Host defence peptides in cervicovaginal fluid as predictors of preterm birth

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Host Defence Peptides in Cervicovaginal Fluid as Predictors of Preterm Birth

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I. Abstract

Introduction: Clinicians have a limited screening and diagnostic repertoire with which to identify which pregnant women will complete their pregnancy to term (37 weeks of gestation and beyond), and who are destined to give birth prematurely, the leading cause of death in children below 5 years old (Liu et al. 2015). Within the preterm birth (PTB) scientific community, there is much interest in the role of innate immune system proteins (host defence peptides [HDPs] or antimicrobial peptides [AMPs]) in the inflammatory process associated with spontaneous PTB (sPTB). Following promising pilot work, which suggested that early pregnancy cervicovaginal fluid concentration of HDPs elafin and cathelicidin are associated with cervical shortening (Abbott et al. 2014), this PhD project was designed to explore and validate these findings in a large powered observational cohort of women at both high and low risk of sPTB (n=619).

Hypothesis: My working hypothesis was that host defences of the cervicovaginal environment contribute to an individual pregnant woman's sensitivity to ascending infection and risk of sPTB, and that these defences are influenced by maternal characteristics/phenotype as well as the vaginal microbial environment.

Methods: Protein concentrations of elafin and cathelicidin, and the enzyme human neutrophil elastase (HNE) were measured in over 1,000 cervicovaginal fluid (CVF) samples (10 to 24 weeks' gestation), and relationship with cervical shortening, sPTB and maternal and fetal adverse outcomes were investigated using statistical modelling, including stratification according to ethnicity. CVF samples were analysed for bacterial vaginosis (BV) using microscopy and gram staining, which was correlated with HDP concentration and pregnancy outcome. The relationship between HDP expression and quantitative fetal fibronectin (qfFN) concentration was also investigated.

Results: Adjusted CVF cathelicidin and HNE concentrations (but not elafin) were raised in high-risk women who developed cervical shortening and who delivered prematurely and were predictive of sPTB < 37 weeks, with an area under the curve (AUC) of 0.75 (95% CI 0.68 to 0.81) for cathelicidin concentration at 14 to 15^{+6} weeks. Elafin concentrations were affected by gestation, body mass index and smoking. CVF elafin in early pregnancy was modestly predictive of sPTB < 34 weeks (AUC 0.63, 0.56–0.70). Ethnic disparity (Black vs. White women) in both HDP expression and relationship with pregnancy outcome was particularly notable within this cohort, when stratified analysis was undertaken. Black women had higher CVF concentration of elafin and cathelicidin across gestation (10 to 24 weeks of gestation), yet low CVF elafin was associated with cervical shortening in Black women. In contrast, high CVF elafin was associated with cervical shortening and sPTB in White women. High CVF cathelicidin predicted sPTB in White women, but not in Black women.

BV was associated with low elafin concentration, but high cathelicidin concentration, particularly in high-risk Black women.

Cathelicidin was positively correlated with CVF fFN concentration, and there appeared to be predictive merit in combining earlier measured cathelicidin and fFN measured after 18 weeks of gestation, though qfFN remained the best predictor of sPTB <37 and <34 weeks of gestation, superseding most other demographic risk-factors (including ethnicity).

Conclusions: There is evidence to support the hypothesis that alterations in innate immune response proteins in early pregnancy contribute to the mechanisms of some sPTB, and that they display distinct ethnic profiles. This may range from a failure to mount an appropriate immune response (e.g., low concentration of elafin) and increased susceptibility to infection and inflammation related sPTB, which may be related to vaginal dysbiosis (including BV), to an exaggerated host response (e.g., high elafin or cathelicidin) driven by infection and inflammation, and/or a hypersensitive

host response leading to tissue damage, cervical shortening and sPTB. Further investigation is warranted to understand the drivers for this, and their potential contribution towards clinically useful prediction techniques. In particular, mid-trimester cathelicidin has promising early pregnancy biomarker potential which may prove useful in the future.

II. Lay Summary

Spontaneous preterm birth before 37 weeks of completed gestation is responsible for the majority of baby deaths and long-term disability worldwide. Researchers still do not fully understand why some women give birth prematurely nor how to accurately predict who is most at risk, and thus how to prevent it from occurring. Our preliminary work in this field discovered that in a small group of women who had previously had premature babies, the concentrations of innate immune system proteins (called cathelicidin and elafin) measured in the vaginal mucus during early pregnancy, were higher in women who developed a short cervix (a precursor to many preterm births), and those who birthed premature babies. This research project was designed to evaluate, in a larger study, whether these proteins were indeed associated with premature birth, and how they are influenced by demographic characteristics of the mother (e.g., ethnicity) and the local bacterial composition of the vagina. This could provide both insight into the contribution of the mother's cervicovaginal immune system to the disease process of preterm birth, and potentially markers which could identify women at high-risk of premature birth

I measured the concentration of immune system proteins elafin and cathelicidin, and a related enzyme human neutrophil elastase in the cervicovaginal fluid collected from 619 women, including women at both high and low risk of premature birth, assessed them for the presence of bacterial vaginosis, a change in the natural balance of bacteria in the vagina.

Cathelicidin and human neutrophil elastase were raised in the vaginal mucus of high-risk women who birthed babies spontaneously before 37 weeks of gestation. Elafin concentrations were mildly raised in women who delivered early. Elafin was influenced by gestation, body mass index and smoking status. Innate immune proteins were influenced by ethnicity; Black women had higher concentrations of elafin and cathelicidin, yet low elafin was associated with cervical shortening in Black

women, whereas high elafin was associated with cervical shortening and premature birth in White women. Women with bacterial vaginosis had lower elafin but higher cathelicidin. High cathelicidin was associated with high concentrations of fetal fibronectin, an established protein predictor of preterm birth. Given that it can be measured earlier than fetal fibronectin, it may be of value in earlier prediction of premature birth.

In conclusion, I have demonstrated that changes in the innate immune system early in pregnancy may contribute to some premature births, and that there are distinctive patterns of expression according to ethnicity. We suggest that this may range from a failure of an immune response to infection or inflammation (low concentrations of elafin), leading to preterm birth (which may be related to bacterial imbalance including bacterial vaginosis), to an excessive immune response (high elafin and/or cathelicidin) which may also result in premature birth. This requires further investigation to fully understand these mechanisms, as well as to further evaluate the potential for cathelicidin to be used as a predictive test for preterm birth.

III. Declaration

I declare that the content of this thesis is my own work and that all contributions and collaborations have been explicitly acknowledged in the text. No material presented in thesis has been submitted to any other university or for any other degree.

Name: Natasha Hezelgrave

Date: 14/7/2021

IV. Acknowledgements

This work would not have been possible without the generous funding via a Doctoral Fellowship from the National Institute for Health Research (NIHR), as well as financial support from Tommy's the Baby Charity and the Rosetree's Foundation.

I am hugely indebted to my supervisors for their guidance over the past decade, for inspiring me to develop my research and clinical work in the field of preterm birth and for supporting me throughout my extended research period, and during maternity career breaks. Professor Tribe, thank you for supporting my development as a scientist and critical thinker, for your generous friendship, patience, creative thinking and eagle-eye for detail. Professor Shennan, thank you for believing in me, pushing me out of my comfort zone and for modelling the skilful, thoughtful and questioning clinical academic that I hope to emulate.

I extend huge gratitude to Mr Paul Seed who has provided statistical teaching, guidance and support throughout this PhD. You are patient and kind, and I will be forever grateful to you and your brilliant mind. Thank you to my lab colleagues, particularly Evonne-Chin Smith who painstakingly taught me meticulous lab technique, was instrumental in guiding Insight sample processing and was a friendly ear when things did not go to plan. To the staff in St Thomas's prematurity clinic; without your energy, passion for clinical care and robust research, diligence and excellent humour we would never have been able to recruit so well to this challenging study. Thanks particularly to midwives Dr Jenny Carter, Judy Filmer, Falak Diab, Vicky Robinson and Deborah Finucane.

To the women involved in the Insight study, many of whom have experienced the heart-wrenching pain of pregnancy loss, thank-you for your generosity, and I commit to continuing to work to make pregnancy safer.

Finally, thank you to my husband Mathew for his unfaltering support and love, particularly when this task felt impossible. And to Dora, Rafferty and Molly, all born during this project, I am so sorry that this took me away from you, I hope to make you proud.

V. Statement of own work

I was the Insight study coordinator. With support from my supervisors, I was responsible for developing the idea, preparing, and submitting the Fellowship funding application, writing the protocol, developing the laboratory standard operating procedures, designing the consent forms and patient information sheets, obtaining regulatory approval, database design, development, and modification (incorporating Insight into an existing preterm birth studies database) recruiting and supporting new sites, and trial management. With support from a team of dedicated research midwives and research assistants, I contributed to patient recruitment, sample processing, outcome collection and data monitoring. I performed the ELISA measurements for elafin and cathelicidin. Human neutrophil elastase measurement by ELISA was performed by Evonne Chin-Smith and Alexandra Ridout. BV slide assessment was performed by me and the nursing team at Burrell St. Sexual Health Clinic. Mr Paul Seed, study statistician, provided training, support and supervision of the statistical analysis performed.

VI. Abbreviations

ACTH	Adrenocorticotrophic Hormone
AF	Amniotic Fluid
AMP	Antimicrobial peptide
ARDS	Acute Respiratory Distress Syndrome
aOR	Adjusted Odds Ratio
AUC	Area Under the Curve
BMI	Body Mass Index
BV	Bacterial Vaginosis
CI	Confidence Interval
CIN	Cervical Intraepithelial Neoplasia
CL	Cervical Length
CLIC	Cervical Length and Inflammatory Changes
CRH	Corticotrophin Releasing Hormone
CRP	Cell Reactive Protein
CVF	Cervicovaginal Fluid
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FDCS	Full Dilatation Caesarean Section
FUT2	Fucosyltransferase-2
fFN	Fetal fibronectin
G-CSF	Granulocyte Colony Stimulating Factor
GWAS	Genome Wide Association Study
HDP	Host Defence Peptide
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
HRP	Host Response Peptide
HNE	Human Neutrophil Elastase
IL	Interleukin
IUGR	Intrauterine Growth Restriction

LLETZ	Laser Loop Excision of the Transformation Zone
LR	Likelihood Ratio
MMP	Matrix Metalloproteinase
NAP	Natural Antimicrobial Peptide
NET	Neutrophil Extracellular Trap
NF-kB	Nuclear Factor Kappa B
NPV	Negative Predictive Value
OR	Odds Ratio
PAMPS	Pathogen Associated Molecular Pattern
PI3	Peptidase Inhibitor 3
PPROM	Premature Prelabour Rupture of Membranes
PPV	Positive Predictive Value
PRKCA	Protein Kinase C- α
PTB	Preterm Birth
qfFN	Quantitative fetal fibronectin
RANTES	Regulated upon Activation Normal T Cell Expressed and presumably Secreted
RAP	Interleukin-1 Receptor Associated Protein
ROC	Receiver Operating Characteristic
RCT	Randomised Controlled Trial
RR	Relative Risk
SNP	Single Nucleotide Polymorphism
sPTB	Spontaneous Preterm Birth
SVD	Spontaneous Vaginal Delivery
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitor of Metalloproteinase
TLR	Toll-Like Receptor
TNF-α	Tumour Necrosis Factor alpha
TVUS	Transvaginal Ultrasound
US	United States

WES	Whole Exome Sequencing

VI List of publications and abstracts arising from this thesis

Primary research

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Oral Presentations

Hezelgrave NL. Ethnic variations in cervicovaginal natural antimicrobial protein expression in pregnancy and association with spontaneous preterm birth. Annual Preterm Birth Conference, Birmingham, 2019 (1st prize winner).

Hezelgrave NL. Host defence peptides, ethnicity and prediction of spontaneous preterm birth. 3rd European Spontaneous Preterm Birth Congress, Edinburgh 2018.

Hezelgrave NL. Elafin in cervicovaginal fluid as a predictor of spontaneous preterm birth. British Maternal and Fetal Medicine Society, Brighton, 2018.

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Hezelgrave NL. Quantitative fetal fibronectin modifies the risk of preterm birth in asymptomatic high-risk women with a short cervix. Annual Academic Meeting/Blair Bell Research Meeting, RCOG, London 2015 (1st prize winner).

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Invited lectures

Hezelgrave NL. The Insight Study; The cervicovaginal environment, innate immune system and spontaneous preterm birth. Society of Reproductive Investigation, Paris, 2019.

Poster presentations

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VII Manuscripts in preparation

Hezelgrave NL, Hadingham A, Seed P, Chin-Smith E, Shennan A, Tribe R. Ethnic variations in cervicovaginal natural antimicrobial expression in pregnancy and association with spontaneous preterm birth, 2021.

Hezelgrave NL, Seed P, Chin-Smith E, Shennan A, Tribe R. Cervicovaginal fluid cathelicidin and its relationship to fetal fibronectin ad prediction of spontaneous preterm birth, 2021.

VIII List of related publications during research period

Primary research

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Gülmezoglu AM, Lawrie TA, **Hezelgrave NL,** Oladapo OT, Souza JP, Gielen M, Lawn JE, Bahl R, Althabe F, Colaci D, Hofmeyr GJ. Interventions to reduce maternal and newborn morbidity and mortality. In: Black RE, Laxminarayan R, Temmerman M, Walker N, editors. Reproductive, Maternal, Newborn, and Child Health: Disease Control Priorities, Third Edition (Volume 2). Washington (DC): The International Bank for Reconstruction and Development/The World Bank. 2016.

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Contents

List of Figures

List of Tables

1 Introduction

1.1 Background to thesis

Clinicians have a limited screening and diagnostic repertoire with which to identify which pregnant women will complete their pregnancy to term (>37 weeks of gestation) and who are destined to give birth prematurely, the leading cause of death in children below 5 years old (Liu et al. 2015).

It is widely accepted that infection and inflammation are important contributors to preterm birth (PTB) (Romero et al. 2007; Bastek, Gómez, and Elovitz 2011; Talati, Hackney, and Mesiano 2017; Romero, Dey, and Fisher 2014) and that some women are more at risk due to alterations in their immune response to infection. In particular, there is currently much interest in characterising the role of the innate immune response in spontaneous PTB (sPTB), both to elucidate its pathogenesis, and to identify candidate biomarkers which may prove helpful to identify women at risk. Natural antimicrobial peptides/proteins (NAPS), often (and hereafter) referred to as human defence peptides/proteins (HDPs), are multifunctional components of the innate immune system produced by epithelial and inflammatory cells. They have broad spectrum anti-microbial activity together with immunomodulatory action and are potential early pregnancy predictors of sPTB associated with inflammation and infection (representing at least 40% of PTBs) (Agrawal and Hirsch 2012). HDPs are known to play an essential role in responding to vaginal infection (Stock et al. 2009; Frew and Stock 2011), and there have been a few studies, though often with conflicting findings, exploring vaginal HDP expression (e.g., protein concentration in cervicovaginal fluid) in relation to sPTB (Bastek et al. 2013; Manning et al. 2019; Elovitz et al. 2019; Abbott et al. 2014; Para et al. 2020). However, the temporal profile of HDP expression in the CVF has not been defined, and relationships between HDP expression and sPTB are still far from understood.

This PhD project builds on findings from a promising pilot study (the Cervical Length and Inflammatory Changes Study: CLIC) which suggested that concentrations of the HDPs elafin and cathelicidin in CVF during early pregnancy are associated with

cervical shortening (Abbott et al. 2014). However, the study cohort was small (n=74 high-risk women) and lacked power to detect a clinically meaningful outcome (i.e., sPTB). Therefore, this research project was designed to investigate and validate these findings in an appropriately powered cohort of pregnant women and evaluate the potential role of these molecules (alone and in combination with other risk factors and markers of sPTB) as predictive biomarkers for sPTB. We hypothesised that raised HDP concentrations may represent a primary response to infection, leading to cervical shortening, and/or that women predisposed to developing cervical shortening could also have constitutively raised CVF HDP concentrations as part of a heightened immune response. If validated as an early gestation predictor of sPTB for high-risk women, we may be able to identify those women who would most benefit from increased surveillance and prophylactic treatment during pregnancy. Furthermore, insight into the pathophysiology of sPTB could inform development of novel therapeutics (e.g., anti-inflammatory therapy). To complement the primary aim, this project also sought to define the temporal profile of HDP expression in the CVF starting from early pregnancy (10 weeks of gestation) until 24 weeks of gestation and explored the *in vivo* interactions between HDPs and host characteristics (gestation and demographics) and the vaginal environment (bacterial vaginosis, [BV]).

This first chapter is focussed critical review of the literature relevant to this study. It begins by outlining the importance of sPTB, and what is currently known about risk stratification. It discusses the role of infection and inflammation in sPTB, before focussing on in-depth evaluation of the current literature and knowledge-gaps surrounding the role of the innate immune system in PTB and how this may relate to its pathogenesis.

1.2 Definition and epidemiology of sPTB

PTB is defined as delivery prior to 37 completed weeks of gestation or fewer than 259 days from the first date of a woman's last menstrual period (Howson, Kinney, and Lawn 2012). It may be iatrogenic (physician initiated for maternal or fetal health indication) or spontaneous (spontaneous onset of uterine contractions resulting in

delivery, or premature pre-labour rupture of membranes [PPROM]). This research project focuses on sPTB, which represents approximately 70% of all PTBs (Goldenberg et al. 2008), though there are indications that the contribution of iatrogenic delivery to PTB is increasing in high-income environments (Richter et al. 2019; Lisonkova, Hutcheon, and Joseph 2011).

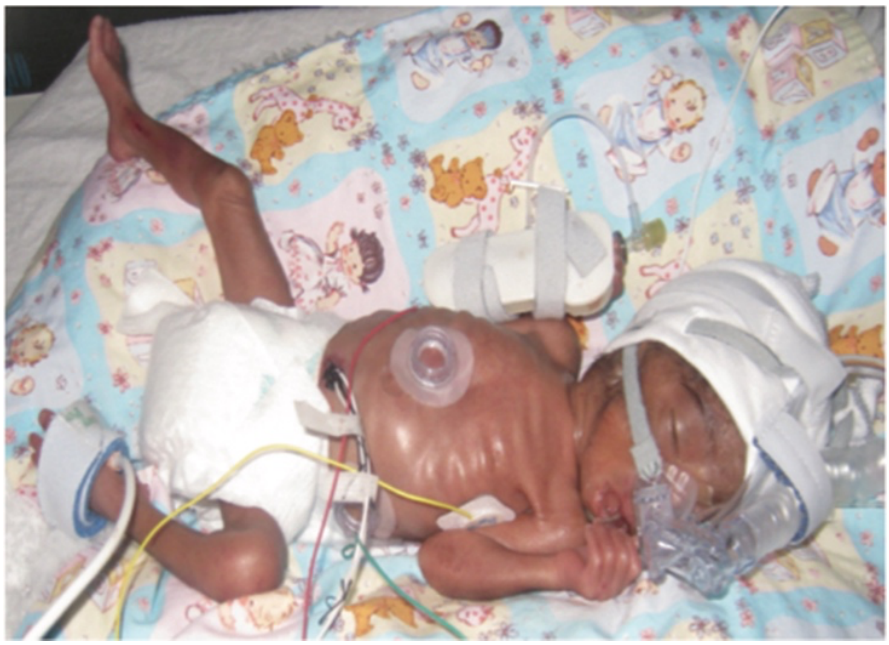

Figure 1-1. Image of a preterm infant born at 23 weeks of gestation. With kind permission from the mother.

Globally, the incidence of PTB (for 2014) was estimated by the World Health Organisation (WHO) to be 10.6% (9.0-12.0%), ranging from 8.7% in Europe, to an estimated 13.4% in North Africa (Chawanpaiboon et al. 2019), an estimated 14.8 million infants born prior to full term per year. 60% of these births occur in low and middle-income countries (Blencowe et al. 2013). Responsible for 1.1 million deaths a year, it is the leading cause of neonatal mortality and morbidity globally (Blencowe et al. 2012). Focussing on England and Wales, most recent statistics estimate that around 7% of live births are preterm (Office for National Statistics 2015). Worryingly, the incidence of PTB is thought to be increasing in all global regions with reliable data (Blencowe et al. 2012; Chawanpaiboon et al. 2019). This increase may be due to improved reporting, classification issues (e.g., utilising a reduced threshold of fetal viability), reductions in still birth rates with corresponding increase in liveborn prematurity rate, increased multiple birth rate with associated risk of prematurity, or

increased iatrogenic PTB for maternal or fetal conditions. Environmental factors such as pollution and maternal stress may also play a role.

Seminal data from the 1995 UK EPICure study (Wood et al. 2000) on preterm neonatal morbidity and mortality showed that one quarter of surviving babies born prior to 25 weeks' gestation will suffer serious physical and/or neurodevelopmental disability. In high income countries such as the UK, survival of extremely preterm infants (both survival to discharge and 'intact' survival with absence of long term neurodevelopmental disability) has dramatically improved due to improved neonatal care. The EPICure study was followed up with data from 2006 (Figure 1-2, for 2006 survival rates according to gestation at birth) which demonstrated slightly improved outcomes for these gestationally immature infants (Moore et al. 2012), though mortality was still high. Since these studies was performed, in high income countries such as the UK, further advances have been made in both obstetric and neonatal practice and intact survival rates are likely to be higher, though this may be counteracted by shifting lower thresholds of definitions of fetal viability and increasing resuscitation of infants born prior to 23 weeks of gestation (Ishii et al. 2013; Serenius et al. 2015; Mehler et al. 2016).

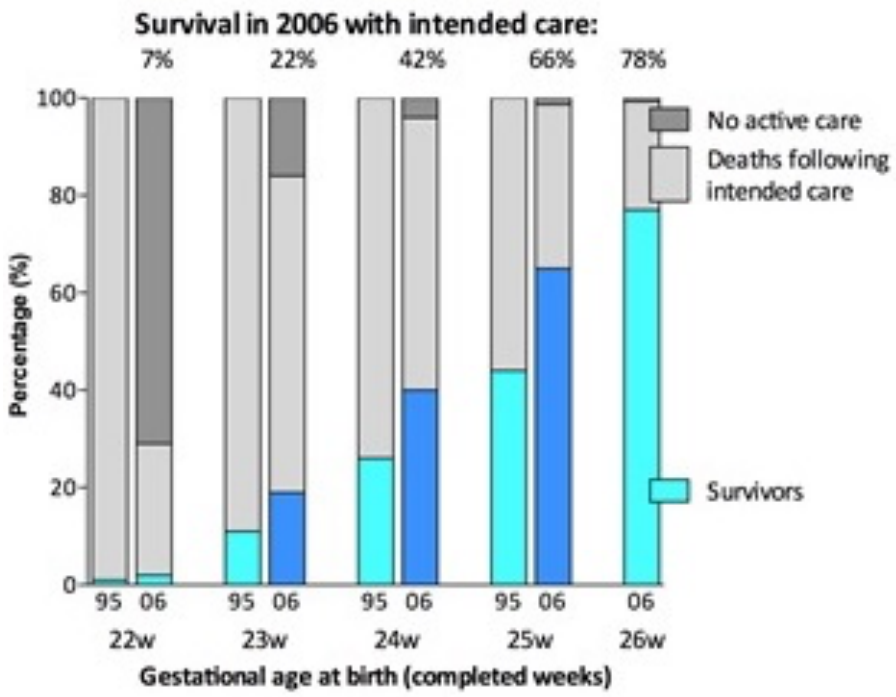

Figure 1-2. Survival of babies involved in the Epicure study according to gestational age, in 2006. Taken from Epicure.ac.uk (n=3133).

As well as the profound physical and emotional effect on affected families, PTB has enormous economic consequence for health systems. In the UK, costs to the NHS in 2006 were estimated at 2.9 billion per year; 92% of these were postnatal hospital inpatient costs (Mangham et al. 2009). These data clearly need updating; nearly 20 years later, with improved technology and increased resuscitation of infants at lower gestational ages, the costs to the NHS and other similar health systems are likely greater (though a more recent similar economic analysis has not been performed). Even infants born in the late preterm period 34 to 37 weeks of gestation incur significant economic costs, with studies performed in high-income countries finding increased initial hospitalization costs of between two and ten-fold for infants born at 34 weeks compared to term, with significant modelled cost savings for delaying birth by even one to two weeks (Petrou 2019). This excess risk of hospitalization for preterm infants likely remains throughout childhood, as well as for those born in the early term period, compared to 40 weeks of gestation (<28 weeks rate ratio 4.92, 95% CI 4.58 to 5.30; 38 weeks 1.19, 1.16 to 1.22), particularly during infancy but even up to age 10 (Coathup et al. 2020). Whilst not able to adjust for all important confounders due to missing data (for example breastfeeding, smoking and maternal illness related to risk of PTB), it is another compelling argument as to the medical and economic benefit to preventing PTB.

The enormous health, social and economic burden of PTB demands that efforts are stepped up to understand the pathophysiological mechanisms underlying PTB, to inform the use and timing of preventative treatment. In particular, if women most at risk of early delivery could be identified in early pregnancy, tailored intervention could carry huge cost benefit. However, despite continued research into aetiology, risk factors and treatment, and introduction into practice of various preventative strategies, we have, so far, failed to make a meaningful impact on rates of prematurity.

1.3 Risk factors for sPTB

Much of the scientific research into sPTB has focused on how to define a high-risk population upon whom we should focus our prediction and prevention efforts. Whilst there are a myriad of socio-demographic characteristics that increase risk of sPTB (see section 1.3.5), for clinical (screening) purposes in most high-income health care settings, 'high-risk' women tend to be defined by their previous obstetric or gynaecological history; those with previous PTB/mid-trimester loss PPROM; known uterine abnormalities; previous full dilatation caesarean section; history of significant cervical surgery. Indeed, in our own UK setting, recent guidelines from the UK Preterm Clinical Network (UK Preterm Birth Clinical Network 2019) and NHS England (NHS England 2019) recommend that these women at high-risk of sPTB should be offered screening tests around 18 to 22 weeks of gestation (cervical length [CL] monitoring and fetal fibronectin [fFN], see section 1.3.7) through a preterm prevention service, with a view for intervention if required (e.g. cervical cerclage, progesterone therapy or, more experimentally, the Arabin pessary). It was also from this list of risk-factors that we defined entry into the high-risk cohort of this PhD study 'Insight'. These risk factors, along with broader socio-demographic and environmental risk factors, are discussed below.

1.3.1 Prior pregnancy history

Whilst a myriad recognised maternal modifiable and non-modifiable pre-pregnancy risk factors exist for sPTB, the most significant and consistently identified in several large studies is a woman's history of previous sPTB. The prospective Preterm Prediction Study (Goldenberg et al. 1998) (n=2929 women) observed that for women with a previous sPTB (pregnancy ending between 18 and 36 weeks), the sPTB <35 weeks recurrence risk was 15.2%, compared to nulliparous (3.3%) and parous women with previous uncomplicated term deliveries (2.4%), a relative risk (RR) of 6.4. This risk was exaggerated (RR 10.6) for women who suffered subsequent early sPTB (<28 weeks of gestation). The highest risk of subsequent sPTB was found in women with previous early (23-27 weeks of gestation) sPTB compared with those closer to term (RR 22.1 for subsequent sPTB <28 weeks' gestation). A larger study of over 50,000 American women (3836 of which were women with a subsequent pregnancy, who

delivered prematurely in their first pregnancy), described an even higher recurrence rate of sPTB <37 weeks of 32% (Laughon et al. 2014). More recently, a systematic review of studies up to 2017 (with the majority in UK, USA or Europe) had a similar pooled recurrence in women with at least one previous sPTB <37 weeks (30%) (Phillips et al. 2017). Many of these included studies evaluated the impact of preventative intervention after previous sPTB, and so the recurrence rates without treatment may actually be higher.

Risk of subsequent sPTB is also elevated with a history of more than one sPTB. In an USA population-based cohort study (McManemy et al. 2007) (n=19,025 third births), recurrence risk ranged from 42% for women with two prior preterm deliveries, 21% for prior term/preterm, 13% with preterm/term, and 5% with previous term/term deliveries (Figure 1-3). A similar modification of recurrence risk was observed according to the gestation of the prior birth, as seen in the Preterm Prediction Study.

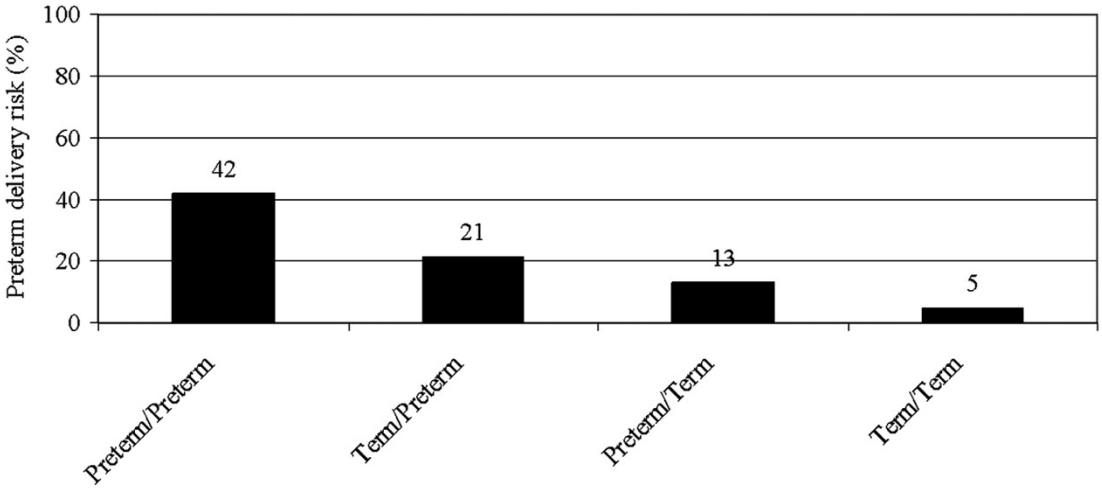

Figure 1-3. Spontaneous preterm birth risk for third births according to prior preterm birth status (McManemy et al. 2007).

After previous sPTB, women with multiple pregnancy are at the highest risk of recurrence (57.0%, 95% CI 51.9-61.9%), vs. those carrying a singleton pregnancy (25%, 95% CI 24.3-26.5%). Similarly, recurrence risk has been shown to be lowest in those who had previous preterm delivery with a twin pregnancy, subsequently pregnant with a singleton pregnancy (10%, 95% CI 8.2-12.3%) (Kazemier et al. 2014). It is worth noting, however, that most women who have had a previous sPTB will go on to have a normal term birth in subsequent pregnancies. In fact, only 18% of PTBs

before 35 weeks in second pregnancies could be predicted by first pregnancy outcome, and recurrent preterm birth accounted for only 10% of all PTBs in an USA hospital-population based study (>15,000 births) (Bloom et al. 2001). Moreover, at-risk nulliparous women (in whom at least 50% of PTBs will occur), cannot not be identified using past obstetric history risk screening, until they have had an adverse pregnancy outcome. For this reason, using obstetric history alone to define risk is inadequate.

1.3.2 Previous invasive cervical surgery

Invasive cervical surgery (laser or cold knife cone biopsy or loop electrosurgical procedures [LLETZ]) performed for treatment of cervical intraepithelial neoplasia (CIN) is a recognized risk factor for sPTB. Whilst several large population-based cohort studies have found an association between sPTB and any excisional procedure (Albrechtsen et al. 2008; Kyrgiou et al. 2006), it is generally accepted that the more invasive the procedure (volume of tissue removed), and the higher number of procedures performed, the higher the risk of PPROM and/or sPTB (Kyrgiou et al. 2015). This may be due to physical weakening of the cervix and/or increased access to the uterine cavity for genital tract microbial infection through a shortened cervix. Alternatively, it has been postulated that surgical treatment to the cervix interferes with the production of the antimicrobial rich mucus plug which protects the uterus against ascending infection (Miyako, Iwanari, and Kitao 1993; Hein et al. 2002), altering the cervicovaginal microflora (Wiik et al. 2019), or host defence mechanisms via alterations in the innate immune system, which could also be related to the underlying human papilloma virus (HPV) infection (Usyk et al. 2020; Cheng et al. 2020), as well as the subsequent treatment. Women may also have distinct background or even immunological characteristics which may predispose to pre-cancerous lesions and sPTB (Castanon et al. 2012). However, a recent large Dutch population-based cohort study of over 45,000 women with CIN (matched to a control group without any cervical anomaly), demonstrated a strong correlation between sPTB and volume of excised tissue, regardless of the severity of CIN (Loopik et al. 2021), implying that it is the treatment rather than the CIN which confers the risk of sPTB.

1.3.3 Previous full dilation caesarean section

Damage to the cervix, not only after excision procedures, but also during caesarian section when performed at full dilatation, may also confer increased risk of sPTB. During the second stage of labour, the cervix is drawn into the lower segment of the uterus and is at risk of inadvertent damage during caesarian section, when a low uterine incision may damage the cervicoisthmic tissue. A retrospective study of over 800 women found that women who had a term full dilation caesarian section (FDCS) had a significantly higher rate of PTB (13.5%) compared to women who had a term first-stage caesarian section (2.3%) (Levine et al. 2015). Watson et al. (2017) found that risk of subsequent recurrent PTB <30 weeks was higher in women who had had term delivery by caesarian at full dilatation, followed by a late miscarriage or sPTB (n=29), compared with women who had had a term vaginal delivery, followed by a late miscarriage or sPTB (n=37) (RR 3.06, 1.2 to 7.7). Figure 1.4 illustrates the Kaplan Meir survival curve depicting the proportion of women who remained undelivered at each gestational timepoint for those who had a term FDCS vs those who had a term vaginal delivery. The mechanism underlying this increased risk is unknown, as is whether preventative treatment (e.g., cerclage or progesterone) may impact on future risk. This is the topic of an ongoing RCT and associated observational studies (Carlisle et al. 2020).

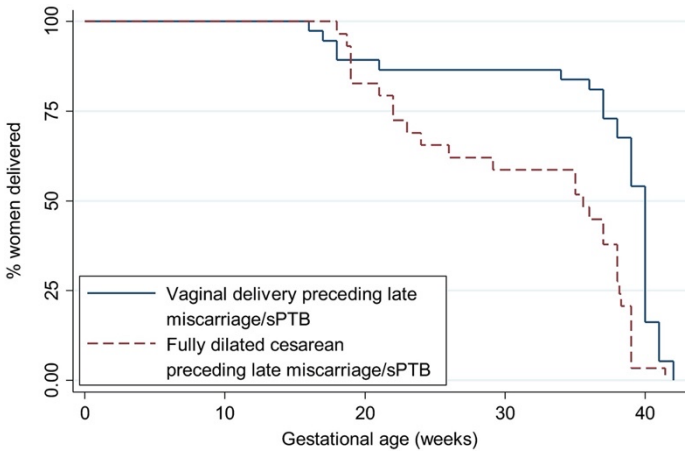

Figure 1-4. Kaplan Meir survival curve depicting the proportion of women undelivered in pregnancy at each gestational week according to past pregnancy history (Watson et al. 2017)

1.3.4 Mülllarian tract abnormalities

Müllarian duct abnormalities are associated with risk of sPTB. The OR for sPTB for

women with a bicornuate or double uterus or uterine septum in the Preterm Prediction study (Goldenberg et al. 1998) was 7.02 (1.69-29.15). It is possible that pre-pregnancy correction of the abnormality if possible (e.g., removal of the uterine septum) may reduce the risk of sPTB in women with a previous sPTB, though studies are generally small and present conflicting results.

1.3.5 Genetic predisposition

Individual genetic predisposition may modulate risk of sPTB (Strauss et al. 2018; Varner and Esplin 2005; Wu et al. 2019; Zhang, Feenstra, et al. 2017; Bezold et al. 2013). Compelling evidence for familial aggregation exists; Swedish women whose sisters had PTB have an increased risk of PTB (80%) (Winkvist, Mogren, and Högberg 1998) and likewise if their mother had sPTB (OR 1.4) (Shah and Shah 2009). Twin studies have demonstrated heritability ranging from 17-40% (Strauss et al. 2018). Whilst many genome-wide association studies (GWAS, analysis of single nucleotide polymorphisms [SNPS] across the entire genome designed to identify chromosomal loci responsible for phenotypic traits) performed to investigate this association have failed to identify any consistent variants associated with sPTB, cohorts are often underpowered (Wu, Clark, Manuck, et al. 2013; Zhang, Baldwin, et al. 2015). However, Zhang, Feenstra, et al. (2017), in a large European cohort of >43,000 women reported a number of association regions/loci which were associated with gestational duration and PTB, in both the discovery and replication set of data, containing genes associated with uterine development (decidualization of the endometrium and embryo implantation), nutrition (particularly the selenocysteine pathway) and uteroplacental circulation. Other polymorphisms have also been shown to be associated with sPTB (Engel et al. 2005; Menon et al. 2006), particularly those associated with inflammatory pathways (Couceiro et al. 2021) (see section 1.3.6 below, and 1.4.1), often displaying distinct ethnic differences (see below section 1.3.6).

