incubated for 30 minutes with SYTO nucleic acid stain. Green-fluorescent bacteria was detected in all cases.

The gating strategy depicted in Figure 5.14. was used to establish the percentage of bacteria bound to IgG, IgA and C3b. The population of interest was identified by sequentially gating on the singlets (FSC-A vs FSC-H) and the bacterial population (FSC-A vs SSC-A). The population of interest was the double positive population of Syto with the relevant anti-human antibody (SYTO+, IgG+; SYTO+ IgA+; SYTO+ C3b+). A tight gate was drawn, and the MFI was measured within the gate.

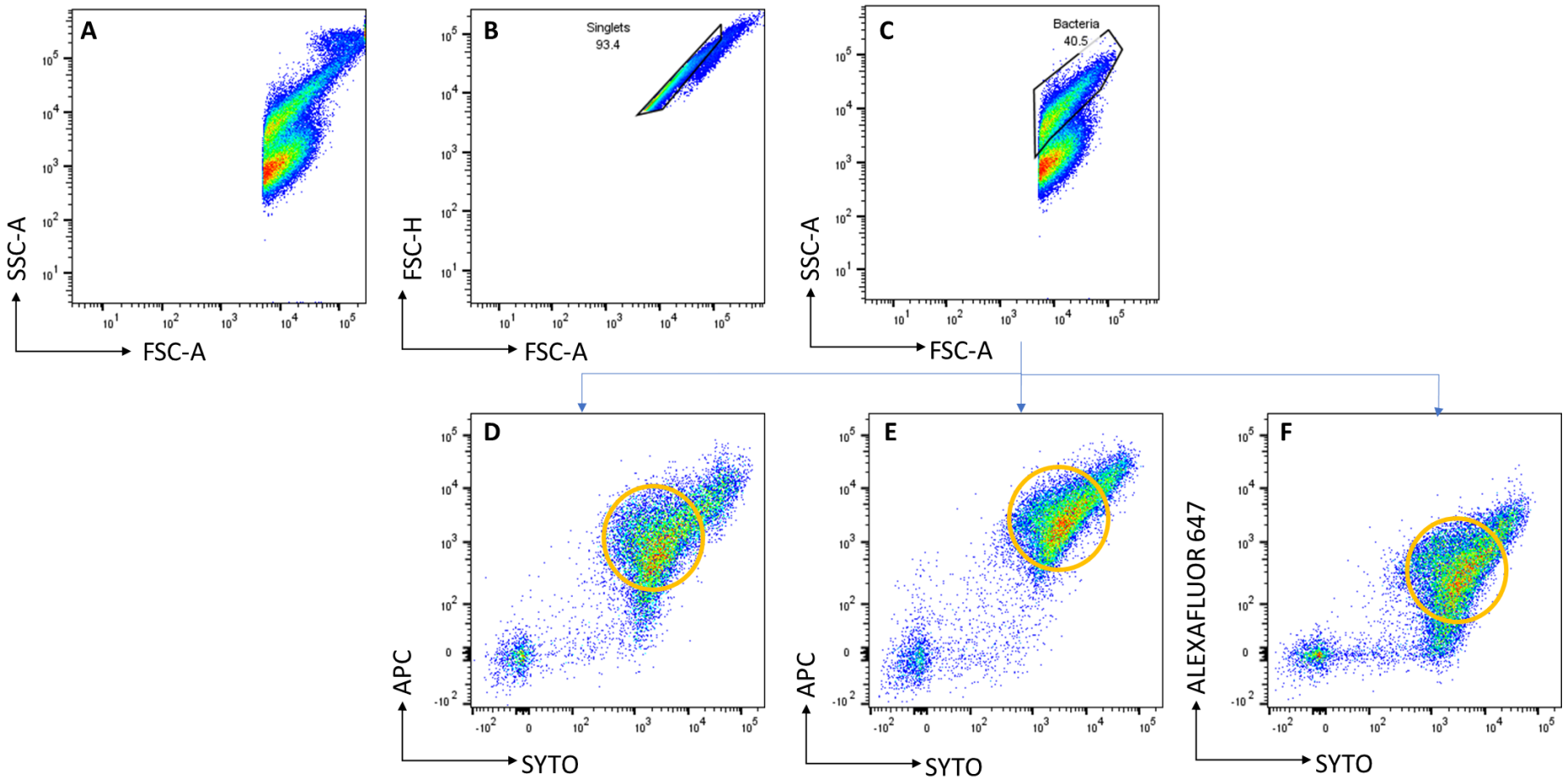


Figure 5.14. Gating strategies for the analysis of IgA, IgG and C3b bound bacteria in vaginal samples. Events were collected on FSC-A vs SSC-A **(A)**. First singlets were identified on FSC-A vs FSC-H **(B)**. Secondly, a tight gate was drawn on the bacteria population on FSC-A vs –SSC-A **(C)**. Lastly, frequencies of IgA **(D)**, IgG **(E)** and C3b **(F)** bound bacteria was assessed by bounding the double positive population of Syto+ with the respective APC or Alexa Fluor 647 anti-antibodies.

5.6. Results

5.6.1. Study population characteristics

Forty-three women from the VMET2 Research Study were included in this study. Based on their pregnancy outcome, women were divided into two groups: women who had a spontaneous preterm delivery before 37 weeks of gestation (PT, N=10, 23.26%) and women who had an uncomplicated term delivery (TU, N=33, 76.74%). The only statistical difference between groups was the mean gestation at delivery (PT, 30+5; TU, 39+5, $p<0.0001$). Details about the clinical characteristics from both groups are fully detailed in Table 5.4.

No differences were observed regarding ethnicity between PT and TU participants ($p=0.6858$). Most of the participants in both groups were white (approximately 70% in both groups). No differences were observed between groups for age ($p=0.1947$), BMI ($p=0.0872$), or gestation at sampling (timepoint A, $p=0.5443$; timepoint B, $p=0.8931$).

Table 5.4. Study cohort demographics and clinical description

	Preterm (PT)	Term (TU)	p-value
N (%)	10 (23.26%)	33 (76.74%)	NA
Gestation at delivery			Unpaired t test
Mean	30+5	39+5	p<0.0001
Range	24+3-36+5	37+1-42+2	****
Age			
Mean	35.3	33.82	Unpaired t test
Range	23-49	25-41	p=0.1947
BMI			Mann-Whitney
Median	29.15	23.5	
Range	20-47.60	18.20-43	p=0.0872
Ethnicity			Chi-square test for trend
White	7 (70%)	23 (69.7%)	0.6858
Black	2 (20%)	3 (9.1%)	
Other	1 (10%)	7 (21.1%)	
Gravida			Mann-Whitney
Median	4	2	p=0.0352
Range	1-11	1-10	*
Parity			Mann-Whitney
Median	1	1	p=0.1361
Range	0-7	0-4	
Gestation at sampling			
Timepoint A (N)	8	25	Unpaired t test
Mean	15+3	15+1	0.5443
Range	13+4-16.5	12+6-16+6	
Timepoint B (N)	6	25	Unpaired t test
Mean	22	21+4	0.8931
Range	20+2-24+5	19+3-23+5	
Longitudinal samples			NA
Samples (women)	8 (N=4)	32 (N=16)	
Risk factor			NA
Cervical treatment	1	14	
Cervical treatment + PTB	0	1	
Cervical treatment + PPROM	0	1	
PTB	4	1	
PTB+PPROM	2	9	
PTM/MTL	1	0	
MTL	2	2	
MTL+PPROM	0	5	

Women who underwent cervical shortening (≤25mm) at some point during their pregnancy were grouped and compared to woman who maintained a normal cervical length (>25mm) across pregnancy. Clinical characteristics about both groups are available in Table 5.5. Out of the 43 women included in this study, 7 (16.3%) experienced cervical shortening, whereas 36 (83.7%) did not short. The median shortest cervical length was shortest in women who experienced cervical shortening (19mm) compared to the median in the normal cervical length group (31.5mm) ($p<0.0001$). The mean gestational age at which women shorten was 23+5. The latency period from shortening to delivery was on average 68 days (9+5 weeks), although it ranged from 17 days (2+3 weeks) to 95 days (13+4 weeks). Finally, the majority of women who underwent cervical shortening had a preterm delivery (5/7, 71.4%), whereas only 5 women with a normal cervical length (5/36, 13.9%) ended delivering preterm ($p=0.0042$).

Table 5.5. Clinical characteristics of the study population according to the detection of cervical shortening

	Cervical shortening	Normal CL	p-value
N (%)	7 (16.3%)	36 (83.7%)	n.a
Shortest CL (mm)			Mann-Whitney
Median	19	31.5	p<0.0001
Range	9-25	26-40	****
Gestation at shortening			
Mean	23+5	n.a	
Range	15+5- 32+2	n.a	
Latency delivery			
Mean	9+5 (68 days)		
Range	2+3 (17d)- 13+4 (95d)		
Ethnicity			Chi-square
White	5 (71.4%)	25 (69.4%)	p=0.9167
Black	2 (28.6%)	3 (8.33%)	
Other	0 (0.0%)	8 (22.22%)	
Outcome			Fisher's exact
Preterm	5/7 (71.4%)	5/36 (13.9%)	p=0.0042
Term	2/7 (28.6%)	31/36 (86.11%)	**
Gravida			Mann-Whitney
Median	3	2	p=0.4131
Range	1-11	1-11	
Parity			Mann-Whitney
Mean	1	1	p=0.3704
Range	0-4	0-7	
Gestation at sampling			
Timepoint A			Unpaired t-test
Mean	15+6	15+2	p=0.0583
Range	14+4-16+6	12+6-16+5	
Timepoint B			Unpaired t-test
Mean	21+4	22+0	p=0.4378
Range	20+2-22+1	19+3-24+5	

CL: cervical length. D: days.

5.6.2. Immunoglobulin coating of bacteria in cervicovaginal fluid

Cervicovaginal microbial composition

A total of 63 vaginal swabs from 43 women were analysed in the current study. Samples were collected at two different timepoints A: 12-16 weeks (n=33) and B: 20-24 weeks (n=31). Prior to the analysis of immunoglobulin coating bacteria in CVF, the frequency of assigned Community State types (CSTs), diversity and richness was evaluated in the overall population (Table 5.6) and across pregnancy (Table 5.7.).

As summarised in Table 5.6., the 63 CVF samples were assigned to different CST according to the dominating species. The frequency of assigned community state types revealed same pattern as previously shown in chapter 3, being CST I the most common in the overall population (31/63, 49%), followed by CST III (8/63, 25.4%) and CST IV (8/63, 12.7%). The alpha diversity was measured by the Shannon Index. Samples classified as CST IV had a higher alpha diversity compared to samples classified as CST I (p=0.0004) and CST III (p=0.0243). The richness (species observed) median was also higher in women with a CST IV vaginal dominance compared to CST I (p=0.0015) and CST III (p=0.0022).

Table 5.6. Vaginal microbial composition and structure

CST	N samples (%)	Shannon Diversity Index	Sobs
CST I *L. crispatus*	31 (49%)	0.03862[a] (0.009-0.1371)	4[c] (3-7)
CST II *L. gasseri*	4 (6.34%)	0.7342 (0.2581-0.9293)	12.5 (6.5-18.5)
CST III *L. iners*	16 (25.4%)	0.1525[b] (0.01186-0.4888)	4[d] (3-6.75)
CST IV Heterogeneous	8 (12.7%)	1.373 [a, b] (0.3913-2.351)	28 [c, d] (14-42.25)
CST V *L. jenseniii*	4 (6.34%)	0.7306 (0.3772-0.7337)	7.5 (7-11)

Measure of alpha diversity measuring Shannon Diversity Index, and richness (species observed). Median and interquartile range are indicated. Statistical analysis was performed using Kruskal Wallis test with Dunn's multiple comparison post hoc test. The letters a, b, c, and d indicate statistically significance difference at $p<0.05$ within each row comparison.

When assessing the vaginal microbial composition across pregnancy (Table 5.7.), the most common CST at both timepoint A and B was *L. crispatus* CST I (51.5% and 46.4%). At timepoint A, CST IV was the second most prevalent CST (18.2), followed by CST III (15.2%). At timepoint B, samples were classified more frequently to CST III (36.67%) than to CST IV (6.67%). In this case also, no differences were observed in the Shannon Index (median Shannon Index timepoint A, 0.1632; median Shannon Index timepoint B 0.09903; p=0.8967) nor the richness (species observed) (median Sobs 5 vs 5.5., p=0.8501) (Table 5.7.). Of note, transitions between timepoint A and B were not evaluated since not only longitudinal matched A+B were analysed; however, within the 20 women with matched longitudinal samples (n=40 samples), women classed as CST I and CST II had stable vaginal microbial compositions, whereas women classed as CST IV transition towards a CST III (n=2/3) and one women classed as CST V also transition towards a CST III (1/2).

Table 5.7. Vaginal microbial composition and structure across pregnancy

CST	Timepoint A	Timepoint B
CST I *L. crispatus*	17 (51.5%)	14 (46.67%)
CST II *L. gasseri*	2 (6.1%)	2 (6.67%)
CST III *L. iners*	5 (15.2)	11 (36.67%)
CST IV Heterogeneous	6 (18.2%)	2 (6.67%)
CST V *L. jenseniii*	3 (9.1)	1 (3.33%)
Shannon Diversity Index	0.1632	0.09903
SObs	5	5.5

Measure of alpha diversity measuring Shannon Diversity Index, and richness (species observed) in the overall cohort at timepoint A (12-16 weeks) and timepoint B (20-24 weeks). Median is indicated. Statistical analysis was performed using Mann-Whitney two-tailed test.

Cervicovaginal bacteria bound to immunoglobulins across pregnancy

A total of 63 vaginal swabs from 43 women were analysed by flow cytometry. Only four swabs were not included for statistical analysis after not detecting enough bacterial cells (no positive syto signal on the fluorescence channel). Cervicovaginal bacteria bound to immunoglobulins was evaluated in 59 CVF samples.

First, the potential changes in immunoglobulin binding of bacteria across pregnancy was analysed. High percentages of bacteria were bound to IgG (median >55% at both timepoints, range 20-66%), IgA (median >60% at both timepoints; range 11-78%) and C3b (median 57% at both timepoints; range 13-67%). But the percentage of IgG, IgA or C3b bound bacteria did not significantly change across pregnancy (p=0.4535, p=0.6676, and p=0.5818, respectively between timepoint A and B) (Figure 5.15.) The intensity of the binding (MFI) and the coating index (% of immunoglobulin bound bacteria *MFI) did not statistically change across pregnancy either (Figure 5.15.).

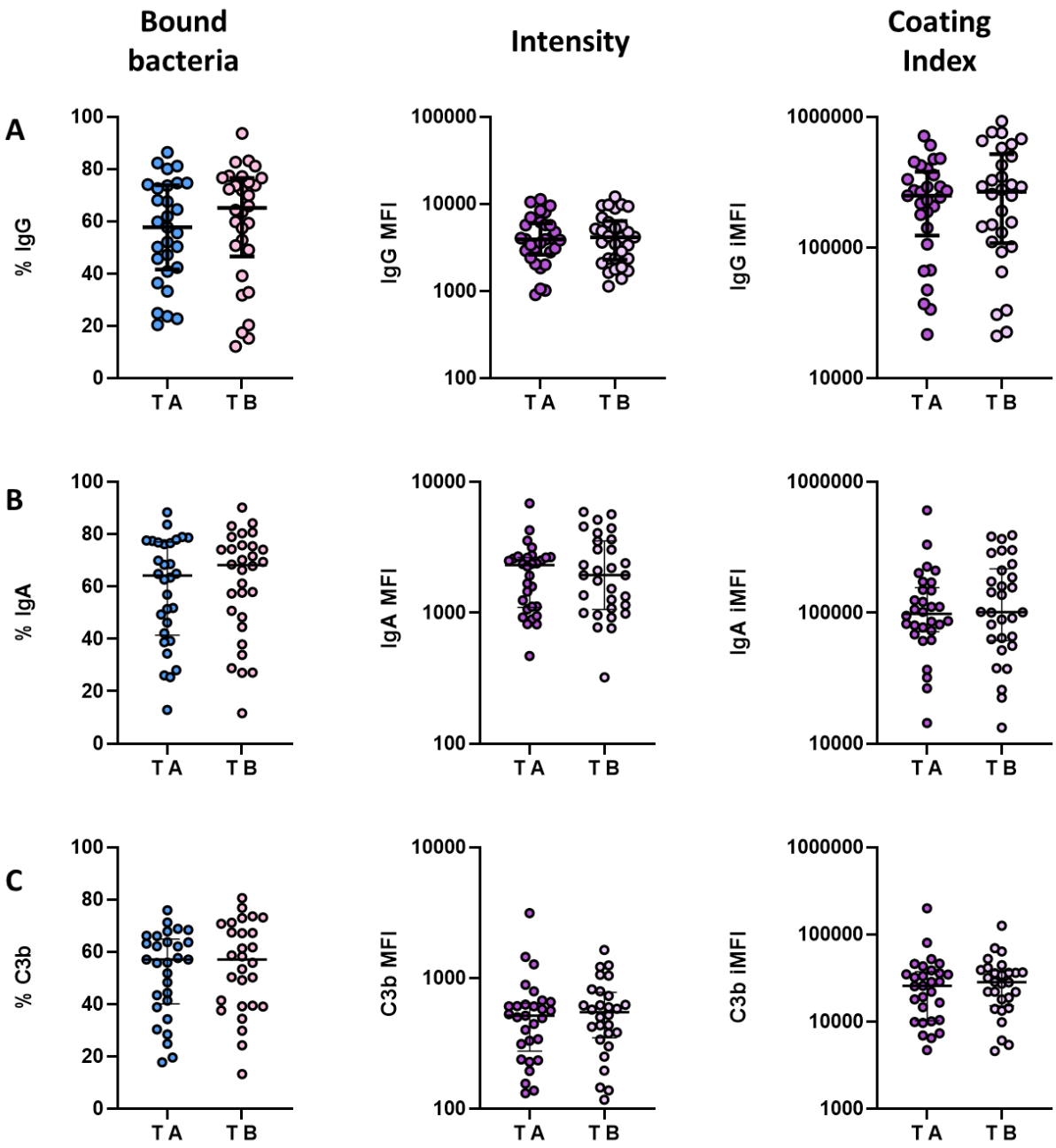


Figure 5.15. Coating of bacteria in cervicovaginal fluid across pregnancy. Coating of bacteria with **A)** IgG, **B)** IgA and **C)** C3b represented as percentage of bacteria with bound immunoglobulin or C3b (first column); intensity (MFI) of bacteria with bound immunoglobulin or C3b (second column); and integrated MFI of bacteria with bound immunoglobulin or C3b (third column). Measured across pregnancy; timepoint A (TA, n=29): 12-16 weeks of gestation; timepoint B (TB, n=30): 20-24 weeks of gestation). Data is presented as median values with interquartile range.

Correlation analysis revealed a very strong positive correlation between the percentages of immunoglobulins bound to bacteria (Figure 5.16.). The strongest correlation was observed between bound IgA and IgG (r=0.8492, p<0.0001) (Figure 5.16.A). But the correlations between bound to immunoglobulins with bound to C3b was also strong: r=0.7014 for %IgA-%C3b (Figure 5.16.B) and r=0.6692 for %IgG-%C3b (Figure 5.16.B.) (both p<0.0001).

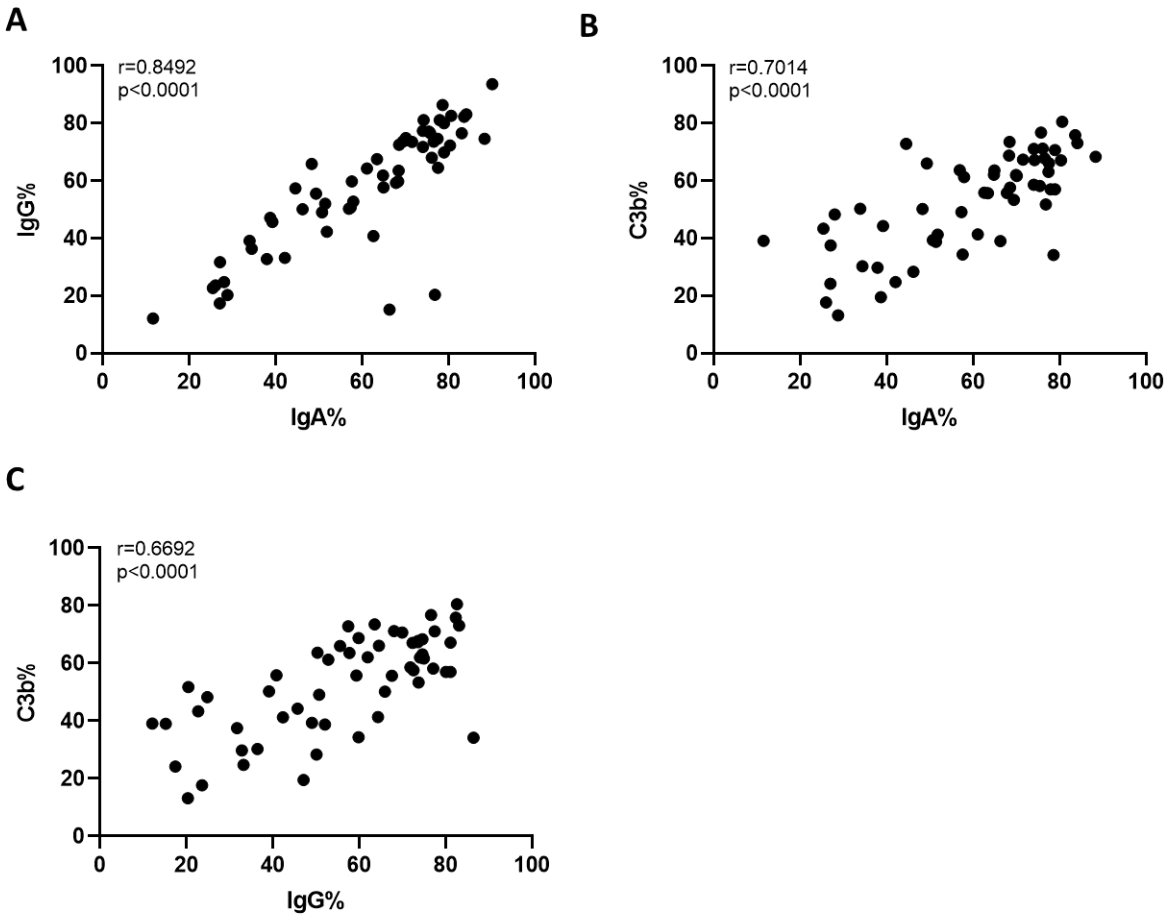


Figure 5.16. Correlation between percentage of immunoglobulins and C3b bound to bacteria. A) Correlation between percentage of IgG and percentage of IgA bound to bacteria. **B)** Correlation between the percentage of C3b and percentage of IgA. **C)** Correlation between the percentage of C3b and percentage of IgG. The r coefficient of correlation (from Spearman's correlation of determination) and the respective p-value is shown in each pair.

Cervicovaginal bacteria bound to immunoglobulins and the local microbial composition

Next, the percentages of bacteria bound to immunoglobulins depending on CST was analysed. Overall, higher percentages were observed in samples who were classified as a CST I vaginal composition.

The percentage of bacteria bound to IgG was higher in women with a *L. crispatus* vaginal dominance (CST I) (median, 73.5) compared to women with a diverse CST IV vaginal composition (median, 20.40) (p=0.0008) (Figure 5.17. A). The IgG MFI was higher in women dominated by *Lactobacillus* spp. A decrease in the MFI of women with a CST IV composition was seen compared to women with a CST I (p=0.0038) and CST III (p=0.0082) (Figure 5.17.B). The coating index (also referred to as integrated MFI) followed the same pattern of higher bacteria bound in women with a CST I and CST III compared to CST IV (Figure 5.17.C).

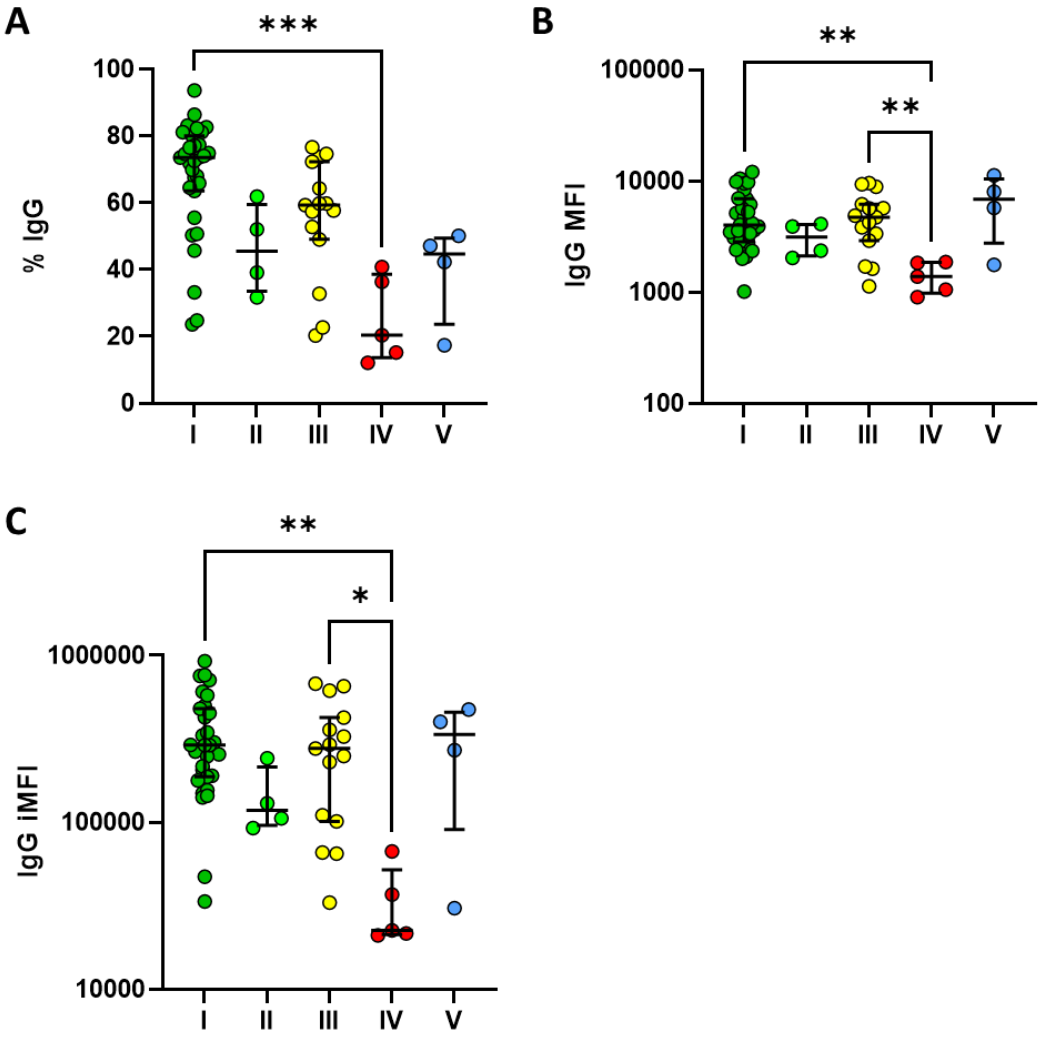


Figure 5.17. Bacteria bound to IgG according to CST vaginal microbial composition. A) Percentage of bacteria bound to IgG. **B)** IgG MFI and **C)** Integrated MFI (coating index), according to the local cervicovaginal microbial composition: CST I, *L. crispatus* (n=31); CST II, *L. gasseri* (n=4); CST III, *L. iners* (n=15); CST IV, heterogeneous (n=5); CST V, *L. jensenii* (n=4). *p<0.5; **p<0.01; ***p<0.001. Statistical analysis was performed using a Kruskal-Wallis test with Dunn's multiple comparison post hoc test; and data is presented as median values with interquartile range.