1.3.6 Ethnicity

Ethnic differences in risk of sPTB have been consistently observed. In both the UK and USA, women of Black-African, Caribbean and African-American race are consistently

found to have increased risk of sPTB compared with Caucasian women. In England and Wales, a population-based study of over four million births revealed that babies born to women in all minority ethnic groups except 'other-White' had increased odds of being born preterm compared to White British babies, with the highest risk being in Black Caribbean women (aOR 1.25, 95% CI 1.21-1.30) (Li et al. 2019). The Preterm Prediction Study reported a relative risk of 1.5 (1.2-1.9) for African-American women vs. White women delivering spontaneously prior to 37 weeks of gestation (Goldenberg et al. 1998). Schaaf et al. (2013) in a large systematic review of 30 studies reported a doubling of risk of sPTB for Black women vs White women (95% CI 1.8-2.2), whilst other studies have shown that Black women are up to four times more likely to deliver prematurely than White women (Reagan and Salsberry 2005; Simhan and Bodnar 2006; Zhang and Savitz 1992; Goldenberg, Cliver, et al. 1996). A similar story emerges for recurrent sPTB; in a population-based study of American women (N=644,462), Black women were over four times as likely to experience recurrent PTB (aOR 4.11, 95% CI 3.78 to 4.47), and over six times more likely to experience recurrent PPROM (aOR 6.4, 95% CI 3.7 to 11.0), compared to White women (Kistka et al. 2007).

Studies evaluating sPTB in other ethnic populations have less consistent findings. In the above-described American systematic review, inconsistent results were seen in 12 studies of women of Asian ethnicity (Schaaf et al. 2013). Compared to non-Hispanic White women, seven studies showed an increased risk of sPTB, whilst five studies showed no significant increased risk (notably, population rates of sPTB in these studies ranged from 2.3% to 16.3%, making them hard to compare) (Manuck 2017). Whilst differing study outcomes result from different populations studied, the complexity and varied classification of race (most ethnicity studies are based on self-reporting rather than genotyping), as well as multiple definitions of PTB phenotype, there is no doubt that racial disparity in risk of sPTB exists. Whether this is due to genetic and biological differences, and/or social and economic disparity remains to be untangled.

Biologically, it is possible that Black women manifest different PTB phenotype compared to White women. Manuck et al. (2015) defined nine clinical PTB phenotypes within 1025 America women based on clinical data;

infection/inflammation, maternal stress, decidual haemorrhage, uterine distention, cervical insufficiency, placental dysfunction, premature rupture of the membranes, maternal comorbidities, and familial factors. Many of these women (78%) had multiple phenotypes identified, and a small number (<5%) had no phenotype identified. Black women with early sPTB (<34 weeks) were more likely to have cervical insufficiency and maternal stress, compared with White women, who had higher incidence of placental dysfunction and decidual haemorrhage. Indeed, some researchers (Manuck et al. 2015; Esplin 2016; Villar et al. 2012) argue that precise phenotyping (rather than solely defining spontaneous vs. iatrogenic PTB) and personalised medicine are vital to identify sPTB mechanism and improve surveillance and application of preventative measures (which may have variable responses according to clinical phenotype).

Multi-layered socioeconomic determinants also contribute to ethnic disparity. Poorer access to health care, lower maternal education (McGrady et al. 1992), short pre-pregnancy intervals (Hogue et al. 2011), economic deprivation (Ncube et al. 2016), more common in general among Black women, all confer higher risk of sPTB. Yet disparities exist even after correction for identified risk factors such as these. This may, in part, be due to the differential impact of risk factors upon women of different ethnicities (Manuck 2017); for example, Torloni et al. (2012) analysed 447 sPTB cases and concluded that obesity conferred a lower risk for sPTB in African-American women vs. non-obese African-American women, compared with increased risk of sPTB for obese Caucasian women vs. non-obese Caucasian women. Ncube et al. (2016) found that the influence of socioeconomic deprivation on rate of sPTB was more pronounced for African-American women vs. White women after controlling for other known risk factors. Experience of stress or racism could also play a part (section 1.3.9).

Biological risk factors such as the higher incidence of urogenital infection in Black women (particularly BV) and fundamental differences in the vaginal microbiome are also likely to contribute. As described in more detail in section 1.4.1, the presence of BV is a known risk factor for sPTB. Many studies have demonstrated that Black women are more likely to be diagnosed with BV both inside (Meis et al. 1995;

Goldenberg, Klebanoff, et al. 1996) and outside (Ness et al. 2003) of pregnancy. Most studies defining the vaginal microbiome with precision next generation sequencing (see section 1.4.3) have found Black women in general have more diverse vaginal microbiome, compared to the *Lactobacillus crispatus* dominant microbiome of White women (Ravel et al. 2011; Fettweis et al. 2014), and that this persists into pregnancy (MacIntyre et al. 2015). Although studies are conflicting in part due to different sampling and sequencing techniques and classification systems, it is generally accepted that lactobacillus dominant vaginal environments confer some protection from sPTB (antimicrobial defence) whereas the absence of lactobacillus is associated with amniotic infection and sPTB (section 1.4.3 for more detail and references), which may well relate to the ethnic disparity in sPTB.

It is likely that there are also genetic factors behind race-dependent risk of sPTB. As described earlier (section 1.3.5) there is some heritability in risk of sPTB, though candidate genes have not been consistently identified (Strauss et al. 2018). What has been more clearly identified are the presence of important genetic polymorphisms in White and Black women which, among others, may be responsible for immune system regulation and the maintenance of a term pregnancy (Menon et al. 2010). Whilst many studies are limited by small sample sizes and ethnic heterogeneity, genetic polymorphisms in genes related to infection and inflammation (see section 1.4.1 for the inflammatory mechanisms associated with sPTB) are frequently found to be associated with sPTB. For example, a case control study (1165 sPTB cases, and 3830 controls) found association between the CC polymorphism of the rs1800795 genotype (interleukin-6 [IL-6] gene) and term birth, but only in White women, not Black American women (Wu, Clark, Stoddard et al. 2013). Similarly, Menon et al. (2010) examined the interplay between ethnicity, genetic variants, birth outcome (sPTB case vs. control) and amniotic fluid IL-1B, IL-10, and IL-8 concentrations (obtained during labour by before spontaneous membrane rupture). A total of 166 SNPs in cytokine-related genes in White women (145 sPTB cases, 194 controls) and Black women (76 cases, 191 controls) were examined. The authors identified key differences between ethnicities in the interaction between SNPs, plasma cytokine concentrations and pregnancy outcome. For example, in White women heterozygosity (CT) of the rs9817203 SNP of the IL1-RAP (receptor associated

protein), involved in many cytokine-induced immune and inflammatory reactions, was associated with higher IL-1B amniotic fluid concentrations in cases vs controls (p=0.02) but not in homozygotes (CC). This association was not found in African-American women. Black women, however, had many more SNPS in IL1-related genes compared to White women (e.g., maternal IL1- RAP 1024941, p<0.001) with the minor allele frequently associated with increased cytokine concentration and sPTB. In another larger study of 1536 SNPs, Frey et al. (2016) studied 833 African-American women (77 sPTB, 756 term births). They identified a number of SNPS related to seven genes involved in inflammation, extracellular remodeling and cell signaling. Many were associated with protein kinase C-α (PRKCA), which has a central role in cell signalling and uterine contractility/quiescence, as well as matrix metalloproteinase-2 (MMP-2, cell remodelling) and tissue inhibitor of MMP-2 (TIMP-2).

Whilst these studies hold interesting mechanistic clues behind the racial disparity seen in sPTB, no consistent genes or polymorphisms have been identified as clinically useful to predict premature birth, nor do these SNPs consistently emerge as significant in larger GWAS studies (e.g., Zhang et al. 2017). Given the complex and heterogenous pathophysiology underlying the common sPTB phenotype (see section 1.4), it is not surprising that, as yet, clinically useful genetic associations have not been implemented.

Of note, racial disparity in birth outcome is not limited to PTB. In the UK, the most recent 'Mothers and Babies reducing risk through audits and confidential enquiries' (MBRRACE) report highlighted higher rates of both infant and maternal morbidity and mortality in Black, Asian and ethnic minority women (Knight et al. 2019), who are more likely to receive lower quality of health care, experience implicit racial bias by care givers, be less likely to participate in clinical research, and have poorer access to health care, in addition to the above described socio-economic and possibly biological factors. Research and policy to address these is of the utmost importance.

1.3.7 Maternal age

A recognised association exists between PTB and advanced maternal age, though this

is lessened when confounding factors such as maternal disease (e.g., hypertension and diabetes) and use of assisted reproductive technology are considered. Nevertheless, a population-based study using independent patient-level data from five high-income countries (including over four million singleton pregnancies) found significantly increased odds of PTB with maternal age >35 years (ORs 1.2 to 1.4 in each country), particularly for women >40 years (ORs 1.3 to 1.8). Whilst some of this increase is accounted for by iatrogenic PTBs, the impact of maternal age was consistent also for sPTB only (Ferrero et al. 2016). Young adolescent women, particularly girls under the age of 16, are at higher risk of sPTB compared with women aged between 20-35, the main increased risk being in girls under the age of 16 (Goffinet 2005).

1.3.8 Behavioural and social risk factors

Women who are overweight or obese are also at higher risk of PTB, largely due to their higher risk of medical complications of pregnancy and iatrogenic preterm delivery. However, women with a body mass index (BMI) >30 kg/m^2 also have higher risk of sPTB, with BMI >40 kg/m^2 conferring highest risk (OR 2.99, 95% CI 2.28-3.92) (Cnattingius et al. 2013). Other modifiable risk factors for sPTB include a short inter-pregnancy interval, low pre-pregnancy BMI, accelerated or poor pregnancy weight gain, low socio-economic status, maternal smoking, and drug use (Goffinet 2005; Ferrero et al. 2016). It may well also be linked to deficiencies such as iron (Shobeiri, Begum, and Nazari 2006; Hämäläinen, Hakkarainen, and Heinonen 2003; Scanlon et al. 2000), zinc and micronutrients (Ramakrishnan et al. 2012; Dunlop et al. 2012) particularly in low- and middle-income countries, though causal links remain unproven (studies in this area are often conflicting, underpowered, and confounded by other socioeconomic risks) (Dunlop et al. 2011). Replacing maternal micronutrients has not been shown to reduce risk of sPTB.

1.3.9 Maternal stress

Whilst maternal ‘stress’ (as well as other psychological adversity during pregnancy such as anxiety, depression, quality of intimate relationships and coping mechanisms) are difficult to measure objectively, evidence points to association with adverse

pregnancy outcomes, including sPTB. The biological mechanisms behind this are not clear, and the presence of multiple confounding factors such as socioeconomic status, ethnicity, experience of racism/racial bias by care givers, low maternal BMI, intimate partner violence, health behaviours such as illicit drugs and smoking, prescription medication and young maternal age among others, makes this association complex.

A systematic review examining pregnancy outcomes in women with varying levels of clinically diagnosed depression or those with anxiety, or stress symptoms during their pregnancy identified 39 robust studies (>134,000 women) (Staneva et al. 2015). sPTB rates varied from 4-23% between studies (though many did not control for potentially confounding health behaviours such as smoking, drinking and drug abuse). They reported ORs for sPTB between 1.13-3.93 in women with depression, including a dose-response relationship between severity of depression and sPTB (even after excluding those participants on antidepressant medication, and controlling for social, demographic, and reproductive confounding factors (n=465) (Li, Liu, and Odouli 2008). Three out of four robust studies associated anxiety with sPTB (OR 1.48-2.73) and two of three studies with low bias demonstrated elevated risk of sPTB with 'perceived stress' (OR 1.14, RR 1.75). Interestingly, one such study correlated the measurement of blood inflammatory markers (tumour necrosis factor-α [TNF α] and IL-6) with stress and outcome amongst 173 women, demonstrating positive correlations between inflammatory markers measured in both early (14-18 weeks) and late (28-32 weeks) gestation, also predictive of a shortened gestational age (Coussons-Read et al. 2012). Low levels of inflammatory markers among women reporting pregnancy stress seemed to partially mediate the effect of stress on gestation at delivery; in some cases, augmenting the effect (e.g., TNF α) and in others, supressing the negative effect (e.g., IL-6).

It is likely that the link between stress and sPTB involves the hypothalamic-pituitary-adrenal axis (Wadhwa et al. 2011). Studies have shown association between elevated corticotrophin-releasing-hormone (CRH), produced by the adult hypothalamus to stimulate cortisol release, as well as by the human placenta, (stimulated by the stress hormones noradrenaline and cortisol), and sPTB. Maternal CRH concentration is

found to be associated with higher risk of sPTB (McLean et al. 1995; Mancuso et al. 2004), also correlating with validated anxiety scores. Given that we know that studies have shown a reduction in sPTB among women who receive antenatal support, in particular continuous care midwifery (Sandall et al. 2016) (though the mechanisms are unclear), it is plausible that stress reduction may contribute towards a more favourable pregnancy outcome.

Could the experience of stress in pregnancy tie into the observed imbalance in sPTB rates between ethnicities? A US population-based study (n=15,915) found an association between acute financial and relationship stressors and sPTB (Almeida et al. 2018), which was attenuated (but not entirely explained) by ethnicity; non-Hispanic Black women both had a higher risk of sPTB than white women, and greater association between stress and sPTB. However, neither stress nor other socio-economic disparity have been proved to account for the ethnic disparity in sPTB. Grobman et al. (2018) performed a prospective study of 9470 pregnant women (60% White, 14% Black, 17% Hispanic) in the US, using self-reported measures of psychosocial stress (stress, depression, racism, anxiety, resilience, and social support). Whilst Black women were more likely to experience sPTB than White women (12.3% vs. 8.1%, $p<0.05$), these differences were not explained after adjusting for differences in self-reported psychosocial factors, nor was psychosocial burden associated independently with risk of sPTB.

1.3.10 Risk factors associated with current pregnancy

Women with multiple pregnancies are at higher risk of PTB (spontaneous and iatrogenic) (Refuerzo 2012), as are women who experience bleeding in pregnancy both in the first and second trimester (Yang et al. 2004), and pregnancies with fetal malformations (Brown 2009). The impact of infection during pregnancy (vaginal, amniotic and systemic) will be explored in section 1.4. Placental disorders/insufficiency with or without intrauterine growth restriction (IUGR) are also associated with sPTB (McElrath et al. 2008; Villar et al. 2012).

1.3.11 Risk factor-based tools to predict sPTB

Whilst we have seen that a number of recognized risk factors are associated with sPTB, the strongest being a prior history of sPTB, risk scoring systems developed to define pre-pregnancy or early pregnancy risk of sPTB have low sensitivity and poor predictive value, particularly in nulliparous women, without a prior pregnancy history. Historically, the most notable scoring system was developed by Creasy, Gummer, and Liggins (1980) utilizing socioeconomic factors, previous obstetric history, daily habits and aspects of the index pregnancy, which has low positive prediction value and limited clinical use, likely due to the multifactorial nature of PTB. Pre-pregnancy and early pregnancy risk factors are, however, used to triage patients to sPTB screening clinics, and also as secondary information contributing to the predictive value of currently used screening tests (see section 1.3.7) and shall be assessed in conjunction with biomarker expression in this thesis.

1.3.12 Clinical tools for prediction of sPTB in asymptomatic women

Risk assessment and management of prematurity has been enhanced in recent years by the knowledge of pathophysiologically relevant biomarkers. Fetal fibronectin (fFN) and CL measurement by transvaginal ultrasound (TVUS) are the best predictors of sPTB available clinically; however, their accuracy for risk screening of asymptomatic women is currently limited to 20-22 weeks of gestation onwards. Moreover, although these tests have high negative predictive values (NPV), they have sub-optimal positive predictive value (PPV) (<50%) (Honest et al. 2002; Revah, Hannah, and Sue-A-Quan 1998; Sotiriadis et al. 2010), limiting clinical accuracy. There is an acknowledged need to identify novel biomarkers that have better positive prediction values and/or have potential to increase the accuracy of existing tests. The results of our recent discovery stage study of elafin in CVF (section 1.6.1.6) indicate that it may be a potentially useful biomarker for prediction of women at risk of cervical shortening and sPTB and could potentially complement/enhance existing screening tools. Below is a summary of the clinically utilised tools for prediction of sPTB in asymptomatic women.

1.3.12.1 *Cervical screening*

The process of labour is characterised by cervical shortening, effacement, and dilatation. Effacement and shortening ("funnelling"), beginning at the internal cervical os can be demonstrated using transvaginal ultrasonography prior to dilatation of the external os palpable on digital vaginal examination. In term pregnancies, this gradual process usually begins after 32 weeks of gestation, and often not until immediately prior to delivery. Measurement of CL using TVUS (Figure 1-5) between 14 and 24 weeks of gestation has been shown to be a sensitive predictor of sPTB in both low-risk and high-risk pregnancies: The risk of sPTB is inversely related to the CL: the shorter it is, the higher the risk of sPTB. High-risk women (those who have had a previous PTB, PPROM or late miscarriage) who have detectable cervical shortening prior to 24 weeks of gestation have a high-risk of subsequent PTB.

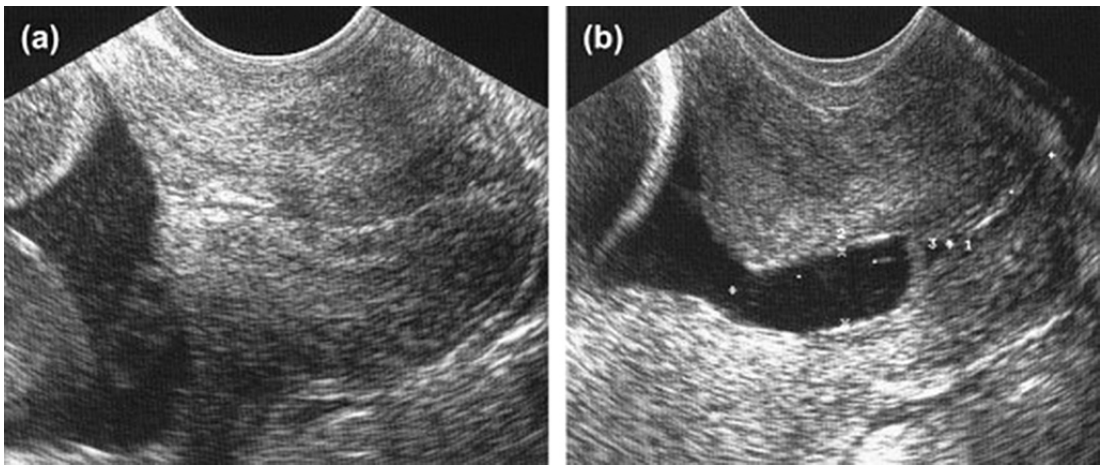

Figure 1-5. Transvaginal ultrasound scan of a) a normal cervix, b) a short cervix with funnelling (Simcox and Shennan 2007).

Several studies have replicated the finding that risk of subsequent sPTB in high-risk women with a CL below 25 mm (approximately the 10th centile according to population centile charts) between 14 and 24 weeks of gestation is substantially greater than women without cervical shortening, and that and that this risk increases exponentially with decreasing CL (Guzman et al. 2001). The incidence of sPTB prior to 34 weeks' gestation was found to be between 19.8% and 76% in women with CL < 25 mm, compared to 7–23% of women with CL > 25 mm (Guzman et al. 2001; Owen et al. 2001; Watson et al. 1999; Berghella et al. 1997; Andrews et al. 2000). In a

prospective study of 183 women, Owen et al. (2001) showed that a single measurement of CL <25 mm at 16–19 weeks' gestation was associated with a relative risk of 3.3 (95% CI 2.1–5.0) for sPTB <35 weeks, which increased to 4.5 (95% CI 2.7–7.6) if serial measurements were performed. The positive prediction for sPTB in women with even shorter cervical lengths of <15 mm is up to 70% for sPTB <35 weeks when detected between 14-18 weeks, and 40% when measured between 18 and 22 weeks (Iams et al. 1996). Importantly for any screening test, intervention (with cervical cerclage, vaginal progesterone or the Arabin pessary, see section 1.7) has been shown to improve outcome (reduction in sPTB) for those women who 'screen positive', though the optimal gestation of screening, cervical length thresholds and regularity of monitoring are not yet clearly defined. Furthermore, women with other risk factors for sPTB (for example previous cervical surgery, or Mullarian duct abnormalities) are often included in cervical screening programmes but the benefit of intervention in these populations is not established. Likewise, although cervical length is proportional to risk of sPTB in low-risk populations (Hassan et al. 2000), the incidence of a 'screen positive' short cervix is so rare amongst low-risk women, and the benefit of intervention questionable, it is rarely performed routinely. Even amongst high-risk women, that many who have a short cervix detected on ultrasound do not go on to have a PTB (sub-optimal PPV/sensitivity), and vice versa (sub-optimal NPV/specificity) make it an imperfect screening tool.

1.3.12.2 *Fetal fibronectin*

FFN a glycoprotein component of the extracellular matrix found in placental tissue, in amniotic fluid, and between the chorion and decidua, is released into the CVF prior to the fusion of the decidua and fetal membranes. After this time (approximately 18 weeks of gestation), CVF fFN concentrations fall and may become undetectable. If fFN is detected in the CVF after this time, it is a result of presumed disruption (inflammatory, infective or mechanical) to the choriodecidual interface (Jackson et al. 1996) and is associated with increased risk of sPTB. CVF fFN is used both independently and together with cervical length measurement in earlier pregnancy, but also in later pregnancy (up to 35 weeks) as a sign of impeding delivery in both symptomatic and asymptomatic women, in order to guide reactive therapies such as antenatal corticosteroids for fetal lung maturation, magnesium sulphate for cerebro-

protection and in-utero transfer to a more appropriate level if neonatal care in the event of delivery. CVF fFN may also be involved in the pathogenesis of sPTB itself (Mogami et al. 2013); *in vitro* demonstrated the biological function of fFN; by treating human amnion mesenchymal cells with fFN they witnessed increased synthesis and expression of matrix metalloproteinases (role in breakdown of cervical collagen) and prostaglandins (role in fetal membrane rupture), mediated by Toll-Like Receptor-4 (TLR-4). Furthermore, fFN could be induced by pro-inflammatory lipopolysaccharide (LPS) and TNF-α, and fFN injection induced sPTB in mice.

Originally measured using enzyme-linked immunosorbent assay (ELISA) containing the FDC-6 monoclonal antibody, fFN concentration is now measured from CVF samples collected from the posterior fornix of the vagina during speculum examination, using a bedside automated instrument. It was originally used as a binary test (positive/negative at a threshold of 50 ng/ml), chosen as the optimal balance between sensitivity and specificity (Lockwood et al. 1991), supported by (Shennan et al. 2005) who demonstrated that fFN >50 ng/mL identified future sPTB <34 weeks in high-risk asymptomatic women with a moderately predictive area under the curve (AUC) of 0.64. Arguably its greatest value lies in its NPV for early delivery (building on the already low prevalence of early delivery even among high-risk women). The Preterm Prediction Study demonstrated NPV of 96% for sPTB <35 weeks of gestation, with a negative (<50 ng/ml) fFN test (2926 women at 24–26 weeks' gestation) (Goepfert et al. 2000). A systematic review of the accuracy of fFN for screening for preterm delivery evaluated 28 studies performed in asymptomatic women, nine in high-risk pregnancies, and eight in low-risk pregnancies (Honest et al. 2002). Pooled positive likelihood ratio (LR, the ratio of the pre- and post-test probabilities) for predicting PTB <34 weeks was 4.01 (2.93–5.49), with negative LR of 0.78 (0.72–0.84). Its sub-optimal LR and PPV (<30%) and thus high false positive has led to may questioning its clinical utility.

Clinical utility, however, has been greatly enhanced by the use of fFN as a continuous variable, quantitative fetal fibronectin (qfFN), rather than a binary one based around one threshold; qfFN concentration correlates directly with risk of sPTB. For symptomatic (Abbott et al. 2013) and asymptomatic (Abbott et al. 2015) women, use

of alternative incremental thresholds (10 and 50, and 200 and 500 ng/mL respectively) enhances PPV for sPTB (an improved "rule in" test) within 14 days and before 34 weeks, while the NPV remains high at every threshold (Table 1-1).

Table 1-1. Predictive value of quantitative fetal fibronectin taken between 22 and 27^{+6} weeks of gestation, for PTB <34 weeks of gestation (n=1433 asymptomatic women) (Abbott et al. 2015).

	Fetal Fibronectin Threshold (ng/mL)			
Predictive Variable	**10 or Greater**	**50 or Greater**	**200 or Greater**	**500 or Greater**
Sensitivity	73.3 (63.5–81.6)	46.5 (36.5–56.7)	28.7 (20.1–38.6)	9.9 (4.9–17.5)
Specificity	72.2 (69.7–74.6)	88.7 (86.8–90.3)	96.4 (95.3–97.3)	99.2 (98.5–99.6)
PPV*	16.7 (13.3–20.5)	23.7 (18.0–30.3)	37.7 (26.9–49.4)	47.6 (25.7–70.2)
NPV*	97.3 (96.1–98.2)	95.6 (94.3–96.7)	94.7 (93.4–95.8)	93.6 (92.1–94.8)
Positive likelihood ratio*	2.64 (2.28–3.05)	4.10 (3.17–5.31)	7.97 (5.27–12.1)	12.0 (5.20–27.6)
Negative likelihood ratio*	0.37 (0.27–0.51)	0.60 (0.50–0.72)	0.74 (0.65–0.84)	0.91 (0.85–0.97)
ROC area		0.78 (0.73–0.84)		

PPV, positive predictive value; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating curve.
Data are % (95% confidence interval) unless otherwise specified.
* All comparisons for each gestational endpoint are statistically significant ($P<.01$) except 10–49 ng/mL compared with 50–199 ng/mL and 200–499 ng/mL compared with 500 ng/mL or greater ($P>.1$ for all gestational endpoints).

More recently, researchers have incorporated qfFN, CL and clinical parameters (risk factor for sPTB, symptoms, singleton or multiple pregnancy) to create and validate a prediction model to create individualised risk of PTB for women at risk with high accuracy (AUC's between 0.75 and 0.90 for prediction of sPTB <30, 34 and 37 weeks (Watson et al. 2019), now available in a mobile application (QUIPP) for clinician use at point of care. Critics of this prediction tool (Goodfellow et al. 2019) argue that use of this algorithm to guide sPTB prevention treatment at a set level of defined risk (e.g., >10% risk of sPTB) would substantially increase intervention without substantial evidence of any benefit. This may stem from a failure to appreciate that applying a fixed threshold to guide treatment is not appropriate, given the lack of evidence that we currently have regarding the utility of most prophylactic interventions (some benefit in women with cervical shortening or recurrent sPTB). In contrast, women with a low risk of sPTB according to the predictive model, are able to avoid treatment (which carries risk) and be reassured by their likely good outcome. Women with a high probability of sPTB can be transferred to and managed in a unit with appropriate neonatal facilities, and treatment to improve neonatal outcome in the event of early delivery can be tailored by clinicians with appropriate and informed consideration of risk and benefit. Further research is needed to evaluate whether interventions to prevent sPTB are beneficial in this population.

Nonetheless, despite the increasing accuracy of prediction of sPTB using models such as that described above, their use is limited to later pregnancy (18 weeks and beyond). An accurate predictor of sPTB detectable in earlier pregnancy could potentially reassure low risk women and reduce anxiety, and potentially allow for earlier prophylactic intervention; for this, a greater understating of the pathophysiology underlying sPTB is vital.

1.4 Mechanisms underlying sPTB

Our knowledge of risk factors for sPTB (section 1.3) has contributed to our scientific understanding of the biological mechanisms of sPTB, and consequently, ways to reduce that risk (section 1.7). Below is a summary which describes in more detail our current understanding of the complex mechanism behind sPTB.

Pathophysiologically, sPTB may be initiated by a number of different causative factors, each with distinct biological pathways (though they may overlap) which may be initiated well before preterm labour is evident, but which culminate in a common effector pathway of myometrial activation, cervical shortening, rupture of membranes and delivery of a gestationally immature infant. These include; infection (ascending from the urogenital tract, or introduced iatrogenically during intrauterine diagnostic or therapeutic procedure), inflammation (sterile or non-sterile), placental immune dysfunction, hormone imbalance (myometrial progesterone functional withdrawal or disruption of the hypothalamic pituitary axis), uterine over-distension (polyhydramnios or multiple pregnancy), cervical insufficiency (pre-existing or secondary to treatment of cervical intra-epithelial neoplasia), and/or vascular causes (poor placentation, ischaemia or haemorrhage) (Romero, Espinoza, Kusanovic, et al. 2006; Goldenberg et al. 2008) (Figure 1-6).

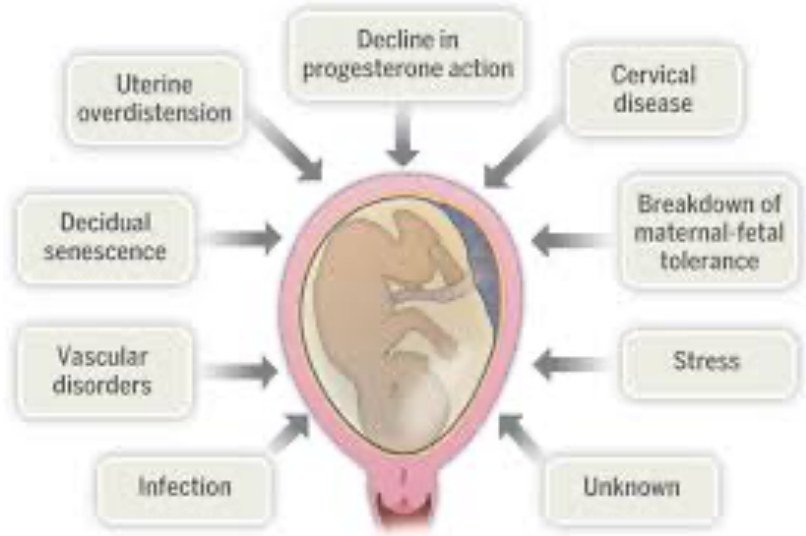

Figure 1-6. Biological pathways implicated in spontaneous preterm birth (Romero, Dey, and Fisher 2014).

More recently, the Interbio-21st study (Kennedy et al. 2019) has been designed to deeply phenotype newborns born prematurely (or small for gestational age) using epidemiological data as well as nutritional, biochemical, 'omic' and histological profiles from large biobank data collected from seven low-, middle- and high-income country sites (2012-2018) to improve the functional classification of this highly heterogenous syndrome. However, it is likely that common to all of these sPTB pathways is activation of the maternofetal inflammatory response. Hereafter, this thesis specifically focuses on inflammatory, and infection mediated sPTB, in an attempt to further understand underlying mechanism, and identify a sub-group of women who may be amenable to early prediction of sPTB and targeted prophylactic and reactive therapies.

1.4.1 Inflammation and sPTB

Inflammation is considered a normal physiological and beneficial process during pregnancy, involving both acute and chronic responses to remodel and reshape uterine tissue, with balance between the pro and anti-inflammatory responses. Labour itself is proposed to be an inflammatory process; inflammatory neutrophils, macrophages and mast cells are present in the cervix, uterus, decidua and fetal membranes immediately prior to and during labour (Thomson et al. 1999; Osman et al. 2003; Mackler et al. 1999; Hamilton et al. 2012; Young et al. 2002). Levels of

cytokines including IL-1ß (Romero et al. 1990) and TNF-α (Romero et al. 1992; Laham et al. 1994) are found in higher concentrations in the amniotic fluid in association with labour, and in animal models, infusions of both of these can stimulate labour (Romero, Mazor, and Tartakovsky 1991; Sadowsky et al. 2006) and cervical ripening (Chwalisz et al. 1994), suggesting that this may be a cause rather than consequence of the labour process.

It is hypothesized (and widely accepted) that the premature disruption of the delicate immune balance during pregnancy in favour of an inflammatory response overwhelms the normal inhibitory response (Rinaldi et al. 2011), leading to irreversible stimulation of the labour cascade, matrix degradation and membrane rupture, responsible for a significant proportion of sPTB and PPROM (Romero, Espinoza, Goncalves, et al. 2007), though specific chemotactic activity and cytokine activation may differ between term and preterm labour/rupture of membranes (Gomez-Lopez et al. 2013). Pathogen-associated molecular patterns (PAMPS) (linked to toxins and infection) are thought to activate the innate immune system, largely via TLRs (upregulated in the decidua, placenta and fetal membranes throughout pregnancy) (Couceiro et al. 2021) but also via IL-1B receptors (Nadeau-Vallée et al. 2016). TLR activation activates NFKB, upregulating genes responsible for uterine contractility and cervical ripening. Rising concentrations of prostaglandins in maternal serum and fetus prior to labour (Olson 2003) activate uterine contractions (Crankshaw and Dyal 1994), upregulate oxytocin receptors (Liggins 1989), stimulate cervical ripening (Kelly 2002) and upregulate matrix metalloproteinases (MMPs) which are implicated in fetal membrane rupture (McLaren, Taylor, and Bell 2000). Chemokines and cellular adhesion molecules, which release pro-inflammatory cytokines and MMPs (Romero et al. 1991; Osmers et al. 1995; Winkler et al. 1999) degrade fetal membranes and are thought to contribute to cervical ripening by collagenolysis of the extracellular matrix (Junqueira et al. 1980; Osmers et al. 1992; Norman et al. 2007; Romero et al. 2007).

More recently, the hypothesis of fetal membrane aging (and associated deterioration of fetal membranes) acting as a trigger for labour has been proposed; it is thought that both infectious and non-infectious risk factors can cause oxidative stress (Menon

2014; Chai et al. 2012; Menon et al. 2016) via damage-associated molecular patterns (DAMPs) which results in damage to cells, cellular organelles and shortening telomeres (Menon 2019; Kawanishi and Oikawa 2004). The resultant inflammation, decidual activation, cervical ripening and myometrial contractility may lead to rupture of membranes and/or labour (whether term or preterm). This inflammatory response may be stimulated by infection (section 1.4.1.2) though may also be sterile (section 1.4.1.1).

Polymorphisms of inflammatory-related genes have been associated with increased risk of sPTB (also see section 1.3.6 regarding ethnicity related inflammatory gene polymorphisms), including for the gene encoding the TLR-4 receptor, thought to affect NFKB activation (Lorenz et al. 2002), and the IL1 receptor agonist (IL1RA) gene (Genç et al. 2002; Murtha et al. 2006) among others identified by candidate gene studies, as well as those identified by larger GWAS such as early B cell factor-1, angiotensin II receptor type 2 and eukaryotic elongation factor selenocysteine (Zhang, Baldwin, et al. 2015).

1.4.1.1 *Sterile inflammation and sPTB*

While microbial invasion of the amniotic cavity is an important mechanism thought to underly sPTB (see following section 1.4.1.2), the concept of sterile intra-amniotic inflammation is an alternative hypothesis for the pathogenesis of some sPTB, given that evidence of infection is not always found in cases of sPTB, despite the triggering of an inflammatory cascade. Romero, Miranda, et al. (2014) evaluated the presence of infection (using culture, polymerase chain reaction [PCR] and mass spectrometry) and inflammation (IL-6 >2.6 ng/ml) in amniotic fluid obtained by amniocentesis (between 20-35 weeks with suspected preterm labour with intact membranes) in 135 women. 26% of women had evidence of sterile inflammation (no evidence of microbial colonisation) vs. 11% who had positive microbial presence. Furthermore, the earlier the gestation at delivery, the higher the prevalence of both sterile and microbial inflammation. Moreover, women with sterile inflammation had similar histological evidence of chorioamnionitis as the women with microbial inflammation. Given that the group used molecular bacterial detection methods in addition to lower sensitivity conventional culture, this is evidence to support the theory that sterile

inflammation, likely resulting from inappropriate activation of the inflammatory cascade, is involved in the pathogenesis of preterm parturition. The same group subsequently published further evidence of sterile inflammation in amniotic fluid in women with a short cervix on TVUS, but who were asymptomatic of PTL (Romero et al. 2015). A total of 10% had evidence of sterile intra-amniotic fluid inflammation (compared with 2% of microbial associated inflammation, $p<0.001$). Furthermore, sterile inflammation in women with short cervices was associated with sPTB; 70% of these women had a sPTB <34 weeks vs. 32% without inflammation ($p<0.001$).

Whilst the absence of detectable microorganisms defining sterile inflammation is a function of the sensitivity of scientific techniques (in the above example, PCR and culture), and more sensitive molecular techniques are now available, the concept of sterile inflammation is strengthened by several epidemiological and biological associations. Though the exact mechanisms are still unclear, many environmental sterile stimuli have epidemiological association with sPTB (section 1.3) such as cigarette smoking, obesity, environmental pollution, or psychosocial stressors, result in inflammation. Adiposity is associated with raised cytokines, particularly IL-1 and IL-6 (Brydon 2011), and heightened cortisol stress responses (Epel et al. 2000). Cigarette smoke is a source of reactive nitrogen species which may increase systemic inflammation. Psychosocial stress is likely to impact on the hypothalamic pituitary axis (section 1.3.7) resulting in increased cortisol and inflammatory cytokines (Wadhwa et al. 2011).

It is likely there are key differences in the inflammatory mechanisms leading to sPTB whether sterile or microbial induced; Menon's group reported a different inflammatory response in a human fetal membranes induced by cigarette smoke (apoptotic mechanisms), compared with bacterial lipopolysaccharide (cytokine and MMP production) (Behnia, Sheller, and Menon 2016). However, given that HDPs have been shown to have roles in the suppression of both sterile inflammation and microbial-mediated inflammation (see sections 1.5-1.6), they remain biologically plausible biomarkers for both sterile and infection related phenotypes of sPTB.

1.4.1.2 *Infection as a cause of sPTB*

Ascending infection (for example from the genitourinary tract) may activate the inflammatory pathways involved in parturition, leading to cervical shortening and/or fetal membrane rupture. Infection initiates host immune response, with enhanced inflammatory immune response measurable in the amniotic fluid, maternal blood and CVF. Cervical shortening may also itself provide a permissive route for microbial colonization of the choriodecidual space, and/or compromising the innate immune protection of the cervical mucus plug (Hansen et al. 2014). Basic scientific and clinical data have consistently shown an association between both systemic and genitourinary infection, and sPTB. Examples of these associations are listed below.

- **Chorioamnionitis.** Animal studies provide evidence that infection leads to sPTB. Administration of microbial products to pregnant animals can result in PTL and delivery (Romero et al. 1988; Hirsch, Saotome, and Hirsch 1995; Gibbs et al. 2004; McDuffie Jr, Sherman, and Gibbs 1992; Gravett et al. 1994), whilst treatment of chorioamnionitis with antibiotics can prevent sPTB (Fidel et al. 2003). In human studies, both clinical and subclinical chorioamnionitis are more commonly seen in preterm vs. term labours. In an observational study of 315 pregnancies ending between 20 and 35 weeks gestation, 53% had chorioamnionitis confirmed from cultured samples obtained via amniocentesis, 92% had histological chorioamnionitis and 12% of the live-born infants had positive blood cultures within 72 hours of birth (Yoon et al. 2000). Hassan et al. (2006) demonstrated, using bacterial culture, intra-amniotic infection in 5/57 (9%) of amniocentesis samples from asymptomatic women with a short cervix, which could imply exposure of the fetal membranes to the organisms in the upper genital tract, though they did not have comparative samples in a matched group of women with a long cervix. Studies of mid-trimester amniocentesis have found that women with intrauterine infection were at higher risk of sPTB compared to those without infection (Perni et al. 2004; Gray et al. 1992; Horowitz et al. 1995).
- **Bacteriuria**. Women with asymptomatic bacteriuria are at higher risk of sPTB compared with women who do not have bacteriuria. In a large retrospective population-based study of 199,093 births, asymptomatic bacteriuria (2.5% of

the study population) was independently associated with sPTB (OR 1.6, 95% CI 1.5-1.7) (Sheiner, Mazor-Drey, and Levy 2009), treatment of which reduced the incidence of low-birth-weight babies (Smaill and Vazquez 2015). Whilst the quality of data was low for the outcome of PTB, this may imply that treatment may reduce the infection/inflammation/bacterial load enough to prevent placental compromise, as well as reducing the consequences of the inflammatory cascade which leads to sPTB.

- **Systemic infection.** Women with systemic infection such as malaria (Kalanda et al. 2006; Steer 2005), pyelonephritis (Wren 1970) and pneumonia (Madinger, Greenspoon, and Gray Ellrodt 1989; Goodnight and Soper 2005) are at higher risk of sPTB than their non-infected counterparts.
- **Bacterial Vaginosis.** A diagnosis of BV, abnormal vaginal bacterial flora, with an overgrowth of mixed anaerobic bacteria species such as *Gardnerella, Prevotella* and *Atopobium* is associated with a two to five times increase in risk of sPTB (Gravett, Hummel et al. 1986, Hill 1998, et al. 2005; Klebanoff et al. 2005), as well as having associations with PPROM and chorioamnionitis. Puzzlingly, the majority of women with BV do not deliver early, and there remains controversary over the effect of treating BV on subsequent PTB; whilst a number of individual trials have reported reduction in PTB in women with previous PTB who were screened and treated for BV in early pregnancy (Hauth et al. 1995) and also in the general obstetric population (Ugwumadu et al. 2003), many others report no effect, consistent with the most recent Cochrane systematic review (Brocklehurst et al. 2013) (> 7000 women). Furthermore, the most recent large trial of over 5000 women with BV (from a population of 84000 screened pregnant women), randomized to treatment (clindamycin) or placebo showed no benefit (Subtil et al. 2018). This may fit with our hypothesis that the underlying host vaginal environment (including innate immune response or microbiome) which determines susceptibility to BV may also determine risk of sPTB; treating BV with antibiotics does therefore not alter the underlying susceptibility to sPTB.

Intra-uterine infection may be identified in the decidua, between the amnion and chorion or reach the amniotic cavity to the fetus. Access to the amniotic cavity for microorganisms may be by a number of routes (Goldenberg et al. 2008):

a) ascending infection from the vagina, and crossing intact or ruptured fetal membranes

b) haematogenous spread through the placenta

c) iatrogenic introduction during invasive procedures e.g., amniocentesis

d) retrograde spread via the fallopian tubes.

Infection and inflammation associated with sPTB is often sub-clinical, with only a minority of proven cases of chorioamnionitis presenting with symptoms or signs of infection (Romero, Espinoza, Gonçalves, et al. 2006). Activation of this inflammatory cascade, triggered by infection, is believed to be associated with up to 40% of sPTB (Romero et al. 2007a), with a likely larger contribution at the earliest gestations. This figure may even be higher, underestimated due to the difficulties in detecting intrauterine infection with conventional culture-based techniques, and also the difficulty in accurately sampling and measuring appropriate markers of infection (e.g., in amniotic fluid), prior to, or at the time of, preterm labour. Commonly reported microorganisms reported in the amniotic cavity originate from genital tract, e.g., *Mycoplasma spp*, particularly *Ureaplasma Urealyticum* (Perni et al. 2004; Yoon et al. 2003). Others include *Gardenella vaginalis, Mycoplasma hominis, Bacteroides* spp, Group B *Streptococcus (GBS)* and *Escherichia coli* (Goldenberg et al. 2008; Nelson et al. 2007). Suff et al. (2018) demonstrated that intravaginal administration of a bioluminescent strain of pathogenic *E. coli* into a pregnant mouse model induced pre-term parturition. They later demonstrated how inoculation of pregnant mice with an adenovirus gene vector containing the human beta defensin 3 (HBD-3) gene, coding for expression of HBD-3, an antimicrobial peptide component of the innate immune system (section 1.5), reduced bacterial ascent and increased the proportion of mouse pups born alive (Suff et al. 2020).

Many non-genital tract organisms are also associated with sPTB, including oral cavity infection *(e.g., Fusobacterium nucleatum*) with presumed haematogenous spread across the placenta (Aagaard et al. 2014). It must be noted, however, that whilst a

number of studies using 16S RNA gene amplification techniques (see section 1.4.1.3) suggest that the nature of bacterial colonization of the placenta (the placental microbiome) may differ in complicated (e.g. preterm) pregnancies and uncomplicated (term) pregnancies, this view has been challenged by those who believe that the placenta is sterile until labour and that spurious microbiome results have resulted from either contamination of laboratory reagents, or acquisition during labour and delivery. Indeed, de Goffeau et al. (2019) reported a study of placental biopsies from 100 cases of sPTB and 198 controls (as well as pre-labour placentas total over 500 placentas) using multiple methods of gene extraction and detection, to control for experimental contamination. They found that a high proportion of the bacteria detected came from contamination or during the labour process (when comparing pre-labour biopsies vs. biopsies taken post labour). Only *S. agalactiae* was confidently detected in the placenta prior to delivery, which was not associated with gestation or pregnancy outcome.

In general, the intra-amniotic infective process is usually believed to be a chronic process. Studies looking at detection of infection by both culture (n=61) (Yoon et al. 2003) and PCR techniques (Cassell et al. 1982) (n=257) at the time of mid-trimester genetic amniocentesis, discovered that many more who were positive for *U. Urealyticum* went on to have sPTB or PPROM many weeks after the procedure, compared with those who tested negative. That infection tends to be with microorganisms with low virulence may account for the chronic nature of most infection, as well as frequently absent clinical signs and symptoms. Mechanistically bacterial ligands bind to TLRs in decidual, amniotic, cervical and placental cells, triggering the release of activated neutrophils, macrophages and pro-inflammatory chemokines (e.g., IL-8, chemokine [C-C] motif ligands 5), as well as cytokines (e.g., TNF-α and β, IL1-α and β, IL-2, IL-6, and granulocyte colony stimulating factor [G-CSF]). A positive-feedback maintained immune cascade drives the synthesis and release of prostaglandins (via cleavage of arachidonic acid from glycerophospholipids in the cell membrane by phospholipase A2) and genital tract proteases e.g., matrix metalloproteinases (MMP), which degrade the extracellular matrix proteins of the cervix and gestational tissues (Fortunato, Menon, and Lombardi 2002; Oner et al.

2008), and enhance myometrial contractility (Gibb 1998). This may lead to continued cervical shortening, and eventually rupture of the fetal membranes.

Biochemical evidence of chorioamnionitis is consistently associated with sPTB. Elevated levels of IL1-β, IL-6, IL-8, TNF-α and CRP have been found in maternal serum, amniotic fluid and cervicovaginal discharge at the time of sPTB (Engel et al. 2005; Hagberg, Mallard, and Jacobsson 2005; Wenstrom et al. 1998; Menon et al. 2008; Kramer et al. 2010). Indeed, such cytokines have been evaluated extensively as predictive biomarkers for subsequent PTB (Jia 2014; Perales-Puchalt et al. 2013), though none have reliably made it into clinical practice. Promisingly, IL-6 (Wei, Fraser, and Luo 2010), IL-1β and platelet secreted RANTES (Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted) (Amabebe et al. 2018) have been found to be raised in late-mid-trimester CVF of women who subsequently deliver spontaneously and prematurely, and correlate with raised fFN, however such studies are still limited to small numbers of high-risk women (n=63). Granulocyte-macrophage colony-stimulating factor (GM-CSF), and monocyte chemotactic protein-1 (MCP-1) were raised in CVF in the pilot study by our research group (n=78) in women 16 to 24 weeks gestation who developed a short cervix, but not other cytokines such as IL-6, IL-8 and TNF-α (Chandiramani et al. 2012). In general, these inflammatory biomarkers are raised at the start of sub-clinical or overt clinical infection and once elevation occurs, the time to delivery is short, limiting their use as early biomarkers to target prevention strategies. Should these findings be replicated in larger studies, their clinical utility may be limited by the relatively small test to delivery period. However, should a related perturbation in the earlier reacting innate immune system be found in women destined to deliver early, as is investigated in this thesis, a greater time-window for intervention may be permitted.

To add an additional layer of complexity, it may be that a reduction in the reproductive tract immune response may increase susceptibility to genitourinary tract infection and/or permit ascending infection in women destined to have sPTB; Kalinka et al. (2005) collected mid-trimester CVF samples from 114 pregnant women (mean gestational age 29 weeks) alongside Gram-stained swabs for vaginal pathogens. Whilst they found no difference in overall CVF cytokine levels between

term and PTB, women with pathological flora (N=49, *M. Hominis, U. Urealyticum* or BV) who delivered prematurely had lower IL-1a, IL-1β (OR 10.7 and 4.9 respectively) as well as IL-6 and IL-8 compared with those who delivered at term. Similarly, Simhan et al. (2003) in a cohort of 403 women, found that women with at least one low CVF cytokine concentration (IL-1β, IL-6 or IL-8) were more likely to develop clinical chorioamnionitis, and that this likelihood increased if two or three depressed cytokine concentrations were found. It is therefore possible that inadequacy of the immune response (both immune hypo and hyper-responsiveness) may put a woman at risk of sPTB. Thus, it is increasingly theorized that underlying disorders of maternal innate or acquired immunity (the body's ability to react to infection), as well as the presence of ascending genitourinary infection, are responsible for many infection-related sPTB.

Researchers are looking closely at other mechanisms by which infection may be related to sPTB (see also section 1.4.1.3 the vaginal microbiome). For example, maternal H-antigen secretor status is determined by the fucosyltransferase [FUT2] gene and inherited in an autosomal dominant pattern. H-antigen is a mucosal cell surface glycan, thought to influence infection susceptibility via host pathogen interactions. Women who do not express the H-antigen are at increased risk of certain infections, and in a mixed ethnicity North American cohort of 300 women, lack of H-antigen production (measured using ELISA from a saliva sample) was associated with sPTB, after controlling for known confounders, including ethnicity (Caldwell et al. 2020). If such findings are validated, glycans such as H-antigen may be useful biomarkers to predict preterm birth, as well as representing potential targets to manipulate the vaginal microbiota composition and modulate the maternal host immune response. Work is currently ongoing by the research team at Imperial College (data presented but unpublished), to relate these findings to FUT2 polymorphisms and the vaginal microbiome.

1.4.1.3 *The vaginal microbiome*

As discussed above, while microbe/pathogenic-induced inflammation is understood to be an important cause of sPTB, more subtle alterations and variations in an

individual's vaginal microbial community structure, the 'vaginal microbiome', is likely to influence the vaginal environment (including pH and innate host immune response) and is thought to be another important contributing factor to pregnancy outcome.

The human vaginal microbiome (community of organisms, mainly bacteria, coexisting within a species) is a highly balanced community and is a first line of defence against foreign pathogens (Ma, Forney, and Ravel 2012). It is composed largely of commensal *Lactobacillus* sp. which anaerobically metabolise glycogen produced by the epithelia, producing lactic acid, creating an unfavourable environment (normally pH 3.5-4.5) for other bacteria/pathogens which demand a more alkaline host environment. The lactobacillus-rich vaginal microbiota is associated with protection against sexually transmitted infections (chlamydia, gonorrhoea, HPV and Human immunodeficiency virus [HIV]) (Martin et al. 1999; Abdool Karim et al. 2019), *Candida* (Parolin et al. 2015) and UTIs (Gupta et al. 1998), as well as influencing fertility and pregnancy outcome (Kroon, Ravel, and Huston 2018). The composition of the vaginal microbiome is highly dynamic, influenced greatly by host-environment interactions including sex-hormone levels (e.g., oral contraceptive pill/stage of menstrual cycle/menopause and pregnancy), sexual activity, diabetes mellitus and antibiotic drug therapy. As discussed preciously, racial differences in the composition of vaginal microflora (in the absence of disease) have also been demonstrated; Black and Hispanic women more frequently have vaginal environments lacking in *Lactobacillus* sp., with corresponding higher pH (Ravel et al. 2011; Fettweis et al. 2014).

Until relatively recently, the ability to characterise the vaginal (and other body site) microbiome has been limited by the use of culture-based techniques which allows characterisation of only a minority of the microbes in the genital tract, failing to give an accurate picture of microbial diversity. However, the development of culture-independent metagenomic technology 'next generation sequencing', specifically, the adoption of 16S rRNA gene amplification by PCR, followed by cloning and sequencing, has allowed for a more comprehensive and broad evaluation of vaginal microbial diversity. The bacterial 16S ribosomal gene contains regions that are conserved between bacterial species and areas that are variable between species. By using

primers that target the conserved regions of the gene it enables amplification of the DNA from most bacterial species. The bacterial DNA is then sequenced to facilitate identification of bacteria. As well as improving the assessment of microbial prevalence and diversity over traditional methods (particularly given the slow growing nature of many microorganisms), it allows identification of organisms even if they are no longer viable (e.g., after antibiotic administration) (Jones et al. 2009). Indeed, studies comparing culture dependent and PCR analysis of amniotic fluid from women in PTL and PPROM detected 11% of total microbes in 'culture negative' samples (Gardella et al. 2004). A criticism of the technique, however, is that despite detecting the bacteria, it is unable to distinguish between alive and thus actively pathogenic bacteria, or dead bacteria, nor quantify the bacterial load, and thus may over or under-estimate the importance of the presence of particular findings. Moving towards evaluating functional read-outs of bacteria (e.g., measurement of the metabolites that they produce) may overcome some of these problems (Stafford et al. 2017; Amabebe et al. 2019).

Most of the original pregnancy-related vaginal culture-independent pyrosequencing studies focused on describing the vaginal microbiome in normal pregnancy. They report key differences between gravid and non-gravid vagina microbiota (with a higher proportion of lactobacillus species noted in pregnant samples), as well as differences by gestational age and proximity to the cervix (Aagaard et al. 2012; Romero, Hassan, et al. 2014), with a decrease in the alpha diversity (the within sample microbial diversity measured using the Shannon Index) and increase in stability as pregnancy progresses (Romero, Hassan, et al. 2014).

1.4.1.4 *The vaginal microbiome and sPTB*

Many research groups have examined the relationship between the vaginal microbiome and risk of sPTB, implicating alterations in the host vaginal microbiome in the pathogenesis of infection-related sPTB. However, they have produced mixed, and often conflicting results, likely due to differences in populations studied, as well as microbiome collection and sequencing techniques, which have advanced rapidly in recent years, as well as different classification systems.

Hyman et al. (2013), using 16S pyrosequencing technology, sequenced the vaginal microbiome in each trimester of 46 high-risk and 42 low-risk women, 17 of whom delivered prematurely. They noted that there was strong correlation with the vaginal microbiome and ethnicity. Amongst the Caucasian women, the mean Shannon Diversity index differed between Caucasian women with sPTB and term gestation, with reduced diversity amongst the preterm patients (n=7). Numbers were not sufficient to repeat this analysis for other ethnicities. The ROC area for microbial diversity and sPTB was 0.70, performing better than risk of sPTB by previous history (ROC 0.52). Interestingly, this study had an unusually high PTB rate (19% overall, 17% amongst 'low risk women'), higher than the national average (though not statistically different). Furthermore, sPTB was only classified as above or below 37 weeks. It would be interesting to know how results varied according to early or late sPTBs (though in this study, the sample size would be inadequate).

In contrast, Professor Romero's group examined 18 sPTB cases (median gestation at delivery was 30 weeks') and 72 controls (term deliveries) using similar techniques, though with higher depth sequencing that Hyman's group (Romero, Hassan, et al. 2014), finding no variation in the diversity or composition of the microbiome between term and sPTB. They concluded that it is possible that the perturbation of the vaginal microbiome leading to intra-amniotic infection is transient and therefore difficult to detect using the sample frequency employed in their study. Alternatively, differences in the microbiome between term and preterm women might be related to function of microorganisms (not detected using this technique), bacterial load or genetic modification of bacteria within the same species. Larger sample sizes are needed to make definitive conclusions, possibly using indices of relative abundance.

Specific bacterial species have been shown to be associated with sPTB. Kidinger et al. (2017) sampled CVF from the posterior fornix of 161 women (65% White, 19% Black) at high-risk of sPTB (previous history of sPTB <37 weeks) at approximately 16 weeks of gestation. Women who delivered spontaneously <34 weeks were significantly more likely to have an *L. iners* dominated vaginal microbiome (67%) compared to later preterm (31%) and term births (29%), which persisted after accounting for

ethnicity, maternal age, BMI and gestation at sampling. In contrast, those with *L. crispatus* dominance were more likely to have a term birth.

Similar findings were reported in a study (n=158) by Stafford et al. (2017), who found that (predominantly White) women who experienced sPTB were less likely to have a microbiome dominated by *L. crispatus*, and more likely to be dominated by *L. jenseni.* They also correlated these findings with an associated increase in the metabolites succinate and decrease in lactate, in those samples from women who delivered prematurely. Fettweis et al. (2019) conducted a case-control study of 45 preterm, and 90 term births in American women predominantly of African ancestry (78%). Like Kidinger's results, in the earliest vaginal samples taken at approximately 18 weeks of gestation (taken by self-sampling or collection without a speculum, so potentially lower in the vagina than the posterior fornix and thus further away from the cervix), lower levels of *L. crispatus* characterized the vaginal microbiome of women who delivered prematurely, as well as higher concentrations of other anaerobes including BV associated Bacterium 1 (BVAB1), *Sneathia amnii*, and *Prevotella* species.

Elovitz et al. (2019) made further attempts to clarify the potential impact of ethnicity of the vaginal microbiome and subsequent risk of sPTB in a larger US cohort; 107 cases of sPTB and 432 women delivering at term, using longitudinally collected CVF samples. They described the differences in microbiome composition between US Black and White women; as seen in other studies (Callahan et al. 2017; Romero, Hassan, et al. 2014), a larger proportion of African American women had a microbiome that was non dominant in *Lactobacillus* spp, many of whom still delivered at term. Whilst overall, CST IV (mixed bacterial anaerobes) was associated with sPTB, this association was not seen in Black women. Similarly in non-African American Women, an association between CST IV (*L. jensenii* predominance) was associated with term pregnancy (in contrast to the above study by Stafford), but the converse was shown in Black women. Dominance of *L. iners* were associated with sPTB in non-Black women, but even in these women, the predictive value was relatively low. Furthermore, while predominance of *Lactobacillus spp*. in the presence of high-risk anaerobes such *as Mobiluncus curtsii/mulieris* conferred some protection against sPTB, predominance of *Lactobacillus* in general did not guarantee

a delivery at term. Thus, they concluded that the contribution of the vaginal microbiome to sPTB risk is different according to ethnicity and introduced the idea that risk may be modulated by other factors including expression of HDP (in this case, they examined HBD, discussed later).

In general, there is a lack of consistency, as well as actively contradictory results between studies, likely related to small sample sizes and even smaller event numbers, differing methodology, restricted populations (most frequently US women and not always ethnically stratified) and evolving techniques. A recent systematic review of studies examining the microbiota and sPTB found nine relevant but heterogenous studies, six of which showed an association between the composition of the vaginal microbiota and sPTB (Peelen et al. 2019). Whilst most confirmed some association between vaginal dysbiosis and sPTB, a clinically useful tool has yet to emerge.

1.5 The innate immune system and pregnancy

During pregnancy, maternal-fetal immune competence is achieved by suppression of the adaptive immune system, ensuring differentiation by the immune system between foreign pathogens and the immunologically-distinct fetus, preventing rejection. The traditional explanation for this is the 'fetal allograft model' (Medawar 1953) whereby pregnancy confers an immunosuppressive state e.g., maternal TH1 lymphocyte functional suppression by progesterone (Piccinni et al. 1995), or lack of fetal-antigen presentation to maternal lymphocytes (the trophoblast does not express MHC class I or class II molecules). However, it has been observed that leucocytes both increase in numbers and activity at the maternal fetal interface from the first trimester onwards, and pro-inflammatory cytokines, granulocytes and monocytes proliferate in the maternal circulation with increased expression of cell surface endotoxin receptors (Sacks et al. 1998) and complement activation (Richani et al. 2005; Sacks, Sargent, and Redman 1999). This implies that the suppression of the maternal response to the fetal-antigen is accompanied, or even compensated for, by up-regulation of components of the innate immune system.

Robust prevention of intrauterine infection is essential to the maintenance of fetal health and delivery at term. The innate immune system, comprising surface defences (mainly epithelial barriers, a physical barrier against pathogen entry) and constitutively expressed HDPs predominantly produced by at epithelial surfaces (e.g. skin and vaginal mucosa as well as the epithelial layer of the cervix, fetal membranes, placenta and decidua (King, Kelly, et al. 2007) by leucocytes, is responsible for the initial rapid, 'first defence' non-specific inflammatory response to foreign antigens (Horne, Stock, and King 2008). As well as being a vital system to prevent microbial penetration, the innate immune system has also evolved to facilitate and regulate specialised functions including menstruation, fertilisation, implantation, pregnancy and parturition (Horne, Stock, and King 2008).

There is a wide range of natural defence peptides and proteins forming part of the human innate immune system; over 3,200 antimicrobial peptides/HDPs have been described (http://aps.unmc.edu/AP; accessed 10 August 2020). Over 1200 of these are within the gastrointestinal, respiratory and reproductive tract (Lai and Gallo 2009), and many are implicated in the pathogenesis of inflammatory conditions and infections, including sPTB. They include cathelicidin, elafin (skin-derived anti-proteinase), defensins, bacterial permeability increasing protein (BPI) and secretory leukocyte protease inhibitor (SLPI) (Boix and Nogues 2007; Stock et al. 2009). Via pathways of cytokine and complement activation and phagocytic responses (Janeway Jr and Medzhitov 2002), HDPs disrupt the membranes of microbial pathogens (recognised by TLRs at mucosal and phagocytic cell surfaces), initiate and sustain inflammatory responses, and can be up-regulated following exposure to microbes, epithelial cell released mediators, and phagocytes (Li and Huang 2009). Many of the phagocytic cells involved in this cascade act as antigen presenting cells to T-lymphocytes, a vital link to the adaptive immune response (not discussed this this report). Figure 1-7 is a pictorial representation of the direct and indirect functions of HDPs in the female reproductive tract.

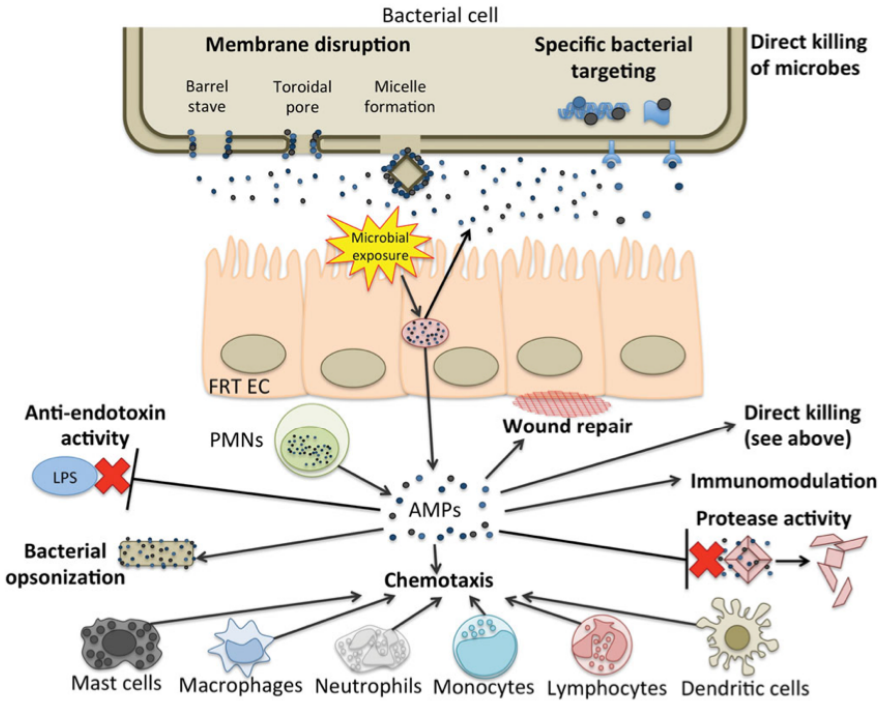

Figure 1-7. Host defence peptides in the female reproductive tract are secreted by epithelial cells and neutrophils and have a wide variety of direct and indirect antimicrobial and anti-inflammatory functions (taken from Yarbrough et al. 2015). FRT=female reproductive tract; EC=epithelial cell; PMN=polymorphonuclear leukocyte; AMP=antimicrobial peptide.

1.6 Host defence peptides

Expressed throughout the female genital tract, HDPs are small cationic host defence molecules functioning within the innate immune system. Key mediators of the inflammatory response, they are released at epithelial surfaces, causing damage to the membranes of microbial pathogens with the electrostatic forces resulting from their positively charged amino acid residues opposing the negative charges exposed on prokaryote cell surfaces. They also demonstrate antiviral and antifungal activity. Many also exhibit anti-protease action, able to counteract the effect of protease mediated inflammation and tissue destruction (Sallenave 2000), as well as immunomodulatory functions, and various roles in tissue homeostasis (Figure 1.8). The activation of these innate defence molecules is promoted by binding of the pathogen associated molecular pattern to pattern recognition receptors, such as TLRs, via NF-kB dependent or independent pathways. Alternatively, TLR independent pathways include induction by pro-inflammatory cytokines (Sallenave 2010) and stimuli generated by inflammation, infection or tissue trauma such as human neutrophil elastase or proteinase-3, PAMPS and DAMPs (Rzepka et al. 2014).

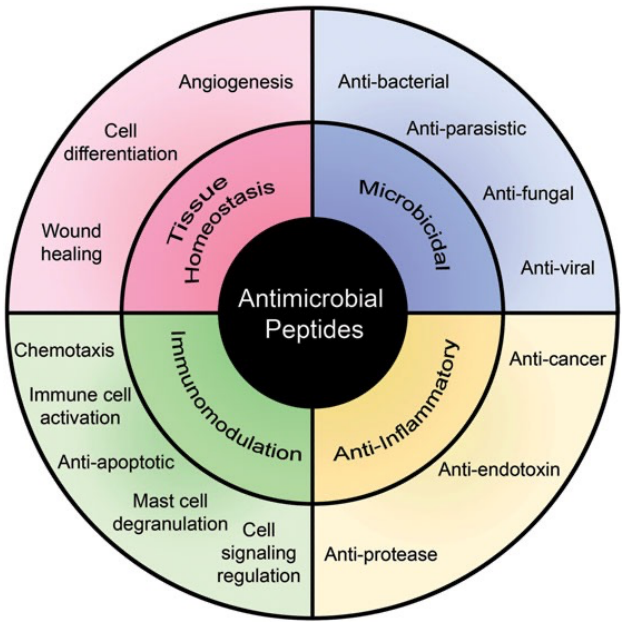

Figure 1-8. The diverse functions of antimicrobial peptides/Host defence peptides (Yarbrough et al. 2014)

Based on their size, composition and structure, HDPs are classified into several categories; peptides with alpha helix structures, beta sheet structures or loop structures. Trappin2/Elafin (also known as peptidase inhibitor 3 (PI3) or skin-derived antiproteinase SKALP), SLPI, HBDs 1-3 and cathelicidin are amongst the HDPs already identified in the reproductive tract (Stock et al. 2009) and most, including elafin, can be induced by the presence of inflammatory cytokines.

Following pilot studies by our group (see section 1.6.1.6), which demonstrated that expression of elafin, a member of the whey acidic protein (WAP) family distributed throughout the human reproductive tract (vagina, cervix, cervical plug, uterus and fallopian tubes) (Horne, Stock, and King 2008) and cathelicidin, a cationic peptide stored in the secretory granules of neutrophils and macrophages in the lower genital tract, were associated with cervical shortening, this PhD project will focus on these HDPs alone. To complement this, the expression of human neutrophil elastase (HNE), an enzyme co-released alongside cathelicidin and targeted by elafin will also be studied.

1.6.1 Trappin 2/Elafin

Trappin 2/Elafin is a HDP produced by the epithelial cells of the cervico-vaginal tract and is measurable in the CVF (as well as expressed constitutively in epithelial surfaces

such as skin, airway and intestinal mucosa). It is a pleiotropic protein possessing anti-proteinase and anti-elastase properties, but also has anti-microbial and immune-modulatory functions at the mucosal surface (Williams et al. 2006).

The PI3 gene (2.3 kb) codes for a molecule composed of 117 amino acid residues (12.3kDa), which is cleaved intracellularly to a 9.9kDa protein (Trappin 2) losing the hydrophobic signal peptide. The cementoin domain (see Figure 1.9) of this molecule forms covalent bonds with the extracellular matrix binding it to tissues. It can be further proteolytically cleaved into a 6kDA molecule (elafin) losing the 'cementoin' domain, which is unattached to the extracellular matrix. While it was traditionally thought that cleaved elafin dominated the anti-proteinase effects of the molecule, Zani et al. (2004), using recombinant DNA coding for trappin-2 and elafin incorporated into a yeast expression system, found both molecules to be fast acting inhibitors of pancreatic elastase, HNE and proteinase 3, by forming an enzyme inhibitor complex. Furthermore, in animal models tissue-bound pre-elafin (9.9kDa) is more effective at inhibiting neutrophil elastase related lung injury compared with commercial synthetic elafin (6kDa) (Tremblay et al. 2002). Both Trappin-2 and elafin can be detected using commercially available antibodies. Few papers, however, distinguish between which form of the peptide that they are measuring, and the term elafin is frequently used indiscriminately to mean both the larger trappin-2 and smaller elafin molecule, which makes interpreting its specific biological actions difficult. The ELISA used for protein measurement in this study cross reacts with the 9.9 kDa pre-elafin, hereafter referred to as 'elafin'.

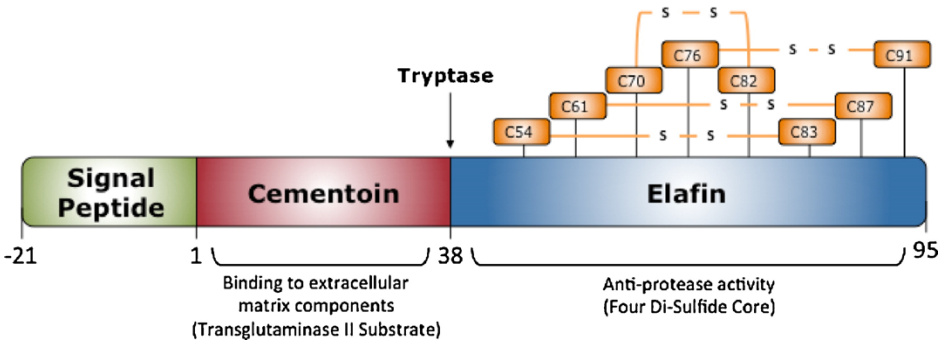

Figure 1-9. Representation of Trappin-2 (Verrier et al. 2012).Trappin-2 binds to the extracellular matrix via its cementoin domain, whilst the elafin domain exerts its anti-proteinase effects. The 22 amino acid hydrophobic signal peptide has a role in transport of trappin-2 to the cell membrane.

Elafin proteins are constitutively expressed in low concentrations at epithelial surfaces. Raised elafin expression is well documented in the epidermis in inflammatory conditions such as psoriasis, correlating with degree of neutrophil influx (Nonomura et al. 1994; Alkemade et al. 1994). Elafin has been identified in bronchial secretions, as well as intestinal epithelium in the colon (Williams et al. 2006). Plasma elafin concentration has been postulated as a biomarker to predict survival in acute respiratory distress syndrome (ARDS); elafin concentration is negatively correlated with ARDS survival (Tejera et al. 2009), and low serum and mucosal expression of elafin mRNA are associated with active inflammatory bowel disease, despite a concurrent elevation in serum IL-8 (negatively correlated with disease activity), suggesting that it may play a protective role in these conditions (Zhang, Teng, et al. 2017). Yet, elafin mRNA is more highly expressed in tissue from colorectal cancer (compared with non-cancerous samples) (Liu et al. 2019), potentially an adaptive reaction to the early inflammation associated with colorectal carcinoma.

In the human reproductive tract, elafin is found in uterine tissue (placental syncytiotrophoblast, chorion trophoblast, amnion epithelium and decidua) during normal pregnancy, as well as expression in the vagina and cervix in pregnant (and also non-pregnant women) (King, Kelly, et al. 2007). It is likely that elafin plays a key antimicrobial role in preventing uterine infection, but also an anti-inflammatory role in pregnancy, preventing excessive inflammation and tissue remodelling during pregnancy. Yet its longitudinal temporal profile during pregnancy has not been described.