The percentage of bacteria bound to IgA also varied depending on the local cervicovaginal composition (Kruskall-Wallis p=0.0166). Women with a CST I vaginal composition had a higher median percentage of bacteria bound to IgA, however significance was lost after Dunn's multiple comparison test (CST I vs CST IV, p=0.0544) (Figure 5.17.A). In contrast to IgG MFI, there was higher intensity binding in women with a CST III *L. iners* dominance compared to a CST I *L. crispatus* (median 3023 vs 1355, p=0.0225) (Figure 5.18.B) and no differences in the integrated MFI.

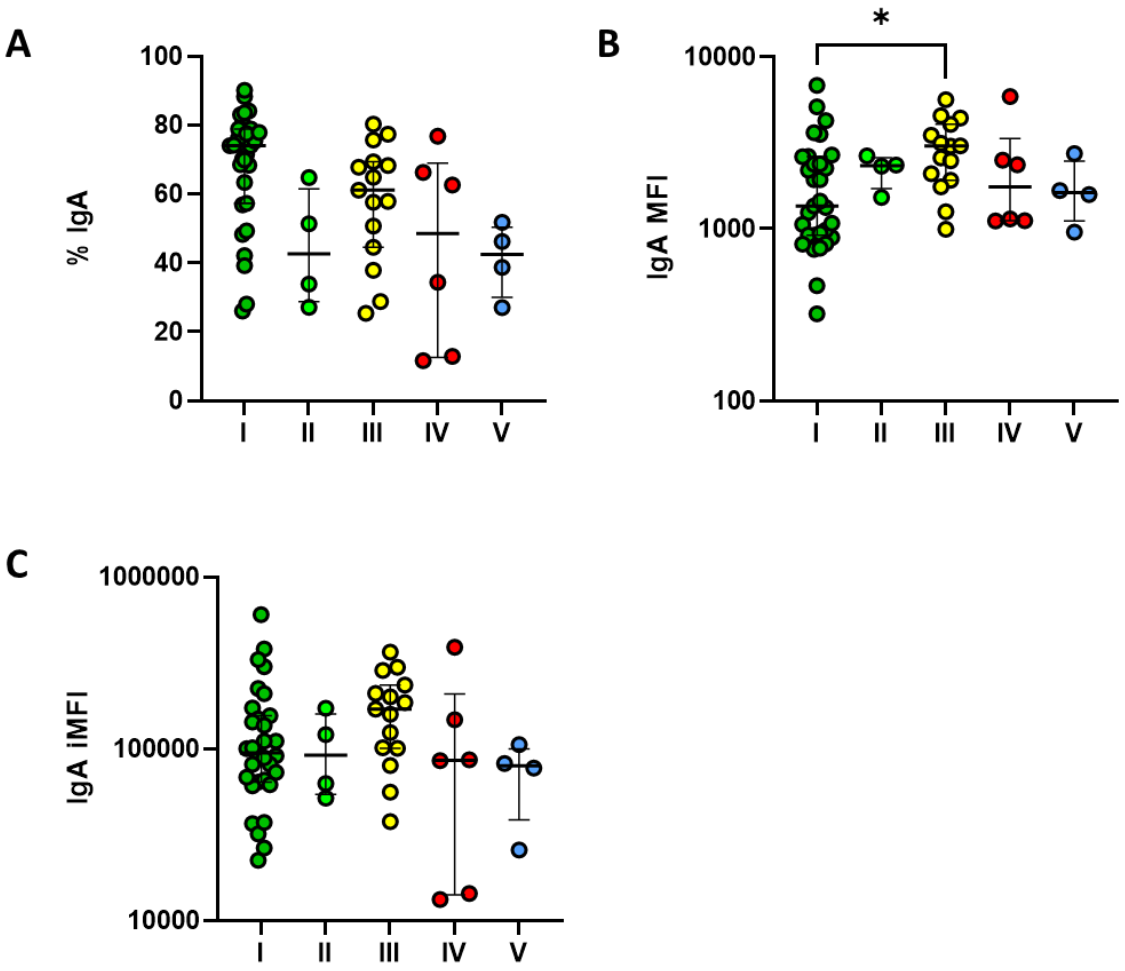


Figure 5.18. Bacteria bound to IgA according to CST vaginal microbial composition. A) Percentage of bacteria bound to IgG. **B)** IgA MFI and **C)** Integrated MFI (coating index), according to the local cervicovaginal microbial composition: CST I, *L. crispatus* (n=31); CST II, *L. gasseri* (n=4); CST III, *L. iners* (n=15); CST IV, heterogeneous (n=6); CST V, *L. jensenii* (n=4). *p<0.05. Statistical analysis was performed using a Kruskal-Wallis test with Dunn's multiple comparison post hoc test; and data is presented as median values with interquartile range.

Finally, the highest percentage of bacteria bound to C3b was seen in women with a CST I vaginal composition. Some associations between the percentage of bacteria bound to C3b might appear in response to the vaginal dominance of *Lactobacillus* (Kruskal Wallis p=0.0419), however, statistical significance between groups was lost after Dunn's post hoc test. (Figure 5.19. A). The intensity of the binding was considerably decreased in women with a CST IV composition compared to women dominated with *Lactobacillus* spp., including CST I (p=0.0150) and CST III (p=0.0001). Women with a CST III also had a higher MFI than women with a CST I (p=0.0471) (Figure 5.19.B.). A decrease C3b coating index was also observed in women with a CST IV vaginal composition compared to CST I (p=0.0058) and CST III (p=0.0007). (Figure 5.19.C).

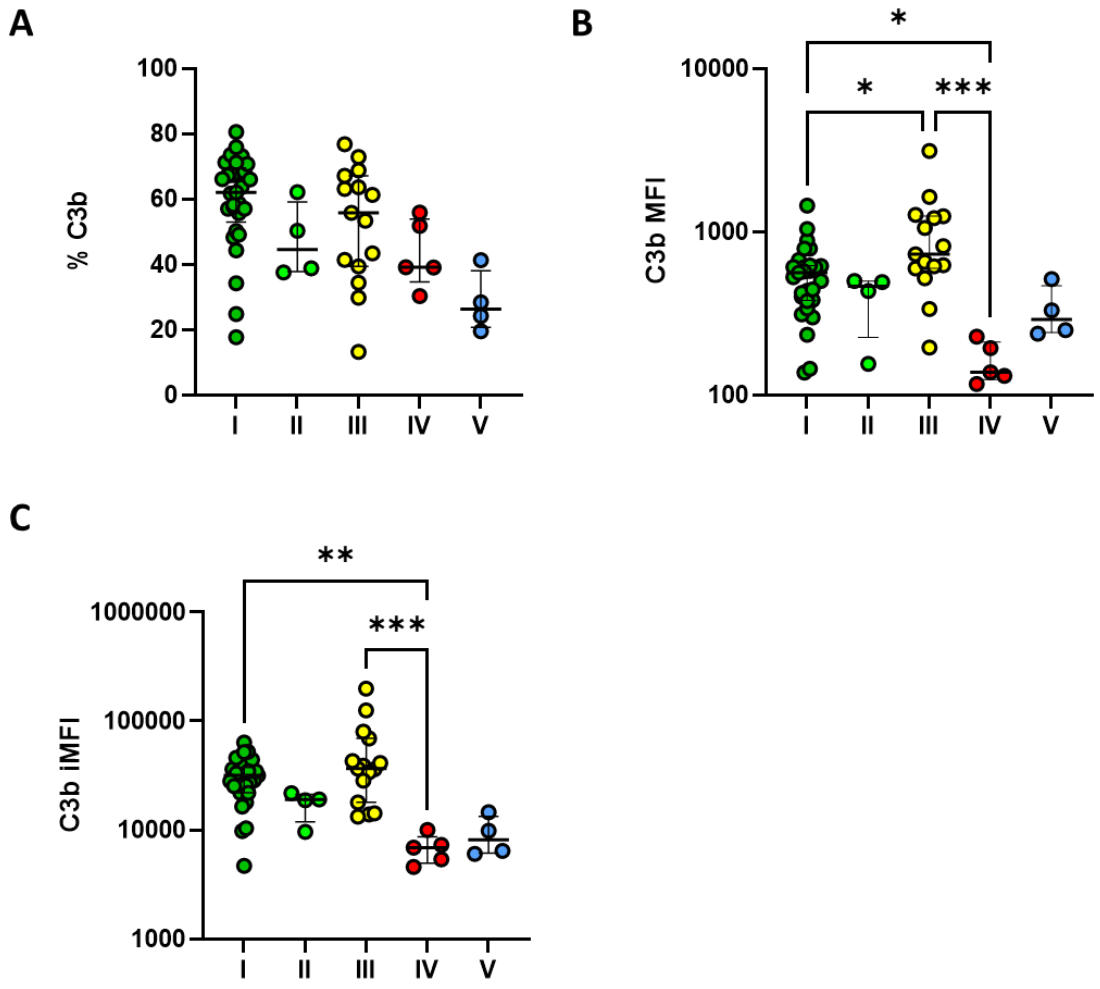


Figure 5.19. Bacteria bound to C3b according to CST vaginal microbial composition. A) Percentage of bacteria bound to C3b. **B)** C3b MFI and **C)** Integrated MFI (coating index), according to the local cervicovaginal microbial composition: CST I, *L. crispatus* (n=29); CST II, *L. gasseri* (n=4); CST III, *L. iners* (n=15); CST IV, heterogeneous (n=5); CST V, *L. jensenii* (n=4). *p<0.05; **p<0.01; *** p<0.001. Statistical analysis was performed using a Kruskal-Wallis test with Dunn's multiple comparison post hoc test; and data is presented as median values with interquartile range.

5.6.3. Coating of bacteria and local immune response

Correlations between percentages of bound bacteria and concentrations of inflammatory mediators (cytokines and complement proteins) were also assessed (Table 5.8.). Although no correlation was seen with any of the cytokines, there were significant correlations observed between opsonisation status and complement protein concentrations. The percentage of bacteria bound to IgG was the condition with more significant correlations associated. In all cases, the majority of the correlations were weak ($r<0.5$) and negatively correlated. For example, the percentage of binding to IgG, but not to IgA, was negatively correlated to the concentration of C1q, a protein from the classical pathway required for the activation of this pathway after recognising IgG-complexes, whereas IgA-complexes don't have the ability to trigger the classical pathway.

Table 5.8. Correlations between percentage of immunoglobulins or C3b bound to bacteria and levels of inflammatory mediators

The r coefficient of correlation from Spearman's correlation and p-value is indicated for each pair. Statistical significant pairs were highlighted in orange

	%IgG		%IgA		%C3b	
	r coefficient	p-value	r-coefficient	p-value	r coefficient	p-value
[IL-1β]	r=-0.2101	p= 0.1102	r=-0.1563	p=0.2331	r=-0.1839	p=0.1708
[IL-6]	r=0.0063	p=0.9620	r=0.0399	p=0.7618	r=-0.1675	p=0.2130
[IL-8]	r=0.1605	p=0.2245	r=0.1828	p=0.1620	r=0.0380	p=0.7789
[C1q]	r=-0.3360	p=0.0114	r=-0.1708	p=0.2081	r=-0.0739	p=0.5952
[C2]	r=0.1071	p=0.4194	r=0.0024	p=0.9850	r=-0.0057	p=0.9662
[C4]	r=-0.3850	p=0.0028	r=-0.3055	p=0.0186	r=-0.3087	p=0.0206
[C4b]	r=-0.1092	p=0.4105	r=-0.0362	p=0.7835	r=-0.0586	p=0.6652
[MBL]	r=-0.0329	p=0.8043	r=-0.0319	p=0.8091	r=-0.0605	p=0.6547
[Factor B]	r=-0.4472	p=0.0005	r=-0.3113	p=0.0195	r=-0.2997	p=0.0277
[Factor D]	r=-0.5314	p<0.0001	r=-0.3868	p=0.0023	r=-0.3420	p=0.0092
[C3]	r= 0.4109	p=0.0014	r=-0.2058	p=0.1180	r=-0.2614	p=0.0517
[C3a]	r=-0.015	p=0.9104	r=-0.1151	p=0.3810	r=-0.1292	p=0.3380
[C3b]	r=-0.3969	p=0.0020	r=-0.2881	p=0.0269	r=-0.1689	p=0.2133
[C5]	r=-0.2474	p=0.0589	r=-0.1222	p=0.3523	r=-0.1436	p=0.2865
[C5a]	r=0.2174	p=0.0981	r=0.0981	p=0.4560	r=0.0972	p=0.4718
[Factor I]	r=-0.4174	p=0.0010	r=-0.2718	p=0.0357	r=-0.2893	p=0.0290
[Factor H]	r=-0.2127	p=0.1090	r=-0.2114	p=0.1079	r=-0.2611	p=0.0519

Rank of percentage bound to IgG and the local immune response

A similar pattern was observed when grouping the concentration of inflammatory mediators based on the lowest and highest percentile of percentage bounding. For the percentage of bacteria bound to IgG, the bottom 25% percentile was 42.30%; and the top 75% percentiles was 74.6%. No differences were observed in the concentration of any of cytokines (IL-1β, IL-6 or IL-8), based on the percentage of bacteria bound to IgG (Figure 5.20).

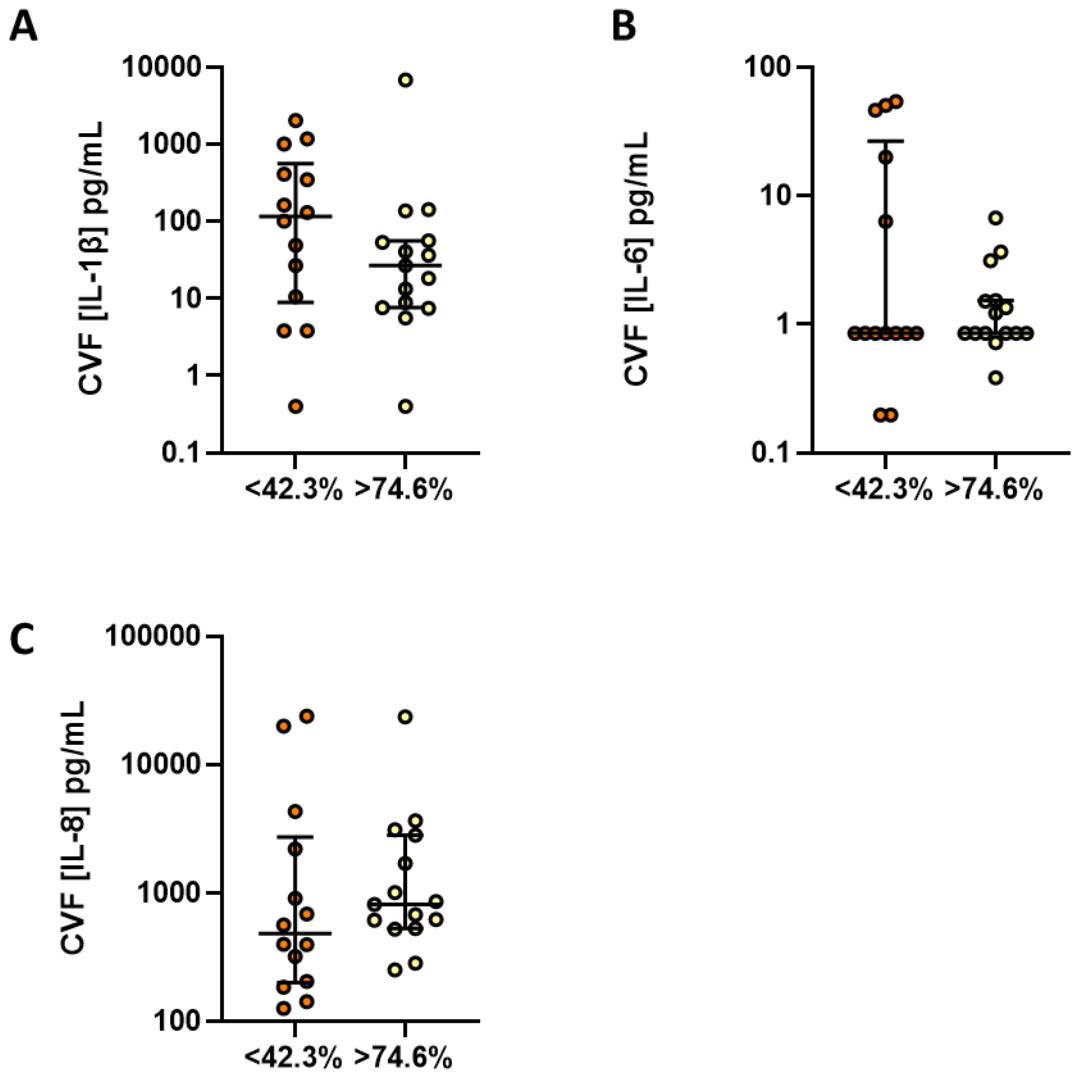


Figure 5.20. Concentration of CVF cytokines based on the lower and higher percentile rank of bacteria bound to IgG. Scatter dot plots of CVF concentration of cytokines according to the lowest (<42.3%, n=14) and the highest percentile (>74.6%, n=15) of percentage of IgG bound to bacteria. Concentration of **A)** IL-1β, **B)** IL-6 and **C)** IL-8. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

When assessing complement proteins from the classical pathway (Figure 5.21), the concentration of C1q and C4 were increased when the percentage bound to IgG was lower (p=0.0126, p=0.0035, respectively).

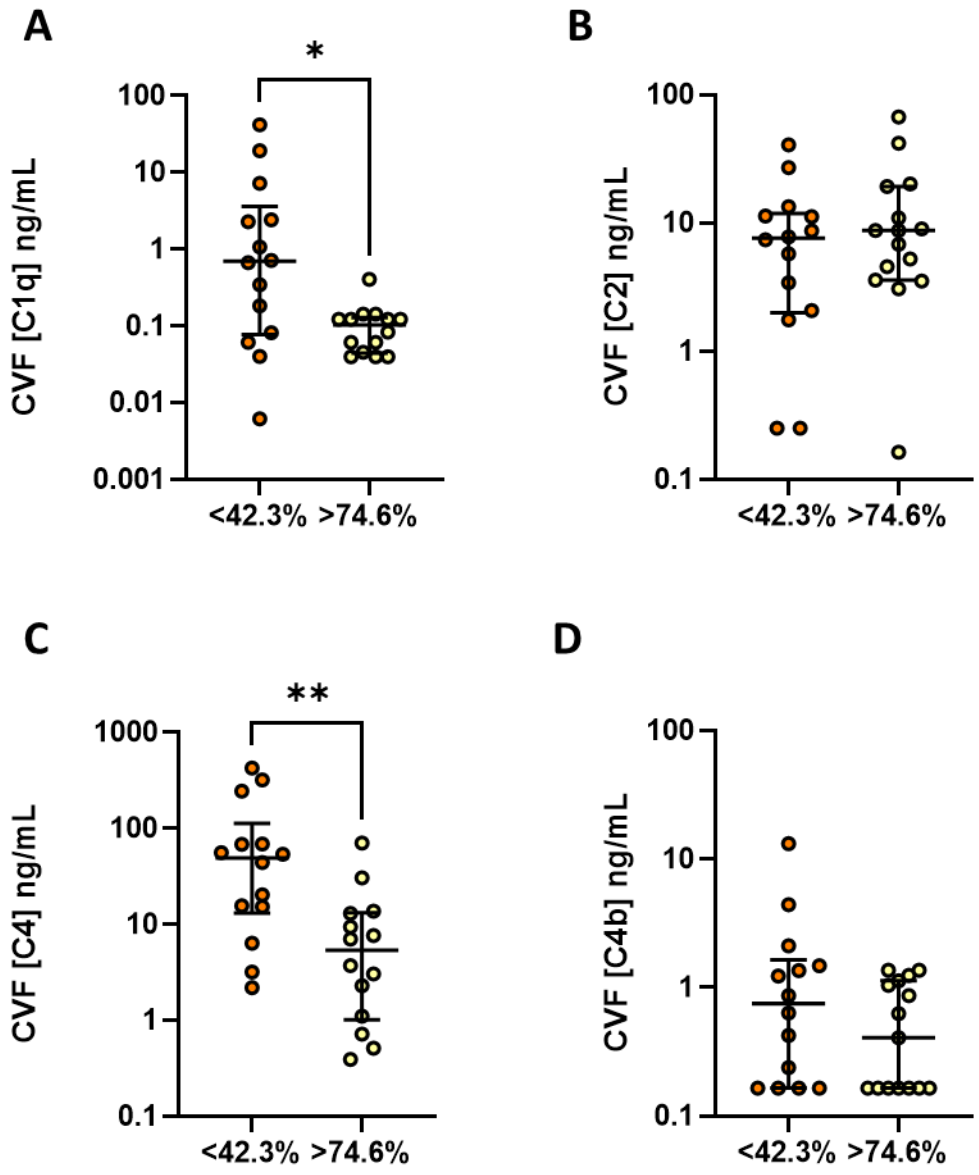


Figure 5.21. Concentration of CVF complement proteins from the classical pathway based on the lower and higher percentile rank of bacteria bound to IgG. Scatter dot plots of CVF concentration of cytokines according to the lowest (<42.3%, n=14) and the highest percentile (>74.6%, n=15) of percentage of IgG bound to bacteria. Concentration of **A)** C1q, **B)** C2 **C)** C4 and **D)** C4b. *p<0.05; **p<0.01. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

With reference to the lectin pathway, the concentration of MBL was not influenced by the percentage of bacteria bound to IgG (Figure 5.22.A). However, when the binding percentage was low, compared to a higher binding, the concentration of both Factor B (median 0.3796 vs 0.026 ng/mL, p=0.0012) and Factor D (median 0.1994 vs 0.0305 ng/mL, p=0.0003) from the alternative pathway was increased (Figure 5.22.B, C).

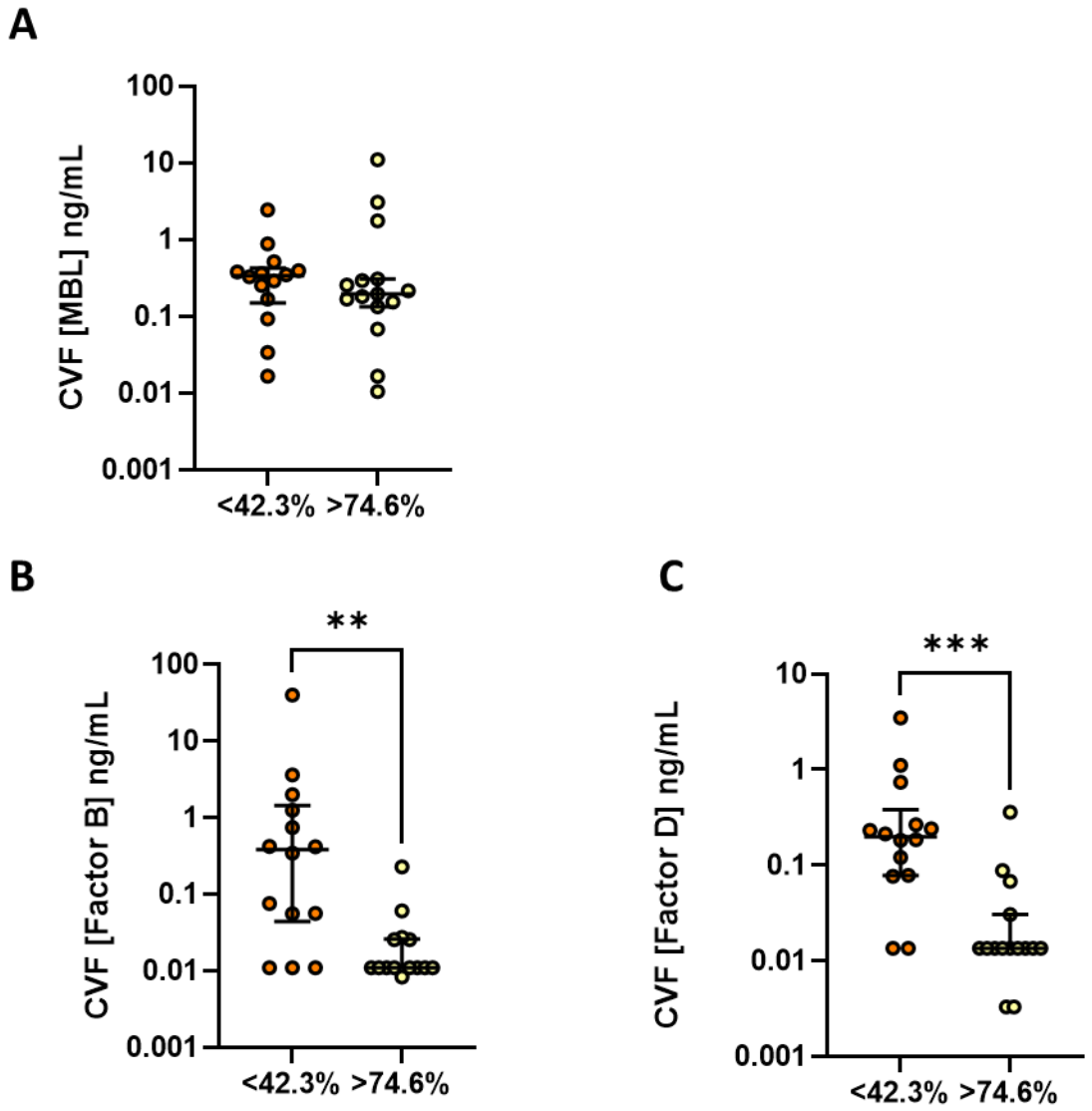


Figure 5.22. Concentration of CVF complement proteins from the lectin and the alternative pathways based on the lower and higher percentile rank of bacteria bound to IgG. Scatter dot plots of CVF concentration of cytokines according to the lowest (<42.3%, n=14) and the highest percentile (>74.6%, n=15) of percentage of IgG bound to bacteria. Concentration of **A)** MBL, **B)** Factor B and **C)** Factor D. **p<0.01; ***p<0.001. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

The complement cascade converges on the activation of C3 and C5 convertases. The concentration of C3 and C3b were higher in women who had lower percentages of bacteria bound to IgG, compared to higher bindings (p=0.0055, p=0.0039, respectively) (Figure 5.23. A, C). At the level of C5 convertase action, C5a was also higher in women with lower percentages bacteria binding to IgG (p=0.03230) (Figure 5.23.D).