1.6.1.1 *Elafin as an anti-microbial*

Elafin exerts considerable antimicrobial action *in vitro* and *in vivo*. Its cationic properties allows elafin to disrupt and penetrate cell membranes (disruption of lipid bilayers) and bind to targets such as DNA to inhibit bacterial growth (Williams et al. 2006). In contrast to mammalian cell membranes, which have high cholesterol and phospholipid content and no net overall charge (negatively charged phospholipids tend to face inwards) bacterial cell membranes have negatively charged outward facing phospholipids (Frew & Stock, 2011). These are disrupted by positively charged

antimicrobials; both physically disrupting the membrane, as well as other mechanisms such as hydrolase induction, damage to critical intracellular proteins (Zasloff 2002, Lai & Gallo 2009), and modulation of bacterial virulence factors (Bellemare et al. 2010).

This antimicrobial action has been demonstrated to be independent from its protease inhibitory function (see section 1.6.1.2 below); using a variant of elafin/trappin-2 'A62D/M63L' produced as a recombinant protein lacking protease properties, Baranger et al. (2008) demonstrated significant activity against *Klebsiella pneumonia, Haemophilus influenza, Streptococcus pneumonia, Staphylococcus aureus* and *Pseudomonas aeruginosa* as well as fungal infections such as *Candida albicans and Aspegilllus.* That cell-free supernatants of *P. aeruginosa* have the ability to induce elafin production by human keratinocytes (Williams et al. 2006), further demonstrates the functional importance of elafin as an antimicrobial. Notably, bacterial proteases (such as pseudolysin produced by *P. aeruginosa)* can cleave elafin of its protease-binding loop, inactivating the anti-neutrophil elastase activity of elafin (though its antimicrobial function is retained after this cleavage) (Guyot et al. 2010).

The antimicrobial properties of elafin are not restricted to bacteria. Elafin (and other HDPs) have been shown to exert antiviral properties*,* including against HIV. Ghosh et al. (2010) demonstrated that recombinant elafin dose-dependently inhibited HIV-1 cell lines, after incubation of the virus with elafin. Furthermore, HIV negative women (n=15, mixed ethnicity) had slightly higher concentrations of secreted elafin (measured by ELISA) in cervicovaginal lavage (CVL) samples, compared with HIV positive women (n=32, also mixed ethnicity), though this did not reach statistical significance, possibly because of the small numbers in each group (P=0.09), with a similar picture found when stratified by race. Drannik et al. (2012) did however demonstrate significantly elevated levels of trappin-2 and elafin (ELISA) in CVL samples in HIV resistant groups of commercial sex workers, compared to HIV-susceptible workers. I*n vitro*, when pooled CVL samples were depleted of trappin2 and elafin using specific antibodies (depletion confirmed by Western Blot) and incubated with genital epithelial cells treated with the HIV-1 virus, infection of the ECs was significantly increased, compared to the mock-depleted CVL samples

(P=0.004), with elafin exerting more potent antiviral activity that Trappin-2. The same research group later demonstrated similar *in vitro* and *in vivo* actions of elafin against Herpes simplex virus Type 2 (Drannik et al. 2013).

1.6.1.2 *Elafin as an anti-inflammatory agent*

As well as direct antibacterial action, HDPs such as elafin have anti-inflammatory functions. Uncontrolled inflammation can have deleterious effects on tissue, resulting in tissue damage and destruction. This is in part mediated by proteases produced by activated neutrophils (Dallegri and Ottonello 1997). Elafin exerts inhibitory action particularly against neutrophil elastase and proteinase 3 (Sallenave and Ryle 1991; Wiedow, Luademann, and Utecht 1991) among other enzymes, protecting tissue from enzymatic degradation.

Elafin demonstrates other anti-inflammatory actions that cannot be attributed only to its anti-proteinase function. Elafin acts as an opsonin, aiding macrophage clearance of bacteria. Wilkinson et al. (2009) demonstrated the clearance by alveolar and bone derived macrophages of a strain of *P. aeruginosa* (PA01) incubated with trappin-2, but not the control PA01 (not incubated with the AMP). They were able to visualise the presence of trappin-2 on the bacterial cell surface of the AMP incubated PA01 strain using fluorescent microscopy, and CD14 was identified as the vital macrophage pattern recognition receptor mediating this process (CD14 receptor depleted bone marrow derived macrophages were resistant to the Pseudomonas opsonising effects of Trappin-2 and were unable to clear the bacteria as effectively).

In models of myocardial infarction, atherosclerosis and viral myocarditis, elafin expressed by the endothelium has been shown to supress inflammation; transgenic mice modelled to overproduce elafin demonstrated improved cardiac function after myocardial infarction, with elevated serine elastase activity in the control mice, but not the transformed mice after infarction, together with significantly increased inflammatory cell infiltration in the control population. Cardiac performance at 28 days post infarction was significantly improved in the transgenic mice (Ohta et al. 2004). This could imply a specific anti-chemotactic role for elafin and/or generalised reduction in inflammation via reduced serine elastase (serine elastase contributes to

inflammatory cell migration through the release of growth factors which induce chemokines).

Indeed, HDPs have a key role in upregulating pro-inflammatory cytokines and chemokines (including IL-8, IL-6 and MCP-1) which results in further recruitment of leucocytes, as well as conversely inducing production of anti-inflammatory cytokines such as IL-10 and TGF-ß (Lai & Gallo, 2009). Moreover, elafin itself is upregulated by microbial products (LPS) (Simpson, Cunningham, et al. 2001) and proinflammatory cytokines such as IL-1B and TNF (Sallenave et al. 1994) with a positive feedback loop. It is also possible that elafin may inhibit the recruitment of further inflammatory cells. For example, elafin has been shown to reduce the influx of inflammatory cells in the lung and arterial walls in response to a wide range of inflammatory stimuli (Williams et al. 2006). Furthermore, elafin has been shown to inhibit cleavage of the macrophage CD14 receptor by HNE, facilitating phagocytosis of apoptotic lymphocytes. It thus may act later in the inflammatory cascade to promote resolution of infection and inflammatory damage, as well as playing an active part in tissue remodelling and healing.

1.6.1.3 *Elafin as an immunomodulator*

Following acute production of elafin in response to infection or inflammation, elafin has been shown to modulate the subsequent adaptive immune response. In the lung, elafin favours the development of a TH1 immune response; overexpression studies of elafin in a transgenic mouse model results in increased accumulation of lymphocytes in the lung in response to adenovirus. Levels of IgG antibodies and lung adenoviral IgA (both cellular and humoral responses of adaptive immune system) were increased in elafin treated mice (Williams et al. 2006).

1.6.1.4 *Elafin in the non-pregnant female reproductive tract*

Elafin, and other host defence peptides, are synthesised and secreted by the female reproductive tract epithelium and immune cells. However, compared to other tissues, there is relatively limited information concerning its role. Neutrophils in endometrial tissue, for example, demonstrate cyclical expression of elafin during the

menstrual cycle, with peak expression associated with menstruation (when circulating oestrogen and progesterone concentrations are low) (King, Paltoo, et al. 2007). Exposure to oestrogen of vaginal epithelial cells *in vitro* likewise dramatically reduces elafin secretion (Patel et al. 2013).

Consistent with HDP studies in other epithelial tissues, endometrial elafin mRNA levels can be upregulated by pro-inflammatory cytokines; elafin mRNA levels in primary endometrial epithelial cells were increased over four-fold using a combination of IL-1β and TNF-α (King et al. 2003). Elafin has also been immunolocalized to the non-pregnant fallopian tube (King et al. 2009), cervix (Pfundt et al. 1996) and vagina (Narvekar et al. 2007). Elafin mRNA is endogenously expressed by both endocervical (END1 cells), ectocervical (ECT E6/7 cells) and vaginal cell (VK2) lines *in vitro* and can be induced further by treatment with LPS (Horne, Stock, and King 2008), and inflammatory cytokines IL-1β (Stock et al. 2009) and TNF-α (Sallenave et al. 1994).

1.6.1.5 *Elafin in pregnancy*

In pregnancy, elafin (protein) expression has been found at low levels in first trimester endometrium and decidua (King 2000, Dalgetty, Sallenave et al. 2008), and in the amnion, epithelium, chorion trophoblast and decidua in later pregnancy (immunolocalisation and mRNA expression) (Tromp et al. 2004; Hein et al. 2002; King, Paltoo, et al. 2007; Stock et al. 2007). Similarly to the non-pregnant cell cultures, elafin was expression upregulated in vitro culture (primary trophoblast cells) by the proinflammatory cytokine IL-8 (King, Paltoo, et al. 2007). As well as demonstrating elafin mRNA production by primary amnion cells in vitro (and SLPI and HBD 1-3), Stock et al. (2009) later demonstrated elafin protein in CVF fluid from asymptomatic pregnant women, though did not investigate temporal changes in pregnancy.

1.6.1.6 *Elafin and sPTB*

There is some evidence to suggest that altered expression of elafin (as well as other HDPs) is associated with sPTB. In a small case-control study of 88 patients, measuring elafin mRNA expression from fetal membranes at delivery using real-time PCR, Tromp

et al. (2004) demonstrated decreased trappin2/elafin protein immunohistological expression in cases with PPROM(n= 14), compared with preterm labours with intact membranes. In contrast, however, patients with chorioamnionitis had higher expression of PI3 mRNA, regardless of membrane status at term and preterm. Referring to previous studies that have shown elevation of neutrophil elastase in the amniotic fluid of women with PPROM (Helmig et al. 2002), the authors propose that production of elafin in the membranes (particularly its anti-protease action) may protect the tissue from damage from HNE, and that patients who are not capable of producing adequate amounts of PI3 (in relation to the elevation of neutrophil elastase) may be predisposed to PPROM. Notably, although they did not measure elafin, Helmig et al. (2002) described a concurrent reduction in amniotic fluid concentration of the other whey acidic natural antimicrobial protein SLPI, in those women with PPROM (without chorioamnionitis). It is possible that reduced expression of elafin (or SLPI) may reduce the protection against harmful pathogens, permitting ascending pathogens into the uterine cavity causing PPROM, though clearly there is a theoretical conflict here with data demonstrating elevated levels of elafin (and other SLPI) in overt cases of chorioamnionitis.

Stock et al. (2009) investigated elafin production in the CVF during pregnancy and the relationship with BV status. Elafin concentrations were determined in CVF secretions of 112 pregnant women (below 20 weeks of gestation) and compared to women with normal vaginal flora (n=65), those with intermediate bacterial flora (abnormal flora, but less florid changes than those in bacterial vaginosis (BV) (n=20), and those with BV (n=27). Elafin expression was examined *in vitro* using cervical and vaginal epithelial cell lines. Elafin protein was lower in the CVF secretions of women with BV (43 ng/mg; IQR 32-58 ng/mg) compared to women with normal flora (56 ng/mg; IQR 43-78 ng/mg; $P < 0.05$) or intermediate flora (52 ng/mg; IQR 39-91 ng/mg; $P < 0.05$). These are interesting findings, given that BV, a common vaginal condition characterised by a deficit of vaginal lactobacilli and overgrowth of mixed anaerobic bacteria (see Section 1.4), is associated with late miscarriage, premature rupture of membranes and PTB (Leitich et al. 2003). It is not yet clear whether the presence of BV induces a reduction in elafin concentrations, or whether elafin deficiency predisposes to BV. Concentrations of other host defence peptides were also not assessed in this study.

However, Valore, Wiley, and Ganz (2006) measured HBD 1,2, and 5, SLPI (but not elafin), and human neutrophil peptide (HNP), an alpha defensin, from fluid obtained after vaginal lavage (n=64). In a similar pattern to the above elafin study, women with BV (n=17) had lower concentrations of AMPs as well as reduced antimicrobial activity against pathogenic *E. coli* (measured by change in colony forming units after addition to the vaginal lavage fluid and incubation). Furthermore, after patients were treated for BV, levels of HDPs (alpha defensins, beta-defensins and SLPI) normalised to levels expected in healthy women, which suggests that reduction is a result of disease rather than the other way around. This adds further evidence to the hypothesis that BV may be associated with impairment of the innate immune pathways.

The potential association between HDPs, infection and sPTB has led researchers to pursue the idea that CVF concentration of HDPs such as elafin may be clinically useful as an early predictor of sPTB. Our research group measured CVF protein expression in longitudinally collected CVF samples from women (n=74) at high-risk of SPTB (previous history of late miscarriage or SPTB). Elafin protein expression was determined in longitudinally collected CVF samples from women CVF concentrations of elafin were significantly higher in women who developed a short cervix group (cases) compared to high-risk women without cervical shortening (controls), regardless of gestation and treatment (Figure 1-10, ratio 2.71, CI 1.94 to 3.79, $p<0.0005$) (Abbott et al. 2014). CVF elafin concentrations predicted cervical shortening from 14 weeks of pregnancy (Figure 1-11, n=11, ROC area = 1.00, p=0.008) and remained three-fold higher when cervical shortening was first detected (ratio 3.03 CI 1.92-4.81, $p<0.001$). Elafin concentrations were unaltered by treatment (insertion of a cerclage or vaginal progesterone daily; ratio 1.28, CI: 0.88-1.87, p=0.196).

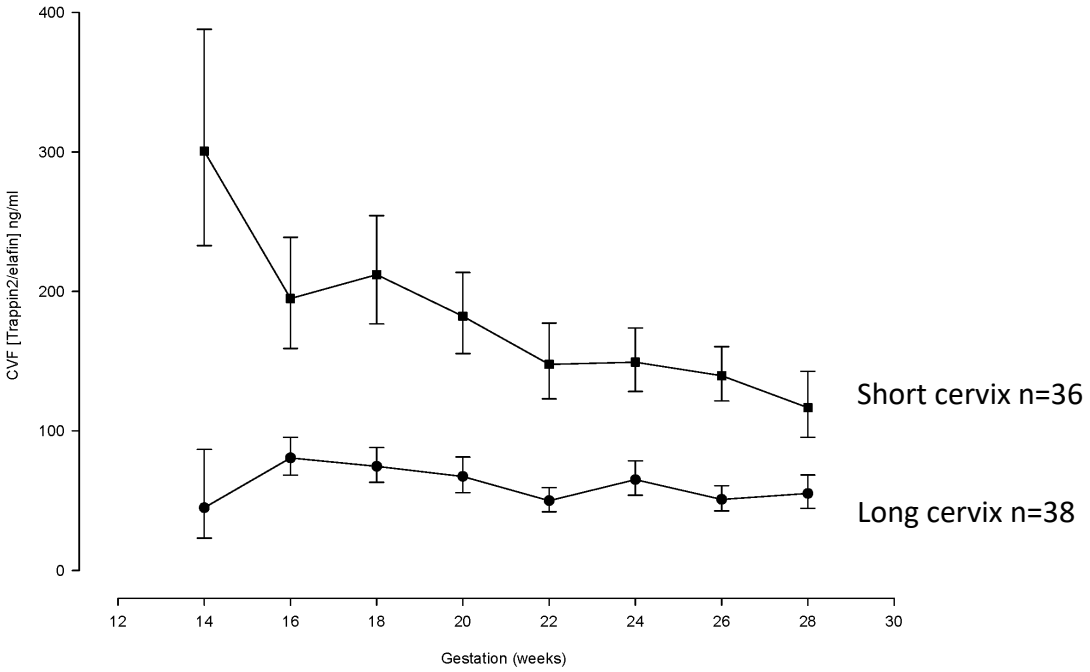

Figure 1-10. Longitudinal cervicovaginal fluid concentrations of elafin (ng/ml) measured in high-risk pregnant women (n=74) who either did or did not develop a short cervix (< 25 mm before 24 weeks of pregnancy). Average gestation that a short cervix was detected was $19^{+1.5}$ weeks (Abbott et al. 2014).

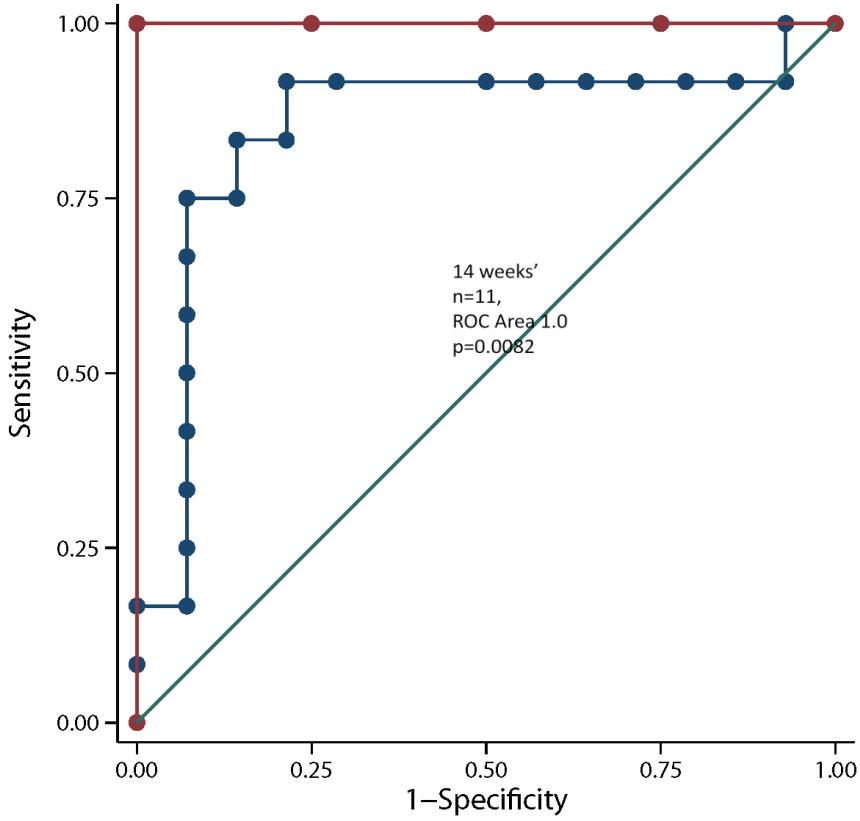

Figure 1-11. Cervicovaginal fluid concentrations of elafin (> 200,000 pg/ml) measured at 14 weeks of pregnancy predict cervical shortening in high-risk women (n=11) (Abbott et al. 2014).

Whilst this small study was not designed nor powered to assess prediction of sPTB (and all women were offered treated once a short cervix had been diagnosed), raised elafin concentrations (<24 weeks of gestation) were associated with SPTB (<37 weeks'; ratio 1.79, CI: 1.05-3.05, p=0.034). On the basis of these results, it was proposed that CVF elafin has potential as a positive predictor of risk of cervical shortening and also SPTB. This finding clearly required validation in a larger cohort of high-risk pregnant women; this PhD project has been designed to do this.

While it is slightly counter-intuitive that high elafin should predict sPTB, given that low elafin concentration are thought to be associated with BV (which in turn is associated with higher risk of sPTB), as well as PPROM, it is likely that different pathological mechanisms behind early delivery may have differential elafin responses. Furthermore, increased elafin production is a recognised response to local infection and inflammation. In women with BV, reduced elafin expression may be an indicator that host-defence responses are suppressed, resulting in abnormalities in bacterial flora. There is thus a clear need to include detailed microbiology in future studies to ascertain the relationship between endogenous elafin production and microbiota profiles, including BV status.

Since this study was published, further data supporting the role of elafin in sPTB has emerged. Stalberg et al. (2017) described elafin expression profiles in fetal membranes from six women with PPROM (mean gestation at delivery 30.1 weeks) and six women who delivered at term. In contrast to the previously described study from Tromp et al. (2014) who observed reduced elafin in PPROM cases, they demonstrated a two-fold increase in elafin mRNA expression in the PPROM membranes, compared to those at term. This is unsurprising given that PPROM is characterised by increased levels of proinflammatory cytokines which are known to induce elafin expression. Furthermore, the comparison group in the Tromp study were women with preterm labour, and not term births. Similarly, a case control study of 68 women of Japanese ethnicity examined elafin and SLPI expression in cervical epithelial cells of pregnant women (women with TPTL who delivered preterm n=19, women with TPTL who delivered at term n=23 and gestationally matched controls n=26) (Itaoka et al. 2015). Using real time PCR they revealed that cervical mRNA

expression of elafin was increased in women in preterm labour who delivered prematurely compared with gestationally matched controls, and women in threatened preterm labour but who did not deliver prematurely.

A less promising result for elafin was achieved by Bastek et al. (2013) who evaluated the predictive role of a number of biomarkers in the CVF for prediction of sPTB in 104 high-risk asymptomatic women. Using CVF collected by vaginal swab, and flash frozen (unlike Abbott when the samples were spun before freezing) they found no relationship between elafin protein concentration (measured by ELISA) and subsequent sPTB. However, the investigators took samples only between 20-24 weeks, and then 24-28 weeks, rather than earlier in pregnancy as our group did. Furthermore, flash-freezing swabs prior to spinning and extracting elafin prior to freezing could theoretically release intracellular pre-trappin2 protein due to lysis of cellular material collected on the swab (Abbott et al. 2014). Given the potential utility of an early pregnancy biomarker to predict sPTB, further evaluation of this promising peptide is essential.

1.6.2 Cathelicidin

Cathelicidins are a family of basic natural antimicrobial peptides/HDPs which share a common pro-region (the cathelin domain). The only known human cathelicidin, human cationic antimicrobial protein [hCAP18] (an 18 kDA precursor) and LL-37 (the C-terminal domain and active form) (Figure 1-12) is found throughout the body, including in cervicovaginal secretions.

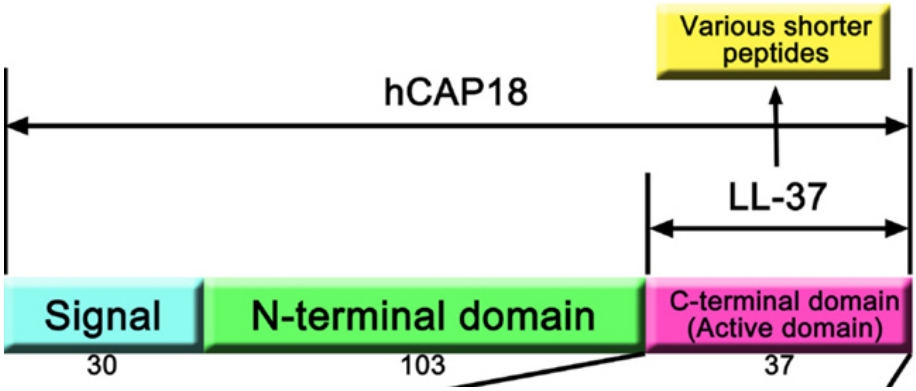

Figure 1-12. Representation of human cathelicidin protein adapted from (Kuroda et al. 2015)
Human cathelicidin protein (HCAP18) consists of signal peptide (30 amino acids), and N-terminal domain (103 amino acids) and C-terminal domain (37 amino acids, the active LL-37).

Cathelicidin is predominantly secreted by neutrophils (and also by other cells of the innate immune system such as natural killer cells, mast cells and macrophages) where it is stored in neutrophilic granules as an inactive precursor (pre-propeptide). It is released extracellularly as an active peptide (LL37) when required, after being cleaved of the N-terminal pro-domain by enzymes such as neutrophil elastase, proteinase 3 and kallikrein, depending on cell or tissue type (Lai and Gallo 2009). Peptide cleavage products of hCAP18 may vary in length and the hCAP18 gene has been shown to yield at least 3 different mature peptides (Murakami et al. 2004); though it is LL37 which is usually released from human neutrophils and shall be referred to as cathelicidin in this discussion hereafter. Epithelial cathelicidin may be further cleaved by microflora bacterial proteases into peptides such as RK-31, KS-30, and K20 (Murakami et al. 2004) which also demonstrate a variety of antimicrobial activities. Cathelicidin is also produced by many other cell types (particularly those exposed to the environment) including skin epithelial cells, epididymis, throughout the gastrointestinal tract (squamous epithelia in the mouth, tongue and oesophagus), and bronchial mucus epithelium, where they are constitutively secreted in low levels, and upregulated in response to infection (Nell et al. 2004) and inflammation (Erdag and Morgan 2002). Our research group demonstrated the expression of cathelicidin (together with elafin) mRNA from freshly collected endocervical cells (Chin-Smith, Hezelgrave, and Tribe 2018).

1.6.2.1 *Cathelicidin as an antimicrobial*

Like, elafin, the highly cationic cathelicidin has direct antimicrobial properties (Overhage et al. 2008) whereby its positive charge interacts with a negatively charged bacterial cell membrane, and hydrophobic residues interfere with the membrane and causes cell death. Antifungal (Murakami et al. 2004; Wong et al. 2011) and antiviral (Barlow et al. 2011; Howell et al. 2006) properties have also been described. Mice lacking the cathelicidin gene demonstrate increased susceptibility to bacterial and viral infection, leading to higher mortality and morbidity (Vandamme et al. 2012). Chromek et al. (2006) demonstrated that cathelicidin (cathelin related antimicrobial peptide) gene deficient mice infected with uropathogenic *E. coli* had higher bacterial bladder attachment, higher urinary infection rate, and higher bacterial survival compared to control mice. The antimicrobial properties of cathelicidin antimicrobial activity may be affected by pH. López-García et al. (2005) demonstrated in vitro that the antifungal properties of human cathelicidin (against *C. albicans*) was pH dependent, whereby the increasing acidity reduced the growth inhibiting capacity of LL37. Given the relatively acidic (and narrow) pH range in the vagina, the effects of small alterations in vaginal mucous pH on cathelicidin function have yet to be explored. However, that CVF cathelicidin concentration does appear to be higher in women with BV (Frew et al. 2014) (see section 1.6.3.4) compared with lactobacillus dominant acidic vaginal environments, may be related to its enhanced antimicrobial function.

1.6.2.2 *Cathelicidin as an immunomodulator*

Inflammatory and immunomodulatory functions of cathelicidin operate largely via signalling of tissue and cell damage, stimulating receptor mediated release of cytokines (e.g., IL8 and Il 6) and chemokines (e.g., CXCL8/IL-8) from cellular components of the innate and adaptive immune system (Elssner et al. 2004; De et al. 2000; Yang, Chertov, and Oppenheim 2001; Vandamme et al. 2012). Similarly to elafin, as well as exerting a proinflammatory role (such as cytokine stimulation and inflammatory cell chemotaxis (Scott et al. 2002)), cathelicidin also has anti-inflammatory actions; it has been shown to inhibit TLR-mediated induction of cytokine release and maturation of dendritic cells (Di Nardo et al. 2007) and inhibit LPS mediated cytokine release from monocytes (Mookherjee et al. 2006). Human

cathelicidin also promotes wound healing by stimulating angiogenesis (recruitment of endothelial progenitor cells and induces their proliferation) (Koczulla et al. 2003) and promoting re-epithelialisation (Carretero et al. 2008).

1.6.2.3 *Cathelicidin and Vitamin D*

As well as responding to microbial and inflammatory stimuli as previously described, production of cathelicidin is stimulated by vitamin D3. *In vitro*, the active form of vitamin D, 1,25 (OH) D3 has been shown to have a directly upregulating effect on the transcription of LL37/hCAP18, as well as indirectly increasing hCAP18 expression by upregulating inflammatory signalling markers such as Toll-like receptors (TLRs) and CD14 (Bucki et al. 2010). CAMP gene expression in endocervical (ED E6/E7) and ectocervical cell lines has been demonstrated to be upregulated by 1, 25 hydroxyvitamin D3 and 25 hydroxyvitamin D3 (Frew et al. 2014), and in END1 cells, a human endocervical epithelial cell line, our research group found that cathelicidin was induced only by the active form of vitamin D (1,25-(OH)2) and calcipotriol and not by IL-1β or lipopolysaccharide (Chin-Smith, Hezelgrave, and Tribe 2018). However, studies present mixed pictures when *in vivo* correlations are investigated. Frew et al. (2014*)* found no correlation between serum 25 (OH) D3 levels (n=122, 118 of whom were characterised as vitamin D insufficient or deficient <75 nmol/ml) and CVF cathelicidin concentration, corroborated by our research group (Abbott et al. 2014). The authors postulate that this finding may be related to the relative vitamin D deficiency in their population which may mask any effect. However, Dixon et al. (2012) showed a positive relationship between plasma cathelicidin concentration and serum 25 hydroxy-vitamin D3 in healthy adults (n=19), only in those who were relatively vitamin D deficient (25(OH)D levels ≤ 32 ng/ml equivalent to ≤ 80 nmol/ml. Similarly Bhan et al. (2011) describe a positive association between serum cathelicidin concentration and serum 25(OH) vitamin D levels (n=60 healthy subjects) only in those with 25(OH)D levels ≤32 ng/ml (r=0.45, P=0.005), whose levels of cathelicidin actually increased after treatment with high dose vitamin D. It would be interesting to know whether vitamin D affected the production and/or antimicrobial/anti-inflammatory function of cathelicidin in response to pathogens. For example, a study of cathelicidin expression in the urinary tract found that while vitamin D supplementation did not affect cathelicidin gene expression in the serum

or bladder biopsy tissue (from 36 women), the ability of bladder biopsy cells *in vitro* to produce cathelicidin (gene and protein expression) in response to *E. coli* was augmented after vitamin D supplementation. It would be of interest to explore whether this response to pathogens could be replicated in the reproductive tract, and whether this varies between women of different baseline vitamin D status.

1.6.2.4 *Cathelicidin in the reproductive tract*

In the reproductive tract, cathelicidin is secreted in CVF of non-pregnant (Levinson et al. 2012; Valore et al. 2002) and pregnant women (Frew et al. 2014). Frew et al (2014) demonstrated expression of hCAP 18/LL-37 protein in over 60% of 130 cervicovaginal secretions collected in the first trimester of pregnancy using ELISA and Western blot. Furthermore, in 116 women with singleton pregnancies at 11-14 weeks of gestation, of mainly White ethnicity (95%), CVF cathelicidin concentration was increased in women with bacterial flora characteristic of BV infection (median concentration 0.35 ng/ml vs. 0.2 ng/ml, $p<0.01$), unlike elafin, which was reduced in women with BV. They hypothesise that this could reflect an increased response to facilitate bacteriocide, or that increased expression of LL37 may predispose to BV. Additionally, the relatively higher pH of the vaginal environment may promote cathelicidin production. That other studies (Valore, Wiley, and Ganz 2006) have demonstrated normalisation of HDPs (alpha and beta defensins and SLPI which are reduced in BV) after BV treatment suggests that it is likely to be the former, however this has not been investigated specifically. It could also be a lack of normal flora in women with BV which stimulates LL37, rather than the presence of specific bacterial species.

Cathelicidin has been identified in amniotic fluid (Yoshio et al. 2003) and immunolocalised to fetal membranes and myometrium in term women biopsied when undergoing caesarean section (Lim, Barker, and Lappas 2015). In this study, increased HCAP18 staining in both myometrium and fetal membranes was observed in women who were biopsied at caesarean section after undergoing spontaneous labour, compared with those who underwent caesarean section prior to labour onset. This is unsurprising given that term labour is considered an inflammatory process, and thus the increased LL37 is likely related to infiltrating leucocytes and associated inflammatory response. The authors were also able to induce gene and

protein expression of inflammatory molecules such as IL-6, IL-8, TNF alpha and MCP 1 in both myometrium and fetal membrane samples upon treatment with recombinant LL37, showing the pro-inflammatory effects of cathelicidin.

1.6.2.5 *Cathelicidin and sPTB*

As with the other HDPs, the precise role of cathelicidin in pregnancy is unknown. However, given that sPTB is likely preceded by infection, and that cathelicidin can potentially induce a responsive inflammatory response, a role for cathelicidin in sPTB is plausible. To demonstrate this, in an established mouse model of labour (LPS induced PTB), Boeckel et al. (2019) showed that injection of LPS in the mouse uterus induced cathelicidin RNA expression from uterine epithelial cells, and cathelicidin protein expression in uterine epithelium, stroma and neutrophils. Furthermore, cathelicidin gene-deficient mice were less susceptible to LPS-induced PTB than wild type mice (40% vs. 82% delivered within 24 hours of intrauterine injection, p=0.015), and had lower levels of circulating IL-6 after LPS injection, demonstrating the role of cathelicidin in mediating the proinflammatory response to LPS in a murine labour model.

In vivo, alongside CVF elafin measurements, our research group (Abbott et al. 2014) demonstrated that in 64 women at high-risk of sPTB (36 developed cervical shortening prior to 24 weeks of gestation, matched to 38 women who did not develop cervical shortening) that cathelicidin CVF concentration rose around 18-19 weeks of gestation in women who subsequently developed cervical shortening. They also demonstrated a strong relationship between CVF cathelicidin and cytokine concentration. Although these women were treated with vaginal progesterone or cervical cerclage, there was no significant effect of treatment on cathelicidin concentration; given the known role of cathelicidin in inflammation and wound healing, the rise is thus potentially attributable to the cervical shortening itself. Although not powered for this outcome, cathelicidin was not found to be predictive of sPTB itself. As with elafin, this requires investigation and validation in a larger cohort, for which this PhD has been designed.

1.6.3 Human neutrophil elastase

HNE is a 218 amino-acid long serine-protease (member of the chymotrypsin family, encoded by the ELA2/ELANE gene on chromosome 19) stored in cytoplasmic granules (Pham 2006), and released predominantly from neutrophils (Weissmann, Smolen, and Korchak 1980), but also mast cells and monocytes in smaller amounts (Weissmann, Smolen, and Korchak 1980; Fitch et al. 2006). It functions to degrade microorganisms once they have been phagocytosed by neutrophils (degrading bacterial cell membranes and cleaving bacterial virulence factors). However, once released into the extracellular space following exposure to inflammatory stimuli, contained within neutrophil extracellular traps (NETs) it can inactivate and clear bacteria (particularly Gram-negative bacteria) degrade extracellular matrix proteins, remodel tissue, and promote inflammation by encouraging neutrophil migration. Furthermore, it has been shown to upregulate inflammatory cytokines (e.g., IL-6, IL-8, GM-CSF) and conversely, degrade cytokines including IL-1, IL-2, TNF-α (Fitch et al. 2006), as well as potentially downgrading the adaptive immune response e.g. cleaving dendritic cells and reducing T cell lymphocyte stimulatory capacity (Roghanian et al. 2006) and cleaving T cell surface proteins (Döring et al. 1995). Thus, although it contributes to intracellular and extracellular defence, it may cause tissue damage and worsen pathogen invasion. HNE is an enzyme target for proteinase inhibitors such as elafin. Elafin (and others such as α-1 proteinase inhibitor, and SLPI) inhibit HNE by forming complexes with the extracellular HNE, inhibiting further HNE mediated proteolysis, and preventing further recruitment of inflammatory cells (Pham 2006).

In the airway, HNE has been shown to be associated with increased mucus production, reduced ciliary activity and direct damage to airway epithelium (Amitani et al. 1991). Unsurprisingly, therefore, high concentrations of HNE are seen in a number of inflammatory conditions, mostly respiratory, including cystic fibrosis, chronic obstructive pulmonary disease, acute respiratory distress syndrome pulmonary fibrosis and exacerbations of asthma (Gramegna et al. 2017); interest is growing in the potential therapeutic use of neutrophil elastase inhibitors (such as AMPs) to treat such conditions. HNE is also thought to be involved in the

pathogenesis of acute pro-myelocytic leukaemia and the autoinflammatory Wegeners graulomatosis (Pham 2006). The role of HNE in pathogenesis of disease is consistently shown; absence of neutrophil elastase protected mice from the inflammatory dermatosis bullous pemphigoid (Pham 2006). Conversely, compared with wild- type mice, mice deficient in neutrophil elastase are more susceptible to infection with several Gram-negative bacteria, with a reduction in inflammatory cell recruitment noted (Pham 2006).

Whilst the role of HNE in the male reproductive tract has been explored (HNE in male semen is a marker of reproductive tract inflammation, has a detrimental effect on semen quality and is a potential marker of fertility (Feng et al. 2011), few studies have examined its role in the female reproductive tract, and relationship with relevant pathologies. As described earlier (elafin section 1.6.1.6), Helmig et al. (2002) retrieved amniotic fluid from 380 patients in preterm labour (n=48), women with PPROM (n=85) and 221 term pregnancies, with and without labour. Using immunoassay to measure amniotic fluid HNE (and SLPI), they noted that HNE was associated with PPROM and sPTB with and without evidence of microbial invasion; women with PPROM, and those with preterm labour but without PPROM who had confirmed microbial invasion of the amniotic cavity (defined by positive microorganism culture) had higher amniotic fluid HNE concentration. In the absence of microbial invasion, both term and preterm labouring women had higher amniotic fluid HNE compared to non-labouring women. Women in preterm labour who delivered prematurely had higher HNE than women in preterm labour but who subsequently delivered at term.