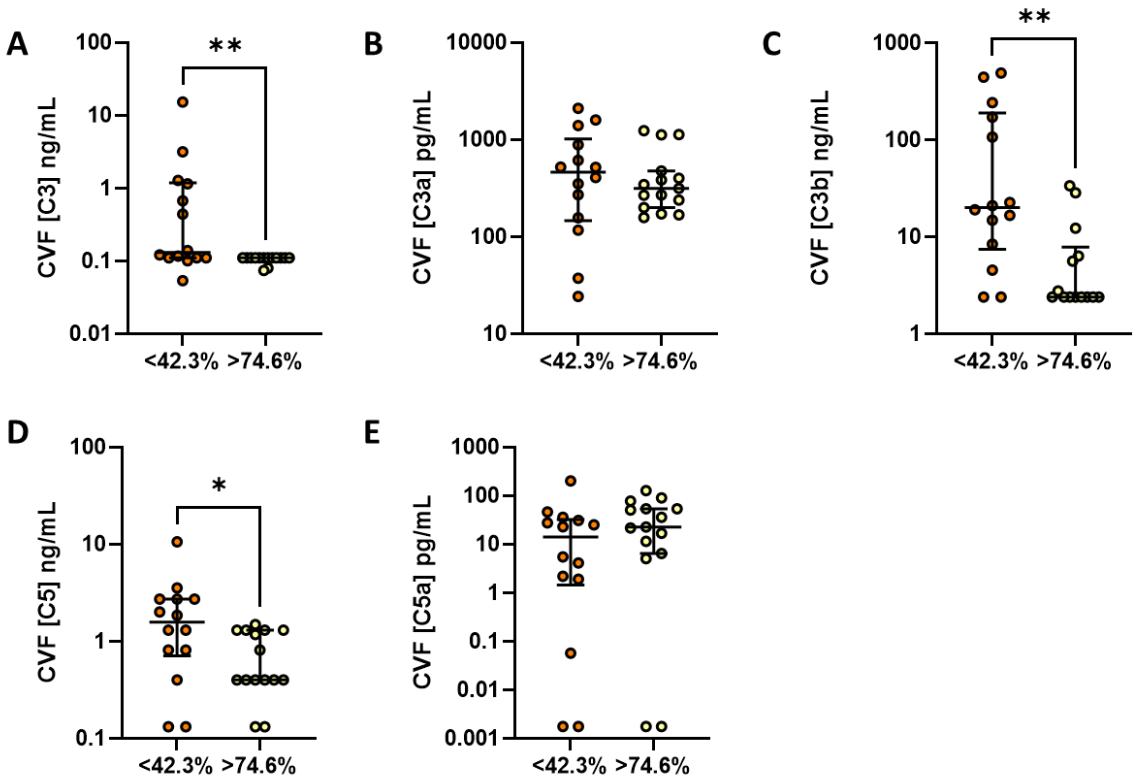


Figure 5.23. Concentration of CVF complement proteins from C3 and C5 convertases based on the lower and higher percentile rank of bacteria bound to IgG. Scatter dot plots of CVF concentration of cytokines according to the lowest (<42.3%, n=14) and the highest percentile (>74.6%, n=15) of percentage of IgG bound to bacteria. Concentration of **A)** C3, **B)** C3a, **C)** C3b, **D)** C5 and **E)** C5a. *p<0.5; **p<0.01. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

When measuring complement regulators (Figure 5.24), increased concentrations of Factor H and Factor I were seen in women with lower percentages of bacteria binding to IgG, however this was only significant for Factor I (p=0.0006) (Figure 5.24.B).

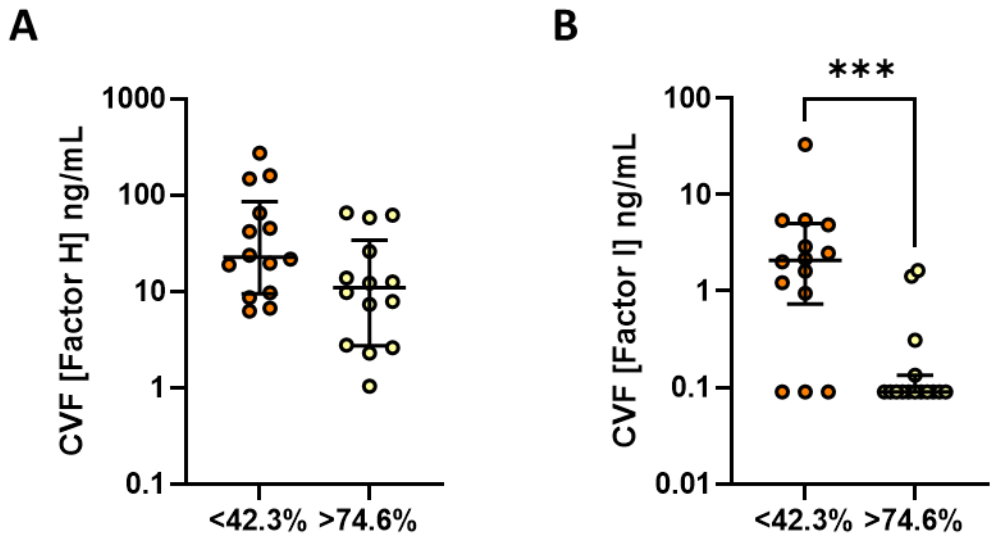


Figure 5.24. Concentration of CVF complement regulators based on the lower and higher percentile rank of bacteria bound to IgG. Scatter dot plots of CVF concentration of cytokines according to the lowest (<42.3%, n=14) and the highest percentile (>74.6%, n=14) of percentage of IgG bound to bacteria. Concentration of **A)** Factor H and **B)** Factor I. ***p<0.001. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

Rank of percentage bound to IgA and the local immune response

The percentage of bacteria bound to IgA in relation to inflammatory mediators was also examined. The 25th centile of IgA binding was 44.93%, whereas the 75th centile was 76.4%. Firstly, when assessing the CVF concentrations of cytokines IL-1β, IL-6 and IL-8, no differences were observed between women with high or low percentage of bacteria bound to IgA (Figure 5.24.).

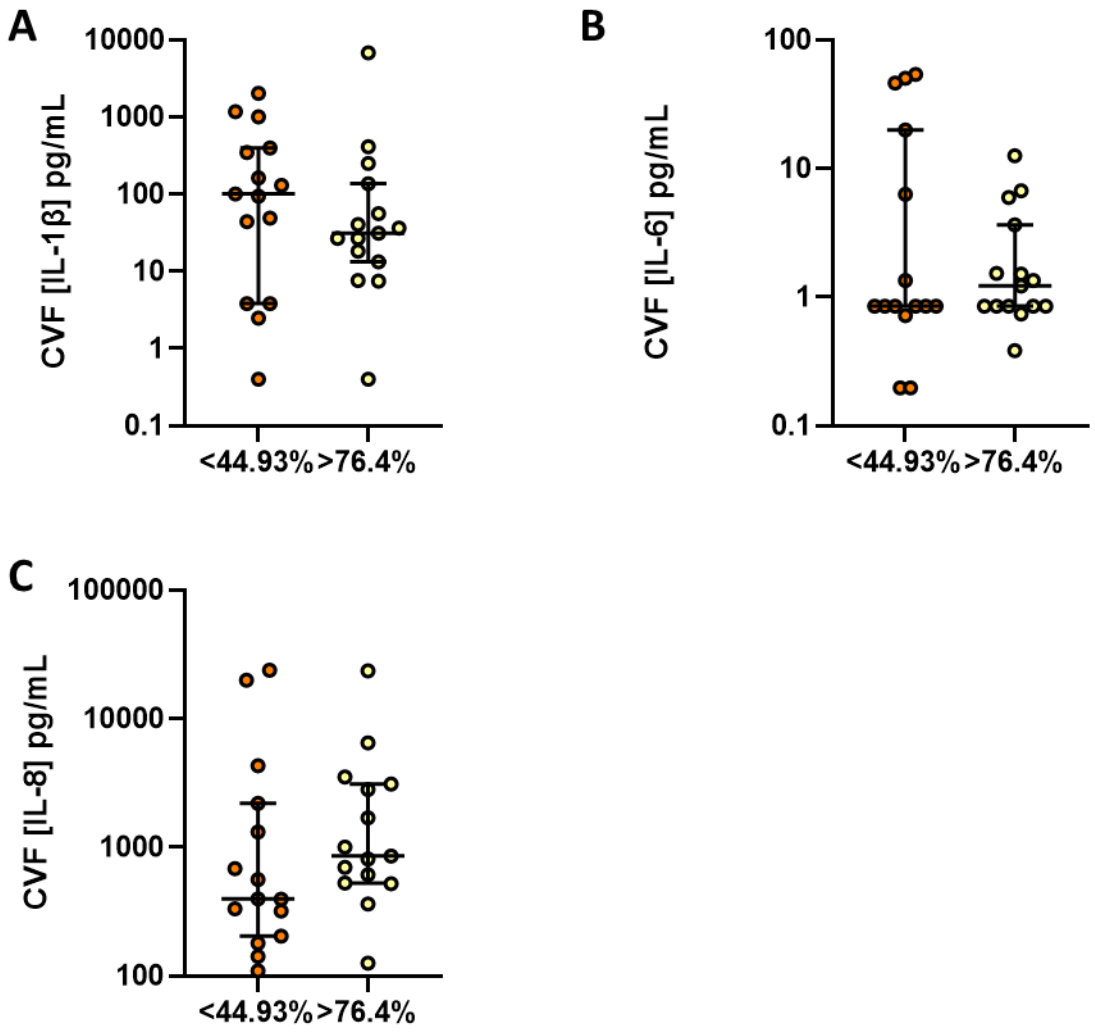


Figure 5.25. Concentration of CVF cytokines based on the lower and higher percentile rank of bacteria bound to IgA. Scatter dot plots of CVF concentration of cytokines according to the lowest (44.93%, n=15) and the highest percentile (76.4%, n=15) of percentage of IgA bound to bacteria. Concentration of **A)** IL-1β, **B)** IL-6 and **C)** IL-8. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

The complement cascade was relatively inert in response to bacteria bound to IgA, compared to what was observed in binding to IgG. In relation to the classical pathway (Figure 5.26), only the concentration of C4 was significantly different between women with a low binding to IgA compared to a higher bacterial binding to IgA (p=0.0411) (Figure 5.26.C). No differences were observed in the lectin pathway as reflected by MBL (Figure 5.27 A) or in the alternative pathway (neither the concentrations of Factor B or D were altered) (Figure 5.27 B, C).

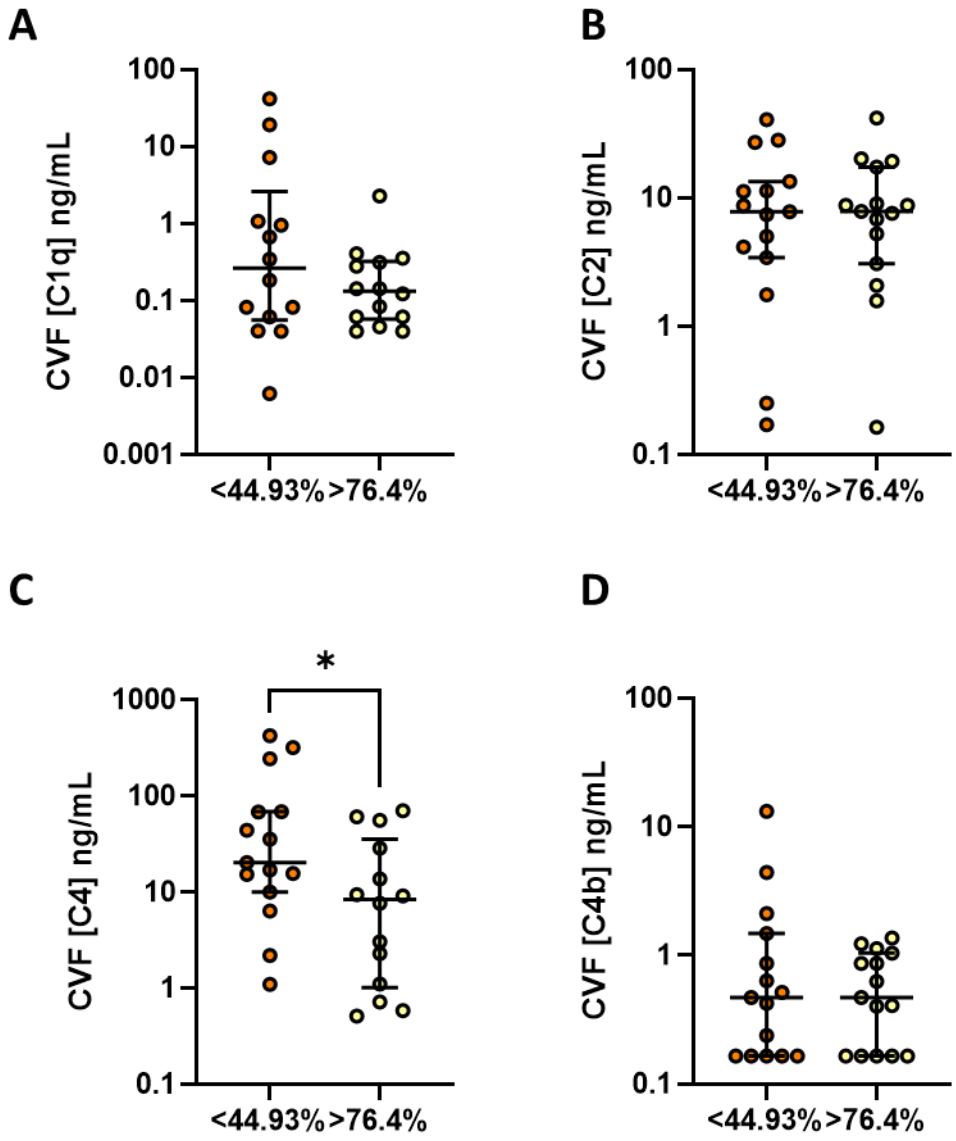


Figure 5.26. Concentration of CVF complement proteins from the classical pathway based on the lower and higher percentile rank of bacteria bound to IgA. Scatter dot plots of CVF concentration of cytokines according to the lowest (44.93%, n=15) and the highest percentile (76.4%, n=15) of percentage of IgA bound to bacteria. Concentration of **A)** C1q, **B)** C2 **C)** C4 and **D)** C4b. *p<0.05. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

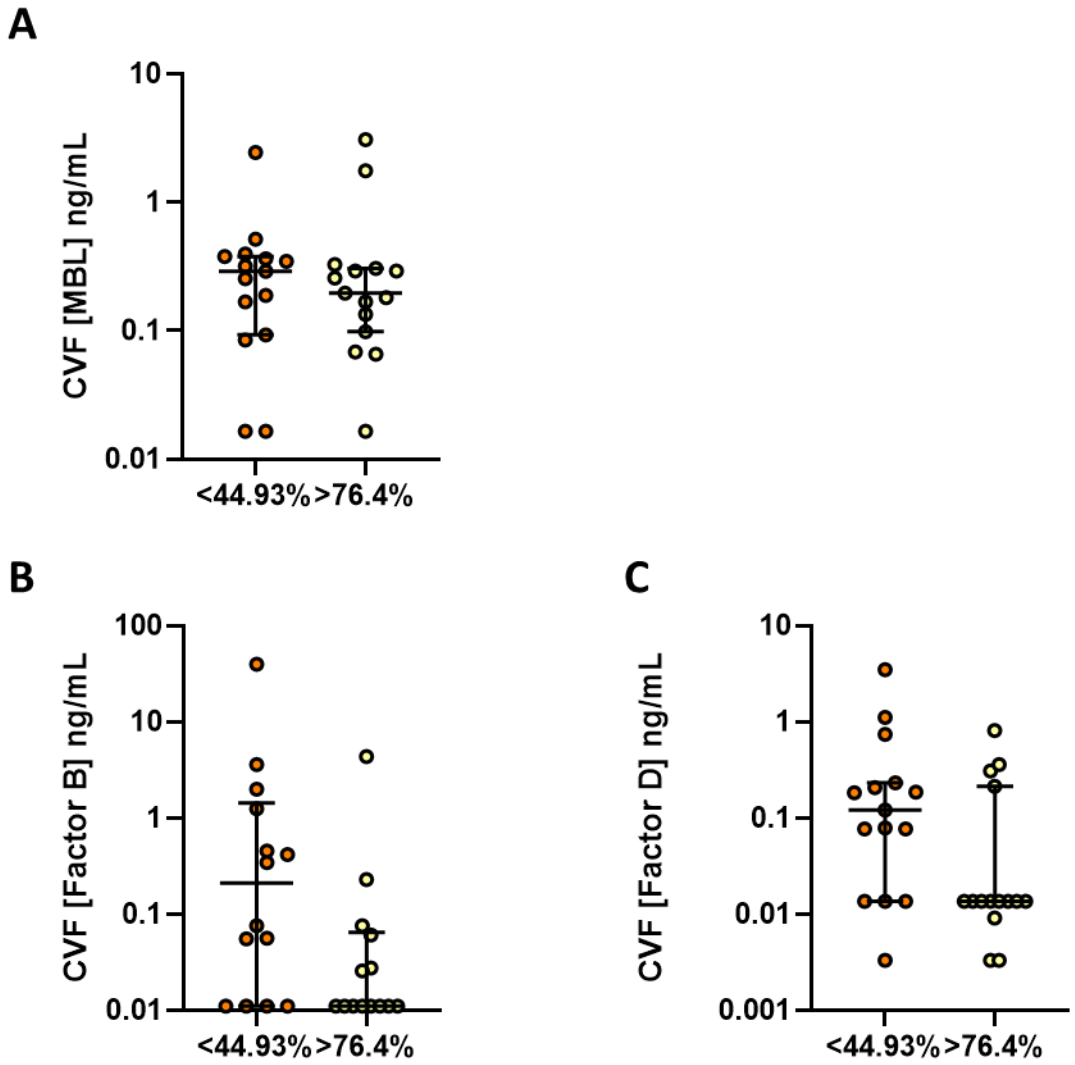


Figure 5.27. Concentration of CVF complement proteins from the lectin and the alternative pathways based on the lower and higher percentile rank of bacteria bound to IgA. Scatter dot plots of CVF concentration of cytokines according to the lowest (44.93%, n=15) and the highest percentile (76.4%, n=15) of percentage of IgA bound to bacteria. Concentration of **A)** MBL, **B)** Factor B and **C)** Factor D. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

When exploring the pathway at the point of convergence on C3 (Figure 5.28), only the concentration of C3b was higher in women with a lower binding of bacteria to IgA (median 17 ng/mL) compared to women with a higher binding of bacteria (median 2.5ng/mL) (p=0.0449) (Figure 5.28 C). No differences were observed in the concentration of complement proteins associated with regulation of the cascade (Figure 5.29).

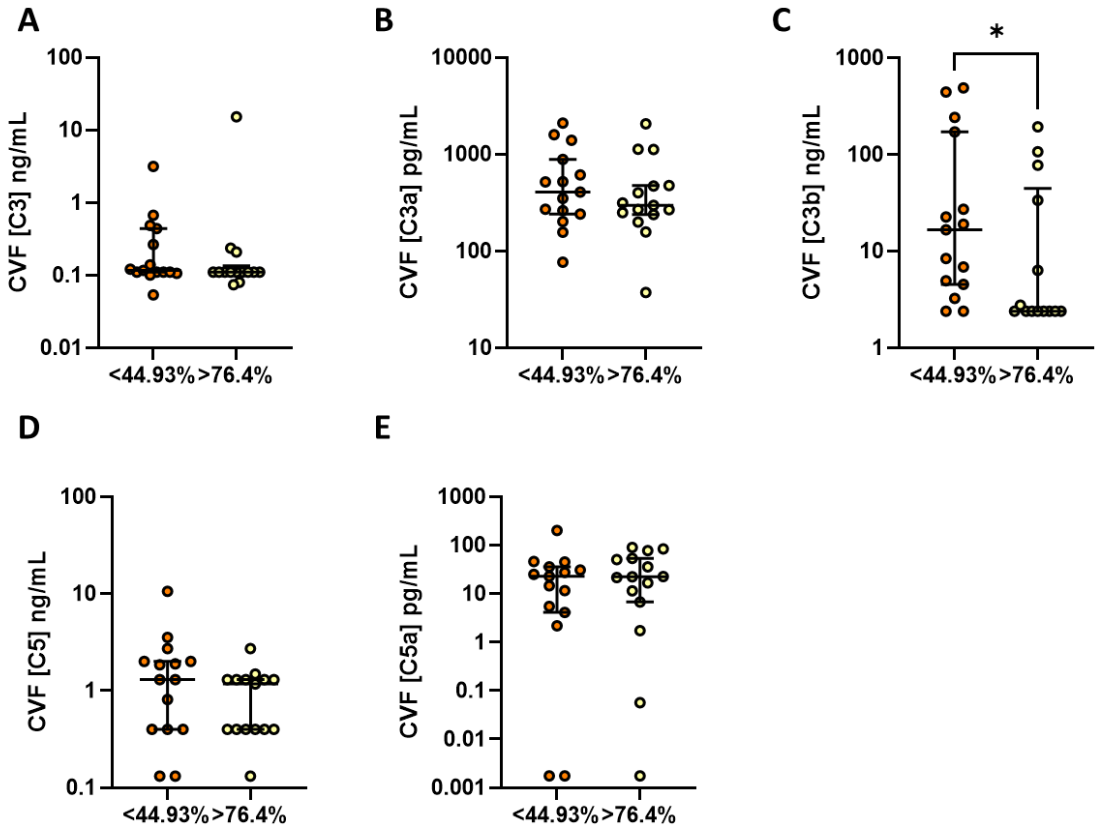


Figure 5.28. Concentration of CVF complement proteins from C3 and C5 convertases based on the lower and higher percentile rank of bacteria bound to IgA. Scatter dot plots of CVF concentration of cytokines according to the lowest (44.93%, n=15) and the highest percentile (76.4%, n=15) of percentage of IgA bound to bacteria. Concentration of **A)** C3, **B)** C3a, **C)** C3b, **D)** C5 and **E)** C5a. *p<0.05. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

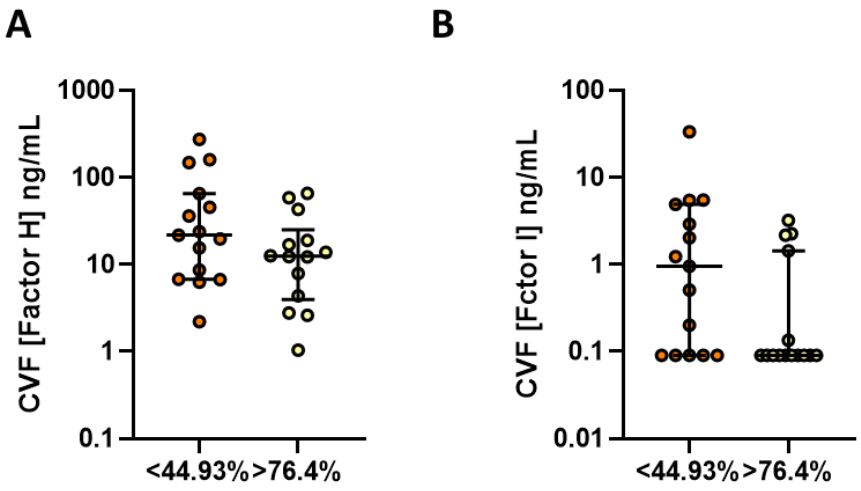


Figure 5.29. Concentration of CVF complement regulators based on the lower and higher percentile rank of bacteria bound to IgA. Scatter dot plots of CVF concentration of cytokines according to the lowest (44.93%, n=15) and the highest percentile (76.4%, n=15) of percentage of IgA bound to bacteria. Concentration of **A)** Factor H and **B)** Factor I. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

Rank of percentage bound to C3b and the local immune response

Finally, the concentration of cervicovaginal cytokines and complement was also assessed based on the percentiles of bacteria bound to C3b (lowest 25th centile rank, <39.25%; and highest 25th centile, >67.17%). In women with high percentages of C3b bacterial binding, the concentration of IL-1β and IL-6 was significantly decreased compared to women with a low C3b binding (p=0.0030, p=0.0317, respectively) (Figure 5.30.)

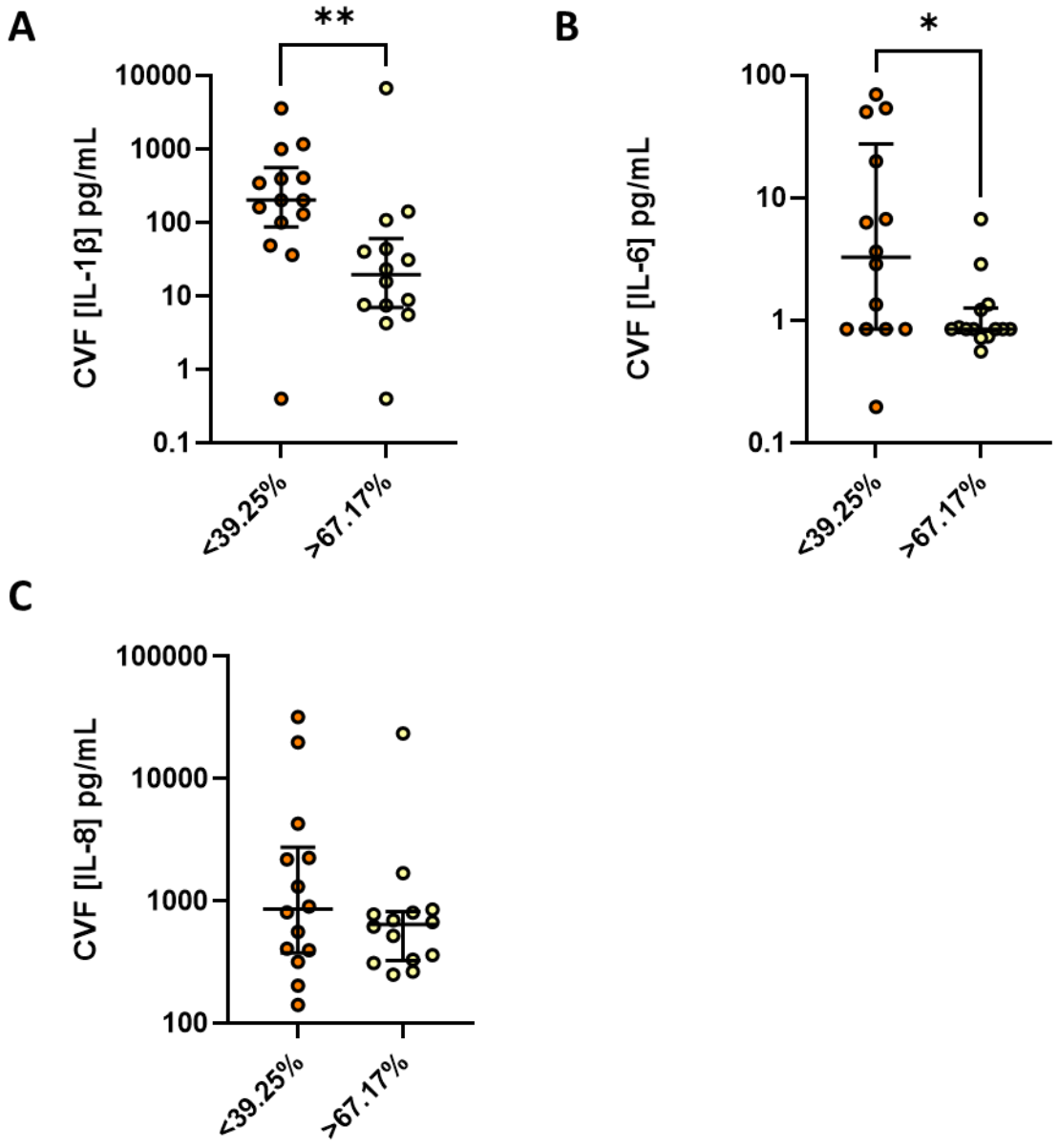


Figure 5.30. Concentration of CVF cytokines based on the lower and higher percentile rank of bacteria bound to C3b. Scatter dot plots of CVF concentration of cytokines according to the lowest (39.25%, n=14) and the highest percentile (67.17%, n=14) of percentage of C3b bound to bacteria. Concentration of **A)** IL-1β, **B)** IL-6 and **C)** IL-8. *p<0.05; **p<0.01. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

Different analytes from the classical pathway were analysed in terms of the percentage of binding to C3b. Some proteins (C1q, C2) appeared to have a higher concentration when the percentage of binding was low (Figure 5.31., A, B). C4, but not C4b, was the only analyte in which the concentration significantly differed between women with a low and a high binding percentage, with lower binding associated with a higher concentration of C4 (median concentration 44.98 and 16.87 ng/mL, respectively, p=0.0012).

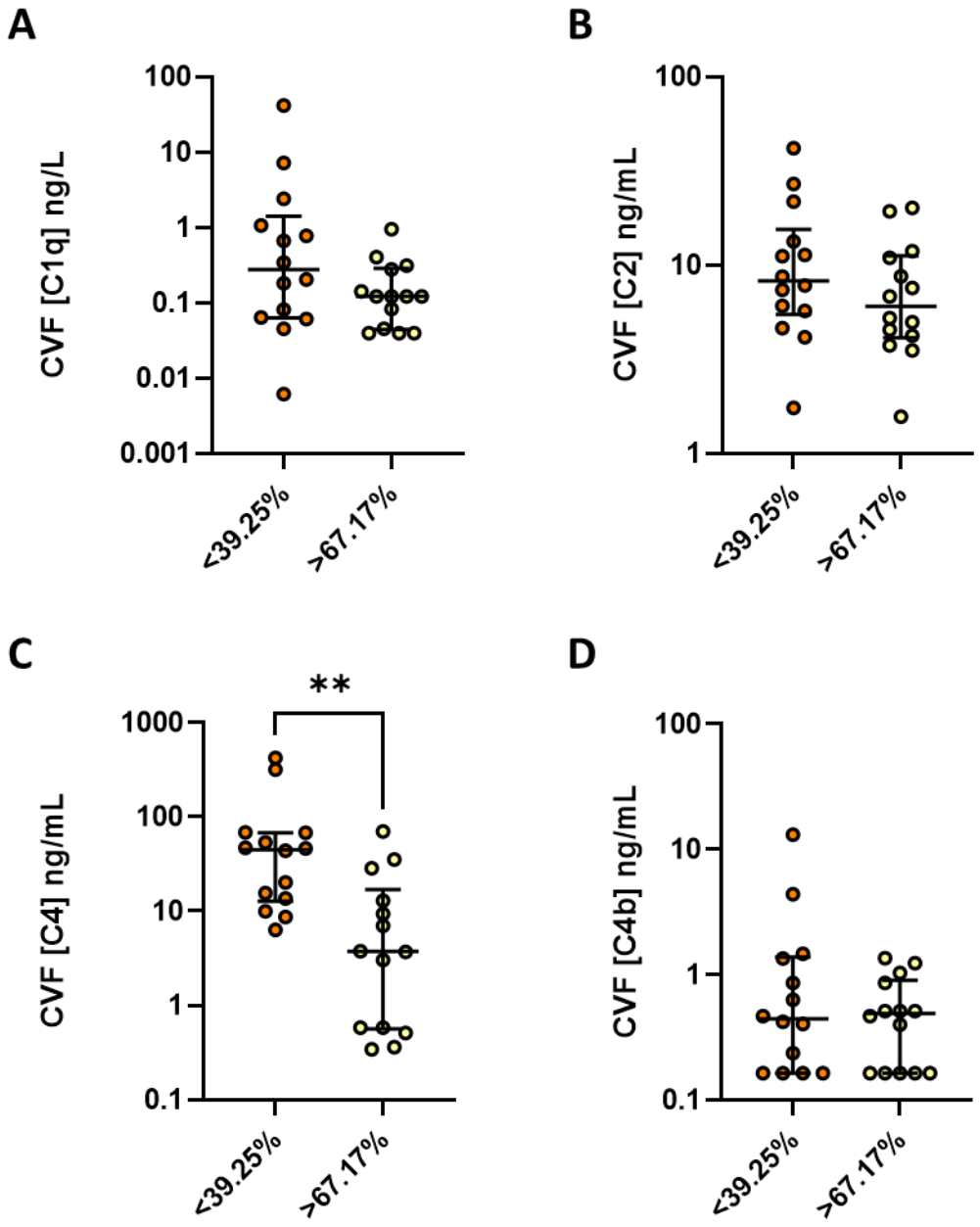


Figure 5.31. Concentration of CVF complement proteins from the classical pathway based on the lower and higher percentile rank of bacteria bound to IgA. Scatter dot plots of CVF concentration of cytokines according to the lowest (39.25%, n=14) and the highest percentile (67.17%, n=14) of percentage of C3b bound to bacteria. Concentration of **A)** C1q, **B)** C2 **C)** C4 and **D)** C4b. **p<0.01. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

In the lectin pathway, a higher concentration of MBL was detected in women with lower binding to C3b, compared to higher binding (median 0.3176 vs 0.1661, ng/mL, p=0.1139) (Figure 5.32.A). However, both mediators analysed from the alternative pathways had a higher concentration in women were the percentage of bacteria binding to C3b was on the lowest 25th percentile (Figure 5.32. B, C). The median concentration of Factor B decreased from 0.1993 ng/mL (in the lower 25th percentile of bacterial binding to C3b) to 0.011ng/mL in the top 75th percentile (p=0.0247). Similarly, the concentration of Factor D was higher in the lower 25th percentile of bacterial binding compared to the higher 25th percentile (0.1518 ng/mL vs 0.01350 ng/mL, p=0.0122).

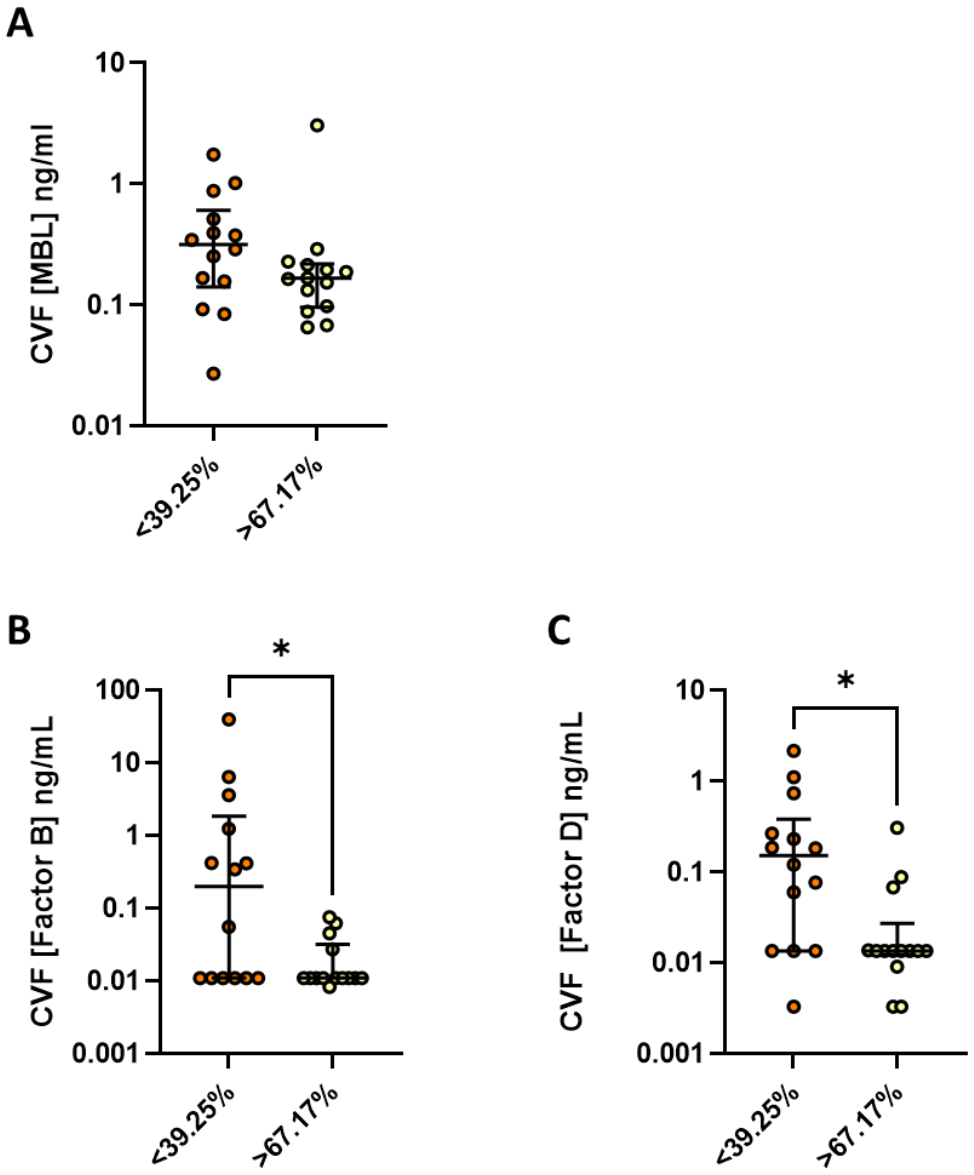


Figure 5.32. Concentration of CVF complement proteins from the lectin and the alternative pathways based on the lower and higher percentile rank of bacteria bound to C3b. Scatter dot plots of CVF concentration of cytokines according to the lowest (39.25%, n=14) and the highest percentile (67.17%, n=14) of percentage of C3b bound to bacteria. Concentration of **A)** MBL, **B)** Factor B and **C)** Factor D. *p<0.05. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

The target of C3 convertases is the defragmentation of C3 into C3a and C3b. No differences were observed in the concentration of C3 according to binding to C3b opsonin. However, the concentration of C3a was increased in women with a lower binding of bacteria to C3b, compared to women on the top percentile (>67% binding) (p=0.0091). Concentration of C3b tended to also be higher in women in the low percentile, however this did not reach significance (p=0.1428) (Figure 5.28 A-C). The concentration of downstream proteins C5 and C5a was not associated with percentage of bound bacteria (Figure 5.28. D, E).

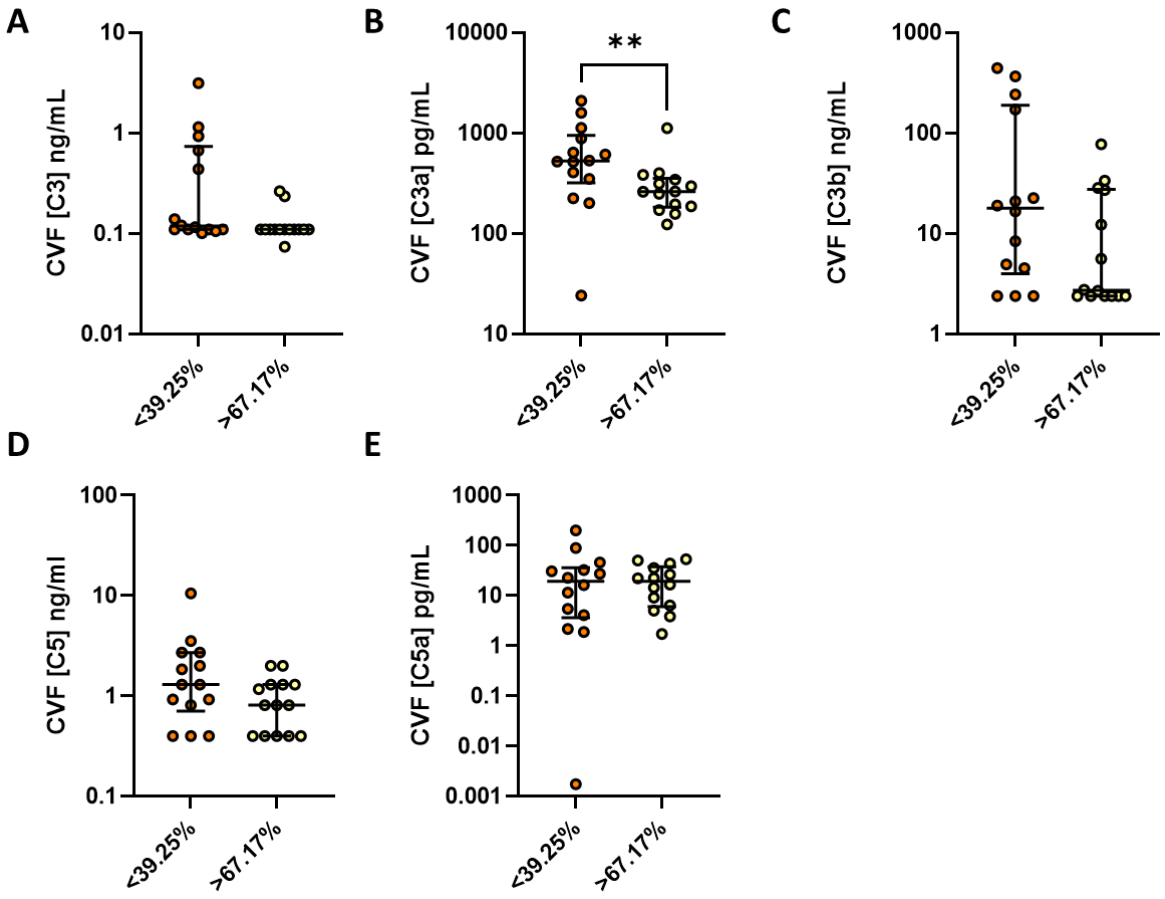


Figure 5.33. Concentration of CVF complement proteins from C3 and C5 convertases based on the lower and higher percentile rank of bacteria bound to C3b. Scatter dot plots of CVF concentration of cytokines according to the lowest (39.25%, n=14) and the highest percentile (67.17%, n=14) of percentage of C3b bound to bacteria. Concentration of **A)** C3, **B)** C3a, **C)** C3b, **D)** C5 and **E)** C5a. **p<0.01. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

Finally, the concentration of complement regulators Factor H and Factor I was significantly increased in women with lower percentages of bacteria bound to C3b, compared to women with higher percentages of binding (p=0.0012; p=0.0071, respectively) (Figure 5.34.).

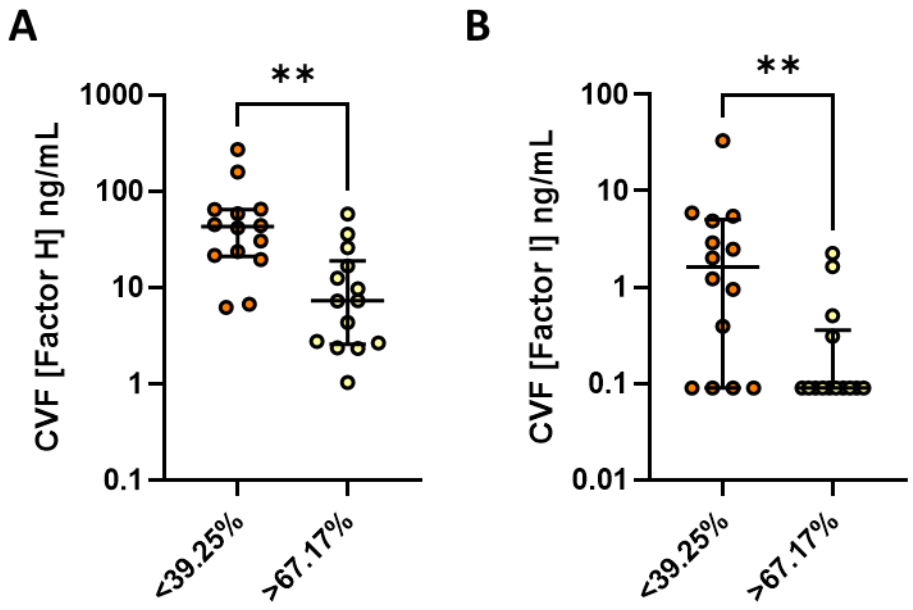


Figure 5.34. Concentration of CVF complement regulators based on the lower and higher percentile rank of bacteria bound to C3b. Scatter dot plots of CVF concentration of cytokines according to the lowest (39.25%, n=14) and the highest percentile (67.17%, n=14) of percentage of C3b bound to bacteria. Concentration of **A)** Factor H and **B)** Factor I. **p<0.01. Statistical analysis was performed using a two-sided Mann-Whitney test. Data is presented as median values with interquartile range.

5.6.4. Coating of bacteria and pregnancy outcome

Cervicovaginal microbial composition by pregnancy outcome

Due to the relative small numbers included in this study, and as previously shown in the full cohort set in Chapter 3, no differences were observed in the proportions of CSTs vaginal microbial composition and pregnancy outcome, including women who undergo cervical shortening (p=0.1196) (Figure 5.35.A) and women who delivered preterm (p=0.3859) (Figure 5.35.B). Despite not statistically significant differences, it is obvious that women who underwent cervical shortening had higher proportions of CST III *L. iners* dominance (33%) and CST IV dominance (25%), compared to women who maintained a normal cervical length (CST III and IV were only 23% and 10% respectively, majority being CST I 54%) (Figure 5.35.A).

Women who delivered preterm had less frequently CST I *L. crispatus* dominance (29%), whilst CST III (36%) and CST IV (14%) enrichment was more common compared to woman who delivered at term. Women from the latter group were more often classed as CST I (55%) than to CST III (22%) or CST IV (12%) (Figure 5.35.B).

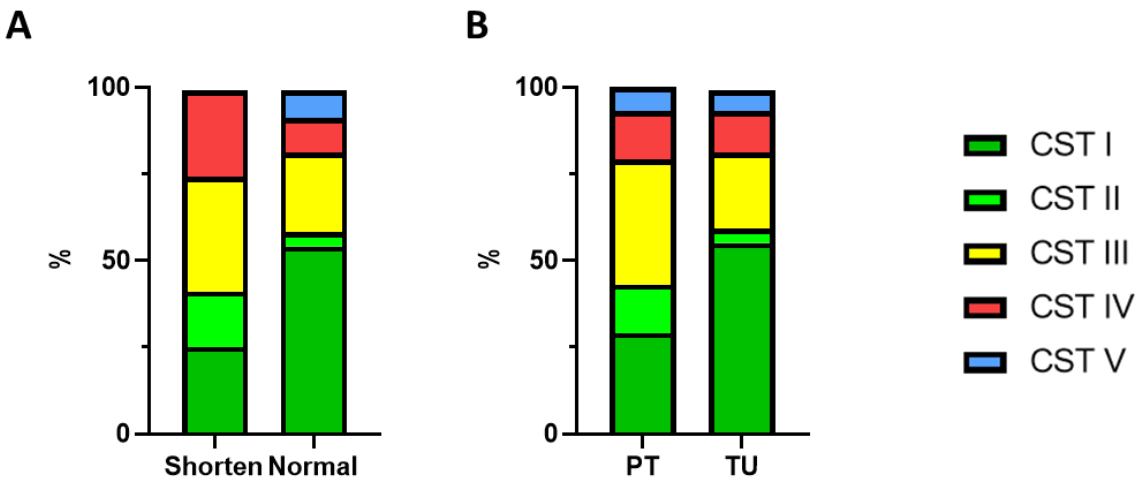


Figure 5.35. Cervicovaginal microbial composition by pregnancy outcome. CVF samples were assigned to CST I (dark green; dominated by *Lactobacillus crispatus*), CST II (light green, dominated by *L. gasseri*), CST III (yellow, dominated by *L. iners*), CST IV (red, heterogenous anaerobic bacteria) or CST V (blue, *L. jensenni*). **A)** Comparison of proportion of CSTs in CVF samples from women who experienced cervical shortening (≤25mm) (n=12/63) compared to woman who maintained a normal cervical length (>25mm). **B)** Comparison of proportion of CSTs in CVF samples from women who delivered preterm (PT, <37 weeks, n=14) and woman who delivered at term (TU, >37 weeks, n=49). Statistical analysis was performed with Chi-square test.

Cervicovaginal bacteria bound to immunoglobulins was evaluated in 59 CVF samples based on pregnancy outcome. Table 5.9. summarises samples from women who experienced cervical shortening and samples from women who experienced preterm birth, according to CST classifications.

Table 5.9. CVF samples based on pregnancy outcome (n=59) for the evaluation of cervicovaginal bacteria bound to immunoglobulins

CST classification	Cervical shortening		Pregnancy outcome	
	Short (≤25mm)	Normal (>25mm)	Preterm	Term uncomplicated
CST I – *L. crispatus*	3	28	4	27
CST II – *L. gasseri*	2	2	2	2
CST III – *L. iners*	4	11	4	11
CST IV – Heterogeneous	2	3	1	4
CST V – *L. jensenii*	0	4	1	3

Cervicovaginal bacteria bound to immunoglobulin in women who undergo cervical shortening

Firstly, the percentage of bacteria opsonisation was compared in samples from women who underwent cervical shortening (cervical length ≤25mm) (n=11) and in samples from women who maintained a normal cervical length (>25mm) (n=48) (Figure 5.36.). No statistically significant differences were observed, however, women who underwent cervical shortening had lower IgG binding compared to woman who maintained a normal cervical length (median 52% vs 65.20%, p=0.1266) (Figure 5.36.A). Cervical shortening was also associated with lower IgA binding (median 51.40 vs 68.50%, p=0.1084) (Figure 5.36.B). The percentage of C3b binding did not differ between women with a short or a normal cervical length (median 55.70 vs 57.90%, p=0.4887) (Figure 5.36.C).

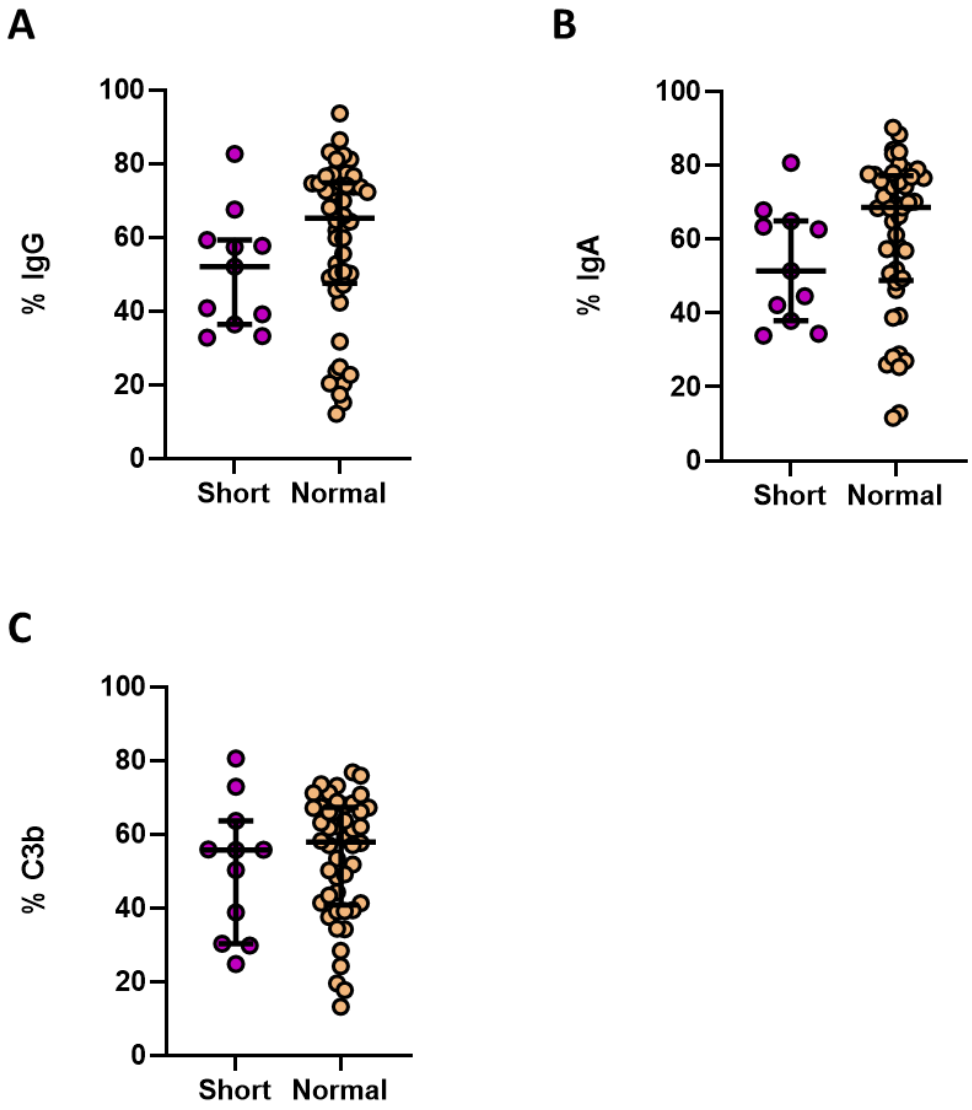


Figure 5.36. Comparison of percentage of opsonins bound to bacteria according to cervical shortening. A) Percentage of IgG. **B)** Percentage of IgA. **C)** Percentage of C3b. Differences between cervical shortening (n=11) and normal cervical length (n=47) samples were assessed with a Mann-Whitney two-tailed test. Data sets of graphs represent dot plots for individual samples; lines indicate the median with interquartile range.

Cervicovaginal fluid bacteria bound to opsonins were also assessed in association with the local cervicovaginal microbial composition and cervical shortening (Figure 5.37). No differences were observed between samples from women who experienced cervical shortening compared to woman with a normal cervical length classed as CST I, CST III or CST IV for any of the opsonins evaluated: IgG (Figure 5.37.A), IgA (Figure 5.37.B) or C3b (Figure 5.37.C).

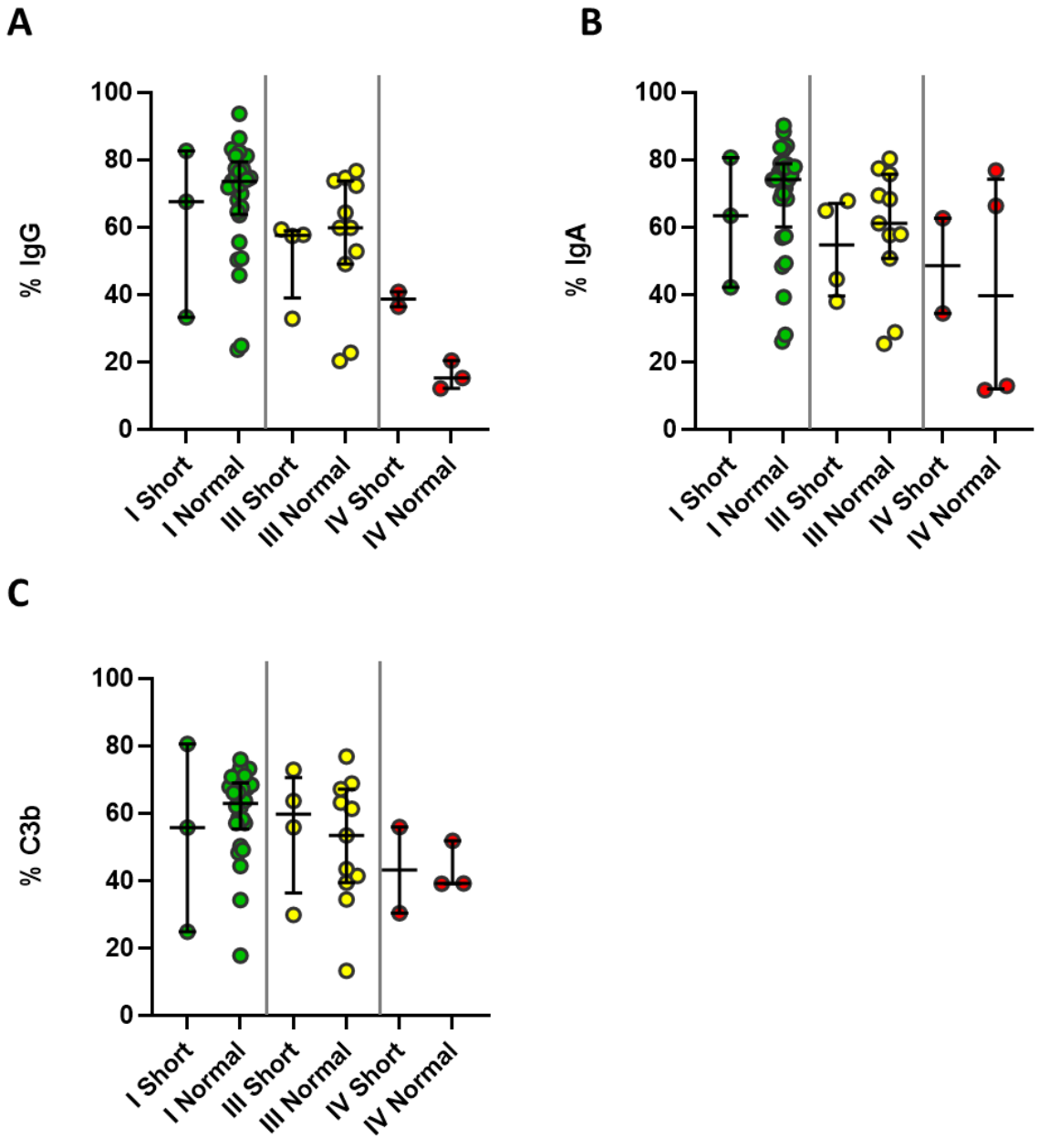


Figure 5.37. Cervicovaginal fluid bacteria bound to opsonins according to CST vaginal composition and cervical shortening. The percentage of bound opsonins was compared in women who shorten and woman who maintained a normal cervical length in those samples classed as abundant in CST I (*L. crispatus*) (n=3 vs 28), CST III (*L. iners*) (n=4 vs 11) or CST IV (diverse) (n=2 vs 3) using a Mann-Whitney one tailed-test. Data is presented as median with interquartile range. **A)** Percentage of IgG. **B)** Percentage of IgA. **C)** Percentage of C3b.