Using similar methodology, a Finnish study of 73 women with singleton pregnancies and intact membranes (27 with preterm contractions and clinically suspected intra amniotic infection and controls undergoing amniocentesis for other reasons), measured biomarkers in amniotic fluid via amniocentesis (22 to 31 weeks of gestation) (Myntti et al. 2017). Although amniotic fluid elafin was higher in cases vs controls, HNE was not significantly higher. However, amongst cases, both HNE and elafin were significantly higher in cases with confirmed microbial invasion of the amniotic cavity (n=7) (positive amniotic fluid culture or bacterial PCR) vs those

without (n=20) albeit in low numbers. Nonetheless, this did equate to a ROC area of 0.98 for prediction of MIAC using amniotic fluid HNE, with high sensitivities and specificities obtained (>95%) with use of a cut-off value for HNE of 257 ng/ml. Whilst arguably not yet clinically useful as a predictor, it demonstrates its response to the presence of infection; should it be raised in a biologically more accessible fluid, clinical utility could be harnessed.

A study of 239 non-pregnant women of reproductive age revealed that women with BV had three times the odds of having increased HNE among proteins in CVF quantified in relative abundance by mass spectrometry, compared to women with normal microbiota (Ferreira et al. 2018). Give that HNE is bactericidal, this observation seems contradictory to its function; however, the authors of this study hypothesise that the presence of HNE in the extracellular space may contribute to the destruction of components of the extracellular matrix and cause tissue damage, leading women more susceptible to overgrowth of BV microorganisms (and potentially other conditions to which women with BV are more susceptible such as sexually transmitted infections, or even sPTB). Yet, although polymorphonuclear lymphocytes and other enzymes such as granulocyte elastase have been shown to be higher in women with PTL (Yamada et al. 1998), no large studies have examined the relationship between HNE and sPTB, nor how it correlates with expression of other HDPs, particularly in pregnant women. Choi et al. (2018) did conduct a small study of 48 women (26 with CL<25 mm and 22 with a normal cervical length at 17 to 24^{+6} weeks of gestation), demonstrating higher CVF HNE (measured by ELISA) in women with a short cervix (concentration of the AMP SLPI did not vary between groups), and higher subsequent incidence of sPTB <34 weeks. While this is a small study, and it is not clear whether elevated HNE preceded cervical shortening (and thus a potential biomarker to predict shortening, allowing earlier intervention e.g., cervical cerclage, section 1.7.1), that HNE may play a role in premature cervical ripening or shortening is suggested by these findings.

Table 1-2 summarises published studies examining the association between reproductive tract HDPs, and sPTB.

Table 1-2 Summary table of published studies examining natural antimicrobial peptides/protein expression and association with sPTB in humans

Paper	Study design	Sample type	Setting	Participants	Host defence Peptide	Main findings
Abbott et al. 2014	Observational cohort study, longitudinal CVF samples taken from 74 women between 10 and 24 weeks of gestation. recruited prospectively. N=25 sPTB.	Cervicovaginal fluid	High-risk antenatal clinic, tertiary hospital, United Kingdom	Pregnant women at high-risk of sPTB (previous sPTB or late miscarriage)	CVF Elafin & Cathelicidin measured by ELISA	Women who developed a short cervix had higher CVF elafin from 14 weeks gestation compared with women who did not. Elafin >200 ng/ml at 14-16 weeks predicted cervical shortening within 8 weeks. Cathelicidin concentration was higher in women who developed a short cervix from 19 weeks of gestation, but was not predictive of cervical shortening at an earlier gestation
Bastek et al. 2013	Prospective cohort study of 104 pregnant women. CVF collected at two timepoints (20 -24 weeks, and 24-28 weeks of gestation). N=26 sPTB	Cervicovaginal fluid	Antenatal setting, United States of America	Pregnant women at high-risk of sPTB (previous sPTB, cervical surgery or uterine anomaly).	CVF Elafin measured by ELISA	There was no association between sPTB and CVF elafin concentration
Elovitz et al. 2019	Nested case control study of 107 women with sPTB and 432 women delivering at	Cervicovaginal fluid	Antenatal setting, United	Pregnant women seen in antenatal clinic setting (cases and	CVF human beta-defensin 2,	Women who had spontaneous sPTB had lower cervicovaginal fluid concentration of human Beta-defensin than those who delivered at term.

	term. CVF collected at 16-20 weeks of gestation.		States of America	controls chosen from a prospective cohort of 2000 women).	measured by ELISA	
Helmig et al. 2002	Observational cohort study; 380 patients in preterm labour (n=48), women with PPROM (n=85) and 221 term pregnancies, with and without labour	Amniotic fluid	Antenatal and labour ward setting, United States of America	Women with threatened preterm labour and uncomplicated term pregnancies	HNE and SLPI in amniotic fluid	Higher HNE in women with PPROM vs preterm labour without infection, and higher HNE in women with TPTL with preterm delivery vs TPTL with term delivery (in the absence of infection). Lower amniotic fluid SLPI in women with preterm and term PROM
Itaoka et al. 2017	Case control study. Cervical epithelial cells swabbed from pregnant women. Controls n= 26 term normal pregnancy; n=23 threatened preterm labour (20 to 35 weeks gestation) who delivered at term, n=19 who delivered preterm after spontaneous preterm labour 20-35 weeks	Cervicovaginal fluid	Antenatal setting, Tertiary hospital, Japan	Pregnant women admitted to hospital with threatened PTB and gestational age matched controls	Elafin and SLPI Cervical mRNA expression measured using real-time PCR	Expression of elafin and SLPI were significantly higher in the PTB group compared with controls, and also compared with women who had suspected preterm labour but who delivered at term.

Manning et al. 2019	Observational cohort study. Single CVF sample taken at 20-24 weeks of gestation from 135 women recruited prospectively. 42 sPTB.	Cervicovaginal fluid	High-risk antenatal clinic, tertiary hospital, United Kingdom	Pregnant women at high-risk of sPTB (previous sPTB or cervical surgery)	CVF Elafin & Human Beta defensin 1 measured by ELISA	CVF concentrations of elafin and HBD1 were not different in women who delivered prematurely vs. those who delivered at term.
Myntti et al. 2017	Observational cohort; 27 women with threatened preterm labour and suspected intraamniotic infection, 46 controls	Amniotic fluid	Antenatal setting, Finland	Women with threatened preterm labour and suspected microbial invasion of the intraamniotic cavity	Amniotic fluid HNE and elafin measured using ELISA	Elafin higher in cases with intraamniotic infection vs. controls, and also in confirmed cases with MIAC vs. cases without MIAC.
Para et al. 2020	Observational cohort study. Amniotic fluid collected from 1) 35 Women at 14-18 weeks who delivered at term. 2) 50 Term pregnancies with or without spontaneous labour.3) Women in preterm labour with intact membranes (subdivided into those who	Amniotic fluid	Antenatal and labour ward setting, United States of America	Pregnant women whose fulfilling case criteria (uncomplicated pregnancies undergoing amniocentesis for genetic reasons, women with threatened preterm	HBD-3 in amniotic fluid measured by ELISA	HBD-3 was higher in women with spontaneous term labour vs no labour, but similar in women with spontaneous preterm labour between those who delivered prematurely and those who delivered at term (in the absence of intrauterine infection). Women with amniotic infection and sPTB had higher HBD-3 than those who delivered at term. Women with PPROM had higher HBD-3 concentration than those who did not have PPROM

	delivered at term (term n=29 vs preterm n=69) 4) 36 women with PPROM.			labour and uncomplicated term pregnancies).		
Varrey et al. 2018	Cross-sectional study of stored biobank samples from pregnant women. Amniotic fluid samples from 1) 35 women who underwent amniocentesis for genetic reasons 14-18 weeks and who delivered at term. 2)50 women undergoing amniocentesis at term. 3) 98 women in spontaneous preterm labour with intact membranes. 4) 36 women who had PPROM	Amniotic fluid	Antenatal and labour ward setting, United States of America	Pregnant women whose fulfilling case criteria (uncomplicated pregnancies undergoing amniocentesis for genetic reasons, women with threatened preterm labour or PPROM	HBD-1 in amniotic fluid measured by ELISA	Among patients with spontaneous preterm labour, amniotic fluid concentrations of HBD-1 were greater in women with intra-amniotic inflammation and/or infection compared to those without intra-amniotic inflammation or infection who delivered preterm and those who delivered at term. No difference between term deliveries and preterm deliveries without evidence of infection or inflammation

PPROM=preterm pre-labour rupture of fetal membranes, ELISA= Enzyme-linked immunosorbent assay, HBD= human beta defensin, SLPI= secretory leucocyte protease inhibitor, sPTB=spontaneous preterm birth, MIAC= microbial invasion of the intraamniotic cavity, CVF=cervicovaginal fluid,

1.7 Summary

sPTB is a process which culminates in cervical shortening, uterine contractions and delivery of a gestationally immature infant. It may, however, be initiated by a number of pathophysiological mechanisms, influenced by various socio-demographic or genetic risk factors. Current prediction and prevention of sPTB is sub-optimal.

The widening body of evidence implicating infection in the pathogenesis of sPTB has signposted us to investigate the role of HDPs in this process; with promising pilot data indicating that elafin and cathelicidin concentration are high in the CVF even in early pregnancy, in women destined to deliver prematurely. They, therefore, may be potential biomarkers to signpost women at high-risk of sPTB, or even as tools to direct preventative therapy.

Elafin in the CVF is a plausible biomarker for cervical shortening and sPTB. If, as scientific evidence would suggest, the process of ascending infection or sterile inflammation could trigger an inflammatory response, elafin as a first line responder within the innate immune system is most likely to be induced to counteract this inflammatory insult. In those women who fail to mount a sufficient response to supress the inflammation, elafin may continue to be secreted via positive feedback, but fail to inhibit the powerful neutrophil proteases (e.g., HNE) which are responsible for tissue damage (as well as cleavage and degradation of elafin), which may contribute to cervical shortening, further intrauterine infection and sPTB. Similarly, given that neutrophil released CVF cathelicidin, is strongly associated with CVF cytokine concentration (Abbott et al. 2014), and that cervical ripening is associated with neutrophil influx and inflammation mediated by chemokines and cytokines (Kelly 2002), it would not be unsurprising to confirm that CVF cathelicidin is raised in women who develop cervical shortening. The responsive role of cathelicidin in responding to tissue damage and promoting wound healing may also be of relevant to this process.

Vaginal dysbiosis (due to BV or possibly other perturbation of the lactobacillus dominated microbiome) can induce local inflammation and may play a part in regulating the vaginal innate immune response. Figure 1-13 represents a simplified representation of the proposed interactions between the vaginal mucosa, host innate immune system and vaginal environment.

This research project and resultant thesis aims to address a number of these unanswered questions; how expression of the innate immune system relates to risk of sPTB, and how this may be affected by the vaginal environments (e.g., dysbiosis caused by BV) as well as exploring the contribution of ethnicity to this risk. It is hoped that the understanding gained here can contribute to further understanding of the mechanism behind sPTB, as well identifying potentially useful clinical tools which may predict an individual's risk of sPTB and inform more targeted preventative intervention early in pregnancy, to those women who may benefit the most.

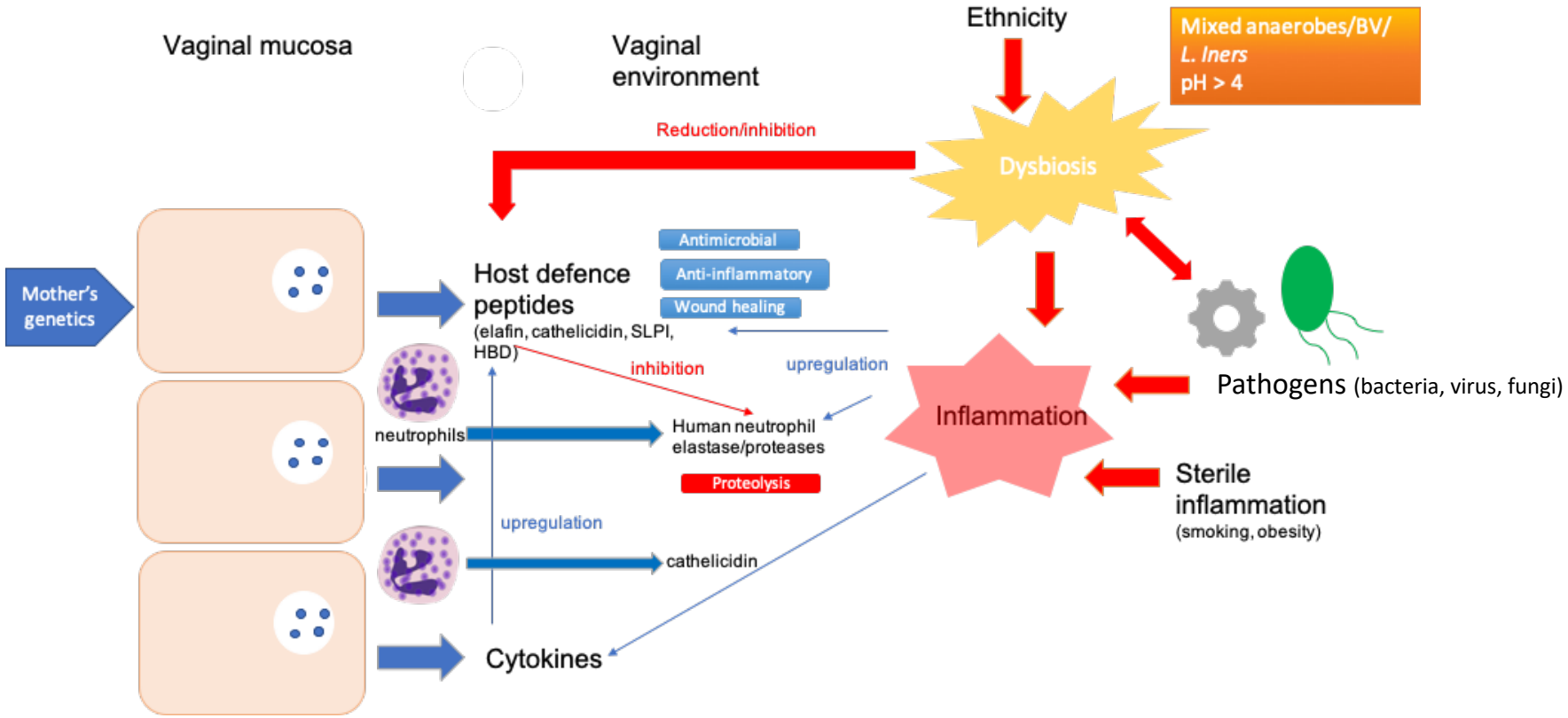

Figure 1-13. Simplified diagrammatic representation of the interactions between the vaginal mucosa, innate immune system, and the vaginal environment

SLPI=secretory leucocyte peptidase inhibitor, HBD=human beta defensin, BV=bacterial vaginosis.

2 Hypothesis, research questions, aims and objectives

2.1 Rationale and hypothesis

Early pregnancy identification of women most at risk of sPTB is needed to ensure women are stratified to appropriate surveillance and antenatal care. Currently, there is no such available test. However, previous research generated by the PTB research group at Kings College London (PIs: Prof R Tribe; Prof A Shennan), identified HDPs, elafin and cathelicidin, as potential early predictors of cervical shortening in women at high-risk of sPTB (Section 1.6.1.6). This PhD, the 'INSIGHT Study', was designed to validate the pilot study findings and address the hypotheses below using CVF and blood samples prospectively collected from a cohort of UK-based women at high-risk of sPTB, as well as from women without risk factors for sPTB.

My working hypothesis was that 'the natural antimicrobial defences of cervicovaginal environment contribute to an individual woman's sensitivity to ascending infection and risk of sPTB, and that these defences are modified by maternal demographics and characteristics, as well as the vaginal environment'.

HDPs, such as elafin and cathelicidin, produced at epithelial surfaces and neutrophils in the reproductive tract, are part of the body's first line immune response to an infective or inflammatory insult. Under healthy circumstances, the constitutive innate immune response of the vaginal mucosa includes low level secretion of HDPs including elafin and cathelicidin which can be amplified in the presence of infection or inflammation. The anti-inflammatory, antimicrobial and wound healing properties of these HDPs defend the cervix and intrauterine environment from infective or inflammatory insult and subsequent risk of sPTB. A vaginal environment rich in lactate producing *L. crispatus* lowers pH, is antimicrobial, and will promote healthy functioning of the innate immune response (i.e., elafin not supressed) (Figure 2.1). In an appropriate response, the inflammation will be supressed easily, the measurable

HDP concentration in the CVF (9.9 KDa Trappin-2 referred to as elafin, and cathelicidin) will be low or moderate, the cervix remains unaffected and risk of sPTB would be low.

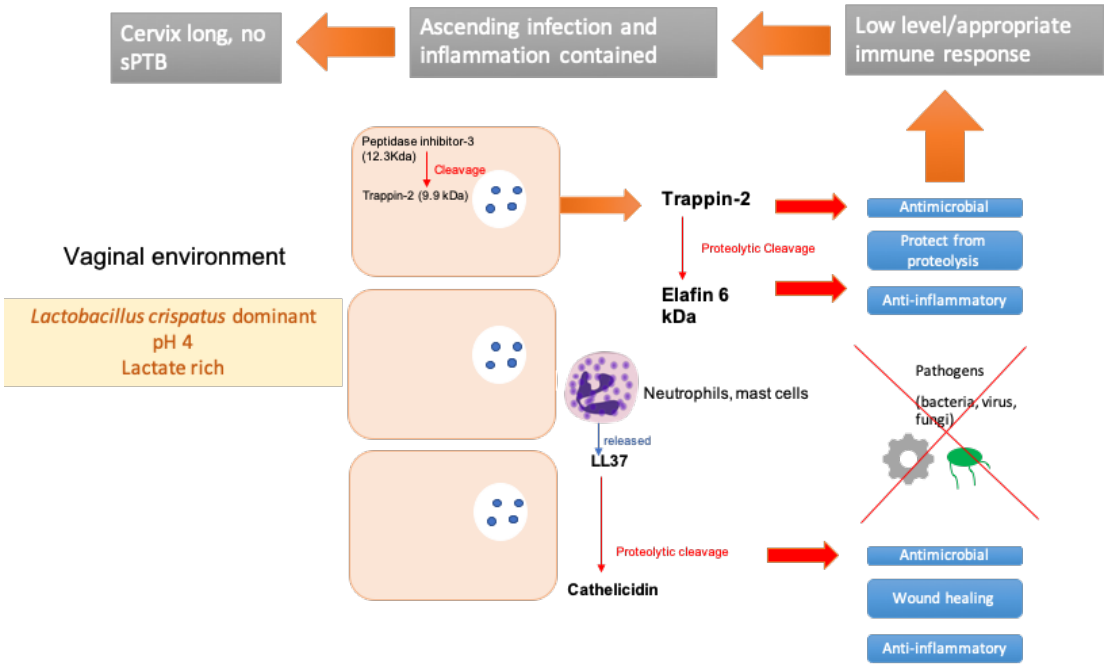

Figure 2-1. Simplified diagrammatic representation of the low-level immune response of the cervicovaginal mucosa and response to infection and inflammation under 'normal' circumstances
sPTB= spontaneous preterm birth.

In contrast, where the infective or inflammatory insult (infective and/or sterile) is overwhelming, or the innate immune system is hyper-responsive, both result in high concentration of HDP secretion into the CVF, tissue damage may occur with amplified inflammation, and infection may ascend to the uterine cavity. High concentrations of HDPs will be stimulated by proteases (encouraging intracellular and extracellular cleavage of elafin to more active forms) and cytokines. This may be more likely in the setting of an altered microbiome, perhaps shifting to an *L. iners* dominated profile. Harmful pathogens may overcome the immune response and ascend to the intrauterine space causing chorioamnionitis. Tissue damage (including to the cervix) will occur via the antimicrobial or inflammatory response. Elafin may also cause

damage through its immunomodulatory actions, via neutrophil recruitment and release of further neutrophil elastase, as can cathelicidin with its role in immune cell infiltration, tissue remodelling and wound healing. Human neutrophil elastase (co-released with cathelicidin from neutrophils) may also contribute to cervical shortening, with its known collagen and elastin proteolytic function.

Therefore, we hypothesise that high cervicovaginal HDP concentrations in early pregnancy will predict cervical shortening and risk of sPTB (Figure 2.2).

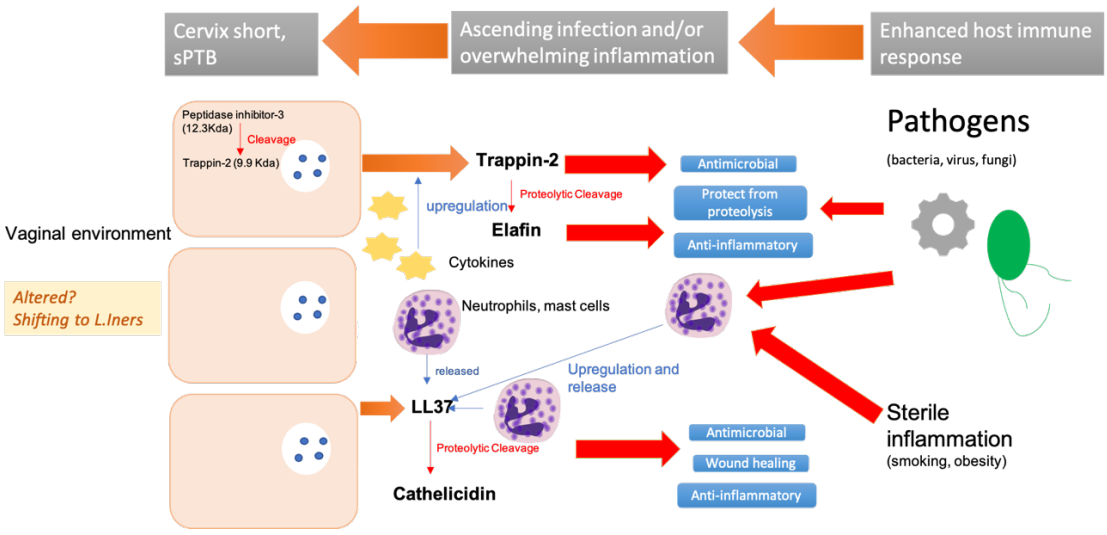

Figure 2-2. Simplified diagrammatic representation of the enhanced immune response of the cervicovaginal mucosa and to infection and inflammation, with subsequent risk of cervical shortening and spontaneous preterm birth.

sPTB=spontaneous preterm birth.

Conversely, given that BV (and general dysbiosis of the vaginal microbiome) appears to be associated with reduced cervicovaginal expression of HDPs, there may be a third scenario which must be taken into consideration. The association between BV and sPTB is well documented, but the mechanisms are unknown. Vaginal dysbiosis can not only induce inflammation but may supress proteins of the innate immune response such as elafin leading to inability to supress infection and tissue damage caused by the associated inflammation.

We hypothesise that, in pregnant women, there will be an inverse relationship between CVF elafin and a dysbiotic vaginal environment, which may relate to risk of sPTB (Figure 2-3).

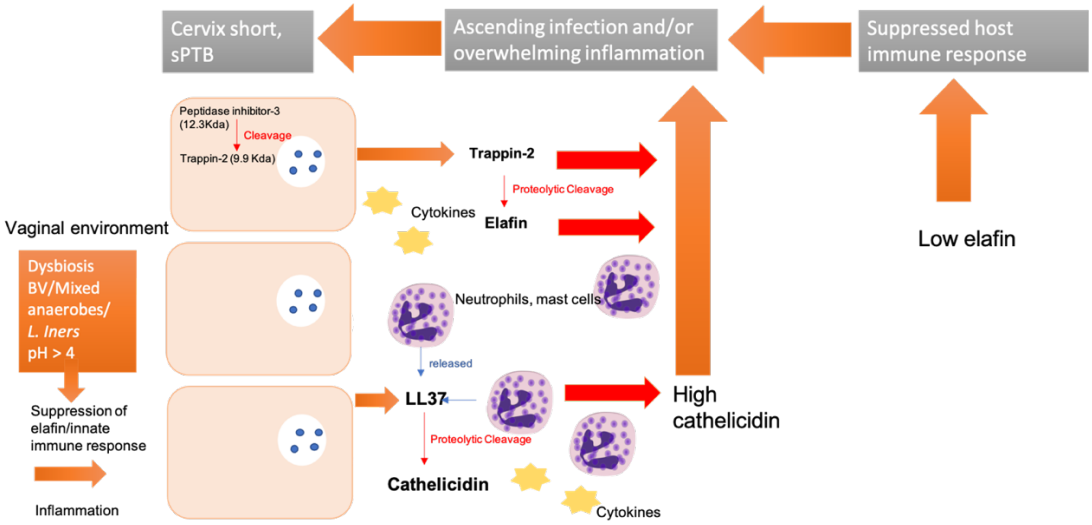

Figure 2-3. Simplified diagrammatic representation of the supressed immune response of the cervicovaginal mucosa to infection and inflammation in the presence of a dysbiotic microbiome, with subsequent risk of cervical shortening and spontaneous preterm birth.

sPTB= spontaneous preterm birth

2.2 Research questions

i) **Profile of HDP expression in pregnancy**: What is the longitudinal pattern of HDP expression in high and low risk pregnancies, and is this modified by maternal characteristics/demography or the vaginal environment? This will be investigated in **Chapter 5 and 7.**

ii) **HDPs as predictive biomarkers**: What is the relationship between CVF HDPs and pregnancy outcome? Can CVF elafin, cathelicidin and human neutrophil elastase be used *in vivo* as potential early pregnancy predictors of sPTB, and can they be combined with existing biochemical screening tools (qfFN). This will be investigated in **Chapter 6 and 8.**

2.3 Study aims and objectives

The **primary** objective of the Insight study was to validate the use of CVF HDP expression as early pregnancy predictors of sPTB, alone, or in combination alone or in combination with each other and qfFN.

The **secondary** objectives of the Insight study were to explore the expression of HDPs in CVF longitudinally from early pregnancy to mid-trimester, and how they are influenced by host demographics and the vaginal environment.

These objectives were addressed by:

1. Prospectively collecting detailed clinical data and a biobank of biological samples of CVF, whole blood and cervical epithelial cells from women at high-risk of sPTB (and compare these to low-risk controls) from 10 weeks to 24 weeks of gestation, at 2-4 weekly intervals.
2. Determining CVF elafin, cathelicidin and human neutrophil elastase concentrations using ELISA in order to interrogate the:
    a. Longitudinal profile of CVF HDP protein expression from 10 weeks of gestation to 24 weeks of gestation, in high and low-risk women.
    b. The relationship with host demographics, cervical shortening and sPTB.
    c. The relationship with vaginal environment (BV).
    d. The value of CVF HDP expression as early pregnancy predictors of sPTB and develop one or more clinical algorithms based on multiple marker (e.g., CVF HDPs, qfFN, demographics).

3 Methods

3.1 The Insight study

3.1.1 Cohort studies in this field

A huge number of individual small cohort studies have been contributing and supporting the scientific hypothesis that early preterm labour pathophysiology is largely driven by inflammation or infection. The relative rarity of the event sPTB, particularly amongst a general antenatal population, has meant that these cohorts are usually small, or at least have a small number of women with the outcome of sPTB. Studies examining HDPs, the vaginal microbiome and pregnancy outcome frequently have small numbers of women/sPTB events and few include longitudinal data. For example; neutrophil elastase and cervical shortening total cohort n=48 with 26 short cervices (Choi et al. 2018); human B-defensin-1 and sPTB total cohort n= 219 (though they did include 105 sPTB/PPROM) (Varrey et al. 2018); vaginal microbiota, cervical length and sPTB n= 161 with 34 sPTB (Kindinger et al. 2017). With such small groups of women, not only are results more vulnerable to type 1 and type 2 errors, subtleties regarding variation in subgroups (e.g., ethnicity) as well as mechanisms behind different sPTB phenotypes cannot be fully understood. More recently larger sPTB cohorts, particularly those evaluating the vaginal microbiome, are being published, e.g., 450 samples from women including 94 women with sPTB (Tabatabaei et al. 2019), as well as a nested case control study of 107 sPTBs, and 432 term births investigating the microbiome and relationship to the antimicrobial peptide human beta defensin (Elovitz et al. 2019). We identified the need to create a large and reliable biobank of biological samples from pregnancy women from early gestation in order to validate the findings of these smaller studies, and to generate new hypotheses and discoveries about the mechanism behind sPTB. This particular study, which recruited 619 women and collected >1500 cervicovaginal fluid samples over three years (with ongoing collection), is the largest cohort evaluating HDP expression that we are aware of currently.

3.1.2 Overview of the Insight study

The Insight study is a large multicentre observational cohort study of pregnant women designed to investigate the complex biochemical events and underlying genetic influences that predispose women to go into preterm labour, particularly the changes that occur in biological cervicovaginal (CVF) and plasma markers throughout pregnancy which are associated with cervical shortening and sPTB.

The study was funded by an NIHR Doctoral Research Fellowship (N Hezelgrave DRF-2013-06-171), Tommy's Charity (no. 1060508); National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' National Health Service Foundation Trust and Rosetree's Trust (Charity no. 298582); Chief Investigator Rachel Tribe; Co-investigators Andrew Shennan, Natasha Hezelgrave. The overarching aim of the study is to validate the role of HDP expression in the pathogenesis of sPTB and generate a deeper understanding of the mechanisms behind sPTB.

The Insight study has generated a large biobank of longitudinally sampled biological fluid [CVF] and plasma as well as cytobrushings, from over 2000 pregnant women at high-risk of sPTB, and low risk controls, together with a linked database of prospectively and rigorously collected clinical data.

3.1.3 My contributions to the Insight study

I was the Insight study coordinator. With support from my supervisors, I was responsible for developing the idea, preparing and submitting the funding application, writing the protocol, developing the laboratory standard operating procedures, designing the consent forms and patient information sheets (Appendix 1 and 2) obtaining regulatory approval, database design, development and modification (incorporating Insight into an existing PTB studies database) recruiting and supporting new sites, and trial management. With support from a team of

dedicated research midwives and research assistants, I contributed to patient recruitment, sample processing, outcome collection and data monitoring. I performed the ELISA measurements for elafin and cathelicidin. Human neutrophil elastase measurement by ELISA was performed by research colleagues Evonne Chin-Smith and Alexandra Ridout. BV slide assessment was performed by me and the nursing team at Burrell St. sexual health clinic. Mr Paul Seed, study statistician, provided training, support and supervision for the study statistical analysis.

3.2 Insight study: population

A cohort of women with singleton pregnancies considered at high-risk of sPTB (Section 3.2.3) between 10^{+0} and 24^{+0} weeks of gestation were prospectively recruited from four tertiary UK high-risk antenatal clinics. Low-risk pregnant women were recruited from routine antenatal or ultrasonography clinics. Biological samples (CVF and blood) were taken with consent at each visit and each high-risk participant underwent a routine TVUS scan if clinically indicated. Recruitment at the main site (St Thomas' Hospital, Guy's and St Thomas' NHS Foundation Trust) commenced on 11/9/2013 once regulatory approvals were in place.

3.2.1 Participating centres

The involvement of more than one study site was desirable both to achieve the pre-defined sample size, but also to increase the generalisability of the study. Sites were selected if they had a PTB clinic/high-risk pregnancy in which women at risk of sPTB could be seen early in pregnancy (ideally before 13^{+6} weeks of gestation), and who had CLRN funded research midwives capable of identifying potential study recruits, coordinating data entry and sample collection, arranging appropriate research follow up, and collecting outcome data. Of pragmatic importance was relatively easy accessibility of the site, for site monitoring and trouble-shooting visits. Sites expressing an interest (via the CLRN portfolio or word-of-mouth) were initially

assessed for suitability as above. Once chosen to participate, their study start date was phased in at different timepoints once local governance approvals were obtained.

Sites involved in the Insight study were:

- Guys and St Thomas' NHS Foundation Trust, London (coordinating centre; start September 2013)
- Manchester Royal Infirmary (October 2014)
- Poole Hospital NHS Foundation Trust (August 2014)
- West Middlesex University Trust, London (April 2015)

3.2.2 Identification of participants

3.2.2.1 *High-risk women*

Women at high-risk of sPTB (see section 3.2.3) with a singleton pregnancy between 10^{+0} and 24^{+0} weeks of gestation attending specialist prematurity surveillance appointments (or equivalent high-risk antenatal care appointments) were invited to participate in the study (aiming to recruit the woman as early as possible within this gestational window). For centres which did not routinely invite high-risk women to specialised clinics, case notes of pregnant women booked for delivery at the hospital were reviewed by study midwives and potentially eligible women were informed about the study at routine antenatal visits and invited to participate.

3.2.2.2 *Low-risk women*

Any pregnant woman who did not have risk factors for sPTB (see section 3.2.3) attending the maternity unit (antenatal clinic, ultrasound scan department, early pregnancy unit) between 10^{+0} weeks and 15^{+6} weeks of gestation were invited to participate in the study. This was done by communication with direct heath care providers and fetal medicine team members. It is currently routine practice that all women booking for pregnancy care at the study hospital are offered screening for

chromosomal abnormalities in the first trimester; this involves a scan and a blood test. In practice, the majority of the low-risk women were recruited at their first trimester 'dating' ultrasound (11^{+0} to 13^{+0} weeks of gestation) and consented at the time or given a number to call if they preferred some time to consider participation.

3.2.3 Eligibility criteria

3.2.3.1 *High-risk women*

Women between 10^{+0} weeks' gestation with a singleton pregnancy (dated by ultrasound or LMP and adjusted for ultrasound estimated date of delivery once ultrasound performed if no miscarriage prior to dating ultrasound) until 24^{+0} weeks of gestation with one or more of the following risk factors for sPTB:

- History of previous sPTB or second trimester loss (≥ 16 weeks or ≤ 37 weeks of gestation)
- Previous PPROM (≤ 37 weeks of gestation)
- Short CL (≤ 25mm) on TVUS at 18-0 to 24^{+0} weeks of gestation
- Any cervical procedure to treat abnormal smears (e.g., large loop excision, laser conisation, cold knife conisation or radical diathermy
- Any uterine anomaly considered to increase risk of sPTB (e.g., unicornuate or bicornuate uterus)

3.2.3.2 *Low-risk women*

Women between 10^{+0} and 15^{+6} weeks of gestation at recruitment (dated by ultrasound or LMP if no ultrasound yet performed) with no risk factor for PTB, and no current known complication of pregnancy related to risk of sPTB.

3.2.3.3 *Exclusion criteria (both groups)*

- Women with vaginal bleeding evident on speculum examination.
- Suspected or proven rupture of the fetal membranes at the time of recruitment.

Women with vaginal bleeding and/or ruptured membranes were excluded due to the potential interference with CVF biomarker measurement by blood or amniotic fluid.

3.2.4 Consenting participants

All potential participants were provided with a written patient information leaflet with verbal translation available for non-English speaking women (via Language Line where available). Women agreeing to take part in the study were consented by an appropriately trained midwife, sonographer, doctor or a member of the research team. All women were provided an opportunity to discuss the research with one of the senior members of the research team if requested. Written consent was obtained from all participants according to principles of Good Clinical Practice (GCP). If a participant elected to withdraw from the study, no further samples were collected. Identifiable data or tissue already collected with consent would be retained and used in the study if they women consents to this. Consent was also sought to collect and use the participant's delivery details (if delivery had not yet occurred).

At the time of recruitment, a unique study number was generated and allocated to the participant. One copy of patient identifiable data linked to this number was recorded on a password-protected computer in order that recruits could be contacted, and delivery outcomes recorded. All electronic records were anonymised at time of data entry in accordance with the Data Protection Act 1998.

3.2.5 Sample size

3.2.5.1 *Sample size calculation*

Based on the pilot data (elafin as a predictive biomarker for sPTB), we calculated that 300 (high-risk) women would need to be recruited to the study to provide 40-50 cases of sPTB <37 weeks of gestation. Assuming a threshold was set in 250 controls to give 90% specificity, and elafin would be measured in 45 cases, this would give our study

83% power to distinguish a relatively weak test (sensitivity 60%) from a relatively strong test (sensitivity 80%).