Cervicovaginal bacteria bound to immunoglobulins in women who delivered preterm

Finally, the percentage of bacteria opsonisation was compared between women who delivered preterm with women who delivered at term. Women who delivered preterm had lower percentages of bacterial binding to immunoglobulins and C3b compared to women who had a term uncomplicated pregnancy. (Figure. 5.38.). Specifically, considerably lower binding of IgG (median 43.10% vs 74.90%, p=0.0174), lower binding of IgA (median 46.75% vs 68.95%, p=0.0283) as well as lower binding of C3b (median 38.90% vs 58.60%, p=0.0042) was demonstrated.

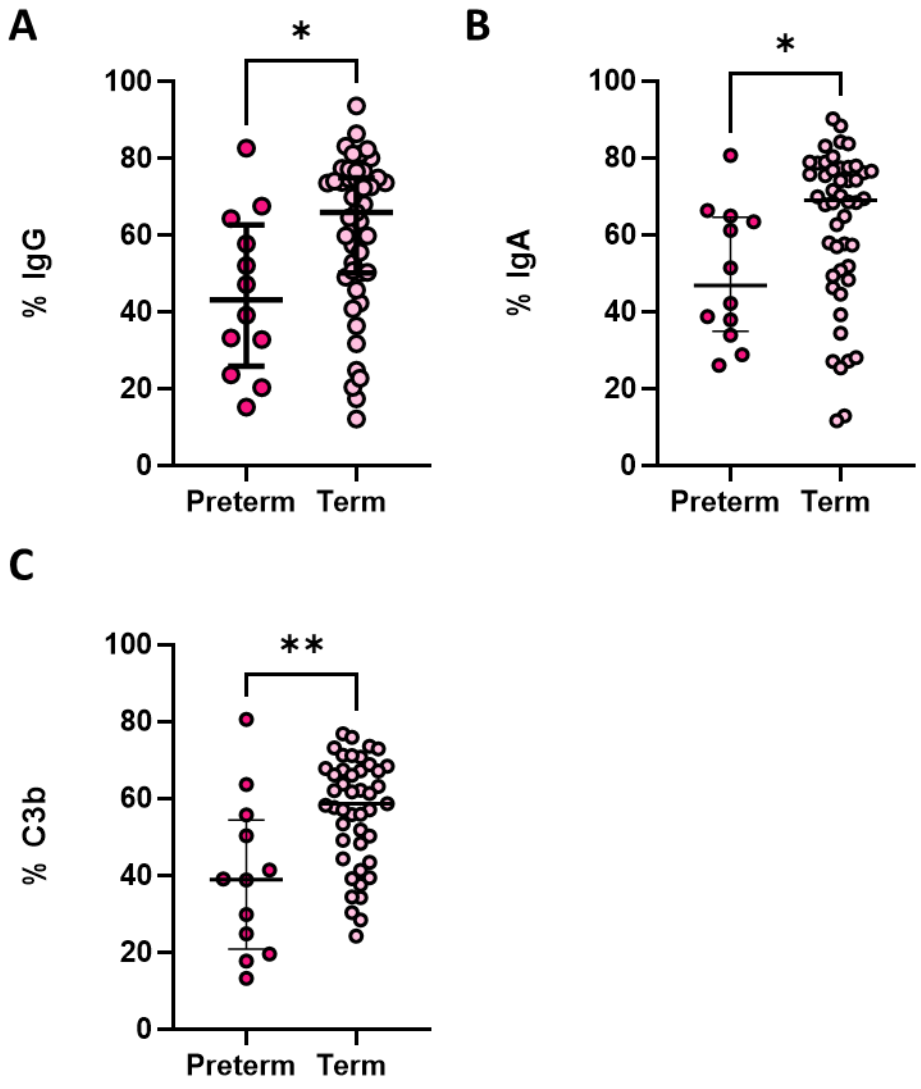


Figure 5.38. Comparison of percentage of antibodies bound to bacteria according to pregnancy outcome. A) Percentage of IgG. **B)** Percentage of IgA. **C)** Percentage of C3b. Differences between preterm (N=12) and term (N=47) were assessed with a Mann-Whitney two tailed test. Data sets of graphs represent dot plots for individual samples; lines indicate the median with interquartile range. *p<0.05; **p<0.001.

When assessing cervicovaginal fluid bacteria bound to opsonins in association with the local cervicovaginal microbial composition and pregnancy outcome (Figure 5.39), no differences were observed between samples from women who delivered preterm compared to woman with a term uncomplicated pregnancy classed as CST I, CST III or CST IV for any of the opsonins evaluated (Figure 5.39.). The median percentage of bound to IgG was increased in women who delivered at term and were classed as CST I (73.60 VS 50.35%), CST III (59.80 vs 45.25%) and CST IV (28.40 vs 15.20%), compared to their preterm counterparts, however this did not reach statistical significance (p=0.2346, p=0.2147, respectively) (Figure 5.39. A). No differential binding patterns were observed either for IgA (Figure 5.39.B) or C3b (Figure 5.39.C).

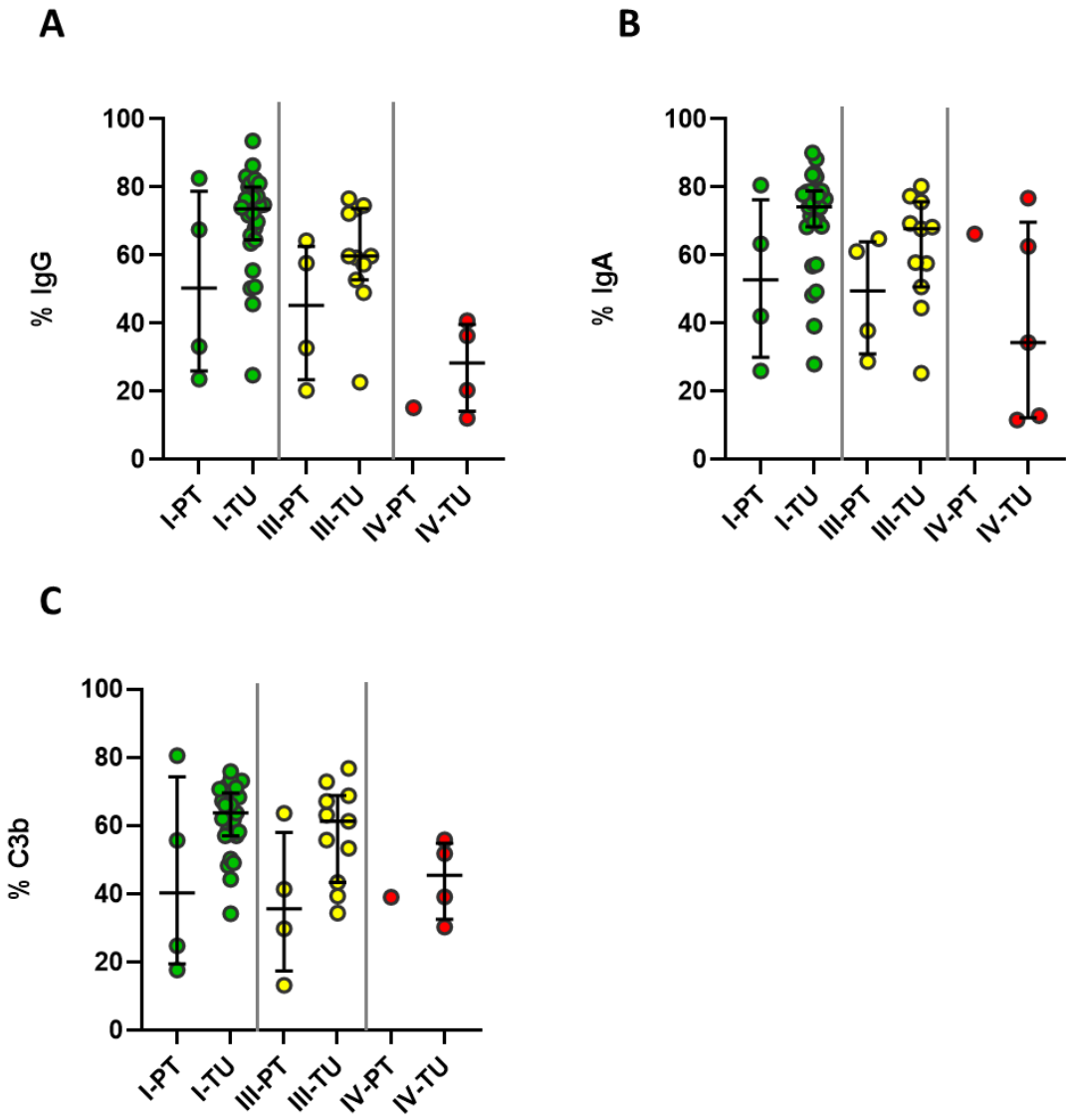


Figure 5.39. Cervicovaginal fluid bacteria bound to opsonins according to CST vaginal composition and pregnancy outcome. The percentage of bound opsonins was compared in women who delivered preterm and woman with a term uncomplicated delivery in those samples classed as abundant in CST I (*L. crispatus*) (n=4 vs 27), CST III (*L. iners*) (n=4 vs 11) or CST IV (diverse) (n=1 vs 4) using a Mann-Whitney one tailed-test. Data is presented as median with interquartile range. **A)** Percentage of bound IgG. **B)** Percentage of bound IgA. **C)** Percentage of bound C3b.

5.7. Discussion

It was hypothesised that opsonins would interact with cervicovaginal microbiota to promote mucosal homeostasis and/or trigger a proinflammatory response. Identifying the interactions by which certain taxa are targeted by immunoglobulins or other opsonins could reveal key dynamics of host-microbial interactions that are important determinants for pregnancy outcome. In Chapter 5, the optimisation of a flow cytometry technique and its novel application for investigating the role of bacterial opsonisation in pregnancy and adverse outcomes has been presented.

Protocol optimisation and troubleshooting

Antibodies have the ability to specifically bind to a target antigen. During the protocol optimisation process it was found that the use of monoclonal antibodies provided more specificity and reliable results over polyclonal antibodies. Antibodies can be polyclonal or monoclonal. Polyclonal antibodies are heterogeneous and can recognise and bind to different epitopes, whereas monoclonal antibodies are homogeneous and consistently raised against a single specific epitope. The main advantage of using monoclonal antibodies is their homogeneity and consistency to evaluate protein-protein interactions, which are ideal characteristics for flow cytometry analysis (Lipman et al., 2005).

Specificity and clear discrimination is highly important in flow cytometry since the measurements of the results rely on the ability to distinguish between positive and negative populations of cells for each fluorescent marker. The negative and positive discrimination can be affected by autofluorescence, spectral overlap and non-specific antibody binding. Spectral overlap can be minimised by optimising multicolorimetric panels and spectrums (Hulspas et al., 2009). This was taken into account when choosing antibodies conjugated to fluorochromes that are far apart on the spectra (Appendix 5.2.), to have none or minimal compensation correction. Non-specific binding occurs when there is binding to a different epitope than the one it was raised against (Hulspas et al., 2009). There are several ways in which an antibody can bind the antigen, including non-specific binding to an antigen not of interest, to an Fc receptor but not as ligand interaction, to the antigen but not to the epitope it was supposed to bind, also via the fluorochrome (Hulspas et al., 2009). Strategies were undertaken to reduce background non-specific binding including the use of Fc receptor blocking reagents (which in our case did not make any difference) and titration of antibody concentration to optimise the amount required (Hulspas et al., 2009; Maecker and Trotter, 2006) which we did for both the antibodies and isotype control. Whilst these strategies did not help to reduce the non-specific binding of the isotype control of the immunoglobulins, the use of monoclonal antibodies instead of polyclonal antibodies did.

Isotype controls are antibodies that have been raised against an antigen not found on the sample to be analysed. The main aim of isotype controls is to ensure that the observed fluorescence is truly due to the antibody binding to the desired target. The most appropriate isotype matches host species, Ig subclass and fluorophore, and should ideally be used in the same concentration as the target antibody (Hulspas et al., 2009). After changing the antibodies to monoclonal non-specific binding with isotype control was not observed. Despite investing a significant amount of time optimising the use of the isotype control, there is some controversy with the actual value that isotype controls provide (Herzenberg et al., 2006; Maecker and Trotter, 2006). It has been argued that titration of target antibodies is sufficient to demonstrate specificity (Herzenberg et al., 2006; Hulspas et al., 2009; Maecker and Trotter, 2006), together with the use of biological controls (e.g., to include an unstimulated population in stimulation assays to distinguish between positive and negative events) (Hulspas et al., 2009). In this study, access to pure biological bacterial cultures with the assistance of the Marchesi laboratory allowed for the most accurate and relevant positive and negative controls. Firstly, the detection of the nucleic acid stain by both flow cytometry and microscopy in pure bacterial isolates served as a as positive control. Secondly, they also served as a negative control, since these bacterial isolates had not been exposed to human immunoglobulins; therefore, as expected there was no opsonisation detected and this confirmed the absence of non-specific staining with the antibodies used.

Opsonisation of bacteria in cervicovaginal fluid in pregnancy

There was no difference in the percentage of IgA, IgG or C3b bound bacteria, or the MFI or the coating index (integrated MFI) across pregnancy. This was not surprising as the microbial composition did not vary across pregnancy (Table 5.7.). CVF samples taken from non-pregnant women of reproductive age also showed no differences in IgA and IgG bound bacteria across different stages of the menstrual cycle (Breedveld et al., 2022), although it was found the coating index (integrated MFI) of IgA and IgG was higher during menses. Since they did not observe changes on the percentage of bacteria, a variation in the MFI explains the change in the iMFI. The vaginal microbial composition of their study was also stable for most of the women (Breedveld et al., 2022). They suggest that since there are discrepancies between studies reporting no changes in the vaginal microbial composition during the menstrual cycle (Chaban et al., 2014; Jespers et al., 2012), but others detected variation in diversity of species (Gajer et al., 2012), the presence of blood, individual sexual and hygiene practices, together with individual host characteristics can impact the vaginal microbial composition and the host response to it (Breedveld et al., 2022).

The percentages of bacteria bound to all mediators strongly correlated between each other, suggesting that bacteria are likely to be bound to more than one opsonin. Livson et al. detected on CVF samples by western blot staining for both IgG and C3 (Livson et al., 2021). This potentially indicates the formation of C3-IgG complexes, which would explain our positive correlation on the percentages of IgG and C3b binding. However, despite knowing opsonins can obviously bind with the local microbiota and interact with immune receptors, the role of this complex formation is not known.

The percentage of opsonisation is altered depending on the local microbial composition

This study provides novel findings reporting on the percentages of cervicovaginal bacterial opsonisation in the context of microbial-induced inflammation and preterm birth. The percentages of bacteria bound to IgG, IgA and C3b were higher in women who had a CST I compared to CST IV (Figure 5.17.- 5.19). Breedveld et al. also found increased percentages of IgA in non-pregnant women whose vaginal composition was dominated by *Lactobacillus crispatus* (Breedveld et al., 2022).

IgA is one of the most investigated immunoglobulins that binds to bacteria, since it is very abundant in mucosal secretions, and also its role in gut homeostasis has been widely reported. IgA contributes to gut homeostasis via exclusion and inclusion mechanisms. The former makes reference to the ability to agglutinate and anchor microorganisms, enclosing beneficial commensals on the lining of the gut, reducing potential proinflammatory interactions via reducing motility and promoting adhesion. Whereas inclusion mechanisms involve the retention of commensals, potentiating the growth of beneficial bacteria, preventing the colonisation of pathogens (Chen et al., 2020).

The patterns of bacteria bound to IgA have been particularly studied in the gut, especially in inflammatory diseases such as inflammatory bowel disease (IBD). Palm et al. found more bacteria bound to IgA in patients with colitis (Palm et al., 2014), and so did Lin et al. and Rengarajan et al. in patients with colitis and Crohn's disease compared to healthy controls (Lin et al., 2018; Rengarajan et al., 2020). This suggests that inappropriate immune responses can contribute to the pathogenesis of IBD. However, IgA has also been implicated in promoting gut homeostasis. Palm et al., via IgA sequencing, also showed that *Lactobacillus*, among other bacteria typical from the gut, were enriched in the unique IgA+ fraction, but not on the IgA- fraction (Palm et al., 2014). Huus et al., also found interesting associations between binding of IgA and *Lactobacillus* in a murine model of malnutrition. In this case, mice that received a malnourished diet showed a decreased binding of IgA to beneficial bacteria, including *Lactobacillus* and *Bifidobacterium*. They were able to show that when IgA interacts

with commensal strains of *Lactobacillus*, aggregation is induced (Huus et al., 2020), potentiating the aforementioned mechanisms to outcompete non-aggregating strains.

The binding to IgA can enhance anti-inflammatory responses. Mikulic et al. showed that complexes of *L. rhamnosus* together with secretory IgA (SIgA) can prevent TLR signalling via upregulating molecules (higher mRNA expression of regulatory proteins *Sigirr, Tollip* and *Socs1*) that restrain TLR signalling (Mikulic et al., 2017). Also, incubation of this complexes together with dendritic cells induce higher release of anti-inflammatory IL-10 compared to the control (Mikulic et al., 2017).

Potentially, the demonstrated role of IgA promoting homeostasis in the gut could be translated to the cervicovaginal milieu. A trend for a higher percentage of bacteria bound to IgA ($p=0.05$) was seen in women with a CST I vaginal composition (Figure 5.18.). Despite not being the most prevalent Ig in CVF secretions, our group previously demonstrated reduced CVF free IgA concentrations in women with CST I compared to CST IV (Chan et al., 2022). Perhaps, no correlations between free vaginal Ig and percentage of Ig bound bacteria could indicate an increased on the Ig specificity rather than the amount of vaginal antibodies available. Or just simply that less free IgA can be detected as a consequence of already being bound to bacteria. However, this cannot be confirmed until future work incorporates assessment of matched free unbound Ig levels in CVF. In association with the inflammatory response, the percentage of IgA bound was negatively correlated with complement proteins (Table 5.8.). This was expected since IgA does not have the ability to trigger the complement system.

The role of IgG is quite ambivalent: it can also enhance mucosal homeostasis, as well as controlling non-invasive and invasive mucosal bacteria. Less is known about the role of IgG bound bacteria in the gut, but higher percentages were found in IBD patients, and the percentages correlated with the state of the disease (Lin et al., 2018; Rengarajan et al., 2020).

IgG is the predominant antibody in cervicovaginal secretions. There are 4 types of IgG subclasses; IgG1 and IgG3 are considered proinflammatory, complement activators and involved in Fcγ receptor signalling (FcγRI, FcγRIII). In contrast, IgG2 and IgG4 subclasses are limited for complement activation (Chen et al., 2020; Schroeder and Cavacini, 2010). The different subtypes also recognise different antigens: IgG1 and IgG3 respond to proteins from pathogens, whereas IgG2 binds to polysaccharides (Vidarsson et al., 2014). Chan et al. measured free soluble cervicovaginal fluid Ig concentrations and found that IgG1 and IgG3 were significantly increased across pregnancy in women who delivered preterm, but not in women who delivered at term. Both IgG1 and IgG3 concentrations were also increased in women with a CST III and a CST IV vaginal composition (Chan et al., 2022).

In our cohort, the percentage of opsonisation with IgG was higher in women with a CST I composition compared to CST IV. Breedveld et al., also detected a double IgG/IgA coated population, indicating that women with *L. crispatus* dominance was also coated with IgG (Breedveld et al., 2022). It is likely that at the cervicovaginal interface there is an IgG and IgA driven promotion of vaginal immune-microbe homeostasis.

In contrast to what was anticipated, lower percentages of C3b- bacterial opsonisation were seen in women with the diverse CST IV vaginal composition compared to CST I. When opsonins like C3b attach to pathogens, the mechanism of phagocytosis is enhanced (Merle et al., 2015; van Lookeren Campagne et al., 2007). There is *in vitro* evidence that *E. coli* coated with purified C3b can trigger neutrophil phagocytosis (Boero et al., 2023). In chapter 3 it was previously shown how there is free circulating C3b on CVF in women with CST IV vaginal composition, with higher concentration than in women with CST I. Pellis et al. also revealed potential activation of the complement cascade by immunofluorescence evaluation of clue cells form BV patients with deposition of C3 on clue cells, potentially recognising BVAB bacteria (Pellis et al., 2005).

A potential explanation for reduced opsonisation binding seen in association with CST IV type microbes such as *G. vaginalis* or *Atopobium* is that these are actively avoiding recognition by the host immune response, allowing them to thrive in certain microenvironments and avoiding elimination. There are several ways in which pathogens can evade immune response, particularly the complement cascade. Encapsulated bacteria impair the C3 deposition (Sharma et al., 2020) , and for example *Trychomonas* releases cysteine proteases that degrades opsonins (IgG, IgA, C3) (Kalia et al., 2019). However, the list of mechanisms that pathogenic bacteria have evolved to acquire in order to avoid elimination is endless. Another example is how GBS can avoid complement activation by binding the soluble complement inhibitor factor H. GBS exhibits a surface protein known as β or Bac. When it binds to factor H, the interacting site of Factor H with C3b remains accessible. This potentially allows Factor H to bind to C3b to form the inactive iC3b to terminate activation of the alternative pathway (Jarva et al., 2004). The main consequence of pathogens preventing binding and uptake via the immune system is that the recognition, lysis and opsonophagocytosis mechanisms are limited, allowing the pathogen to survive. Different mechanisms are performed by *G. vaginali*s. For example, it secretes the virulence factor vaginolysin, which can bind the regulatory CD59 complement molecule, allowing adherence to host epithelial cells (Gelber et al., 2008). Recently, Amabebe & Anumba reviewed the different mechanisms by which bacteria typical from BV can evade the immune system. Many of this BV bacteria, typically classed in the CST IV group (such as *G. vaginalis*, *Prevotella* spp., *Ureaplasma urealyticum*), secrete sialidases. Secretory IgA (sIgA) is recovered by sialic acid in the terminal residues, therefore, desialylation could compromise the antibacterial properties of sIgA (Amabebe and

Anumba, 2022). Amabebe & Anumba also mentioned how neutrophil infiltration via IL-8 inhibition could be impaired, together with a compromised neutrophil and monocyte chemotaxis via the effect of short chain fatty acids and succinate (Amabebe and Anumba, 2022).

In summary, IgG and IgA opsonisation appears to be implicated in vaginal homeostasis, allowing the colonisation of L*. crispatus*. The reduced opsonin binding in members of CST IV could indicate that non-opsonised bacteria have access to the cervicovaginal interface. If opsonin binding is protective and does not trigger recruitment of leukocytes and clearance of bacteria, the immune mediators that were seen to be increased at the cervicovaginal interface in association with lack of opsonisation would need to come from different sources in response to free bacteria/bacterial products. In chapter 3 the extrahepatic complement source was described and leukocytes such as neutrophils, macrophages and dendritic cells, as well as vaginal epithelial cells can secrete complement components (Botto et al., 1992; Bulla et al., 2010; Faried et al., 1993; Lubbers et al., 2017). Moreover, epithelial cells co-cultured with *G. vaginalis* and *G. vaginalis* supernatant can secrete cytokines and inflammatory mediators (Anton et al., 2022, 2018). Otherwise, the inflammatory response detected could be a consequence of the collateral damage (degranulation, proteases, proinflammatory environment) after phagocytosis enhanced by opsonisation.

Differential bacterial binding influences the local proinflammatory immune milieu

In this study, the correlations between bacterial opsonisation and the concentration of cytokines and complement proteins was assessed. For example, higher percentiles of IgG-bound bacteria were associated with decreased concentrations of C1q and C4, which may reflect consumption and activation of the complement via the classical cascade. But this activation could be a protective inflammatory response necessary to protect the mother and the fetus. However, the highest C3b concentration, which is further down the complement system, was observed with lower IgG binding, potentially triggering a dysregulated complement-dependent immune response. In chapter 3 it was reviewed how complex and intricated the complement system is, so focusing on a single protein cannot be representative of the immune response from the complement cascade.

The range of opsonin binding and range of concentrations of inflammatory mediators suggest that there is patient-to-patient variability in the immune response and that there are other factors (e.g., ABO group, ethnicity, viral composition) that could impact the immune response.

Women who deliver at term have higher percentages of opsonised bacteria

Finally, the percentage of bacteria bound to IgA, IgG and C3b was higher in women who delivered at term, compared to women who experienced a preterm delivery. This implies that opsonisation is a protective mechanism, providing a homeostatic and regulated mechanism for opsonisation. Weis & Round reviewed and summarised how bacteria can differentially bound IgA (by increasing or decreasing) during diseases, varying between different diseases and conditions, including IBD, nutritional status and metabolic diseases (Weis and Round, 2021).

Perhaps our findings are a reflection that the majority of women who delivered at term had a CST I vaginal microbial composition. However, not everyone that delivered at term had a CST I vaginal composition, nor everyone that had a preterm delivery had an adverse vaginal composition. The variation of opsonin binding is evident in both groups, and the reasons for patient-to-patient variability remains unknown.

Strength and limitations

Although this study has been able to demonstrate differential percentages of opsonisation depending on the vaginal microbial composition and pregnancy outcome, results should be interpreted cautiously since the study cohort is relatively small (10 cases vs 30 controls). Further studies with larger cohort numbers are required to confirm these microbial: host interactions.

A strength of the current study is the extensive level of protocol optimisation to confirm and validate our protocol. Firstly, pure bacterial cultures were a high-quality negative and positive controls for the study design. Secondly, the fact that all samples were double stained with Syto and each of the individual opsonins, allowed for robust assessment of bacterial material, without only relying on FSC-SSC distributions to distinguish between real bacteria and debris contained within the swab/sample material. However, no double staining of targets was performed. Therefore, with single staining of targets is possible to detect the percentage of bacteria bound to specific immunoglobulins and C3b, but study is limited to reveal if microbiota from the female reproductive tract is differentially or preferentially bound between immunoglobulins, in terms of establishing any dominance or competitivity between the different opsonins. In this instance for example, Breedveld et al. showed that the majority of samples, no matter they were classified as *L. crispatus* or non-*L. crispatus* dominated were double positive for IgA and IgG, but the single IgA dominant population was more commonly detected in the *L. crispatus* group (Breedveld et al., 2022), again suggesting the potential

homeostatic and favoured binding towards beneficial commensal species. Nevertheless, it is possible to infer from the correlations results (Figure 5.16.) that bacteria are not coated by a single opsonin.