3.2.5.2 *Assumptions behind recruitment target*

We anticipated that 400 women will be screened each year through the STH preterm surveillance clinic, of whom (based on previous experience) 80% would be willing to take part in an observational study involving cervicovaginal and blood samples. We estimated that it would be logistically possible to recruit five new high-risk women every week. This represents 260 women/year. Approximately 14% of high-risk women in our clinic (based on the previous study of fetal fibronectin and sPTB (EQUIPP) deliver spontaneously prior to 37 weeks. Therefore, over 24 months, we anticipated we could recruit 520 women, to obtain 72 sPTBs.

3.2.5.3 *Initial recruitment figures*

Over the first 12 weeks at the primary site, it was clear that the recruitment target of five new women had been ambitious. On average, 2.6 new high-risk Insight women had been recruited per week. This was attributed to the following reasons: Fewer than 80% of women agreed to take part in the study. The figure of 80% had been based on the previous EQUIPP study; fetal fibronectin and sPTB. Women in this study were agreeing to have a vaginal swab, though it was one that they would have had clinically regardless of the study. Women approached for the Insight study were asked to provide a speculum and CVF samples at times when ones would not normally be clinically indicated. Approximately 60% of women approached agreed to be part of the study. Many women did not attend the high-risk antenatal clinic at gestations early enough (<16 weeks) to be included in the Insight study. Logistically, a clinic appointment for a woman included in the Insight study took approximately 20 minutes longer than a standard clinic appointment, and required midwives and doctors trained in sample acquisition. The sample then had to be transported on ice immediately to the laboratory and processed within one hour of sample acquisition. Therefore, there was a limit to how many women could be recruited at one time,

both in terms of clinic appointments (within a busy NHS setting) and laboratory protocols.

The following steps were taken to address these problems:

- All clinical staff within the clinic were trained in Insight consent and sample acquisition to maximise recruitment opportunity.
- Research assistants trained in Insight laboratory sample processing.
- Efforts made to encourage clinical staff to refer women to prematurity clinic at early gestation/at 12 week dating scan.
- Additional sites identified for Insight recruitment

Figure 3.1 shows the actual recruitment numbers per month (absolute and cumulative) for the study.

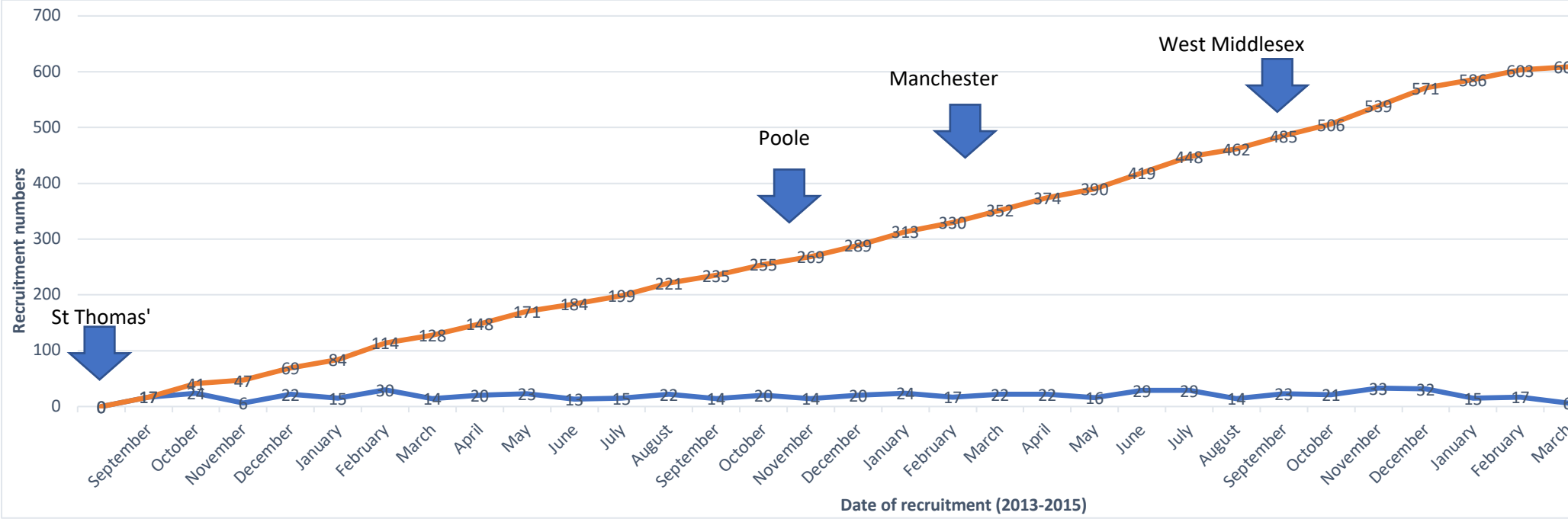

Figure 3-1. Actual (blue) and cumulative (orange) recruitment to the Insight study across 4 study sites. Blue arrows represent the timing at which each new study centre started recruitment

3.3 Visit schedule

3.3.1 High-risk women

The following visits for study purposes were planned, aiming to combine these with their need for clinical care.

Category 1: 10-13^{+6} weeks of gestation
Category 2: 14-15^{+6} weeks of gestation
Category 3: 16-19^{+6} weeks of gestation
Category 4: 20-24^{+0} weeks of gestation

Consent for biological sampling was confirmed verbally at each visit. If consent was obtained, the following procedures were undertaken at each visit:

- One speculum examination and up to 4 cervicovaginal swabs.
- A cervical length assessment assessed by transvaginal ultrasound from 14 weeks of gestation at every visit up until 24 weeks of gestation.
- Two blood samples (2 x 15 ml) at the first visit and between 18-24^{+0} weeks of gestation.

Women were first recruited at any gestation between 10^{+0} and 24^{+0} weeks and followed the visit schedule thereafter according to gestational category (we aimed to recruit the majority of women at category <16 weeks of gestation). Some women only wanted to provide samples at a single visit or only provide one or more of the samples listed. Every routine appointment in the prematurity clinic in most centres involved a transvaginal scan to assess cervical length, and the majority of women had a speculum and a swab taken for clinical purposes (e.g., to exclude infection, to analyse fetal fibronectin), and this was combined with taking swabs taken for study purposes.

If further visits to the clinic were made outside of these gestational time points, relevant clinical information was recorded on the study database, but no further biological samples were taken for study purposes.

3.3.2 Low-risk women

Low-risk women were seen for up to 2 visits for research purposes, planned to coincide with routine antenatal care appointments (usually the early nuchal scan and mid-trimester anomaly scan).

Category 1: 10^{+0} to 15^{+6} weeks of gestation
Category 2: 18^{+0} to 24^{+0} weeks of gestation

Consent for biological sampling was re-confirmed verbally at each visit. If consent was obtained, the following procedures were undertaken at each visit:

- A speculum examination and up to 4 cervico-vaginal swabs
- Two blood samples (2 x 15 ml)

Women who were recruited after 18 weeks of gestation were seen on one occasion only. Some women preferred to provide only one or more of the samples listed.

3.4 Clinical procedures: details

3.4.1 Vaginal swabs

Specimens were taken prior to TVUS or digital vaginal examination to avoid contamination. At each visit two Dacron vaginal swabs were taken from the high vagina/posterior fornix during speculum examination, rotated for ten seconds to achieve saturation, and then transferred into a pre-prepared vial of 750 µl of standard phosphate buffer (PBS) solution containing 1 protease inhibitor cocktail tablet (Complete, Roche Diagnostics GmbH, Germany) dissolved in 50 ml standard phosphate-buffered saline solution (Sigma-Aldrich Company, Ayrshire, UK). Two

microbiology swabs were taken during the same speculum examination from the high vagina/posterior fornix, rotated for ten seconds and then removed and one placed into liquid Amies medium and the other into 1ml of pre-prepared Tris-EDTA (TE) buffer. These swabs were kept on ice, before and during immediate transfer to the laboratory.

Prior to placing the second microbiology swab into the vial of Amies medium, it was pressed lightly onto a pH indicator paper (Machery Nagel pH-Fix range 3.6-6.1), pH was recorded, and then smeared onto a sterile glass slide for gram-staining and microscopy.

If clinically indicated and available at the study site, qfFN sampling was undertaken at the time of sterile examination and HDP sampling, using a polyester swab in the posterior fornix of the vagina, rotated for 10 seconds until saturation. This was placed into a transport tube containing 700 µl of extraction buffer, before it was analysed immediately using the quantitative bedside instrument (Rapid 10Q analyser) (Abbott et al. 2013). Routine qfFn testing was only performed in high-risk asymptomatic women at the St Thomas' Hospital centre (from which the majority of the Insight cohort women were recruited) and Poole Maternity Hospital.

3.4.2 Blood samples

One EDTA bottle and one SST gel bottle were filled using standard venepuncture technique and transported to the laboratory immediately.

3.4.3 CL measurement

CL measurement by TVUS was performed by trained operators in accordance with standardised guidelines (Berghella et al. 2003) as per clinical protocols (at least once between 14 and 24 weeks, usually at every clinical visit), with the woman in supine position and with an empty bladder. A sagittal view of the cervix was obtained with the long axis view of the endocervical mucosa. Transfundal pressure was exerted to

assess funnelling, and the total closed length (linear distance) in all women was measured three times (mm) over a minimum of three minutes using optimal magnification and zoom settings, with the shortest measurement of the three recorded. For analysis purposes, the cervix was classified as 'short' if it measured less than 25 mm prior to 24^{+0} weeks of gestation.

3.4.4 Design of database and data collection

Clinical data and linked sample tracking and storage information was entered onto a dedicated secure online PTB study database (www.medscinet.net/ptbstudies). This database was designed to accommodate multiple PTB studies, and pages specific to the Insight study were specifically designed and created. Figure 3-2 shows the Home page for the database, incorporating the Insight Study.

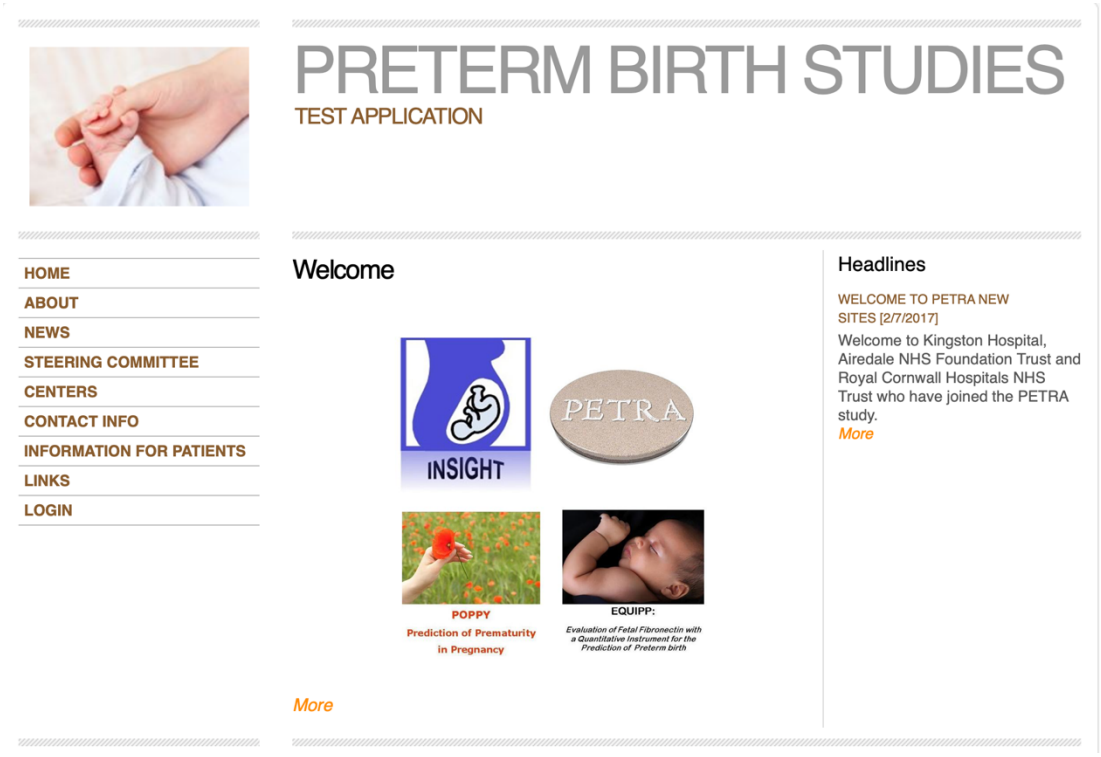

Figure 3-2. Home page of PTB Studies Database.

Authorised users at each site had variable degrees of access to the database; individual sites were only authorised to see data from women at their own site. Users

allocated global access roles were able to see data inputted at every site, enabling data to be monitored, modified if appropriate and extracted. An audit trail monitored all database activity.

Minimal demographic data, along with medical and obstetric history were entered directly onto the database at the time of recruitment. At each visit, visit specific data was recorded (including symptoms, current medications and other potentially interacting factors such as previous sexual intercourse) as well as a record of cervical length (if taken) and biological samples collected. All samples were barcoded, enabling linkage to the study ID number.

Once the participant had delivered, clinical endpoints were measured by retrospective analysis of patient records (via clinical electronic notes or hand-held patient notes) and entered into the database. If the information was not available, the patient was contacted by phone (in case of delivery elsewhere). If this was not successful, every effort was made to gather outcome data via contacting other hospitals or the patient's general practitioner. Regular data monitoring was performed to ensure the integrity of the data entry.

Outcome data was obtained from case note review by trained study team members. Missing data on the database was highlighted in the patient overview pages; those with missing data appeared yellow, and completed pages, green. Every page of data was monitored by an independent trained data monitor (including myself), and queries raised to the original inputter if discrepancies were found. Once a page had been monitored and deemed satisfactory, it was locked by the data monitor.

3.4.5 Clinical endpoints

Primary outcomes were development of a short cervix, and sPTB prior to 34 and 37 completed weeks of gestation. Secondary outcomes included PPROM, composite evidence of infection at delivery (any of maternal pyrexia during labour >38°; raised

CRP >10 or WBC >20 during labour or within 24 hours after delivery; clinical diagnosis of chorioamnionitis; positive maternal blood culture, MSU or HVS during delivery admission), and composite poor neonatal outcome (any of Apgar <5 at 1 or 5 min at delivery; admission to SCBU/NICU; intraventricular haemorrhage; USS brain abnormality; respiratory distress syndrome; NEC, NICU/oxygen at 28 days, hypoxic ischaemic encephalopathy; neonatal death; positive culture of infection in first 48 hours). Women were considered to have had sPTB if they had spontaneous onset of labour or experienced PPROM and delivered prior to 37 weeks of gestation (including late miscarriages), regardless of onset of labour or mode of delivery.

Collection of the INSIGHT cohort is ongoing to develop a biobank. Data analysis for this study was performed on women when final visit data and samples were obtained for all participants whose estimated due dates were on or before 05/03/2017. All high-risk women within this time frame were included if they had donated at least one sample. Women from the low-risk control group were included for analysis if they had provided two longitudinal samples. Women with iatrogenic delivery (including those delivered due to maternal medical conditions and intrauterine fetal demise) before the specific gestational outcome of interest were excluded from the analysis.

3.4.6 Index of multiple deprivation data

Where consent was obtained to do so, each participant provided their postcode which was immediately transformed by the database to become a Lower-layer Super Output Area (LSOA). This is an overall weighted composite measure of multiple deprivation indices (income, employment, education, skills and training, health and disability, crime, barriers to housing and services and living environment), experienced by people living in particular delineated areas of England, which are all ranked relative to that of other areas. Deprivation decile data are publicly available, derived by ranking the 32844 LSOAs in 10% sections of most deprived regions: most deprived (decile 1) to least deprived (decile 10). For our analysis, these were

converted into quintiles. Deprivation index was not available for 52/619 women, due to an invalid or not supplied postcode.

3.4.7 Research governance

3.4.7.1 *Ethical/Research and Development Approval*

Biological samples for analysis were obtained from a sub-set of women recruited to the ongoing prospective observational study; INSIGHT: Biomarkers to predict premature birth. Approval from London City and East Research Ethics Committee (13/LO/1393) and Kings Health Partners Research and Development (RJ113/N173) was granted. Informed written consent was obtained from all participants (Appendix 1 and 2). Annual progress reports were submitted to the research ethics committee (REC). Table 3-1 lists major study ethical amendments to protocol since inception up until data download for thesis (data pertaining to amendments 1, 2 and 4 pertaining to a student project, data which is not included in this thesis).

Table 3-1. Substantial amendments to the Insight Study

Date of substantial amendment approval	Summary of amendment
1. 10/12/2013 Approved 10/2/2014	Validation of the Careplan pH glove in a subset of women
2. 14/04/2014 Approved: 29/4/2014	Amendment to obtain Careplan pH glove measurements from non-pregnant women attending sexual health clinics
3. 29/09/2014 Approved: 15/2/2014	The inclusion of a participation information leaflet in the letter sent to patients for their first appointment at prematurity clinic.
4. 14/03/2016 Approved: 24/3/2016	The addition of a low vaginal swab to be taken from low and high-risk women to assess low vaginal pH, as well as enabling comparison of the vaginal microbiome low in the vagina and high in the vagina

3.4.7.2 *Study management*

Monthly Insight meetings were held with the research team; me as study coordinator, Rachel Tribe (PI), and the Insight research midwifery team, to ensure satisfactory study progress, discuss recruitment and data management and trouble shoot problems arising. Three monthly teleconferences with representatives (lead research midwife and/or principal investigator) from each external study site were conducted, and face to face meetings planned if required (e.g., to provide lab training or recruitment support), following the initial site initiation meeting.

3.4.7.3 *Public involvement in the research*

Around the time of the Insight study conception, the Kings College Department of Women and Children's Health PTB patient public involvement (PPI) panel was created to inform and contribute to this study, as well as other PTB studies within the department. Advice was sought from the group about the study protocol and recruitment strategy, as well as the design and wording of the patient information leaflets and consent forms. The group (consisting of women and their partners who had prior experience of sPTB) meet twice a year, as well as providing advice by email when required. At each meeting the group was kept up to date with the study progress, as well as providing advice where needed.

3.5 Minimising bias in the study design

The need to collect longitudinal cervicovaginal samples from women starting in early pregnancy, before the outcome (term or sPTB) was known meant a prospective study design was essential. It enabled us to collect all the desired information regarding risk factors and potential confounding factors before the outcome of pregnancy was known. The decision to analyse all samples as a cohort (of both high-risk women recruited via high-risk antenatal clinics, and low risk women recruited at routine midwifery or sonography appointments) rather than a nested case-control study

(analysis of samples only from those who had a sPTB, and matched term births), although increasing the cost and effort of study, ensured that selection bias was minimised, regarding the potential non-comparability between selected cases and controls.

Each hospital site operated a weekly specialist prematurity or high-risk pregnancy clinic, to which all women booking their pregnancy within that trust were referred if they had risk factors for sPTB. All women potentially eligible for the Insight study seen in these clinics were approached to participate in the study, and therefore we can feel confident that we did not introduce recruitment bias into the study by only approaching a sub-set of women at high-risk of sPTB by virtue of one characteristic or another.

In general, women who have had a previous premature birth are usually seen in clinic between about 14 and 18 weeks of gestation, and women who have other risk factors for sPTB, may not be seen until after 18 weeks. During study set-up, we encouraged all high-risk clinics to invite all women to clinic as early as possible in their pregnancy (ideally before 16 weeks of gestation) if this was feasible within the constraints of their clinic system, as we aimed to recruit women as early as possible in pregnancy. It may be the case that women who were considered of the highest risk of sPTB were seen earlier in clinic than women who had other risk factors for sPTB and thus there may be over-represented in the cohort of high-risk women, compared to women with other risk factors who may have come to clinic at a later gestation and not be recruited to the study. It may also be possible that women with particular risk factors were more likely than others to agree to participating in the study, e.g., at the St Thomas' site, we found that women who had very poor pregnancy histories were more likely to agree to being part of the study to potentially help others who had been through similar devastating experiences. This may affect the generalisability of the study results, however sub-analysis according to risk factor at study enrolment was carried out to evaluate any potential differences in biomarker expression and

outcome in each of these risk groups. In both the low and high-risk groups, willingness to participate in the study is also affected by socioeconomic and demographic factors, which may also slightly limit the generalisability of findings.

Due to cost limitations, we decided to analyse only samples of CVF from low-risk women who had provided swabs at two timepoints (10 to 15^{+6} weeks and 18 to 24^{+0} weeks) in order to evaluate the longitudinal expression of natural HDPs and how they compare to women at high-risk. This excluded low-risk women who did not attend their second scan appointment, who had changed their appointment without the researcher's knowledge, or who had refused consent for a second swab. By definition, therefore, this excluded women who may have lost their baby before 20 weeks of gestation, and thus the 'low-risk group' may be slightly unrepresentative of the population from which they are representing.

3.6 Laboratory processing of biological samples

3.6.1 Cervicovaginal fluid processing

Once transported to the laboratory on ice, the Dacron swab was removed, placed in a clean tube, vortexed for 10 seconds and centrifuged (2600 g for 10 minutes at 4°C). Resultant fluid was collected and added to the fluid in the original tube. This was mixed and centrifuged for a further 10 minutes to remove cell debris. The target time from collection to storage was <60 minutes. Cell-free supernatants were divided into aliquots (110 µl) into bar-coded tubes and stored at -80°C until analysis. The microbiology swabs in TE buffer and liquid Amies were stored directly at -80°C. The position of each sample within the freezer was recorded directly onto the study database. CVF smeared slides were airdried and stored in slide boxes for later gram-staining.

3.6.2 Blood sample processing

Upon arrival in the laboratory, the SST gel bottle was allowed to stand upright at room temperature for 30 minutes. Both the SST and EDTA bottles were then centrifuged (2,500g for 10 minutes at 4°C). The resultant plasma from the EDTA tube was then aliquoted, as was the serum from the SST-gel tubes (both 750 µl aliquots) into bar-coded tubes and stored for analysis at -80°C. The buffy coat was removed from the plasma EDTA bottle using a Pasteur pipette, and placed into a bar-code labelled tube and frozen at -80°C. These were stored as part of the Insight biobank.

3.7 Measurement of HDPs and HNE by enzyme linked immunosorbent assay

Once target numbers had been achieved, samples were thawed at room temperature, briefly vortexed and analysed by ELISA (Trappin2/elafin, HK318; cathelicidin [LL-37], HK321; HNE, HK319-02, all from Hycult, Biotech Cambridge) in duplicate, according to manufacturer's instructions. While mass spectrometry was considered as a method for protein detection and quantification (particularly sensitive for low abundance proteins) it would have been more expensive, and we had previously demonstrated that the ELISA kits were a reliable and practical method of measuring both elafin and cathelicidin. With hindsight, however, it would, have negated the problems we encountered below regarding the effect of diluting samples to ensure positioning on the ELISA standard curve (as detailed in section 3.2.10.1). At the time of sample analysis, no available Luminex multiplex assay for measurement of HDP, nor HNE was available.

Samples used for elafin measurement were diluted in sample buffer (1:20 and 1:100 for each sample) to ensure positioning within the standard curve, based on results obtained from a pilot study (CLIC). Samples used for HNE measurement were diluted in sample buffer 1:200 for each sample. CVF samples for cathelicidin measurement were undiluted. A consistent ratio of samples from high and low risk women were

included on each plate to minimise bias. A pooled CVF sample was created by combining a random set of 10 CVF samples from the pilot study and included on each plate to correct for inter-plate variability. Final concentrations were calculated from the standard curves using logistic regression. For trappin2/elafin, the detection range of the ELISA was 0.9 to 10 ng/ml. For cathelicidin this was 0.1 to 200 ng/ml and for HNE, 5 to 5000 ng/ml. Elafin was detectable in all but one of the CVF samples (range 3999 to 1062059 pg/ml). Cathelicidin (range 0.001 to 12.6 ng/ml) and HNE (range 0.4 to 7500 ng/ml) were detected in all the samples tested. The mean HDP concentration of the duplicate samples was used in the analysis. Repeat sample analysis was undertaken if the coefficient of variability (CV) between sample duplicates was over 20% and discarded if the repeat sample duplicate CV was also more than 20%.

3.8 Number of CVF samples

Of the 619 women enrolled, there were 1521 CVF samples from 586 women yielded usable elafin measurements (Table 3-2). Cathelicidin was measured in a subgroup of high-risk women (976 samples from 342 women) in whom there was sufficient CVF sample after the elafin analysis was complete. 1495 samples from 577 women were used for HNE. BV status was determined for 1494 samples from 600 women.

Table 3-2. Table to demonstrate the number of valid CVF samples for each host response peptide collected at each gestational category

Risk status	**Peptide**	**Gestation at sampling (weeks $^{+\text{days}}$)***				
High-risk women		Category 1 10-13^{+6}	Category 2 14 to 15+6	Category 3 16 – 19^{+6}	Category 4 20-24^{+0}	Total
	Elafin	241	265	313	297	1116
	Cathelicidin	215	234	268	259	976
	HNE	233	251	317	288	1089
Low-risk women						
	Elafin	193	9	18	185	405
	HNE	194	10	18	184	406

**These figures include all samples which may have been taken more than once in each gestational sampling window*

3.9 Assessment of BV status

CVF obtained from a Dacron swab was smeared onto a sterile glass slide. Slides were air-dried, for subsequent Gram stain, microscopy and scoring according to Hay's criteria (normal flora, intermediate flora or bacterial vaginosis (Hay et al. 1994), Table 3-3).

Table 3-3. Hay (1994) microscopy scoring criteria (Normal, intermediate or Bacterial Vaginosis)

Hay Score (BV status)	Definition
Grade 1 (Normal)	Lactobacillus morphotypes predominate
Grade 2 (Intermediate)	Mixed flora with some Lactobacilli present, but Gardnerella or Mobiluncus morphotypes also present
Grade 3 (Bacterial Vaginosis)	Predominantly Gardnerella and/or Mobiluncus morphotypes. Few or absent Lactobacilli

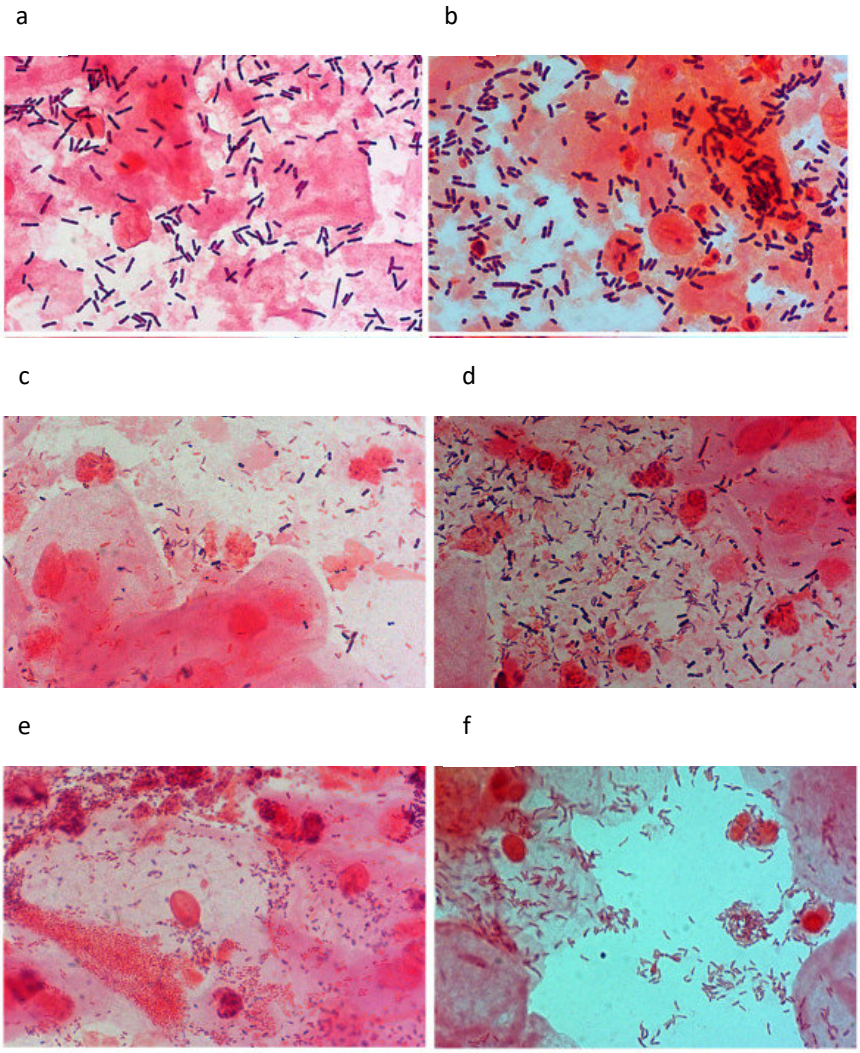

Figure 3-3. Gram-stained vaginal smears illustrating the different categories of vaginal microflora (Verhelst et al. 2005).

a & b: homogenous lactobacilli-dominant normal smears. c & d: Intermediate smears with mixture of lactobacillus but also some BV associated bacteria. e & f: Bacterial Vaginosis- associated bacteria with few lactobacilli

3.10 Statistical methods

All statistical analysis was performed using Stata (version 14.2, Stata Corp, College Station, Texas). Actual p-values are given (usually to 2 decimal places), except for very small values, shown as p <0.001.

3.10.1 Demographic data and pregnancy outcome (Chapter 4)

Descriptive statistics were used to summarize the data, presented as demographic characteristics (frequencies and percentages) according to risk status at enrolment, and pregnancy outcome (sPTB/Term birth). Means (± standard deviation) were compared using students t-test. Categorical variables were compared using Chi-squared tests. For statistical comparison of risk factors between high and low-risk women, results are presented as risk differences/ratios.

For the pregnancy outcome analysis, both single predictor analysis and multi-predictor models were created. Simple regression (single predictor) analysis (also presented as OR) was conducted for all risk factors in the term vs. sPTB groups; quantitative variables were analysed by regression with robust standard errors, and categorical variables analysed with X^2 test. For statistical comparison of risk factors between high and low-risk women, results are presented as risk differences/ratios. For the smaller low-risk group, and for the outcome sPTB <34 weeks, in which the event rates were lower, ORs calculated by exact logistic regression (Mehta and Patel 1995) were used, and only sPTB <37 weeks was analysed as an outcome (only one case of sPTB <34 weeks in low risk cohort).

Multi-predictor analysis was performed separately in the high and low-risk cohorts as they were recruited as two separate cohorts and thus this was clinically and statistically most appropriate, however there were too few events in the low-risk cohort to make multi-predictor analysis useful or valid in this group. To understand how predictors affect outcomes, we divided the predictors into groups for analysis: sociodemographic factors, relevant medical history and obstetric history. Those significant risk factors were included in a multiple variable model to predict prematurity (high-risk group only as too few events in low-risk cohort).

3.10.2 Gestational profile of HDPs (Chapter 5)

Using data from the whole cohort the gestational trends in CVF elafin, cathelicidin and HNE concentrations were analysed to assess the trend in protein expression, as well as the influence of demographic and social factors (risk status, ethnicity, BMI, maternal age and maternal smoking status). Distributions of data were first established by examination of distributional plots for raw and transformed values. Log transformations were applied to trappin2/elafin, cathelicidin and HNE concentrations to achieve approximate normality. Results are presented as geometric means and ratios, and as weekly rates of change as appropriate, with 95% confidence intervals. The average difference between cases and controls was determined using a regression model with correction for effect of gestation (2 weekly categories) and inter-assay plate variation. Adjustments for BMI, maternal age and ethnicity, and current smoking status were considered. When considering multiple measurements from the same woman, a random effects regression model was used allowing for observations to be grouped by participant. For elafin, where values were censored due to measurement limits, a random effects interval regression model was used. Spearman's rank correlations (rs) show association between markers.

3.10.2.1 *Corrections made for dilution effect*

Elafin concentrations, in particular, had a complicated distribution due in part to censoring of low and high values in both the 1:20 and 1:100 diluted samples. Furthermore, due to the extremely high and low concentration of elafin in the CVF samples, and need for positioning on the standard curve, it was necessary to perform the ELISA on samples diluted to concentrations 1:20, and 1:100.

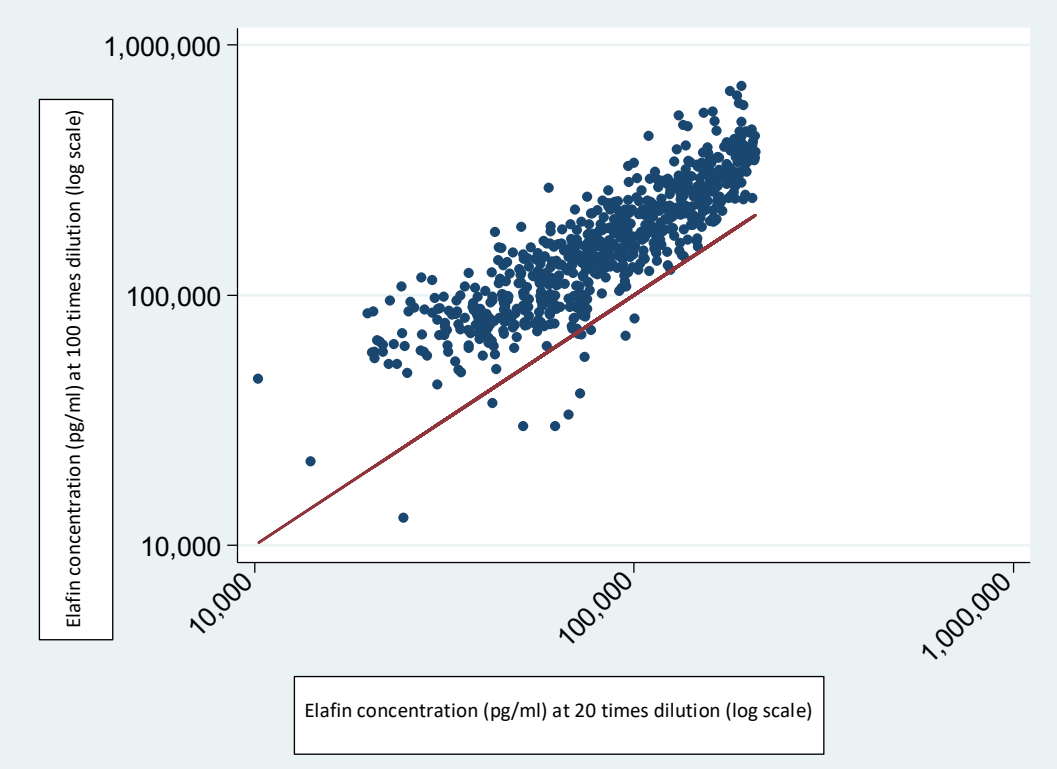

Figure 3-4. Scatter chart to demonstrate the relationship between the (log) elafin concentration at 1:20 dilution factor and 1:100 dilution factor.

When the concentrations were measured, it was clear that they were not comparable once multiplied by the dilution factor. Figure 3-4 demonstrates the relationship between the concentrations measured at 1:20 and 1:100 dilution. It is clear that samples measured at 1:100 lie above the line of agreement i.e., that the 1:100 concentration over-estimates, or the 1:20 concentration underestimates, the true concentration of elafin.

The problem encountered with the sample measurements is referred to as poor linearity of dilution, whereby the sample buffer dilutant affected elafin protein detection differently. It is possible that the less diluted samples have a lower ELISA signal than expected due to interference from other sample components, or the possibility that large aggregates of protein may get washed off the plate. The more diluted samples may have falsely elevated signals due to disruption of the sample matrix and release of bound protein.

We were not able to demonstrate that a sample with a concentration above the upper limit of quantification can be diluted to a concentration within the working range and provide a reliable result. Given we did not have further aliquots of sample with which to measure alternative dilution solutions, we overcame the problem by mathematical modelling of the expected sample concentration to obtain a 'substituted value' of elafin for samples diluted to 1:20 concentration which fell above the upper limit of quantification.

The ratio of the uncensored means of the 1:20 to 1:100 sample concentrations was 0.54 after adjusting for the dilution factor. Thus, the following adjustments were made; where a valid concentration was available for the 1:20 diluted sample, then this value was used in the analysis. Where sample concentrations at the 1:20 concentration were below the limit of assay detection, an interval regression method was used, with the missing values taken as being at an unknown point on the interval between zero and the smallest positive concentration observed (Amemiya 1973). For calculations where a value was required (e.g., when drawing graphs), this was calculated as the lower limit of detection multiplies by 0.5. Where sample concentrations at the 1:20 dilution were above the upper limit of detection of the ELISA, and a valid 1:100 concentration was available, the elafin concentration was taken as the 1:100 estimation, multiplied by the ratio of the uncensored means of the 1:20 and 1:100 dilution (0.535) producing a 'substituted value'. If no valid 1:100 concentration was available, the interval regression method was used, with the missing values taken as being an unknown point above the upper limit of normal. Where an actual value was required for analysis, this was taken as the upper limit of detection of the ELISA, multiplied by 1.5.

3.10.3 Prediction of cervical shortening and sPTB (Chapter 6)

Prediction analysis was performed according to gestational age at the time sample was taken split into 4 categories: 10-13^{+6} weeks, 14-15^{+6}, 16-19^{+6} and 20-24^{+0} weeks of gestation. If multiple samples were taken within a gestational time point, the first

one within each gestational window was used for the prediction analysis. Missing measurements during each of these time-points were due to clinic non-attendance/consent not given for collection, enrolment after a gestational time period had expired, or invalid samples (e.g., vaginal bleeding at the time of swab, high CV duplicate values, or invalid plate and no repeat sample available).

Results are presented as geometric means and ratios, with 95% confidence intervals. The average difference between cases and controls was determined using a regression model with correction for effect of gestation (two weekly categories) and inter-assay plate variation. Adjustments for BMI, maternal age and ethnicity, and current smoking status were considered. When considering multiple measurements from the same woman, a random effects regression model was used allowing for observations to be grouped by participant. For elafin, where values were censored due to measurement limits, a random effects interval regression model was used.

Prediction of sPTB, short cervix and maternal/fetal outcomes are described using receiver operating characteristic (ROC) curves. Confidence intervals for areas ROC curves are calculated using the exact binomial method (DeLong, DeLong, and Clarke-Pearson 1988) and areas under ROC curves compared using the DeLong, DeLong, and Clarke-Pearson (1988) algorithm.

Analysis was repeated in high-risk women only, as pathogenesis of sPTB is plausibly different in women with recurrent sPTB, as well as the highest-risk women (only those with previous sPTB or late miscarriage) to mirror the analysis performed in the pilot study. To assess the impact of intervention (cerclage, progesterone or Arabin pessary) on HDP concentrations, the prediction analysis was repeated with all samples taken after intervention excluded. This was particularly important since the pilot study excluded women from study entry who had intervention in place at study entry and randomised them to intervention only when cervical shortening was detected. In contrast, many of the women in the insight cohort had a prophylactic

stitch in situ from early gestation or were using vaginal progesterone pessaries. Given the noticeable effect of ethnicity on HDP concentration (Chapter 5), and the well-described ethnic disparity in sPTB (section 1.3.6) sub-group analysis was also performed according to ethnicity.

3.10.4 Association between BV, sPTB and HDP concentration (Chapter 7)

Sample characteristics were compared with Anova and X^2. Logistic regression analysis was used to determine the relationship between BV and sPTB in the whole cohort (women were designated to the BV group if they ever had a BV sample at any gestation between 10 and 24 weeks of gestation), and then sub-analysed according to ethnicity. CVF concentrations of elafin, cathelicidin and HNE were compared between women with normal flora, those with intermediate bacterial flora, and those with BV (as diagnosed by smear at the time of testing and adjusted for multiple tests), also sub-analysed according to ethnicity.

3.10.5 QfFN, HDPs and prediction of sPTB (Chapter 8)

QfFN concentrations were categorised into ranges, as suggested by Abbott et al. (2013); <10, 10-49, 50-199, >200 ng/ml, and the first tests taken between 18^{+0} to 19^{+6} weeks of gestation (to coincide with gestational category 2 HDP testing), and 20^{+0} to 24^{+0} weeks of gestation (gestational category 3 and 4 HDP testing) used for analysis purposes. ORs for sPTB at less than 34 and 37 weeks of gestation was calculated using the <10ng/ml category as the reference category, by binomial logistic regression using log [qfFN]. Prediction of sPTB <34 and <37 weeks of gestation are described using ROC curves. Sub-group analysis was performed by ethnicity (Black/White). Confidence intervals for areas ROC curves are calculated using the exact binomial method (Clopper and Pearson 1934). Areas under ROC curves are compared using the algorithm as above (DeLong, DeLong, and Clarke-Pearson 1988).

When combining HDPs with qfFN for ROC prediction analysis and model generation, the gestation at which the HDP had proved most predictive during single marker

analysis was used; for elafin this was 10-13^{+6} weeks of gestation and 16-19^{+6} weeks, cathelicidin and HNE 14 to 15^{+6} weeks of gestation. This was combined with the first qfFN concentration taken after 18 weeks of gestation to generate a combined ROC AUC. The three AUC ROC generated per combination of markers were statistically compared to each other (DeLong, DeLong, and Clarke-Pearson 1988), and also individually to qfFN as the 'gold standard'.

To create a combined model to predict sPTB in high-risk women, we followed a 3-stage procedure:

1) We first identified all potential predictors for sPTB and cervical shortening identified by previous work: Socio-demographics and medical history (age, BMI, ethnicity, sPTB risk factor history of recurrent UTIs, chronic viral infection); CVF HDP concentration at appropriate gestations (logs of elafin, cathelicidin, HNE and CE ratio) and log qfFN. As these predictors would only be available for high-risk women who had results for every biomarker, as well as sociodemographic and outcome data, we increased participant numbers by broadening the gestational categories for which the biomarkers could be used; for elafin (most useful gestational window 16-19^{+6} weeks), if there was not a value for this gestation, then the closest earlier value was used (10 to 15^{+6} weeks); for cathelicidin HNE and CE ratio (most useful 14 to 15^{+6} weeks) a test result prior to this gestation (10-13^{+6}) was used if no appropriate concentration was available; for fetal fibronectin, the first test performed between 18 and 24 weeks of gestation was used.
2) We tested each of these predictors individually using ROC areas and selected the ones with a significant predictive power to go forward to the third stage.
3) Once the significant predictors were identified, stepwise logistic regression was used to reduce the set further. Both forwards and backwards stepwise regression was used to confirm the model's stability. Results are presented as ORs for the individual predictors, and as ROC areas for the resulting composite predictor. This procedure was followed for three outcomes: sPTB

before 37 weeks, sPTB before 34 weeks, cervical length <25 mm before 25 weeks; for all women and for the two main ethnic subgroups (White & Back). This was then repeated using only biomarkers taken prior to 16 weeks of gestation (thus excluding qfFN).

3.10.6 Missing data

There was very little missing data for either demographic and laboratory data. Where this did exist, we did not impute data as we took the view that the missing values were small in number and were unlikely to be related to the outcome of interest (missing at random). The cohort was not a true representative sample of a population, rather an opportunistic mix of cases and controls as available, thus it would be inappropriate to attempt to reconstruct the true underlying distributions from which they came. Therefore, where data were missing, they were omitted from the analysis.

4 Results: Socio-demographic characteristics of the Insight cohort and relationship with sPTB

4.1 Introduction

This chapter describes the Insight study participant flow and population demographics (modifiable and non-modifiable risk factors) analysed according to risk at study entry (high/low risk) and according to pregnancy outcome (sPTB yes/no <34 and <37 weeks). It then describes the multi-predictor analysis of sPTB using socio-demographic data. A full description of methods is contained in Chapter 3.

4.2 Cohort demographics

In total, 619 women with singleton pregnancies were enrolled; 405 women were classified as high-risk for sPTB at enrolment, and the remaining 214 women were classified as low-risk at enrolment. Figure 4-1 shows the Insight study participant flow, displaying recruitment numbers, exclusions, loss to follow up and gestational outcomes. Primary outcome data (sPTB or term birth) was obtained for 611 out of 619 women.

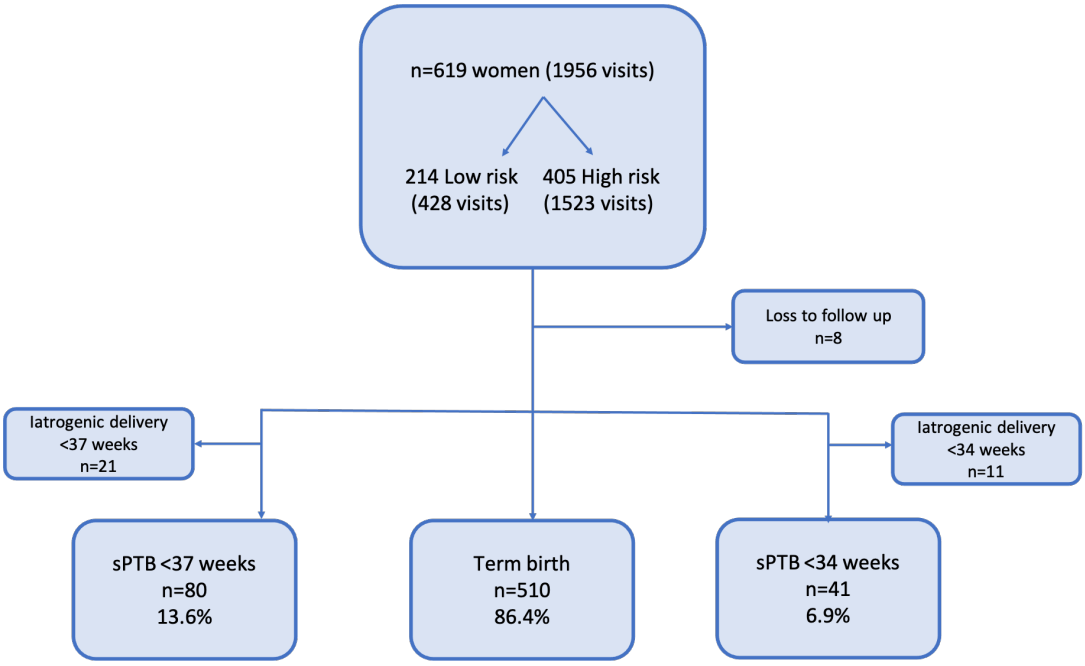

Figure 4-1. Insight study flow chart showing recruitment according to risk status at enrolment, exclusion for iatrogenic delivery, loss to follow up and gestational outcome

4.2.1 Demographic analysis; high vs low risk group

Table 4-1 displays the demographic data with risk differences/ratios for the high vs. low-risk Insight cohorts. Of the high-risk women, 281 women had one or more of previous sPTB, late miscarriage or previous preterm pre-labour rupture of membranes, PPROM), and considered of the 'highest risk'. Of the high-risk women, 34% received a prophylactic or reactive intervention to prevent sPTB (cerclage, vaginal progesterone or Arabin pessary). Black (African/Caribbean) women made up a greater proportion of the high-risk group and White women made up a greater proportion of the low-risk group. None of the women in the low-risk group at enrolment received an intervention to prevent sPTB.

As expected, the sPTB rate in the high-risk group was far greater than in the low-risk group (18.7% vs 2.4% <37 weeks). For those high-risk women who developed a short cervix, the sPTB rate was 22/71 (31.0 %) <34 weeks, and 30/71 (42.3 %) <37 weeks of gestation. The median (quartiles) gestational age of detection of cervical shortening

was 18^{+3} weeks (16^{+3} to 20^{+2} weeks). There was no observed difference in BMI, maternal age, smoking status or deprivation index between the low and high-risk groups. Although absolute numbers were small and confidence intervals therefore wide, women in the high-risk group were 10 times more likely to have experience domestic violence in their current or past relationships. Women in the high-risk group were more likely to report a history of recurrent UTI, and past or present infection with Group B streptococcus (GBS) or BV. Unsurprisingly, high-risk women were far more likely to experience sPTB and/or PPROM in their current pregnancy. No women in the low-risk group experienced late miscarriage <24 weeks, compared to 3.5% of the high-risk group.

Table 4-1 Baseline demographics and clinical characteristics of the total study population (and stratified by risk status)

Characteristic	Sub-category	High-risk women N=405	Low-risk women N=214	Comparison (difference/risk ratio, 95% CI)*	All women N=619
Mean maternal age at booking, Years (SD)		32.7 (5.1)	32.5 (4.7)	0.19 (-0.62-0.99)	32.6 (5.0)
Ethnic group N (%)	White European	246 (60.7)	162 (75.7)	ref	408 (67.0)
	Black African/ Caribbean	114 (28.1)	29 (13.6)	**2.09 (1.44-3.01)**	143 (23.1)
	Asian	37 (9.1)	14 (6.5)	1.89 (0.87-4.11)	51 (8.20
	Other	8 (2.0)	9 (4.2)	0.93 (0.49-1.75)	17 (2.7)
Risk factor N (%)	Previous sPTB	177 (43.7)	-	-	-
	Previous late miscarriage	138 (34.0)	-	-	-
	Previous PPROM	111 (27.4)	-	-	-
	Previous cervical surgery	143 (35.3)	-	-	-
	Incidental finding of a short cervix <25 mm	4 (1.0)	-	-	-
	Uterine anomaly	5 (1.2)	-	-	-
Smoking status N (%)	Never smoked	283 (69.9)	171 (79.9)	ref	454 (73.3)
	Current smoker	22 (5.4)	6 (2.8)	2.13 (0.88-5.15)	28 (4.5)

	Ex-smoker (gave up in current pregnancy)	29 (7.2)	11 (5.1)	1.54 (0.79-3.00)	40 (6.5)
	Ex (gave up before current pregnancy	69 (17.0)	26 (12.1)	1.49 (0.98-2.25)	95 (15.3)
	Unknown	2 (0.5)			
Mean BMI at booking Kg/m^2 (SD)		26.6 (6.9)	24.7 (4.6)	1.90 (0.98-2.81)	25.9 (6.2)
IMD quintile (5=least deprived)	1	63 (16.8)	36 (19.0)	ref	99 (16.0)
	2	141 (37.7)	74 (39.2)	1.03 (0.88-1.20)	215 (34.7)
	3	81 (21.7)	50 (26.5)	0.97 (0.77-1.22)	131 (21.2)
	4	47 (12.6)	18 (9.5)	1.28 (0.83-1.98)	65 (10.5)
	5	42 (11.2)	11 (5.8)	1.71 (0.97-3.02)	53 (8.6)
	Unknown	31 (7.7)	25 (11.7)	-	52 (8.4)
Current or past history of domestic violence N (%)		19 (4.7)	1 (0.5)	**10.31 (1.39-76.45)**	20 (3.2)
Current or past history of recreational drug use N (%)		11 (2.7)	7 (3.3)	0.83 (0.33-2.12)	18 (2.9)
History of 2 or more UTI's in pregnancy		29 (7.2)	4 (1.9)	**3.83 (1.36-10.75)**	33 (5.3)
Past or current infection with BV (at booking)		49 (12.2)	14 (6.5)	**1.86 (1.05-3.29)**	63 (10.2)
Past or current GBS infection (at booking)		38 (9.4)	8 (3.7)	**2.52 (1.20-5.31)**	46 (7.4)
Chronic viral infection (HIV, Hepatitis, other)		4 (1.0)	2 (0.9)	1.06 (0.20-5.74)	6 (0.9)
Pregnancy outcome N (%)	Term delivery >37 weeks	310 (77.1)	200 (95.7)	0.81 (0.76-0.98)	510 (82.4)

	Iatrogenic preterm delivery <37 weeks	17 (4.2)	4 (1.9)	2.21 (0.75-6.48)	21 (3.4)
	sPTB <37 weeks	75 (18.7)	5 (2.4)	**7.80 (3.20-18.98)**	80 (13.1)
	sPTB <34 weeks	40 (10.0)	1 (0.4)	**20.80 (2.88-150.21)**	41 (6.7)
	Late miscarriage	14 (3.5)	0 (0)	-	14 (2.3)
	PPROM	42 (10.4)	5 (2.3)	**4.37 (1.75-10.87)**	47 (7.6)
Cervical shortening <25 mm <24 weeks in current pregnancy N (%)		71 (17.5)	Not measured	-	-
Received prophylactic or emergency intervention to prevent sPTB N (%)	Abdominal or vaginal cerclage	105 (25.9)	0 (0)	**	105 (17.0)
	Progesterone beyond 14 weeks' gestation	26 (6.4)	0 (0)	**	26 (4.2)
	Arabin pessary	6 (1.5)	0 (0)	**	6 (1.0)

*BMI=Body mass index; SD=standard deviation; PPROM=preterm prelabour rupture of fetal membranes; BV=Bacterial vaginosis; GBS=Group B Streptococcus; UTI=Urinary tract infection, sPTB=spontaneous preterm birth; IMD=index of multiple deprivation; HIV=human immunodeficiency virus. *Quantitative variables analysed by regression with robust standard errors. Categorical variables analysed with X^2 test. **Comparison test not performed due to risk of treatment bias. Statistically significant risk differences/ratios are shown in bold.*

4.2.2 Demographic characteristics; sPTB vs. term birth

4.2.2.1 *Whole cohort*

Demographic characteristics of women according to pregnancy outcome (Term/sPTB<37 weeks) in all women, and when stratified by risk status at booking, are shown in tables 4-2 to 4-4. In both the whole cohort, and risk stratified cohorts, there were no statistically significant differences in maternal age, BMI, smoking status or deprivation index between those women who delivered at term vs. those who had sPTB before 37 weeks' gestation. Ethnicity was related to risk of sPTB <37 weeks; Black and Asian women were more likely to experience sPTB than White women. Socioeconomic status (as assessed by IMD quintile) was not related to outcome.

Unsurprisingly, in the whole cohort (Table 4-2), more women in the sPTB group had a history of prior sPTB, late miscarriage but not cervical surgery or PPROM. A higher proportion of women in the preterm group reported a history of recurrent UTI in pregnancy. There was no difference between the groups related to self-reported past or current BV or GBS genital infection at booking. Whilst women with a past or current history of domestic violence were more than two times likely to deliver spontaneously prior to 37 weeks, this did not reach statistical significance, likely due to small numbers. Women with chronic viral infection were over six times more likely to deliver prematurely than women without disclosed/diagnosed viral infection. As expected, a higher proportion of women who delivered spontaneously prior to 37 weeks' gestation received an intervention to prevent sPTB.

Table 4-2 Baseline demographics and clinical characteristics of whole study population by outcome

Characteristic	Sub-category	sPTB <37 N=80	Term delivery N= 510	Comparison (difference/risk ratio, 95% CI)*
Mean maternal age at booking in years (SD)		32.7 (6.3)	32.6 (4.7)	0.08 (-1.36-1.52)
Ethnic group N (%)	White European	37 (46.3)	354 (69.4)	ref
	Black African/Caribbean	27 (33.8)	107 (21.0)	**1.82 (1.30-2.53)**
	Asian	10 (12.5)	21 (4.1)	**3.80 (1.91-7.57)**
	Other	16 (20.0)	49 (9.6)	1.90 (0.84-4.34)
Risk factor N (%)	Previous sPTB	36 (45.0)	131 (25.7)	**1.75 (1.32-2.33)**
	Previous late miscarriage	37 (46.3)	93 (18.2)	**2.54 (1.88-3.42)**
	Previous PPROM	20 (25.0)	84 (16.5)	1.52 (0.99-2.33)
	Previous cervical surgery	14 (17.5)	124 (24.3)	0.72 (0.44-1.19)

	Incidental finding of a short cervix <25 mm	0 (0)	2 (0.4)	-
Smoking status N (%)	Never smoked	62 (77.5)	369 (72.4)	ref
	Current smoker	3 (3.8)	24 (4.7)	0.76 (0.23-2.44)
	Ex-smoker (gave up in current pregnancy)	3 (3.8)	36 (7.1)	0.52 (0.16-1.64)
	Ex (gave up before current pregnancy	12 (15.0)	79 (15.6)	0.92 (0.53-1.60)
	Unknown	0 (0)	2	-
Mean BMI at booking in kg/m^2 (SD)		26.7 (5.0)	25.74 (6.5)	0.98 (-0.24-2.19)
IMD quintile (5=least deprived)	1	14 (19.7)	81 (17.4)	ref
	2	26 (36.6)	179 (385)	0.94 (0.74 to 1.20)
	3	19 (26.8)	106 (22.8)	1.02 (0.74-1.40)
	4	5 (7.0)	56 (12.0)	0.64 (0.30-1.40)
	5	7 (9.9)	43 99.2)	0.96 (0.50-1.84)
Current or past history of domestic violence N (%)		5 (7.0)	15 (3.1)	2.25 (0.84-6.01)
Current or past history of recreational drug use N (%)		1 (1.3)	16 (3.1)	0.40 (0.05-2.95)
History of 2 or more UTI's in pregnancy		10 (12.5)	20 (3.9)	**3.19 (1.55-6.56)**
Past or current infection with BV (at booking)		10 (12.5)	47 (9.3)	1.35 (0.71-2.56)

Past or current GBS infection (at booking)		9 (11.3)	32 (6.3)	1.79 (0.89-3.60)
Chronic viral infection (HIV, Hepatitis, other)		3 (3.8)	3 (0.6)	**6.36 (1.31-30.98)**
Received prophylactic or emergency intervention to prevent sPTB N (%)	Abdominal or vaginal cerclage	33 (41.3)	69 (13.5)	**
	Progesterone beyond 14 weeks of gestation	12 (15.0)	13 (2.5)	**
	Arabin pessary	4 (5.0)	3 (0.6)	**

*BMI= Body mass index; SD=standard deviation; PPROM preterm prelabour rupture of fetal membranes; BV=Bacterial vaginosis; GBS=Group B Streptococcus; UTI=Urinary tract infection; IMD=Index of Multiple Deprivation; sPTB =Spontaneous preterm birth; HIV=Human immunodeficiency Virus; CI= Confidence Interval preterm iatrogenic deliveries excluded n=21. *Quantitative variables analysed by regression with robust standard errors. Categorical variables analysed with X^2 test. **Comparison test not performed due to risk of treatment bias Statistically significant risk differences/ratios are shown in bold*

4.2.2.2 *High-risk cohort*

When the high-risk cohort were analysed separately (Table 4-3) Black women were more likely to deliver spontaneously and prematurely white women, though this did not reach statistical significance (risk ratio 1.36, 95% CI 0.95-1.95). Asian women, however, were significantly more likely to deliver prematurely compared to White women (risk ratio 3.04, 1.40-6.59), despite low numbers of women in each group.

Within the high-risk cohort (as defined by previous obstetric history, uterine anomaly or incidental finding of a short cervix) the risk-factor most strongly associated with sPTB was previous late miscarriage (risk ratio 1.64, 1.24-2.19). Women who had a history of cervical surgery had a lower risk of sPTB compared to women who had not had cervical surgery (but by definition had other high-risk factors such as previous sPTB or late miscarriage). As in the whole cohort, a history of UTI's in pregnancy and chronic viral infection was associated with increased risk of sPTB.

Table 4-3 Baseline demographics and clinical characteristics of high-risk study population by outcome

Characteristic	Sub-category	sPTB <37 N=75	Term delivery N= 310	Comparison (difference/risk ratio, 95% CI)*
Mean maternal age at booking in years (SD)		32.6 (6.44)	32.8 (4.75)	-0.17 (-1.72-1.38)
Ethnic group N (%)	White European	36 (48.0)	199 (64.2)	ref
	Black African/Caribbean	24 (32.0)	83 (26.8)	1.36 (0.95-1.95)
	Asian	9 (12.0)	14 (4.5)	**3.04 (1.40-6.59)**
	Other	6 (8.0)	14 (4.5)	2.17 (0.89-5.33)
Risk factor N (%)	Previous sPTB	36 (48.0)	131 (42.3)	1.14 (0.87-1.49)
	Previous late miscarriage	37 (49.3)	93 (30.0)	**1.64 (1.24-2.19)**
	Previous PPROM	20 (26.7)	84 (27.1)	0.98 (0.65-1.49)
	Previous cervical surgery	**14 (18.7)**	**124 (40.0)**	**0.47 (0.29-0.76)**
	Incidental finding of a short cervix <25 mm	0 (0.0)	2 (0.6)	-
Smoking status N (%)	Never smoked	57 (76.0)	210 (68.2)	ref
	Current smoker	3 (4.0)	19 (6.2)	0.60 (0.18-1.97)
	Ex-smoker (gave up in current pregnancy)	3 (4.0)	25 (8.1)	0.47 (0.15-1.50)
	Ex (gave up before current pregnancy	12 (16.0)	54 (17.5)	0.85 (0.48-1.50)
	Unknown	0 (0.0)	2 (0.6)	-
Mean BMI at booking in kg/m^2 (SD)		26.8 (5.03)	26.5 (7.32)	0.25 (-1.16-1.65)
IMD quintile (5=least deprived)	1	13 (19.4)	48 (16.7)	ref
	2	23 (34.3)	112 (38.9)	0.91 (0.7-1.19)

	3	19 (28.4)	57 (19.8)	1.09 (0.78-1.53)
	4	5 (7.5)	39 (13.5)	0.62 (0.28-1.35)
	5	7 (10.4)	32 (11.1)	0.88 (0.45-1.68)
Current or past history of domestic violence N (%)		5 (7.6)	14 (4.8)	1.57 (0.59-4.20)
Current or past history of recreational drug use N (%)		1 (1.3)	10 (3.2)	0.41 (0.05-3.16)
History of 2 or more UTI's in pregnancy		10 (13.3)	16 (5.2)	**2.58 (1.22-5.46)**
Past or current infection with BV (at booking)		10 (13.3)	34 (11.0)	1.21 (0.63-2.33)
Past or current GBS infection (at booking)		9 (12.0)	25 (8.1)	1.48 (0.72-3.03)
Chronic viral infection (HIV, Hepatitis, other)		3 (4.0)	1 (.3)	**12.36 (1.30-117.16)**
Received prophylactic or emergency intervention to prevent sPTB N (%)	Abdominal or vaginal cerclage	33 (41.3)	69 (13.5)	**
	Progesterone beyond 14 weeks of gestation	12 (15.0)	13 (2.5)	**
	Arabin pessary	4 (5.0)	3 (0.6)	**

BMI=body mass index; SD=standard deviation; PPROM=preterm prelabour rupture of fetal membranes; BV=bacterial vaginosis; GBS=Group B Streptococcus; UTI=urinary tract infection; IMD=index of multiple deprivation; sPTB=spontaneous preterm birth; HIV=human immunodeficiency virus; CI=Confidence Interval

** Quantitative variables analysed by regression with robust standard errors. *Categorical variables analysed with X2 test. Preterm iatrogenic deliveries excluded n=17. ** Comparison test not performed due to risk of treatment bias. Statistically significant risk differences/ratios are shown in bold.*

4.2.2.3 *Low risk cohort*

Amongst the low-risk women only, the absolute numbers of sPTB were small (n=5, 2.4% of cohort), which limited analysis, though Black and Asian ethnicity conferred an increased risk of sPTB (Table 4.4).

Table 4-4 Baseline demographics and clinical characteristics of low-risk study population by outcome

Characteristic	Sub-category	sPTB <37 N=5	Term delivery N= 200	Comparison (difference/risk ratio, 95% CI)*
Mean maternal age at booking in years (SD)		34.0 (3.32)	32.4 (4.72)	1.64 (-1.67-4.35)
Ethnic group N (%)	White European	1 (20.0)	155 (77.5)	ref
	Black African/Caribbean	3 (60.0)	24 (12.0)	**5.59 (2.84-11.01)**
	Asian	1(20.0)	7 (3.5)	**11.57 (2.42-55.28)**
	Other	0 (0.0)	14 (7.0)	-
Smoking status N (%)	Never smoked	5 (100.0)	159 (79.5)	ref
	Current smoker	0 (0.0)	5 (2.5)	-
	Ex-smoker (gave up in current pregnancy)	0 (0.0)	11 (5.5)	-
	Ex (gave up before current pregnancy	0 (0.0)	25 (12.5)	-
	Unknown	0 (0.0)	0 (0.0)	-
Mean BMI at booking in kg/m^2 (SD)		26.1 (3.20)	24.6 (4.59)	1.55 (-1.07-4.17)
IMD quintile (5=least deprived)	1	1 (25.0)	33 (18.6)	ref
	2	3 (75.0)	67 (37.9)	1.12 (0.63-2.00)
	3	0	49 (27.7)	-

	4	0	17 (9.6)	-
	5	0	11 (6.2)	-
Current or past history of domestic violence N (%)		0 (0.0)	1 (0.5)	-
Current or past history of recreational drug use N (%)		0 (0.0)	6 (3.0)	-
History of 2 or more UTI's in pregnancy		0 (0.0)	4 (2.0)	-
Past or current infection with BV (at booking)		0 (0.0)	13 (6.5)	-
Past or current GBS infection (at booking)		0 (0.0)	7 (3.5)	-
Chronic viral infection (HIV, Hepatitis, other)		0 (0.0)	2 (1.0)	-

BMI=body mass index; SD=standard deviation; PPROM=preterm prelabour rupture of fetal membranes; BV=bacterial vaginosis; GBS=Group B Streptococcus; UTI=urinary tract infection; IMD=index of multiple deprivation; sPTB=spontaneous preterm birth; HIV=human immunodeficiency virus; CI=Confidence Interval

** Quantitative variables analysed by regression with robust standard errors. *Categorical variables analysed with X2 test. Preterm iatrogenic deliveries excluded n=17. **Comparison test not performed due to risk of treatment bias. Statistically significant risk differences/ratios are shown in bold.*

4.2.3 Single and multiple risk-factor regression analysis

Data below show the influence of maternal risk-factors, presented as OR on the occurrence of sPTB <37 weeks and <34 weeks, for both single risk factor and multiple risk factor adjusted analysis (Tables 4-5 to 4-7). Multiple risk-factor analysis was organised in a stepwise manner, analysing risk-factors in groups (socio-demographic, medical history and obstetric history) and carrying forward statistically significant risk factors from each group to create the final model (Table 4-8).

4.2.3.1 *High-risk cohort; sPTB <37 weeks of gestation*

Table 4-5 illustrates the univariate and multivariate analysis of the association between sociodemographic risk-factors and sPTB. From this group, only ethnicity was carried forward into the final model.

Table 4-5 Group 1: Sociodemographic factors. Odds ratios and 95% confidence intervals obtained by univariate and multivariate logistic regression analysis (adjusted for all predictors in the table) for sPTB <37 weeks

Independent variables	Sub-category	Univariate analysis			Multivariate analysis		
		OR	**95% CI**	**P value**	**OR**	**95% CI**	**P value**
Maternal age		0.99	0.95-1.04	0.80	0.99	0.93-1.05	0.70
Maternal BMI		1.00	0.97-1.04	0.78	0.99	0.94-1.04	0.58
Ethnicity	White	ref	ref	ref	ref	ref	ref
	Black	1.60	0.90-2.84	0.11	1.23	0.58-2.58	0.59
	Asian	**3.55**	**1.43-8.82**	**0.01**	2.61	0.88-7.75	0.08
	Other	2.37	0.85-6.57	0.09	0.99	0.20-4.77	0.99
Smoking status	Never smoked	ref	ref	ref	ref	ref	ref
	Current smoker	0.58	0.17-2.04	0.40	0.27	0.03-2.24	0.23
	Gave up in pregnancy	0.44	0.13-1.52	0.19	0.43	0.09-2.09	0.30
	Gave up before pregnancy	0.82	0.41-1.63	0.57	1.16	0.55-2.47	0.69
IMD quintile		0.94	0.76-1.18	0.62	0.94	0.72-1.22	0.62
History of recreational drug use		0.40	0.05-3.20	0.39	0.56	0.06-5.03	0.60
History of domestic violence		1.62	0.56-4.66	0.37	2.47	0.77-7.97	0.13

BMI= body mass index; IMD=index of multiple deprivation; OR=odds ratio; CI=confidence interval sPTB=spontaneous preterm birth. Statistically significant risk differences/ratios are shown in bold.

From Group 2 (Medical history), a history of two or more UTIs was carried through into the final model (Table 4-6). Although chronic viral infection was significantly associated with sPTB (both women with HIV delivered prematurely), this was based on n=4, resulting in wide confidence intervals. Table 4-7 displays the on-going model for sPTB <37 weeks, combining significant Groups 1 and Group 2 demographics/characteristics.

Table 4-6. Group 2: Medical History. Odds ratios and 95% confidence intervals obtained by univariate and multivariate logistic regression analysis (adjusted for all predictors in the table) for sPTB <37 weeks

Independent variables	Univariate analysis			Multivariate analysis		
	OR	**95% CI**	**P value**	**OR**	**95% CI**	**P value**
History of 2 or more UTI's in pregnancy	**2.83**	**1.23-6.51**	**0.02**	**2.65**	**1.12-6.19**	**0.03**
Past or current infection with BV (at booking)	1.24	0.58-2.64	0.58	1.02	0.45-2.29	0.97
Past or current GBS infection (at booking)	1.54	0.69-3.46	0.29	1.37	0.58-3.26	0.47
Chronic viral infection (HIV, Hepatitis, other)	**12.83**	**1.32-125.18**	**0.03**	**14.49**	**1.47-142.29**	**<0.001**

BV=bacterial vaginosis; GBS=Group B Streptococcus; UTI=urinary tract infection; HIV=human immunodeficiency virus; OR=odds ratio; CI=confidence interval, sPTB=spontaneous preterm birth. Statistically significant risk differences/ratios are shown in bold.

Table 4-7. Interim regression model combining Group 1 (socio-demographics) and Group 2 (medical risk factors) Odds ratios and 95% confidence intervals obtained by multivariate logistic regression analysis (adjusted for all predictors in the table) for sPTB <37 weeks

		Multivariate analysis		
Independent variables	**Sub-category**	**OR**	**95% CI**	**P value**
Recurrent UTI		**2.99**	**1.24-7.19**	**0.02**
Chronic viral infection		**12.04**	**1.19-121.76**	**0.04**
Ethnicity	White	ref	ref	ref
	Black	1.55	0.85-2.85	0.16
	Asian	**3.12**	**1.23-7.95**	**0.02**
	Other	**2.88**	**1.02-8.13**	**0.05**

UTI=urinary tract infection; HIV=human immunodeficiency virus; OR=odds ratio; CI=confidence interval, sPTB=spontaneous preterm birth. Statistically significant risk differences/ratios are shown in bold.

Addition of Group 3 (risk-factor at booking) required more thought, given that the presence of a sPTB risk-factor at booking was a pre-requisite for entry into the high-risk cohort, and several of the risk-factors were overlapping (e.g., women frequently had a history of sPTB and cervical surgery). Thus, we first evaluated the relative contribution of risk factors to risk of sPTB, in women with one risk-factor only (n=315) (Table 4.8). For the purposes of this analysis, we considered previous PTB and previous PPROM together as one risk-factor, due to the overlapping nature of the condition (most women with PPROM also had sPTB by the definitions used in the Insight study, see Chapter 3). Uterine anomaly and incidental finding of a short cervix were not considered here due to the very small numbers of women in each group. As shown in Table 4-8 cervical surgery carries the lowest risk of subsequent sPTB and having had a late miscarriage carries the highest risk. Thus, when all women were included in the model (including those with multiple risk factors) we assigned risk factor status according to their 'worst risk factor'; previous late miscarriage (n=138); previous sPTB or PPROM (n=143); previous cervical surgery (n=117) and included women with a finding of an incidental short cervix, or uterine anomaly only, as a fourth group (n=7). Univariate analysis for 'worst risk-factor' is displayed below in Table 4-9. Worst risk factor was therefore carried over into the model (Table 4-10).

Table 4-8. Past obstetric history (if one risk factor only present). Odds ratios and 95% confidence intervals obtained by univariate logistic regression analysis for sPTB <37 weeks

Independent variables	Univariate analysis		
	OR	**95% CI**	**P value**
Previous cervical surgery	ref	ref	ref
Previous sPTB or PPROM	**2.88**	**1.32-6.28**	**0.01**
Previous late miscarriage	**4.12**	**1.84-9.26**	**0.001**

PPROM=preterm prelabour rupture of fetal membranes; sPTB=spontaneous preterm birth; OR=odds ratio; CI=confidence interval. Statistically significant risk differences/ratios are shown in bold.