A limitation of this study is the lack of information regarding the concentration of immunoglobulins in CVF. Despite our group previously reporting levels of Igs across pregnancy and in response to adverse vaginal microbial compositions in a similar cohort at high-risk of delivering preterm (Chan et al., 2022), to better understand the role of immunoglobulins in CVF, future work should compare the amount of bound and unbound opsonins within the same set of samples.

Another limitation of the current study is that without *in vitro* functional work, interpretation of the findings should be made cautiously as these results only describe associative mechanisms. This reveals the necessity to further investigate the link between opsonisation, phagocytosis and activation of the complement cascade.

Future work

In this chapter I have presented how percentages of IgG-coated cervicovaginal bacteria are differentially bound depending on vaginal microbial, revealing higher percentages in women with CST I than in women with a CST IV (Figure 5.17-19.) and in women who delivered at term compared to preterm (Figure 5.35). It is widely known that the main role of IgG is as a neutralising antibody, but actually the selective binding of the 4 different subtypes of IgGs have different targets and effector functions. For example, only IgG1-3 activate the complement system, and IgG1 and IgG3 are usually induced in response to protein antigens; whereas IgG2 and IgG4 predominantly target polysaccharide antigens (Schroeder 2010). Therefore, to further complete this work, it would be interesting to establish the percentage of different IgG subtypes coating bacteria. This have been done in the gut in IBD diseases, with different IgG subtypes (specifically IgG1 and IgG3) correlating with different disease states (Masu et al., 2021). Another future direction would be to evaluate IgM opsonisation patterns, since Chan et al. (2022) also demonstrated increased concentrations of CVF IgM in association with microbial driven preterm birth. Future studies could also examine matched serum and CVF immunoglobulin concentrations.

Finally, as previously mentioned, 16s rRNA sequencing provides a relative abundance characterisation, but assessing of bacterial load is the quantitative tool to assess the precise amount of bacteria present. Therefore, to better understand the associations of bacteria bound to immunoglobulins and opsonins, it would be interesting to assess the bacterial load of each sample, to be able to evaluate the precise

associations between microbial composition and the immune response, and quantitatively assess the contribution of each microorganism towards triggering different responses.

5.8. Conclusion

In Chapter 5 I have developed a flow cytometry protocol to accurately identify IgG, IgA and C3b- bound bacteria from vaginal samples. This study is the first to assess the coating of bacteria in CVF samples of pregnant women. Higher percentages of opsonisation and MFI were observed with IgG, IgA and C3b in women dominated with CST I compared to CST IV, and increased binding to opsonins appeared to be associated with term deliveries.

Based on the homeostatic role that IgA performs in the gut, but being IgG the most abundant immunoglobulin in CVF, the data presented in this chapter indicates a similar regulatory mechanism taking place at the cervicovaginal interface, mainly orchestrated via IgG responses. The lower opsonisation percentages observed in women with diverse bacteria could be reflective of already phagocytosed bacteria or mechanisms of immune evasion (Figure 5.40).

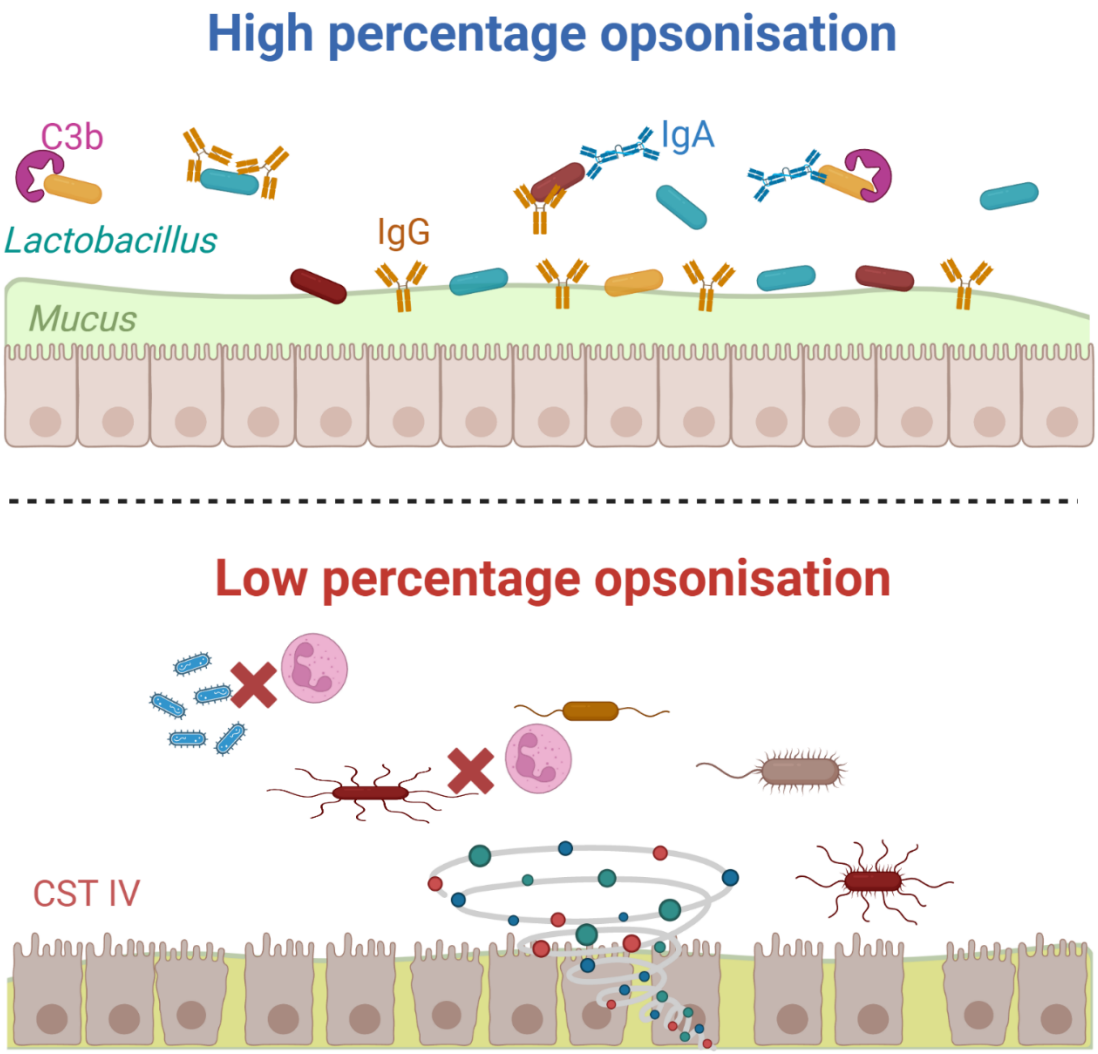


Figure 5.40. Opsonisation of cervicovaginal bacteria. A higher percentage of opsonisation was observed in women with a CST I *L. crispatus* vaginal dominance. This could indicate the role of opsonins (mainly IgG, but also IgA) in vaginal homeostasis. The reduced opsonisation binding in women with CST IV vaginal microbial composition could be due to bacterial immune evasion mechanisms.

6. DISCUSSION

PTB is a common problem affecting 15 million newborns globally each year (Blencowe et al., 2013). There have not been any major advances in prediction or prevention for decades, which is likely to be in part due to the many underlying aetiologies (Romero et al., 2014a), with no "one size fits all" solution. Without being able to effectively stratify women at risk or have deep mechanistic understanding for the different phenotypes of PTB, developing new therapeutic strategies is difficult. Therefore, a better understanding of the mechanisms leading to preterm birth is required to be able to individualise maternity care. Infection and inflammation have been long associated with a vast proportion of PTBs, with growing evidence of associations between certain vaginal microbial communities and PTB. In this context, the purpose of this study was to determine the role of the local cervicovaginal immune response to microbial composition as a key modulator of the risk, to demonstrate if there is potential for immunomodulation as a novel therapeutic strategy.

Local proinflammatory milieu in women with an adverse vaginal microbial composition, cervical shortening and preterm birth

In chapter 3 data was presented showing that proinflammatory cytokines IL-1β, IL-6 and IL-8 concentrations are increased at the cervicovaginal interface in women with an adverse vaginal microbial composition and in women who experienced cervical shortening and preterm birth. These results were consistent with the current literature (Amabebe et al., 2018; Chan et al., 2022; Fettweis et al., 2019; Florova et al., 2021; Jun et al., 2000; Kindinger et al., 2016; Park et al., 2019).

The main novelty of this study is demonstrating the significant contribution of the complement system at the cervicovaginal interface to host-microbial interactions and adverse pregnancy outcome. Until recently, the role of the complement system in pregnancy and parturition had only been studied in the periphery (He et al., 2020; Lynch et al., 2016, 2011, 2008a, 2008b; Richani et al., 2005; Segura-Cervantes et al., 2015; Soto et al., 2005; Vaisbuch et al., 2010), revealing certain associations between complement proteins and PTB and IAI. In the last couple of years, attention has been drawn to cervicovaginal interface, with only Hong, Livson and Chan investigating its role in the human in the context of pregnancy. These studies were focused on a limited panel of complement mediators but started to reveal relationship between complement proteins with pathophysiological events during pregnancy. To investigate the complement system in the most comprehensive way, mediators from the three activation pathways classical (C1q, C2, C4, C4b), lectin (MBL) and alternative (Factor B, Factor D), as well as downstream convergence of pathways on C3 and C5 convertases (C3, C3a, C3b, C5 and C5a), together with complement regulators (Factor H and Factor I) were evaluated. These analytes were dramatically increased in women who experienced cervical shortening, in women who delivered

preterm, and in women with an adverse vaginal composition, specifically women with a CST IV vaginal dominance.

Murine studies also support the contribution of cervicovaginal complement proteins in microbial-driven preterm birth. Gonzalez et al. worked with murine model for infection driven PTB administering lipopolysaccharide (LPS) vaginally. All mice delivered preterm following LPS treatment and were compared to gestational age-matched untreated control mice. They demonstrated increased deposition of C3 in the cervix. Also, via biomechanical evaluation of cervical tissue, LPS treated mice showed greater distensibility and elasticity, as well as a decreased number of collagen fibers. The disorganised collagen net was associated with increased MMP-9. Moreover, mice depleted of the C5a receptor (C5aR) (C5aR-/-) treated with LPS delivered at term. These findings were accompanied by a lower MMP-9 activity, as well as less collagen degradation (Gonzalez et al., 2011). This work supports the role of the complement system in microbial driven cervical remodelling and preterm birth.

Finally, matrix metalloproteinases (MMP-2, MMP-8, MMP-9) were also increased in women with cervical shortening and in women who delivered preterm. However, no associations were observed between concentrations of MMPs and the vaginal microbial composition. Potentially, the elevated concentration of MMPs is not a direct cause of microbial-induced inflammation, but a secondary effect after other inflammatory mediators (cytokines, complement proteins) leading to MMPs release from other sources such as epithelial cells.

The role of neutrophils in parturition

Neutrophils have been demonstrated to be present in the cervix of both complicated and uncomplicated pregnancies (Bokström et al., 1997; Junqueira et al., 1980; Osman et al., 2003; Whitworth et al., 2007). However, there have been limited studies investigating their potential role in preterm birth. Neutrophils are involved in the clearance of pathogens via phagocytosis, producing collateral damage as a result of their effector functions such as degranulation, respiratory burst, and formation of NETs (Figure 1.13., Table 1.4.). Neutrophils can release proinflammatory cytokines (Tecchio et al., 2014), complement proteins (Botto et al., 1992), and also pro-labour mediators such as COX-2 (Maloney et al., 1998), prostaglandins (St-Onge et al., 2007), MMPs (Kelly, 2002; Rath et al., 1998), and neutrophil elastase (Helmig et al., 1995).

In chapter 4, data presented showed that neutrophils accounted for the highest percentage of CD45 cells in cytobrush samples, as previously showed by Hunter and Mohd Zaki (Hunter et al., 2016; Mohd Zaki et al., 2022). However, to the best of our knowledge, this is the first time that the

immunophenotype of cervical neutrophils has been compared directly to matched peripheral neutrophils. Furthermore, this study revealed that cervical neutrophils had an immunophenotype consistent with greater activation.

It is not surprising that cervical and peripheral neutrophils have differential expression of surface markers, as expression will reflect cell function (Elghetany and Lacombe, 2004) in response to different stimuli of the environment and maturation status. Other cell types (e.g., T cells) were found to express higher levels of activation markers between cervical and peripheral populations in non-pregnant women (Prakash et al., 2001). Amsalem et al. found that neutrophils isolated from human decidua expressed higher levels of CD66b compared to peripheral neutrophils (Amsalem et al., 2014). This was consistent with our results that cervical neutrophils also expressed higher levels of CD66b compared to peripheral neutrophils. IL-8 receptors CD181 and CD182 were more highly expressed in peripheral neutrophils compared to decidual neutrophils. The authors suggested that a decreased expression could be a result of the internalisation of the receptor after ligand stimulation (Amsalem et al., 2014). Receptor binding and internalisation may explain our findings of reduced expression of CD35 (C3a receptor) and CD16 (Fcγ receptor III) on cervical neutrophils compared with peripheral neutrophils.

Cervical neutrophils were not present in every woman. There was a subtle increase in the proportion of detection in women with a high-risk microbial composition. Differential expression of activation markers on cervical neutrophils were observed in women with a CST IV vaginal composition compared to CST I (Figure 4.26.). The distinctive reduction of CD16 seen in CST IV could indicate internalisation of the receptor after interaction with Ig-complexes, as well as a simultaneous degranulation and phagocytosis in association with increased expression of CD63 and CD66b. As a result of neutrophil activation, neutrophils could secrete and degranulate the aforementioned pro-labour mediators, including more cytokines (creating a positive feed forward loop), prostaglandins and matrix metalloproteinases, leading to cervical shortening. In support of this, the presence of higher proportions of cervical neutrophils was associated with higher concentrations of IL-1β, IL-8, complement proteins (C1q, C4, Factor D, C3a, C3b) and MMP-8 in CVF (Figure 4.42.- 4.48.). This close association between neutrophil presence and a rich environment of complement proteins is not surprising since neutrophils express several complement receptors: CR1 (CD11b), CR3 (CD35), CR4 (CD11c), DAF (CD55), CD59, C5aR (CD88). The expression of these receptors is dynamic and can vary upon different stimuli and interaction with inflammatory mediators (Elghetany, 2002; Elghetany and Lacombe, 2004; Sengeløv, 1995). Furthermore, neutrophils are highly likely to be contributing to the suggested local complement production via release of C3, factor B, factor D, M-ficolin (Botto et al., 1992; Liu et al., 2005; Wirthmueller et al., 1997; Yuen et al., 2016).

Some inflammatory mediators (IL-1β, C1q, C4, Factor B and D, C3, MMPs) were also detected in the absence of cervical neutrophils, but concentration was obviously highest in women with neutrophils and a high-risk microbial composition present (Figure 4.41., Table 4.4.), perhaps indicating a dysregulated and collateral damage as a result of a massive neutrophil activation. In the absence of neutrophils other leukocytes (e.g., macrophages) or the cervicovaginal epithelium itself could be driving a lower-grade inflammation.

Opsonisation of cervicovaginal bacteria during pregnancy

Despite many studies in the field of PTB describing associations between certain vaginal microbial compositions and the host immune response, little is known about how these two components actually interact between each other. Bacterial taxa can be targeted by different opsonins (immunoglobulins, complement opsonins) triggering different immune responses. Our group previously reported an increase of free unbound IgA and IgG1-4 levels in CVF samples from women with a CST IV *L.* depletion compared to CST I *L. crispatus* dominance (Chan et al., 2022). Ig-complexes have the ability to activate the complement cascade via the classical pathways, binding to C1q. IgA cannot activate the complement cascade, but C3b, the other opsonin evaluated, mediates opsoniphagocytosis. Chan et al. (2022) and I (chapter 3) showed that the concentration of C3b was higher in CVF of women with a CST IV vaginal composition.

In Chapter 5 I presented there was a higher percentage of bacteria bound to opsonins in women with a CST I *L. crispatus* dominance. Breedveld et al. also showed that vaginal microbiota from non-pregnant women is also targeted by both IgA and IgG (Breedveld et al., 2022). These data implies that IgA and IgG opsonisation may be a protective mechanism whereby bacteria are neutralised to prevent immune activation and promoting vaginal homeostasis. This was surprising and completely unexpected, as our hypothesis suggested that women with an adverse vaginal microbial composition would have had higher opsonisation percentages to assist microbial targeting for destruction by the immune response. Instead, this result appears to indicate that opsonin binding, particularly IgG could be beneficial. This is consistent with the literature implicating IgA as being important for gut microbiome homeostasis (Chen et al., 2020). With IgG being the most abundant immunoglobulin in the vaginal secretions (Chan et al., 2022; Johansson and Lycke, 2003)(Johansson 2003, Chan et al 2022), IgG could be implicated in anchoring commensals to the vaginal epithelium, enhancing beneficial bacteria colonisation.

The fact that C3b opsonisation was also reduced in CVF from women dominant with CST IV could indicate that members from CST IV are actively performing immune evasion mechanisms to survive in the cervicovaginal milieu. The lower percentage of opsonin binding was associated with an increase in immune mediators at the cervicovaginal interface. It is likely that the thriving and immune evasion of members of CST IV, makes them "free" to gain access to the cervicovaginal interface, releasing toxins and virulence factors perpetuating an inflammatory microenvironment. Since these members are not recognised by opsonins, perhaps the cervicovaginal epithelium is contributing to the proinflammatory milieu via direct release of cytokines and complement proteins. In support of this, *in vitro* work demonstrating how ectocervical and endocervical cells treated with supernatant from cultured *L. iners* and *G. vaginalis* exhibit a greater release of cytokines compared to *L. crispatus* (Anton et al., 2018). In addition, it was demonstrated that co-cultures of *G. vaginalis* with cervicovaginal epithelial cells resulted in an increased cell death and strong inflammatory response compared to co-cultures with *L. crispatus* (Anton et al., 2022).

The findings described in chapter 3, 4, and 5 confirm the important role that the immune response plays at the cervicovaginal interface. It is probable that neutrophils are major contributors of the inflammatory immune response, although the extent of other participants (macrophages, cervicovaginal epithelial cells) cannot be ruled out. Regardless of which cells are contributing, the end point is a dysregulated proinflammatory immune response in women who undergo cervical shortening and PTB, and that demonstrates a strong rational for immune modulation therapeutics (Figure 6.1.).

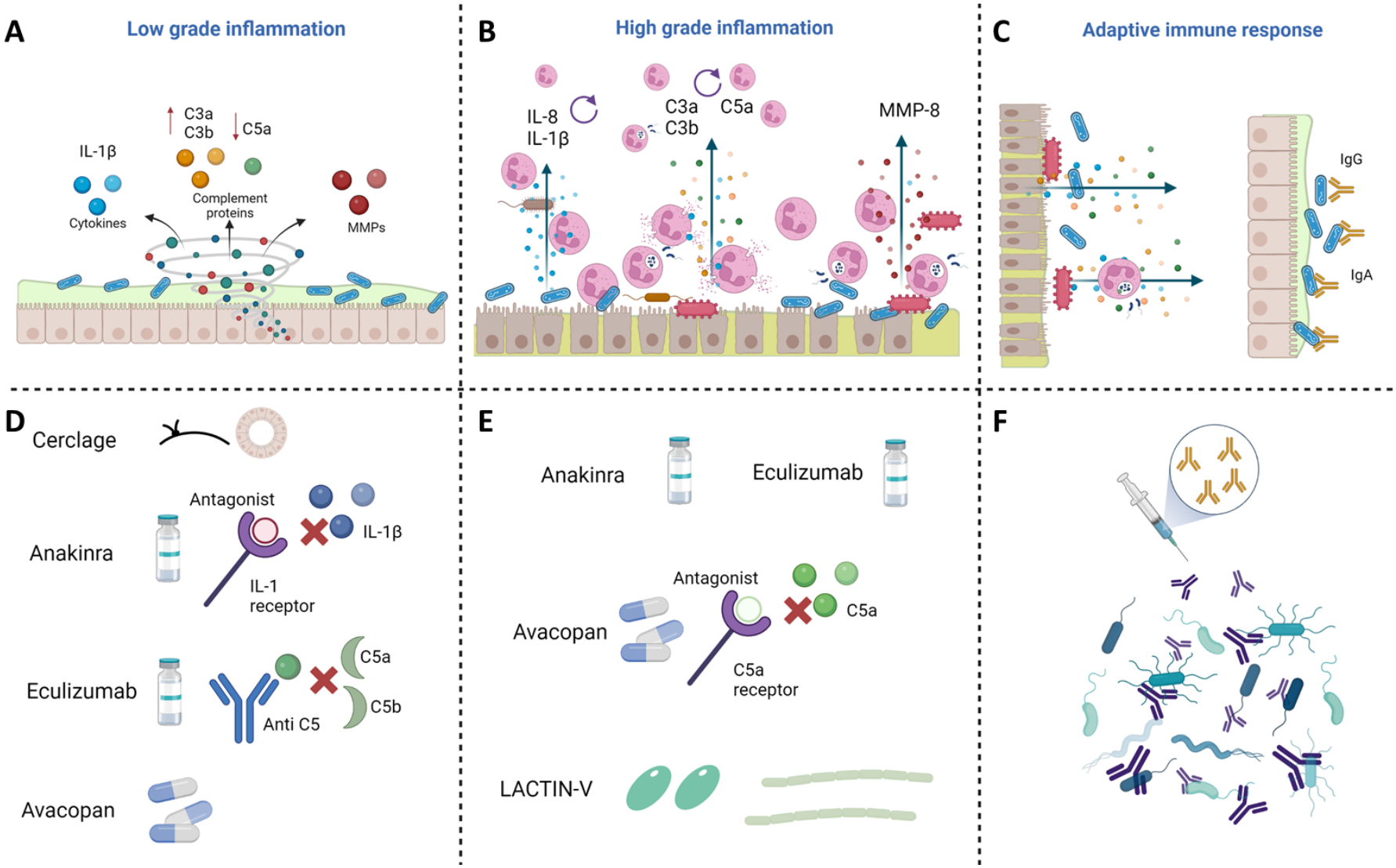


Figure 6.1. A summary of mechanisms triggering immune responses at the cervicovaginal interface as revealed from this study and potential therapeutic strategies. In the absence of adverse vaginal microbial composition and/or cervical neutrophils a "low-grade inflammation" was detected involving cytokines (IL-1β), complement proteins

and MMPs in a proportion of women **(A)**. The potential origin of these inflammatory mediators could be the cervicovaginal epithelium or other leukocytes (e.g., macrophages). The combination of an adverse vaginal microbial composition and cervical neutrophils triggers a "high-grade inflammation" **(B)**. The presence of chemoattractants (IL-8, C3a, C5a) could create a positive feedback loop potentiating the recruitment of more neutrophils; and neutrophils would contribute to the inflammatory immune response releasing its granular contents, including matrix metalloproteinases (e.g., MMP-8). The adaptive immune response can modulate the response to the local cervicovaginal microbiota **(C)**. Opsonin binding appears to be associated with vaginal health homeostasis, with higher percentages of IgG and IgA opsonisation in the presence of *L. crispatus*. While *Lactobacillus* depletion and high diversity communities are less bound to opsonins, likely due to immune evasion mechanisms or because bacteria have already been phagocytosed. Understanding distinct inflammatory mechanisms can lead to developing new prediction and preventive tools, targeted for each of the conditions. Low grade inflammation could be treated with immune modulators and cervical cerclage **(D)**. In a high-grade inflammation situation, cervical cerclage is unlikely to be successful, therefore immune modulation and/or biotherapeutics could be a potential strategy **(E)**. Finally, understanding how the adaptive immune response participates in the cervicovaginal milieu could lead to the development of vaccines targeting specific species and strains of pathogenic bacteria **(F)**. Image created with Biorender.

Potential new biomarkers

This study has demonstrated new associations between a large array of complement proteins and MMPs, that together with proinflammatory cytokines, with the development of cervical shortening and preterm birth. Additionally, these mediators were increased as early as 12-16 weeks of gestation, adding value to their potential as predictive markers. Currently, there is no cervicovaginal biomarker available that can be used to predict preterm birth so early on in pregnancy. Fetal fibronectin and insulin-like growth factor binding protein-1 (Actim partus) have good negative predictive values, and limited positive predictive value, however they can only be used from the 22nd week of pregnancy. Furthermore, these biomarkers do not provide any insight on the mechanism or aetiology of PTB, which also limits their value in providing individualised preventative treatment. In contrast, detection of higher concentrations of complement in early pregnancy offers additional potential for a personalised medicine approach by identifying women at risk earlier in pregnancy, and to identify those who may benefit from immunomodulator therapy.

A limitation to using these biomarkers as predictive markers is the need to use a concentration threshold. Although an overall higher median concentration was detected in many of the analytes of women who developed cervical shortening and who delivered preterm, the range of concentrations varied greatly. Current data has not been analysed to determine if the higher concentrations were associated with shorter latency to delivery for example. However, these findings provide the rational for future work to explore the development of a point-of-care test. An example of the use of varied thresholds to increase prediction performance is seen with the use of quantitative fetal fibronectin using the QUiPP App together with cervical length measurements (Watson et al., 2017). The risk of PTB calculated by the app algorithm will vary depending on the concentration of fFN; for example, a higher concentration of fFN (500ng/mL) implicates a higher risk of PTB compared than someone with a lower concentration (e.g., 60ng/mL). Therefore, a similar strategy could be applied to develop quantitative tools to assess other inflammatory mediators as the ones measured in this project. Another of the main advantages of this work is that not only one marker has been identified in association with cervical shortening and preterm birth, but several. Perhaps a combination of multiple markers, or a ratio between certain complement proteins, could be indicative and have an added predictive value.

Support for the potential to apply these immune mediators as a point-of-care test in the clinical setting comes from the ability of metabolite profiling by DESI-MS for rapid characterisation of microbial composition and several immune analytes already tested (Pruski et al., 2021). Pruski et al. used two independent cohorts to demonstrate the feasibility to identify a specific and unique set of metabolite

markers that can predict the vaginal microbial composition, discriminating between *Lactobacillus* deplete and *L.* dominant compositions, but also to CST level. The immune profiling of 12 different cytokines, 6 immunoglobulins and 4 complement analytes were also mapped by metabolite signature. Therefore, by using DESI-MS, there is potential to develop a bed-side test with a rapid acquisition and assessment (Pruski et al., 2021). After expanding our knowledge on the complement system and MMPs involved in this proinflammatory milieu, this tool could incorporate the additional analytes from this project for improved prediction. This tool could also be used to measure the response to treatments, potentially defining which women would be more suitable for different types of intervention (cervical cerclage, immune modulation therapies or probiotic treatment).

Assessing the role of cervical neutrophils with cytobrush samples had potential for being a feasible screening test, like the cervical smear screening programme. Flow cytometry techniques, that are already available within hospital settings could be used to identify specific cell surface markers such as CD16, CD63 or CD66b. However, since not every woman with an adverse vaginal microbial composition, or who developed cervical shortening or subsequently delivered preterm had cervical neutrophils present, it is concluded that the detection and analysis of cervical neutrophils would not be an effective screening tool for preterm birth prediction.

Potential new therapeutics

The focus of this PhD was to understand the mechanisms underlying microbial-driven cervical shortening and preterm birth, to facilitate the development of novel preventative strategies such as live biotherapeutics, immune modulators and repurposed complement therapeutics.

Targeting the vaginal bacterial composition: live biotherapeutics and probiotics

Since the vaginal microbiota plays an important role in reproductive health, with high diversity and depletion in *Lactobacillus* spp. being associated with higher risk of preterm birth, and *L. crispatus* considered to be protective, modulation of the vaginal microbial composition could provide a novel therapeutic strategy. Probiotics have been defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). The term “live biotherapeutics” emphasises the fact that the live microorganisms are suitable for the treatment and management of specific conditions (Cordaillat-Simmons et al., 2020).

In non-pregnant women, vaginal administration of Lactin-V *L. crispatus* as an adjuvant after standard care (antibiotic treatment) can reduce the risk of recurrent UTI (Stapleton et al., 2011) and BV (Cohen et al., 2020). Results from Daskalakis and colleagues are also very encouraging. 106 women with PPROM were randomised to only antibiotic prophylaxis or antibiotic treatment combined with vaginal probiotic (containing *L. rhamnosus* and *L. gasseri*). In this study, the gestational age, the latency period between PPROM and delivery, and the birth weight were higher in women who received the vaginal probiotic after antibiotic management, compared to women who only received antibiotic treatment (Daskalakis and Karambelas, 2017). In 2019, our group started the first trial administering live biotherapeutics in pregnancy (ClinicalTrials.gov Identifier: NCT03992534), in which pregnant women at risk of delivering preterm were offered *Lactobacillus crispatus* CTV-05 (Lactin-V, Osel) during pregnancy (Bayar et al., 2022). This study included 61 participants and showed that the use of Lactin-V in pregnancy is safe, tolerable and well-accepted. Despite being underpowered to assess clinical outcomes, it was noted that there was a reduction in the rate of PTB in women who received the vaginal probiotic compared to a historical matched cohort (Bayar et al., 2022). Preliminary data about the vaginal microbial composition indicates a shift in the microbial composition in women with a CST III or CST IV prior the vaginal administration to a CST I (post loading dose and maintenance dose) (Bayar et al., unpublished), as well as decreased CVF concentrations of IL-1β, IL-6 and IL-8 (Bayar et al., unpublished).

Targeting inflammation

Although a strong proinflammatory response was seen in women with an adverse vaginal microbial composition, inflammation was also detected in a proportion of women with CST I who experienced cervical shortening and preterm birth. Therefore, a potential way to modulate the risk of preterm birth could involve anti-inflammatory therapeutics, with cytokines as a target. Inhibiting cytokines and/or cytokine receptors could serve to prevent the positive feed forward loop of augmented inflammation to prevent further immune cell influx and to promote immune suppression.

In inflammatory bowel disease, biologics are routinely administered. In 2020, a large systematic review and meta-analysis (48 studies and 6963 patients) revealed that the prevalence of adverse pregnancy outcomes observed in IBD pregnancies with usage of biologics, was comparable to the observed in the general population, without increased risks of adverse pregnancy outcomes such as PTB, low birth weight or malformations (Nielsen et al 2020). Biologics usage in pregnancy such as Adalimumab (anti-TNFα, for chronic inflammatory diseases) and Anakinra or Canakinumab (anti-IL-1β, for inflammatory conditions such as arthritis and lupus) has not being associated with increased

adverse of pregnancy outcomes (Brien et al., 2022; Chambers et al., 2019). In the group of IL-1 receptor inhibitors, Rytvela appears to be the most promising IL-1 biologic (Habelrih et al., 2022; Nadeau-Vallée et al., 2017, 2015).

Tocilizumab is a recombinant humanised antibody that targets the interleukin-6 receptor inhibiting the IL-6 signalling pathways, with approved use for treatment of rheumatoid arthritis and systemic juvenile idiopathic arthritis (Hoeltzenbein et al., 2016). There was evidence from animal models and *in vitro* work suggesting a potential beneficial application in pregnancy. For example, in a mouse model of LPS induced PTB, administration of anti-IL-6 receptor antibody (MR16-1 treatment) reduced the rates of PTB compared to controls (17% vs 53%, p=0.0026). In addition to prolonging pregnancy, no adverse effects were observed in the mice and pups. Moreover, after treating human primary amniotic epithelial cells with IL-6, administration of tocilizumab (human IL-6 receptor) down-regulates the expression of COX-2 and decreases PGE2 concentration in culture media (Wakabayashi et al., 2013). Hoeltzenbein et al. evaluated tocilizumab exposure pre-pregnancy and during pregnancy and although there was no increase in adverse effects/malformations, there was a higher rate of premature birth compared to the overall global rate (31.1.% vs 10-12%) (Hoeltzenbein et al., 2016). However, this study evaluated PTB outcomes as any delivery before 37 weeks of gestation, with no discrimination between iatrogenic or spontaneous, or the aetiologies of PTB. Furthermore, it is important to take into account that autoimmune diseases such as rheumatoid arthritis on its own are associated with higher preterm birth rates, and the severity of the disease correlates with preterm deliveries and low birth weights (de Man et al., 2009). During the COVID-19 pandemic, the WHO recommended the administration of IL-6 antagonists to pregnant women with severe COVID-19 (WHO Rapid Evidence Appraisal for COVID-19 Therapies (REACT) Working Group et al., 2021) the current available data does not suggest an adverse effects or increase in spontaneous PTB rates for women who were treated with tocilizumab (Jorgensen and Lapinsky, 2022)(Jorgensen et al 2022).

However, one of the limitations of monoclonal antibodies as therapeutic agents is the considerable productions costs derived from large cultures, purification steps, following Good Manufacturing Practice conditions, which limits the wide use of these drugs (Chames et al., 2009). Also, to be considered, is that these therapies currently involve intravenous administration of the monoclonal antibodies, since the application is derived from systemic chronic inflammations, and enteral administration would prevent vaginal distribution. Ideally, a local administration in the form of a pessary is required to be able to target the cervicovaginal interface.

Targeting the complement system

Complement therapeutics are a rapidly evolving area of therapeutic development with many targets of the pathway having drugs in phase I-III clinical trials, and several with FDA approval for conditions in non-pregnant population (Zelek et al., 2019). Eculizumab is an anti C5 antibody (humanised immunoglobulin IgG2/4kappa) that prevents the formation of C5a and C5b by blocking C5. It is FDA approved in pregnancy for the treatment of paroxysmal nocturnal haemoglobinuria, atypical haemolytic uremic syndrome and haemolysis elevated liver enzymes and low platelet syndrome. Its use has been considered as safe and efficient for pregnancy (Sarno et al., 2019; Stefanovic, 2019). However, again, this is an expensive monoclonal antibody administered intravenously. With a price of £3150 per 30mL/vial, a total of £170,000 per adult for a 6 month course of treatment would be required.(NICE Press and media, News, n.d.) Despite this quoted expense, dosages and duration of treatment would need to be evaluated for pregnancy, and the cost benefit against the short and long term cost of an extremely preterm birth.

Recently, a new complement therapeutic has been developed and approved for the treatment of antineutrophil cytoplasmatic antibody (ANCA)-associated vasculitis (Jayne et al., 2021). The pathogenesis of ANCA-associated vasculitis involves dysregulation of the complement pathway via the alternative pathway, resulting in production of C5a; and acute lesions are characterised by neutrophil infiltration (Michailidou et al., 2020). Avacopan is an orally administered small molecule C5a receptor antagonist that blocks the C5a receptor (C5aR, also known as CD88), avoiding the effects of C5a, including chemoattraction and activation of neutrophils (Jayne et al., 2021). Despite Avacopan never being used in pregnancy, this molecule presents many advantages that could be translated into pregnancy settings. First, it targets the complement system, which I have shown to be dysregulated in women with adverse microbial composition, cervical shortening and preterm birth. Secondly, via blocking the complement system, neutrophil migration and activity is impaired. Neutrophils are also involved in pregnancy, particularly in the process of preterm cervical remodelling; and Gonzalez et al. showed in a murine C5aR knockout mice that mice did not delivered preterm after administration of LPS (Gonzalez et al., 2011). Thirdly, since it targets the C5aR and not C5 like other monoclonal antibodies, C5b is available to form the membrane complex attack to still provide mechanisms of defense for potential infections. Lastly, but equally relevant, this drug has been developed as a small-molecule, therefore there is a potential translation into a pessary, in order to be administered vaginally, which would permit a local rather than systemic effect.

Targeting opsonisation

The results from chapter 5 were unexpected: a higher percentage of opsonisation was observed in women dominated with *Lactobacillus* spp., particularly *L. crispatus* CST I dominance; whereas women dominated with CST IV heterogenous and diverse communities exhibited lower percentages of opsonin binding. A reassessment of the original hypothesis based on the data presented points to a higher opsonin binding being somehow protective, and not resulting in clearance of bacteria. It is likely that opsonisation carefully regulates promotion of neutralisation, promotion of colonisation and targeting pathogenic commensals for opsonophagocytosis. It is likely that some bacterial taxa classed as CST IV may have reduced opsonisation due to specific immune evasion mechanisms. For example, GBS has developed many mechanisms to avoid the immune system such as avoidance of recognition by neutrophils (Carlin et al., 2009b, 2009a) and avoidance of complement deposition (Jarva et al., 2004; Pietrocola et al., 2016). The polysaccharide capsules that inhibit complement deposition are some of the promising targets that are being currently investigated as potential GBS vaccines (Buurman et al., 2019). It is plausible that a deficiency in maternal antibody responses towards certain bacterial taxa could be the reason for continued colonisation with pathogenic bacteria. A better understanding of opsonin binding patterns could provide new targets for vaccines as a strategy for the prevention of microbial driven preterm birth.

Strengths and limitations

One of the main strengths of this project is that women were sampled more than once during their pregnancy. This allowed for a comprehensive immunophenotype to be assessed that was not restricted to single moment in pregnancy. For example, the earlier pregnancy timepoint allowed the detection of a proinflammatory response before the actual event of cervical shortening occurred.

Another important strength was that cervical shortening was evaluated as an outcome regardless of pregnancy outcome. This allowed the ability to determine the immune phenotype changes seen leading up to cervical shortening, and unlike preterm birth as an outcome, was not influenced by intervention.

In all cohorts, preterm birth cases were compared to term uncomplicated pregnancies as a control. The fact that this is a pure uncomplicated healthy natural pregnancy provides the most appropiate control as potential changes/shifts from the normal trajectory of pregnancy were not included as a result of an intervention such as cervical cerclage. However, this is a constraint at the same time, since

the proportion of women with history of cervical treatment tends to be higher in this TU group, therefore the nature of the risk factors between groups is matched less accurately.

A valuable part of this project is the detailed methodology optimisation for exploring cervical leukocytes by flow cytometry, as well as to evaluate the percentage of opsonisation and microbial-host interactions in cervicovaginal fluid. These protocols can potentially be applied to other cell types, mediators, pregnancy related complications and other women's health studies not related to pregnancy.

There are several limitations to this study. The influence of the microbial compositions on PTB outcome is known to differ depending on ethnicity. This study was not powered to examine host-microbial interactions by ethnicity. In addition, the mechanism of cervical shortening and PTB is likely to differ between women with a history of cervical treatment and a history of MTL/PTB. This study was also not powered to be able to perform a subclass analysis in women depending on risk factor for preterm birth. These limitations will be addressed in future studies.

Future work

Future work will include *in vitro* studies to assess the contribution of vaginal and cervical epithelial cells to the immune response to pathogenic and protective commensals. Work is also underway to determine the potential role of cervical neutrophils and the complement system in women with PPROM. Future work with larger cohorts would also allow to stratify patients by risk factors, and ethnicity. Finally, other proteases (e.g., neutrophil elastase, MPO) and pro-labour mediators (e.g., prostaglandins) are being assessed in CVF to evaluate the influence of microbial composition on these mediators in women who deliver preterm.

Final conclusion

In summary, this study has deepened the understanding of the response of the innate and adaptive immune system to pathogenic and non-pathogenic vaginal commensals. A clear association exists between the presence of cytokines, complement and matrix metalloproteinases and the development of cervical shortening and preterm birth. Furthermore, it is likely that neutrophils are the predominant cell type contributing to the proinflammatory immune milieu. The results from this study support the potential for repurposing complement therapeutics and the use of live biotherapeutics to modulate the local immune response to favour successful term pregnancy. It offers the potential to develop a personalised medicine approach for preterm birth prevention in women where the mechanism is microbial driven inflammation.

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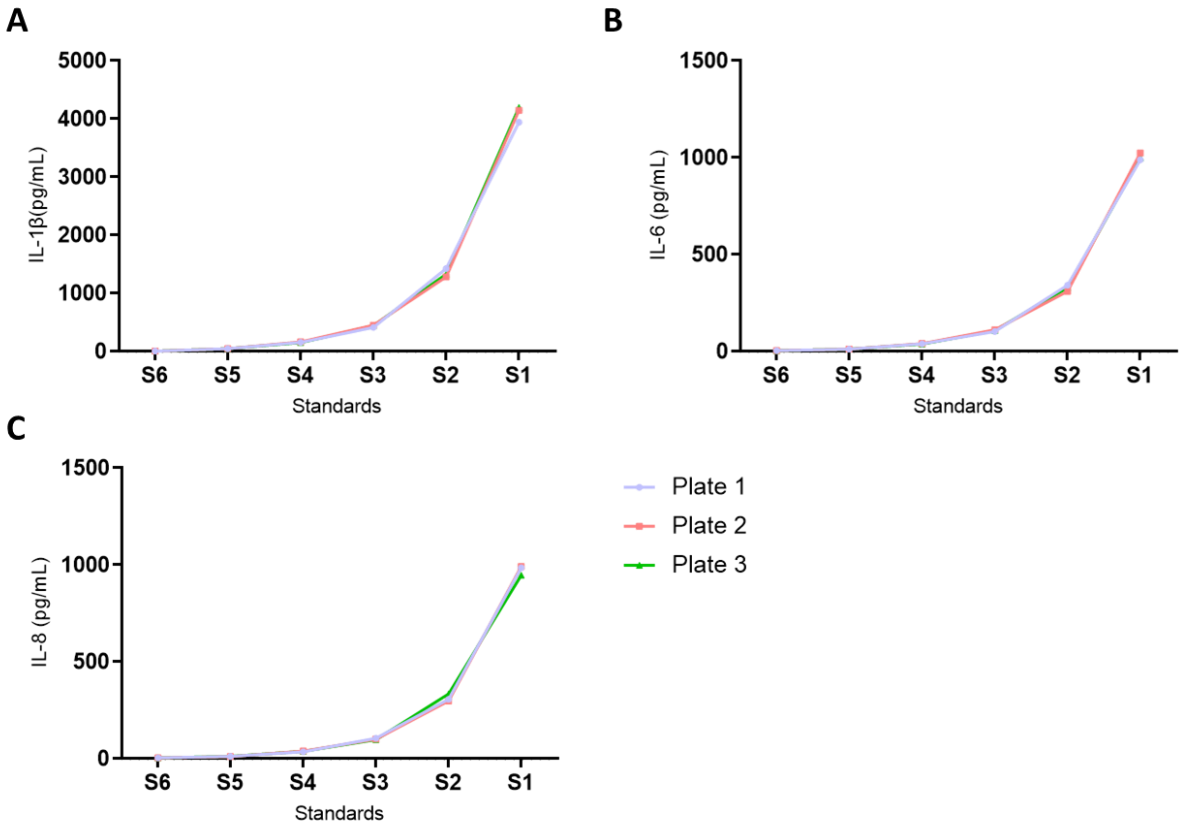
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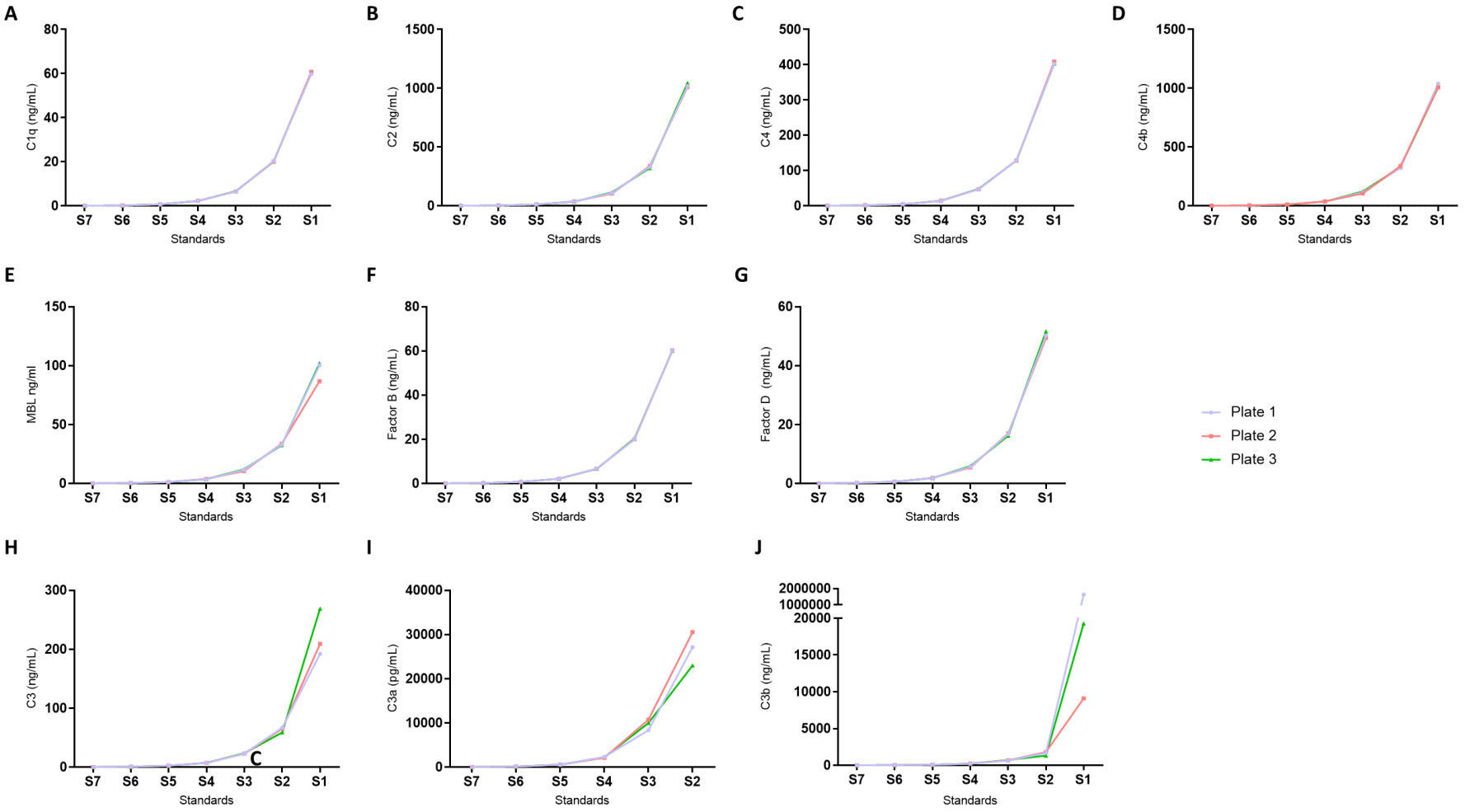
8. APPENDIX

8.1. Chapter 3 Appendixes

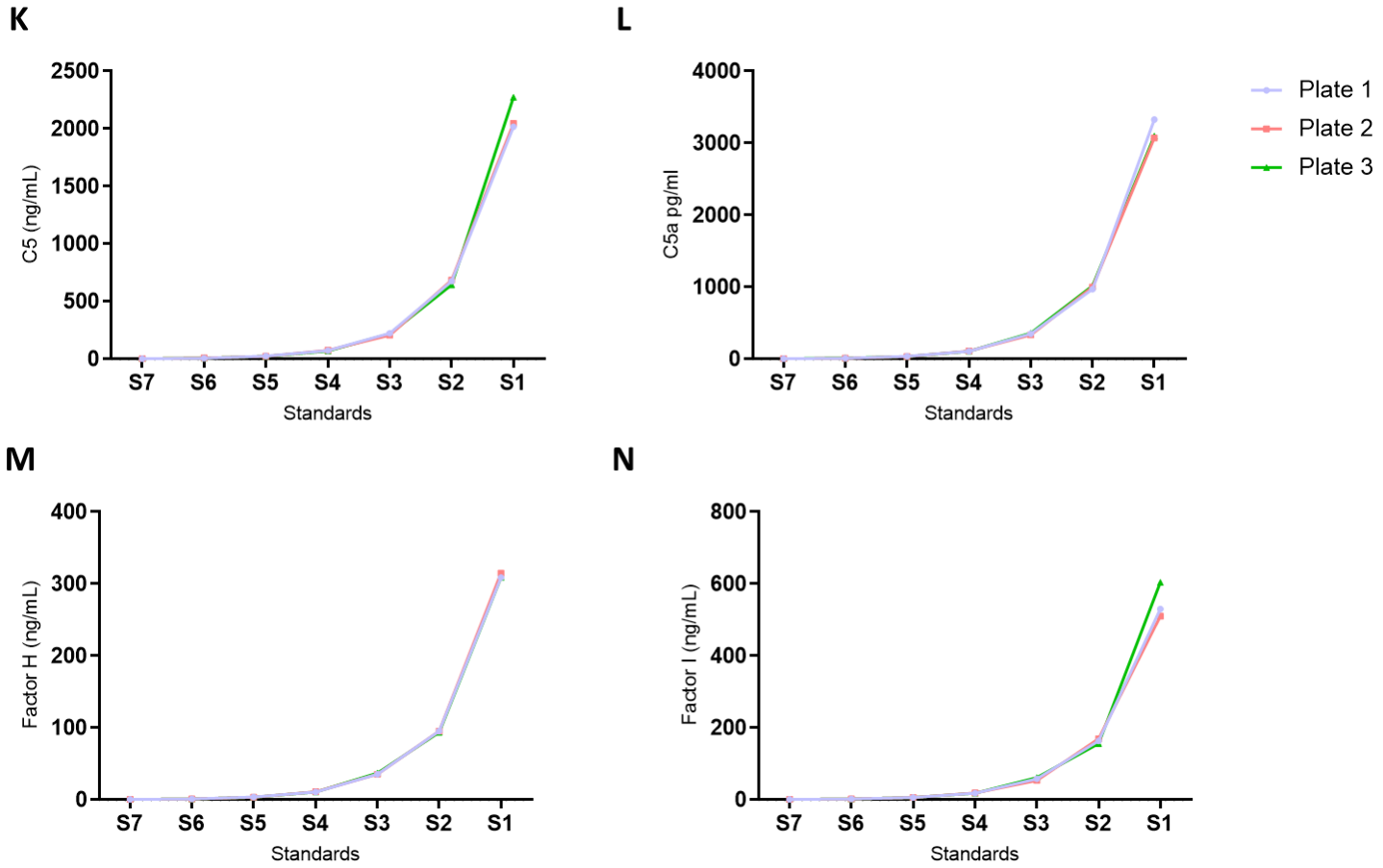
Appendix 3.1. Merged plotting of standard curves across three different plates for cytokines analytes. A) IL-1β, **B)** IL-6 and **C)** IL-8.



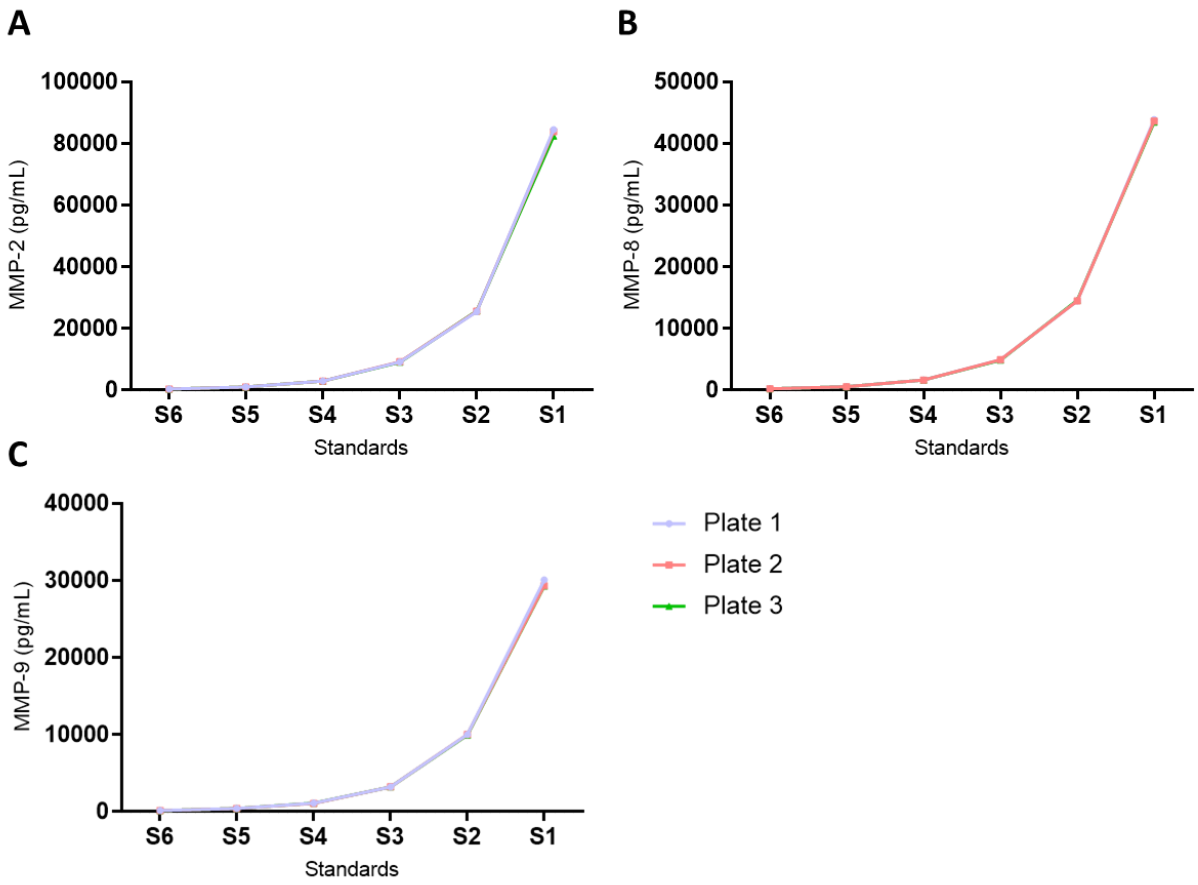
Appendix 3.2. Merged plotting of standard curves across three different plates for complement protein analytes. A) C1q, **B)** C2, **C)** C4, **D)** C4b, **E)** MBL, **F)** Factor B, **G)** Factor D, **H)** C3, **I)** C3a, **J)** C3b, **K)** C5, **L)** C5a, **M)** Factor H, **N)** Factor I.



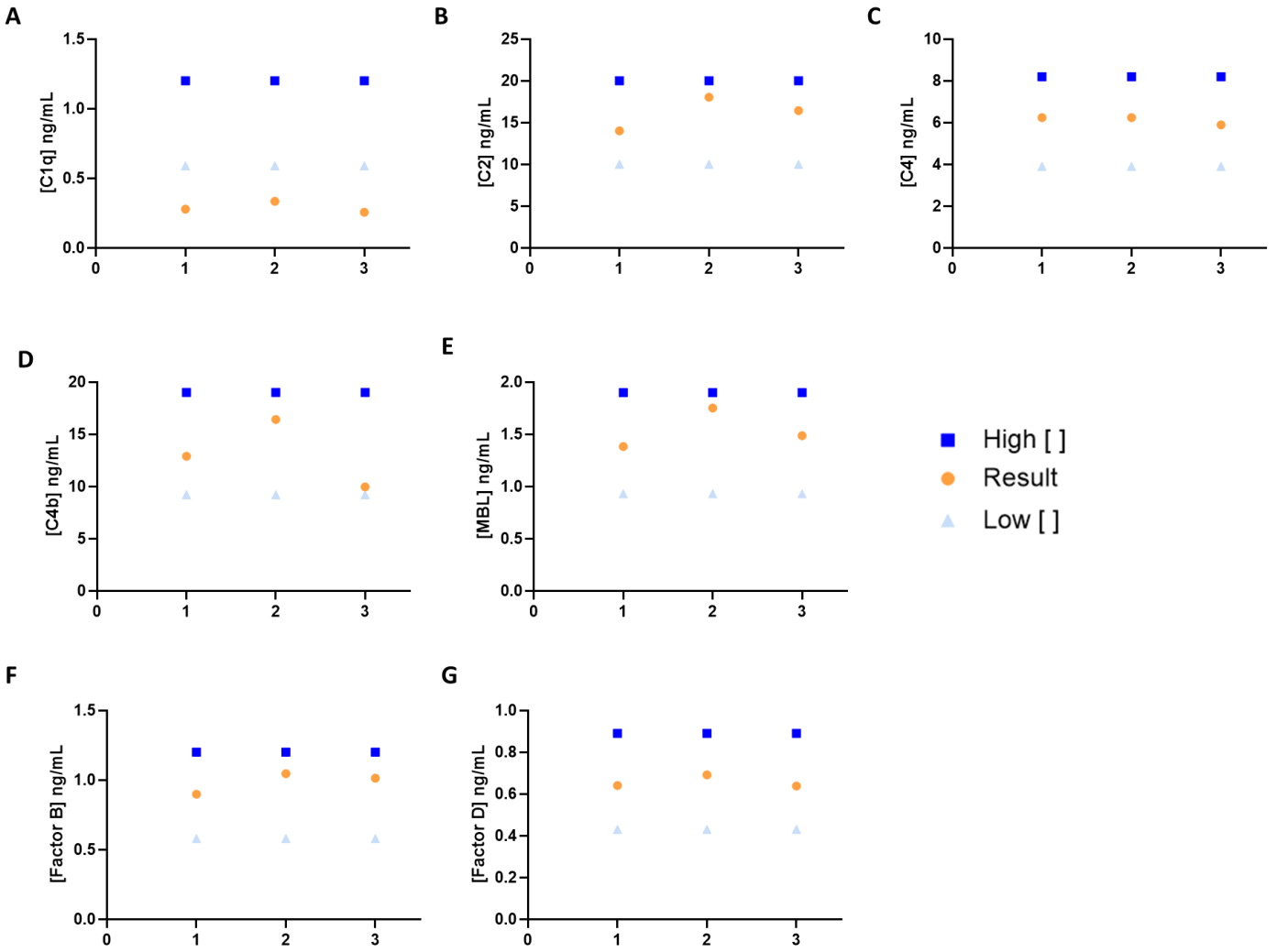
Appendix 3.2. Continuation.



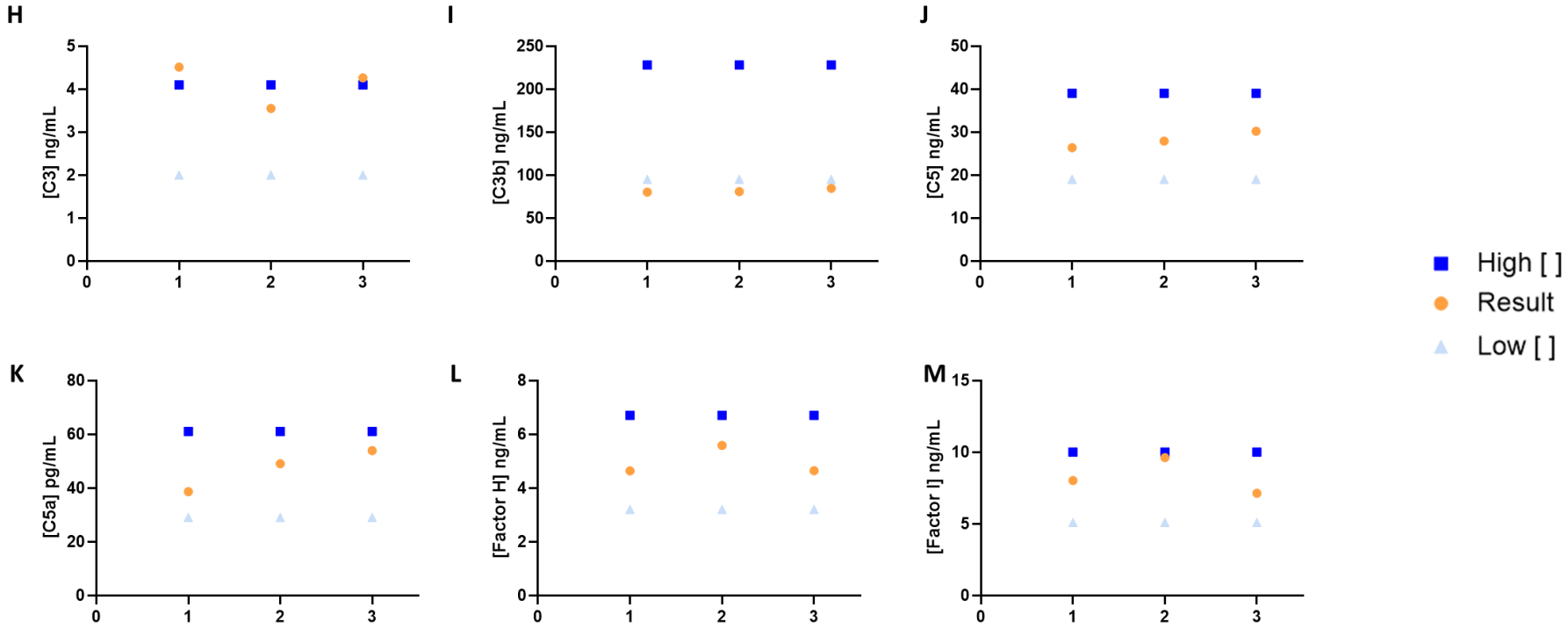
Appendix 3.3. Merged plotting of standard curves across three different plates for matrix metalloproteinases analytes. A) MMP-2, **B)** MMP-8, **C)** MMP-9.



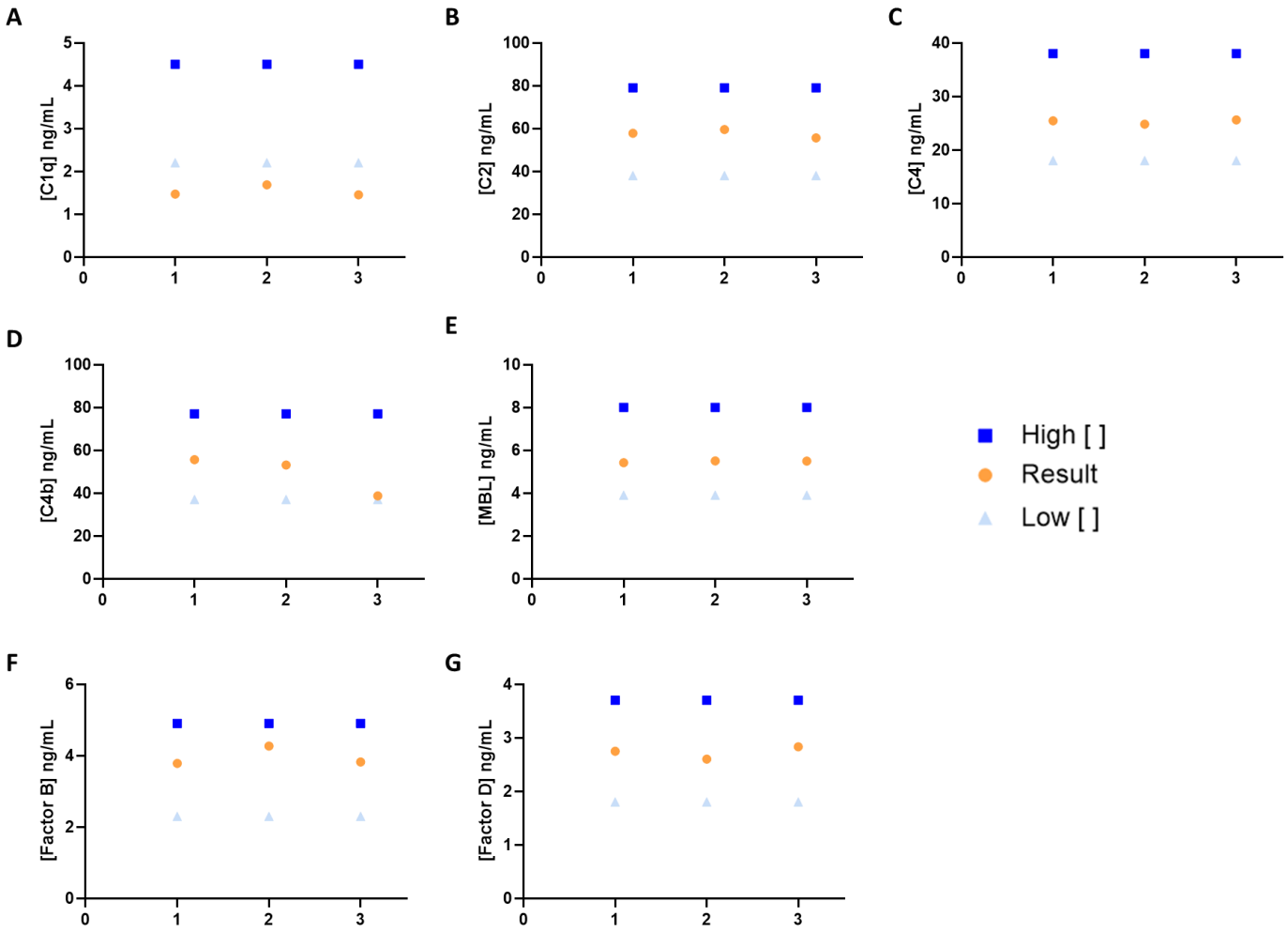
Appendix 3.4. Quality Control 1 test result for Human Complement Panels. Highest concentration expected is plotted as a blue square. Lowest concentration expected is plotted as a light blue triangle. Average of the test result is plotted as an orange dot. QC1 was tested across three different plates (X axis) for **A)** C1q, **B)** C2, **C)** C4, **D)** C4b, **E)** MBL, **F)** Factor B, **G)** Factor D, **H)** C3, **I)** C3b, **J)** C5, **K)** C5a, **L)** Factor H and **M)** Factor I.



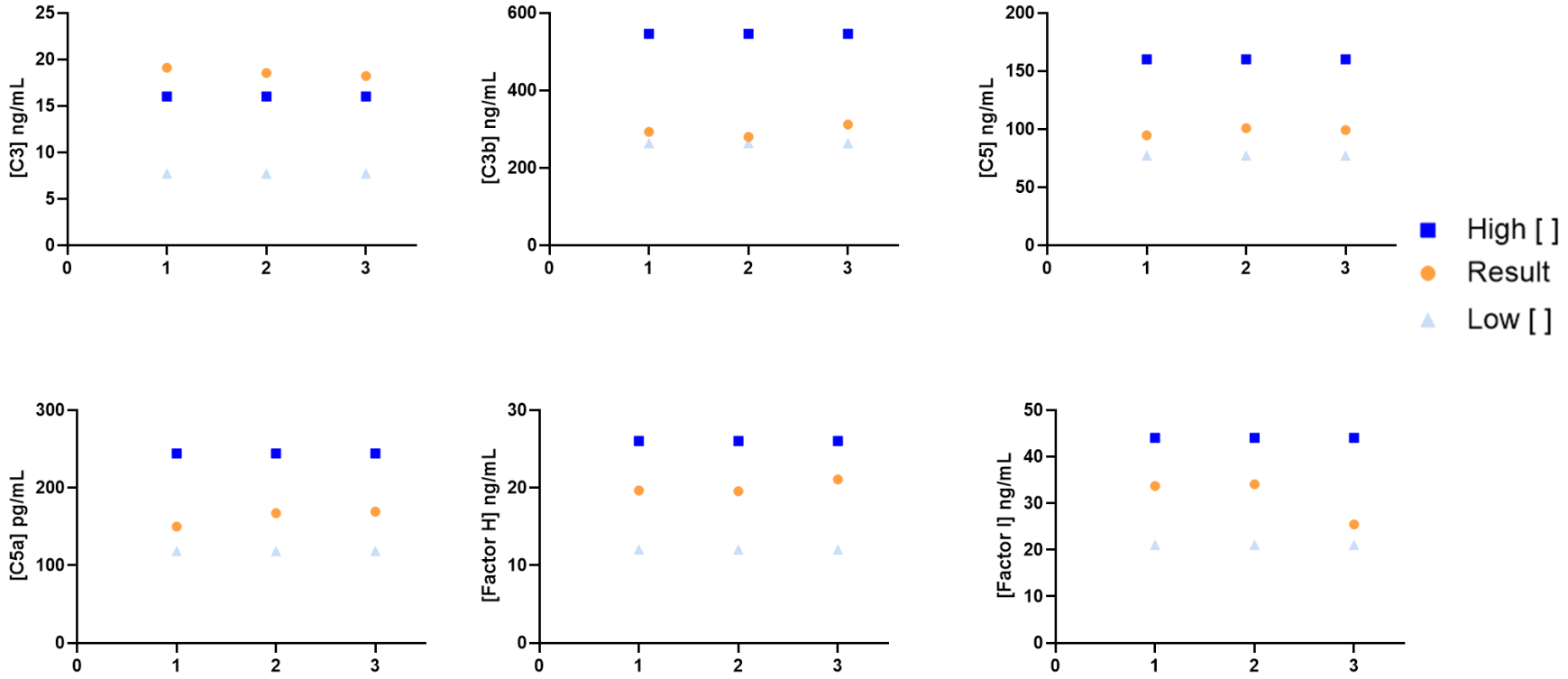
Appendix 3.4. Continuation.



Appendix 3.5. Quality Control 2 test result for Human Complement Panels. Highest concentration expected is plotted as a blue square. Lowest concentration expected is plotted as a light blue triangle. Average of the test result is plotted as an orange dot. QC2 was tested across three different plates (X axis) for **A)** C1q, **B)** C2, **C)** C4, **D)** C4b, **E)** MBL, **F)** Factor B, **G)** Factor D, **H)** C3, **I)** C3b, **J)** C5, **K)** C5a, **L)** Factor H and **M)** Factor I.

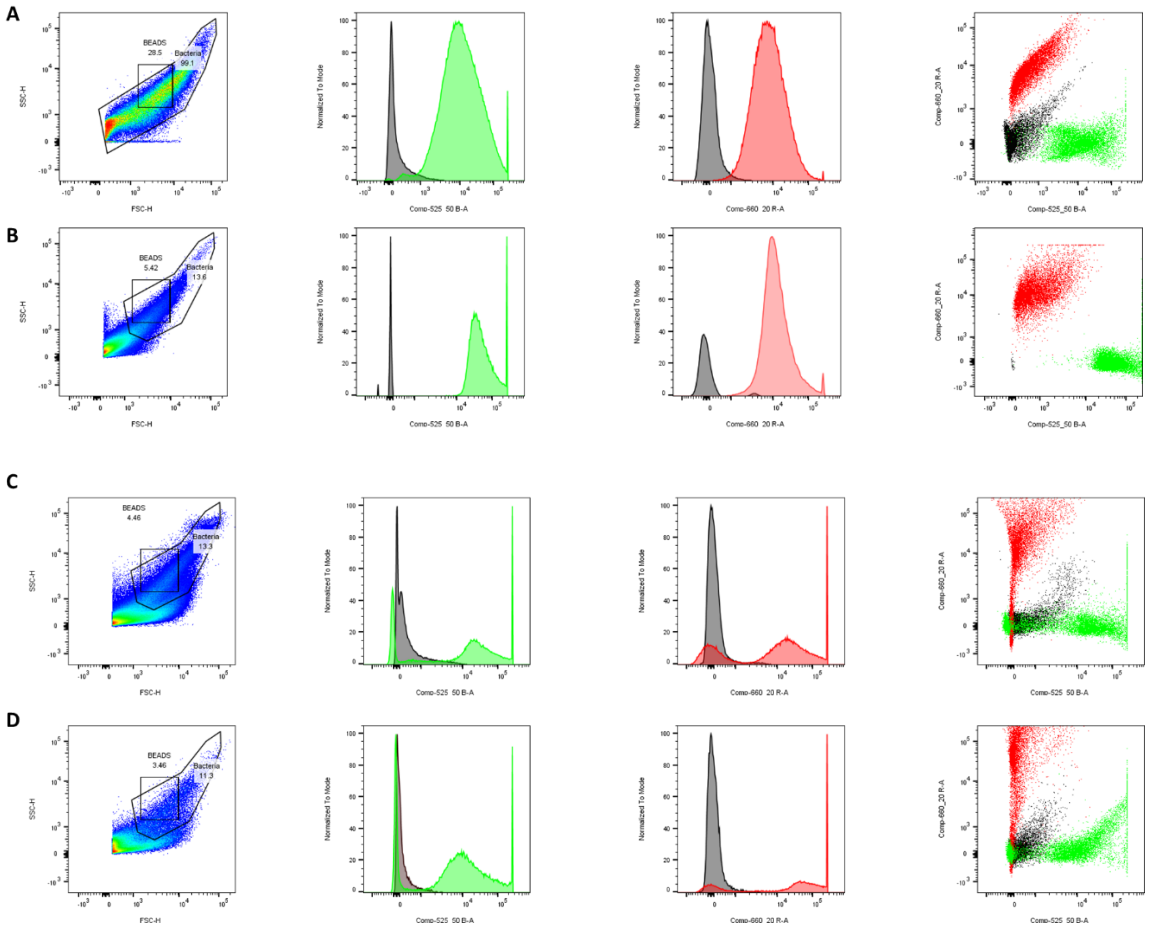


Appendix 3.5. Continuation.

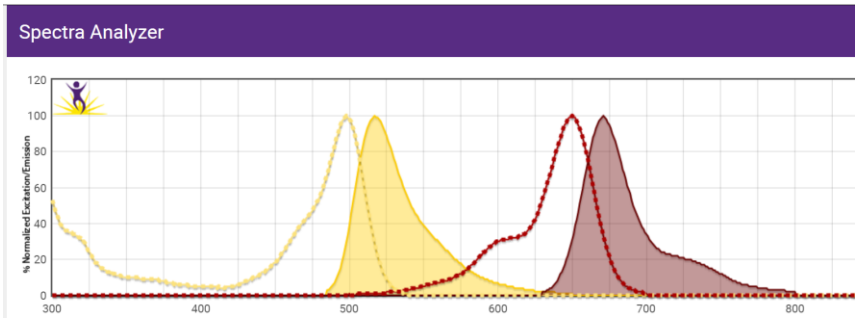


8.2. Chapter 5 Appendixes

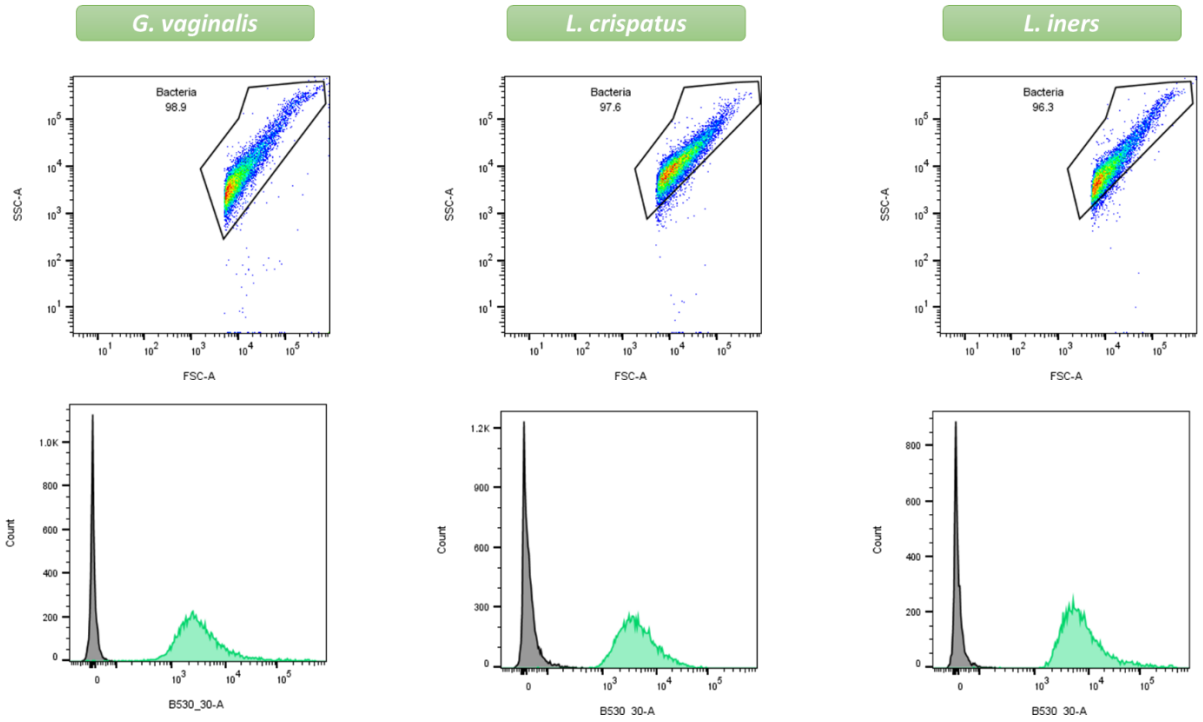
Appendix 5.1. Performance of two different nucleic acid stains. Pure bacterial liquid cultures and samples extracted from vaginal swabs were single stained with SYTO™ BC Green Fluorescent Nucleic Acid Stain and CellTrace™ Far Red. Histogram and pseudocolor plot overlay of unstained reference control (black) and single colour stain (red or green, respectively) for **A)** *L. crispatus,* **B)** *G. vaginalis,* **C)** Swab from non-pregnant donor and **D)** Swab from pregnant donor. All histograms were normalized to the mode.



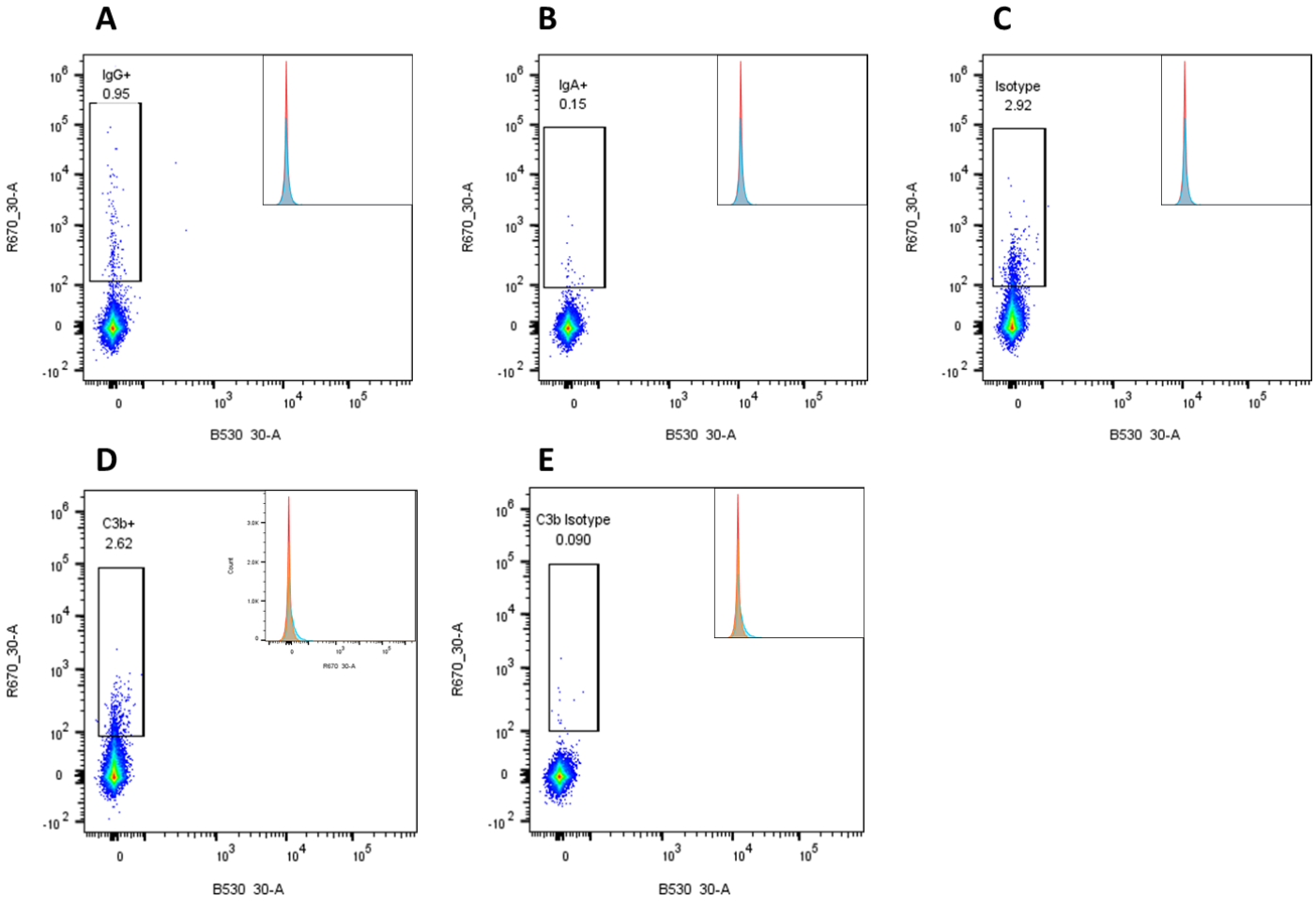
Appendix 5.2. Spectra Analyzer tool from BioLegend. The excitation spectra is displayed as dotted lines. The emission spectra is displayed as smooth and filled lines. The FITC spectra (yellow) was plotted since it mimics and reflects characteristics of Syto™ BC Green, together with Alexa Fluor® 647 (dark red). Tool source: (Spectra Analyzer).



Appendix 5.3. Confirmation of presence of bacteria from pure cultures stained with SYTO™ BC Green dye



Appendix 5.4. *G. vaginalis* pure bacterial cultures as control. *G. vaginalis* overnight cultures were washed three times and fixed overnight. After washing 3 times, bacterial pellet was stained with anti-human immunoglobulins under the same conditions (e.g., concentration, time) that vaginal swabs protocol. Gating strategy consisted in sequentially identifying the singlets (on FSC-A vs FSC-H), the bacterial population (on FSC-A vs –SSC-A) and the percentage of immunoglobulin bound by fluorescent expression of AF-647 (A-E). Samples had negative or negligible binding percentages for **A)** anti-human IgG; **B)** anti-human IgA; **C)** Goat IgG Isotype Control; **D)** anti-C3b; and **E)** Mouse IgG1 κ Isotype Control. Data is shown as both pseudoplots and histogram overlay of unstained (red) and single colour stains for the relevant antibodies (blue) and isotype (orange).



Appendix 5.5. *L. iners* pure bacterial cultures as control. *L. iners* overnight cultures were washed three times and fixed overnight. After washing 3 times, bacterial pellet was stained with anti-human immunoglobulins under the same conditions (e.g., concentration, time) that vaginal swabs protocol. Gating strategy consisted in sequentially identifying the singlets (on FSC-A vs FSC-H), the bacterial population (on FSC-A vs –SSC-A) and the percentage of immunoglobulin bound by fluorescent expression of AF-647 (A-E). Samples had negative or negligible binding percentages for **A)** anti-human IgG; **B)** anti-human IgA; **C)** Goat IgG Isotype Control; **D)** anti-C3b; and **E)** Mouse IgG1 κ Isotype Control. Data is shown as both pseudoplots and histogram overlay of unstained (red) and single colour stains for the relevant antibodies (blue) and isotype (orange).