Table 4-9. Past obstetric history (Worst risk factor) Odds ratios and 95% confidence intervals obtained by multivariate logistic regression analysis for sPTB <37 weeks

Independent variables; worst risk factor	Univariate analysis		
	OR	**95% CI**	**P value**
Previous cervical surgery	ref	ref	ref
Previous sPTB or PPROM	**2.6**	**1.20-5.63**	**0.02**
Previous late miscarriage	**4.14**	**1.95-8.78**	**<0.001**
Uterine abnormality or incidental finding of a short cervix	2.08	0.22-19.60	0.52

PPROM=preterm prelabour rupture of fetal membranes; sPTB=spontaneous preterm birth; OR=odds ratio; CI=confidence interval. Statistically significant risk differences/ratios are shown in bold.

Table 4-10. Interim regression model for prediction of sPTB before 37 weeks.

		Multivariate analysis		
Independent variables	**Sub-category**	**OR**	**95% CI**	**P value**
Worst risk factor	Previous cervical surgery	ref	ref	ref
	Previous sPTB or PPROM	2.21	0.98-4.97	0.06
	Previous late miscarriage	**3.62**	**1.57-8.31**	**0.002**
	Uterine abnormality or incidental finding of a short cervix	2.33	0.24-22.08	0.46
Recurrent UTI		**2.66**	**1.08-6.52**	**0.03**
Chronic viral infection		**11.36**	**1.11-116.19**	**0.04**
Ethnicity	White	ref	ref	ref
	Black	0.97	0.50-1.90	0.93
	Asian	2.13	0.82-5.59	0.12
	Other	2.46	0.84-7.18	0.10

UTI=urinary tract infection; PPROM=preterm prelabour rupture of fetal membranes; sPTB=spontaneous preterm birth; OR=odds ratio; CI=confidence interval. Statistically significant risk differences/ratios are shown in bold.

After adjustment, the effect of ethnicity did not significantly affect risk of sPTB <37 weeks. Table 4-11 displays the adjusted results of the significant risk predictors (worst risk factor, recurrent UTI, and chronic viral infection).

Table 4-11. Final regression model for prediction of sPTB before 37 weeks

		Multivariate analysis		
Independent variables	**Sub-category**	**OR**	**95% CI**	**P value**
Worst risk factor	Previous cervical surgery	ref	ref	ref
	Previous sPTB or PPROM	**2.29**	**1.04-5.03**	**0.04**
	Previous late miscarriage	**3.81**	**1.78-8.13**	**0.001**
	Uterine abnormality or incidental finding of a short cervix	2.17	0.23-20.42	0.50
Recurrent UTI		**2.78**	**1.17-6.61**	**0.02**
Chronic viral infection		9.81	0.99-97.52	0.05

UTI=urinary tract infection; PPROM=preterm prelabour rupture of fetal membranes; sPTB=spontaneous preterm birth; OR=odds ratio; CI=confidence interval. Statistically significant risk differences/ratios are shown in bold.

I subsequently further explored why the effect of ethnicity was reduced when adjustment was made for certain other clinical features (UTI, cervical surgery and viral infection). A higher proportion of Asian women (5/24, 20.8%) reported a history of recurrent UTI compared with White women (21/240, 8.8%) and Black women (3/113, 2.7%), p=0.01. In contrast, a far higher proportion of women in whom cervical surgery was their only risk factor were White (105/240, 43.8%), vs, Black (4/113, 3.5%) and Asian (1/24, 4.2%), p<0.001). Likewise, all women with chronic viral infection were Black (4/113, 3.5%), p=0.02. Whilst these ethnic differences are explored further in the discussion below and in Chapter 5 and 6, clearly these important risk factors are affected directly or indirectly by ethnicity.

4.2.3.2 *High-risk cohort sPTB <34 weeks of gestation*

From Group 1 (Table 4-12) only ethnicity as a risk-factor was carried forward into the final model (OR for sPTB <34 weeks non-White vs. White ethnicity 3.32, 95% CI 1.67-6.59, p=0.001). Only chronic viral infection was carried over into the final model from Group 2 (Table 4-13). When this was combined with ethnicity into an interim model, both chronic viral infection and ethnicity were independently associated with sPTB <34 weeks (Table 4-14) and carried through to the final model.

Table 4-12. Group 1: Sociodemographic factors. Odds ratios and 95% confidence intervals obtained by univariate and multivariate logistic regression analysis (adjusted for all predictors in the table) for sPTB <34 weeks:

Independent variables	Sub-category	Univariate analysis			Multivariate analysis		
		OR	**95% CI**	**P value**	**OR**	**95% CI**	**P value**
Maternal age		0.98	0.92-1.05	0.63	0.99	0.92-1.07	0.84
Maternal BMI		1.02	0.98-1.06	0.41	0.98	0.92-1.05	0.54
Ethnicity	White	ref	ref	ref	ref	ref	ref
	Black	**2.96**	**1.40-6.26**	**0.004**	2.53	0.94-6.82	0.07
	Asian	**4.50**	**1.46-13.92**	**0.01**	**5.16**	**1.37-19.46**	**0.02**
	Other	**4.05**	**1.20-13-75**	**0.03**	1.66	0.19-14.45	0.65
Smoking status	Never smoked	ref	ref	ref	ref	ref	ref
	Current smoker	0.39	0.05-2.98	0.36	1	-	-
	Gave up in pregnancy	0.63	1.14-2.77	0.54	0.65	0.07-5.76	0.70
	Gave up before pregnancy	0.93	0.39-2.23	0.88	1.56	0.58-4.24	0.38
IMD quintile		0.79	0.58-1.10	0.16	0.86	0.59-1.26	0.45
History of recreational drug use		1	-	-	1	-	-
History of domestic violence		1.08	0.24-4.87	0.92	1.63	0.31-8.50	0.56

BMI=body mass index; IMD=index of multiple deprivation; OR=odds ratio; CI=confidence interval, sPTB=spontaneous preterm birth.

Statistically significant risk differences/ratios are shown in bold.

Table 4-13. Group 12: Medical History. Odds ratios and 95% confidence intervals obtained by univariate and multivariate logistic regression analysis (adjusted for all predictors in the table) for sPTB <34 weeks:

Independent variables	Univariate analysis			Multivariate analysis		
	OR	**95% CI**	**P value**	**OR**	**95% CI**	**P value**
History of 2 or more UTI's in pregnancy	1.96	0.71-5.47	0.20	1.64	0.55-4.94	0.38
Past or current infection with BV (at booking)	1.05	0.39-2.84	0.92	0.74	0.25-2.19	0.59
Past or current GBS infection (at booking)	1.82	0.71-4.69	0.21	1.92	0.70-5.30	0.21
Chronic viral infection (HIV, Hepatitis, other)	**9.23**	**1.26-67.46**	**0.03**	**11.14**	**1.49-83.07**	**0.02**

BV=bacterial vaginosis; GBS=group B Streptococcus; UTI=urinary tract infection; HIV=human immunodeficiency virus; OR=odds ratio; CI=confidence interval, sPTB=spontaneous preterm birth. Statistically significant risk differences/ratios are shown in bold.

Table 4-14. Interim regression model for prediction of sPTB before 34 weeks using Group 1 (ethnicity) and Group 2 (chronic viral infection).

			Multivariate analysis	
Independent variables	**Sub-category**	**OR**	**95% CI**	**P value**
Chronic viral infection		6.07	0.79-46.41	0.08
Ethnicity	White	ref	ref	ref
	Black	**2.67**	**1.24-5.76**	**0.01**
	Asian	**4.50**	**1.46-13.92**	**0.01**
	Other	**4.32**	**1.27-14.76**	**0.02**

OR=odds ratio; CI=confidence interval, sPTB=spontaneous preterm birth. Statistically significant risk differences/ratios are shown in bold

Similarly to the <37 weeks analysis, we compared risk of sPTB <34 weeks in women who only had one risk factor, to evaluate which risk factor conferred greatest risk of sPTB <34 weeks (Table 4-15). Late miscarriage conferred the highest risk of subsequent sPTB <34 weeks, and cervical surgery conferred lowest risk of sPTB <34

weeks (within an already high-risk group). Women with uterine abnormalities or incidental finding of a short cervix (and no other risk factors) were excluded from this analysis due to small numbers (n=7). Subsequently 'worst risk factor' analysis was applied to the whole cohort of high-risk women; women with a history of late miscarriage (n=133); previous sPTB or PPROM (=140), previous cervical surgery (n=114). Table 4-16, which was subsequently incorporated into the multivariable risk model incorporating all significant risk predictors for this cohort of high-risk women (Table 4-17). When all risk factors were considered together, a history of a late miscarriage remained the only independent significant predictor of sPTB <34 weeks (OR 4.29, 1.31-14.10, p=0.02) amongst an already high-risk cohort.

Table 4-15. Past obstetric history (if one risk factor only present). Odds ratios and 95% confidence intervals obtained by univariate logistic regression analysis for sPTB <34 weeks

\Independent variables	Univariate analysis		
	OR	**95% CI**	**P value**
Previous cervical surgery	ref	ref	ref
Previous sPTB or PPROM	2.95	0.92-9.42	0.07
Previous late miscarriage	**6.57**	**2.11-20.47**	**0.001**

PPROM=preterm prelabour rupture of fetal membranes; sPTB=spontaneous preterm birth; OR=odds ratio; CI=confidence interval. Statistically significant risk differences/ratios are shown in bold.

Table 4-16. Worst obstetric history; Odds ratios and 95% confidence intervals obtained by multivariate logistic regression analysis for sPTB <34 weeks

		Multivariate analysis		
Independent variables	**Sub-category**	**OR**	**95% CI**	**P value**
Worst risk factor	Previous cervical surgery	ref	ref	ref
	Previous sPTB or PPROM	2.60	0.81-8.29	0.11
	Previous late miscarriage	**6.06**	**2.03-18.03**	**0.001**

PPROM=preterm prelabour rupture of fetal membranes; sPTB=spontaneous preterm birth; OR=odds ratio; CI=confidence interval.

Table 4-17. Final regression model for prediction of sPTB before 34 weeks.

		Multivariate analysis		
Independent variables	**Sub-category**	**OR**	**95% CI**	**P value**
Worst risk factor	Previous cervical surgery	ref	ref	ref
	Previous sPTB or PPROM	2.16	0.65-7.15	0.21
	Previous late miscarriage	**4.29**	**1.31-14.10**	**0.02**
Chronic viral infection		5.63	0.72-44.04	0.10
Ethnicity	White	ref	ref	ref
	Black	1.56	0.67-3.61	0.30
	Asian	2.83	0.87-9.14	0.08
	Other	3.51	1.00-12.41	0.05

PPROM=preterm prelabour rupture of fetal membranes; sPTB=spontaneous preterm birth; CI=confidence interval; OR=odds ratio. Statistically significant risk differences/ratios are shown in bold.

4.2.3.3 *Low risk cohort*

In view of the small number of cases of sPTB <34 (n=5) and sPTB <37 (n=1) a multiple regression model was not attempted to define risk factors in the low-risk cohort. As see in table 4-18, only Black ethnicity was related to sPTB <37 weeks in these low-risk women (X^2, p=0.002). Table 4-18 presents these as ORs (95% CI).

Table 4-18. Odds ratios and 95% confidence intervals obtained by univariate logistic regression analysis for sPTB <37 weeks by ethnicity in low-risk women.

Independent variables	Sub-category	Univariate analysis		
		OR	**95% CI**	**P value**
Ethnicity	White	ref	ref	ref
	Black	**18.83**	**1.45-1023.25**	**0.02**
	Asian	20.78	0.25-1747.92	0.19
	Other	11.14	0- 434.57	1.0

OR: Odds ratio; CI Confidence Interval, sPTB=spontaneous preterm birth. Statistically significant risk differences/ratios are shown in bold.

4.3 Discussion

This chapter described the demographics of the 619 women in the Insight cohort, and the relationship between demographic and clinical risk factors, and risk of sPTB.

4.3.1 High-risk vs low-risk cohort demographics

Women in the high-risk cohort (defined as previous sPTB, late miscarriage, PPROM or cervical surgery) were more likely to be of Black ethnicity compared to women in the low-risk cohort, who were either in their first pregnancy (with no other risk factors for sPTB) or who had previous term births (and no other risk factors for sPTB). This likely represents that Black women are more likely to have had sPTB/PPROM or late miscarriage than White women (Reagan and Salsberry 2005; Simhan and Bodnar 2006; Zhang and Savitz 1992; Goldenberg, Cliver, et al. 1996) and are thus over-represented in the high-risk group. However, Black and other ethnic minority women are under-represented in clinical trials generally (Hamel et al. 2016; Jimenez et al. 2013). It may be that low-risk Black and ethnic minority women were less likely to agree to partake in a clinical trial which required an intimate vaginal swab, and/or that they experienced other barriers to trial enrolment such as cultural and language. In contrast, women in the high-risk group would already have been receiving routine care including intimate vaginal swabs and TVUS, and also may be more willing to consent to study inclusion having previously experienced adverse fetal outcomes, more intrinsically motivated to move research in the field forward, regardless of ethnicity.

High-risk women were more likely to have a previous or current history of domestic violence, compared with low-risk women. That intimate partner violence is a risk-factor for sPTB is well recognised in the literature (Donovan et al. 2016; Sanchez et al. 2013; Berhanie et al. 2019). A systematic review and meta-analysis of 50 global studies (>5 million women) demonstrated an aOR of 1.89 (95% CI 1.43-2.48) for risk of PTB <37 weeks, though studies were heterogenous (Donovan et al. 2016). In

particular a combination of physical and sexual violence in the current pregnancy was associated with highest risk of subsequent sPTB. As with ethnicity, however, in the Insight study, it may also be that low-risk women who experienced domestic instability and domestic violence may be less likely to a) attend their nuchal scan at the appropriate gestation and thus not be approached for recruitment and b) be less likely to agree to be recruited into the study. Similarly, women who were already high-risk, and thus may have an established relationship with the study team who were also providing medical care, may be more likely to disclose a history of domestic violence, and agree to participate in the study, thus such bias cannot be ruled out.

High-risk women were more likely to report previous BV and GBS, as well as a history of recurrent UTI's compared with low-risk women. Whilst these (particularly recurrent UTI and BV) are risk factors for sPTB, this observed result may be a product of recall bias; women with previous poor pregnancy outcome (and thus more likely to give birth prematurely in this current pregnancy) may place greater significance or have memory of the diagnosis of UTIs or BV or have had more contact with health professionals with the potential to diagnose this.

No low-risk woman received an intervention to prevent sPTB. This was expected as intervention is usually based on a previous history of sPTB, or detection of a short cervix; in the UK a practice of universal screening of women for a short cervix is not undertaken, and thus a short cervix would not be detected in low-risk women. Similarly, when analysis was performed according to pregnancy outcome in the index pregnancy, a higher proportion of women who delivered spontaneously prior to 37 weeks of gestation received an intervention to prevent sPTB. This reflects both the practice of inserting an elective cervical cerclage into women with the poorest obstetric histories (and thus the most likely to give birth prematurely) and also the insertion of a cerclage or pessary and/or prescription of progesterone to women with cervical shortening who are therefore at higher risk of sPTB. Unsurprisingly, high-risk

women were more likely to subsequent have sPTB, late miscarriage and PPROM compared to low-risk women.

4.3.2 Risk factors associated with sPTB

Women who delivered prematurely (in the whole Insight cohort) were more likely to have had a history of previous sPTB/late miscarriage, as well as PPROM (although this did not reach statistical significance, likely due to smaller numbers). It is possible that PPROM and subsequent sPTB has a lower recurrence rate than sPTB due to differing pathologies; PPROM may be caused by sporadic infection which has a lower chance of recurrence in a subsequent pregnancy, compared with sPTB which may have more intrinsic pathology and higher recurrence rates. As discussed above, women with sPTB were more likely to be of Black or Asian ethnicity, have a history of UTI's as well as chronic viral infection.

The creation of a 'worse-risk factor' category amongst already high-risk women was useful to assess individual risk within an already high-risk group, where risk factors frequently overlapped. Within this already high-risk cohort, a history of cervical surgery was the least harmful risk-factor for sPTB compared to previous sPTB or late miscarriage. In contrast, a history of a late miscarriage conferred the highest risk of subsequent sPTB. It is likely that late miscarriage represents a continuum of gestational age at delivery with sPTB, and the earlier the gestation at previous delivery, the higher the risk of sPTB, as has been found in other epidemiological studies (Goldenberg et al. 1998).

A history of recurrent UTI was significantly associated with sPTB both in the high and low-risk cohorts, a risk-factor which was retained in the final model after multiple variable logistic analysis. Whilst this was a self-reported measure, and thus subject to recall bias (whereby subjects with the worst outcomes may be more liable to recall diagnosis) or detection bias, whereas the highest risk subjects may undergo more frequent medical visits and urinalysis, there is good evidence that UTI, particularly if

untreated (Smaill and Vazquez 2015) and progressing to pyelonephritis (Kass 1960; Wing, Fassett, and Getahun 2014), is associated with sPTB (Sheiner, Mazor-Drey, and Levy 2009). Recently, David and team at University College London published data showing that women with bacteriuria prior to cerclage insertion were more likely to deliver prematurely, even when treated (Kunpalin et al. 2020). Thus, screening pregnant women antenatally for UTI (often asymptomatic) forms an important part of antenatal sPTB prevention within most antenatal health systems (Goldenberg et al. 2008).

What is less clear is the mechanism by which UTI, most commonly caused by *E. coli* (Córdoba et al. 2017), leads to sPTB. It is likely that the inflammatory cascade associated with UTI, particularly when recurrent, is a potential trigger for parturition. Mouse models of UTI have demonstrated induction of sPTB with *E. coli*-UTI via urethral catheterization, demonstrating that *E. coli* expressing the adhesive molecule 'Dr adhesin' was able to colonise the kidneys of the mothers and transfer to the placenta, delivering small for gestation and preterm, whereas UTI with *E. coli* negative for this molecule did not result in sPTB (Kaul et al. 1999). In women with recurrent UTI, *E. coli* can colonise the vaginal epithelium; this happens more commonly in women with vaginal dysbiosis and a lack of acid producing lactobacilli (Gupta et al. 1998). Thus a 'healthy' vaginal microbiome may be at least partially protective against UTI's.

Perhaps surprisingly, a history of BV was not significantly associated with sPTB in this cohort. Given this was based on patient report at time of booking (see Chapter 7 for correlation with BV clinical diagnosis during the pregnancy), and that BV is often asymptomatic, we may have missed may cases of undiagnosed BV for this analysis or been hindered by poor patient recollection. As BV is more common in Black women, and our high-risk cohort comprised a higher proportion of Black women than our low-risk cohort, BV as a significant risk factor may have been lost amongst the already high-risk nature of the group. Conversely in the low-risk cohort, the event rate of sPTB

may be too small to observe effect. Large cohort, population-based studies e.g. (Purwar et al. 2001) or meta-analyses (Brocklehurst et al. 2013) which demonstrate clear association between BV and sPTB, are more appropriate to draw out risk-factors for sPTB such as BV.

It is hard to draw conclusions regarding the risk of chronic viral infection and sPTB, given the numbers of women with this condition (n=4, all HIV) were so small. Nonetheless, it is not unreasonable to consider HIV as associated with sPTB, a risk factor which persisted as statistically significant in the multiple factor logistic regression. A study (presented in abstract form and not peer reviewed) of 157 women with HIV living in an urban UK setting (Cheshire et al. 2012) found the rate of sPTB <37 weeks to be higher (11%) than that of the general UK population (7.5%), with no association with type of antiretroviral agent, though sociodemographic factors were not considered as possible confounders to explain the different rates, and iatrogenic and spontaneous delivery were not delineated. Many other global studies have similarly found an increase in rate of sPTB in women with HIV infection (Naidoo, Sartorius, and Tshimanga-Tshikala 2016; Ellis et al. 2002), potentially confounded by socio-demographics, nutritional status, antiretroviral drugs and co-existing urogenital infections (Toskine et al. 2004).

In the whole cohort (high and low-risk) smoking, BMI, domestic violence and recreational drug use were not associated with increased risk of sPTB, nor when just high-risk cohort were analysed (too few cases in low-risk cohort). These potentially modifiable risk factors are consistently cited in other (often larger cohorts) as having association with sPTB. It is likely that low prevalence of both risk factors (actual and reluctant recall) and cases of sPTB prevented us from observing a significant effect.

Ethnicity was significantly associated with risk of sPTB; both Black women and Asian women are more likely to deliver spontaneously prematurely than White women (risk differences 1.82, 95% CI 1.30 to 2.53 and 3.80, 1.91 to 7.57 respectively). Given

this (risk of sPTB Black vs. White women) is a consistent finding across populations in the UK as well as Europe and the United States of America (Section 1.3.6), both in high and low risk populations and among women of similar socioeconomic status, this was not unexpected. When the cohorts were analysed according to risk status at booking, a statistically significant association between Black ethnicity and sPTB was lost for the high-risk women, a group already over-represented with Black and Asian women (risk ratio 1.36, 95% CI 0.95-1.95), but was maintained in the low-risk population. Within the high and low-risk cohorts, however, Asian women were still more likely to deliver prematurely compared to White women (high-risk ratio 3.04, 1.40-6.59; low-risk ratio 11.57, 2.42-55.28).

Although our 'Asian ethnicity' group was diverse (comprising women of Indian, Pakistani, Bangladeshi, Far East Asian and South-East Asian self-reported ethnicities) and aggregated comparisons such as these may be misleading, as well as comprising only a small proportion of the cohort (n=31), Asian ethnicity was consistently associated with risk of sPTB in both the low and high-risk cohorts. Although findings such as these are less well established in the literature than those comparing birth outcomes for Black vs White women, they have been echoed by other studies; some finding higher risk (Li et al. 2019; Patel et al. 2004; Steer et al. 1995; Garcia et al. 2017) and some finding equivalent risk (Aveyard et al. 2002; Lyon et al. 1994; Khalil et al. 2013). One of the largest studies, a UK-based population study (Li et al. 2019) evaluating >4 million births between 2006 and 2012 and which disaggregated 'Asian' ethnicity more specifically, revealed that Asian women had a slightly higher risk of PTB <37 weeks than White women (Adjusted ORs Indian women 1.23, 1.20-1.25; Pakistani women 1.16-1.13-1.18; Bangladeshi women 1.21, 1.17-1.25), with ORs increased for sPTB at earlier gestations. They were unable to distinguish between spontaneous and iatrogenic birth in this study. Thus, the authors hypothesise that this may be related to the contribution of the higher prevalence of medical co-morbidities (such as diabetes mellitus, for which they did not adjust) in these groups of women (usually a risk factor for iatrogenic delivery rather than sPTB). However,

that higher ORs were seen for earlier sPTBs does indicate that iatrogenic birth alone is not the sole driver of these results. Indeed, a second UK based population study (n=76,158 births) found that Asian ethnicity was risk factor only for iatrogenic delivery <34 weeks of gestation (Khalil et al. 2013). It is possible that medical conditions, not captured here, contributed to our findings, though iatrogenic PTBs were excluded in our analysis.

When multivariate analysis was performed, significant associations for ethnicity and sPTB were not maintained for prediction of sPTB<37 weeks or 34 weeks in the high-risk cohort (again, likely due to the overwhelming nature of obstetric risk factors). For prediction of sPTB <37 weeks, the final regression model included only worst risk factor and a history of recurrent UTI (aOR 2.78, 1.17-6.61, p=0.04). Why was the effect of ethnicity reduced or removed when these other clinical factors were added? Firstly, there was an ethnic difference observed in women who report a history of UTI, most likely in Asian women, and least likely in Black women. In contrast, a far higher proportion of women in whom cervical surgery was their only risk factor were White. Finally, chronic viral infection was confined to Black women only. Thus it is possible that ethnicity was considered a confounder for these associations and was not included in the final model.

Risk-factors for sPTB have been well described in large population-based cohorts, comprising many more thousands of women than we have in this cohort. Nonetheless, population demographics and potential risk factors change over time (for example changing diets, BMI and exposure to air pollution and external stressors) and it is important to continually review risk factors for sPTB. Furthermore, to examine the relative contribution of risk factors within a given population (for example women 'at high-risk of PTB') is useful to compare the relative contribution of risk factors to enable us to triage women presenting to a surveillance sPTB clinic or incorporate these into a risk prediction tool. For example, the 'Quantitative Instrument for Prediction of Preterm birth (QUIPP) model (now a mobile phone app)

was designed as a risk-predictor specifically for high-risk asymptomatic women (an almost identical population to that included in the Insight study), incorporating sociodemographic information, as well as biomarker results (ultrasound cervical length measurement and qfFN) (Kuhrt et al. 2016). In this predictive model, when qfFN and CL results were incorporated, risk factors such as ethnicity, previous cervical surgery and previous late miscarriage did not add predictive value to the model (only previous sPTB/PPROM, CL and qfFN concentration were included in the final model). However, women frequently had overlapping multiple risk-factors and these were not disaggregated as we have attempted here. We have here used the concept of 'worst risk factor' to delineate which of those risk factors is most important in driving the increased risk; in this cohort of high-risk women it seems that a history of a late miscarriage is associated with the worst subsequent outcomes, which should be factored into existing clinical models. Alternatively, gestation at previous preterm delivery or late miscarriage could be assessed for inclusion as a continuous variable.

It is worthwhile noting that the high-risk cohort in this study was taken from women attending a dedicated and specialist clinic, receiving one-to-one continuity of care and informed lifestyle advice, as well as intervention to prevent sPTB as required (e.g., progesterone, cervical cerclage or the Arabin pessary). This may impact on extending gestation compared to population-based studies where women may not have access to specialist care. It would not be ethically appropriate in settings where preventative intervention is available to conduct a study without intervening if appropriate. Repeating this analysis in a larger population-based cohort of women, particularly primiparous women of multi-ethnic backgrounds, as well as those with pre-existing risk factors and previous adverse pregnancy outcomes, to accurately re-assess the current risk factors for prematurity as other population-based studies have done in the past, would be ideal. We now have the option to test the validity of this model on the entire preterm birth studies database (>3000 women), from which this Insight cohort was taken.

4.4 Conclusions

In this chapter, we have described the key risk-factors associated with sPTB in a high and low risk cohort of women seen in a tertiary hospital setting and introduced the concept of worst risk factor; any previous late miscarriage seemingly a more high-risk pregnancy history than sPTB/PPROM amongst high-risk women, and these women may warrant extra surveillance. Furthermore, a reported history of recurrent UTI's is independently associated with risk of sPTB <37 weeks in high-risk women, which warrants further investigation.

5 Gestational profile of HDPs and relationship with maternal socio-demographic variables

5.1 Introduction

As described in more detail in Chapter 1, HDPs are the body's rapid 'first defence' response to foreign antigens. Both elafin and cathelicidin have been identified as promising biomarkers to predict cervical shortening and sPTB (Abbott et al. 2014), and this will be explored in Chapters 6-8. However, the longitudinal gestational profiles of elafin and cathelicidin are worthy of description in a large cohort of high and low risk women, including how they relate to HNE, which may also emerge as an additional biomarker of interest.

The following analysis was performed on the Insight cohort of 619 women (405 high-risk, 214 low-risk), for elafin and HNE, as well as cathelicidin in high-risk women only. Far fewer low-risk women had CVF sampling between 14 and 19^{+6} weeks of gestation and so results in these gestational categories largely represent samples from high-risk women. We analysed the gestational profiles (10 to 24^{+0} weeks) of CVF protein expression of elafin and HNE in all women to assess the trends in expression, as well as the influence of demographic and social factors (risk status, ethnicity, BMI, maternal age and maternal smoking status). Cathelicidin profiles were assessed in high-risk women only. Full methods are described in Chapter 3.

5.2 Elafin

5.2.1 Gestational profile

Across gestation (10^{+0} to 24^{+0} weeks) there was a systematic decline in elafin CVF concentrations; total cohort mean values fell by approximately one half from 10-13^{+6} weeks of gestation to 20-24^{+0} weeks of gestation (101624.2 pg/ml to 47275.4 pg/ml,

multiplier 0.47 (0.418-0.517, p<0.001). CVF elafin concentration reduced by 8.2% per week (multiplier 0.92, 0.91 to 0.93, p<0.001).

5.2.2 Risk status at enrolment

There was no significant difference in CVF elafin concentration according to risk status at enrolment (Figure 5-1b).

5.2.3 Maternal BMI

CVF elafin concentration was found to be associated with maternal BMI. For every 1 kg/m^2 rise in BMI, CVF elafin concentration rose by 1.7% (1.01, 1.00 to 1.03, p=0.02). This remained significant after adjusting for ethnicity (1.02, 1.00-1.04, p=0.02). When considered categorically, a statistically significant rise in CVF elafin concentration was only associated with obese BMI; compared with BMI <25 kg/m^2, women with BMI of 30-35 kg/m^2 had 52% (1.52, 1.15 to 2.00, p=0.003) higher elafin concentrations and women with BMI >35 kg/m^2 had 65% higher elafin concentration (Figure 5-1a, 1.65, 1.21 to 2.24, p<0.001).

5.2.4 Smoking status

Elafin concentration was strongly related to smoking status. Women who smoked in their current pregnancy, compared to women who reported having never smoked, had double the CVF elafin concentration (multiplier 1.97, 1.28 to 3.20, p=0.002). There was no significant difference between women who gave up smoking before pregnancy, and women who had never smoked (1.05, 0.82 to 1.34, p=0.681). Figure 5-1c illustrates longitudinal CVF elafin concentration according to smoking status.

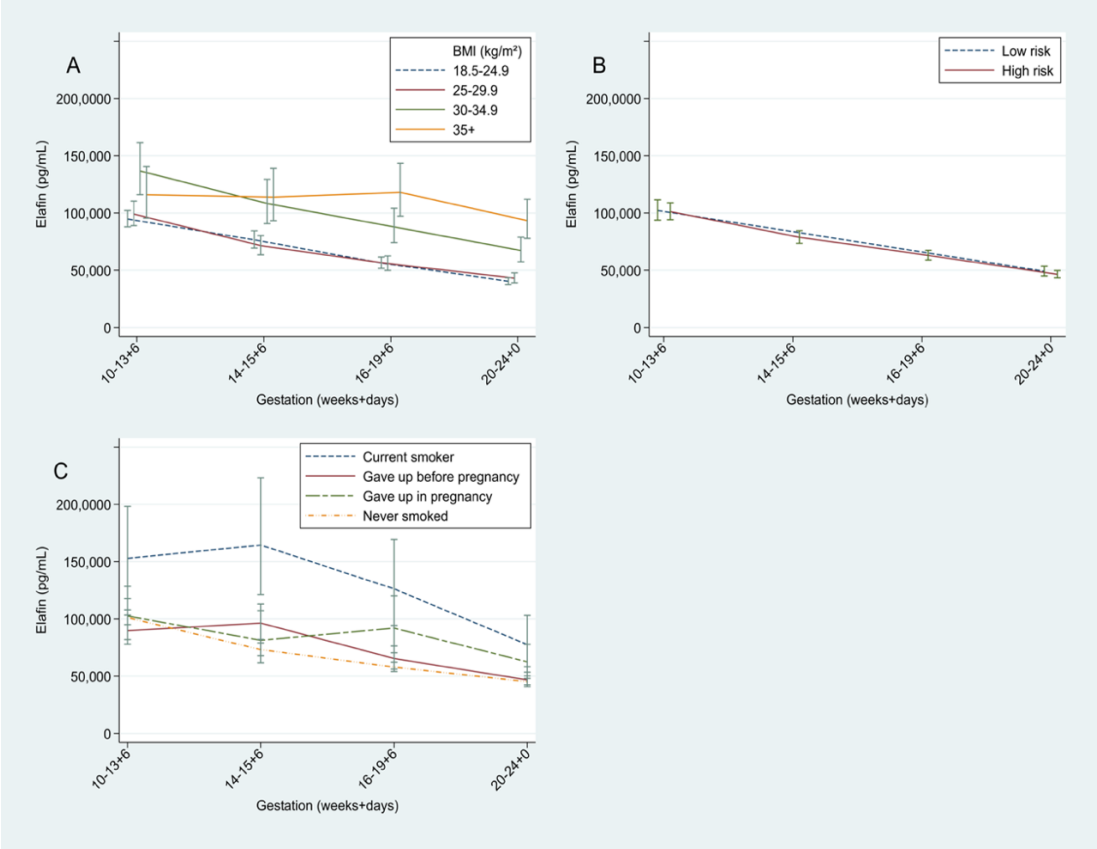

Figure 5-1. Longitudinal measurements of cervicovaginal concentrations of elafin measured by enzyme linked immunosorbent assay during pregnancy.

Geometric means with standard error bars. A. Body mass index (BMI) <24.9 (n= 303), BMI 25-29.9 (n= 163), BMI 30-34.9 (n= 68), BMI ≥35 (n= 48). B. High-risk women (n= 378), Low risk women (n= 207). C. Current smoker (n=28), ex-smoker, gave up before pregnancy (n= 95,), ex-smoker, gave up in pregnancy (n=40), Never smoked (n=454).

5.2.5 Maternal age

There was no association between elafin concentration and maternal age (<25 years vs. 25-29 years ratio 1.38, 0.91-2.09, p=0.13; <25 years vs. 35-39 years 0.87, 0.69-1.09, p=0.24; <25 vs. >40 years 0.89, 0.72 to 1.11, p=0.32).

5.2.6 Ethnicity

Figure 5-2 illustrates longitudinal CVF elafin concentration according to ethnicity. Elafin concentrations were overall 50% higher in Black women compared to White women (1.51, 1.23-1.84, p<0.001). There was no significant difference in elafin concentrations in the Asian (India, Pakistan, Bangladesh) ethnic group compared to

White women, though there was a trend towards lower concentrations in Asian women (0.82, 0.55 to 1.21), and too few women (n=36) in the 'other' category to make meaningful comparisons.

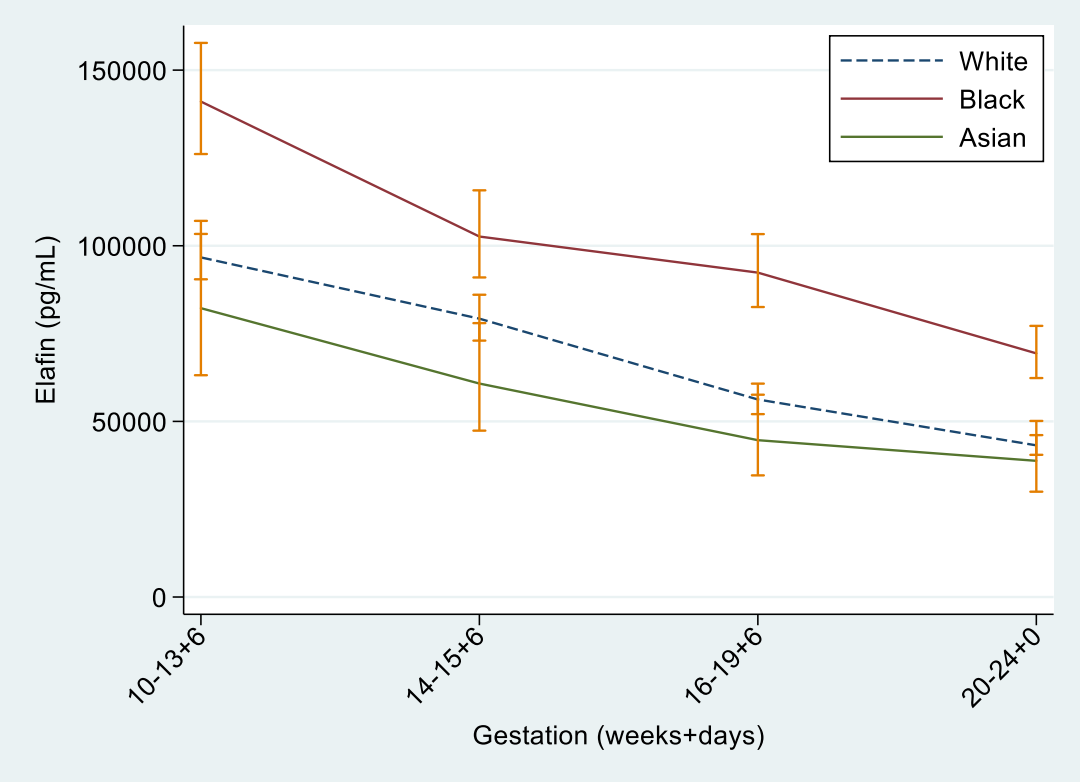

Figure 5-2. Longitudinal measurements of cervicovaginal concentrations of elafin measured by enzyme linked immunosorbent assay during pregnancy, according to ethnicity.

Geometric means ± standard error bars. Black women (n= 139, red solid line), white women (n=384 blue dashed line), Asian women (n=30 green solid line).

When BMI, current smoking status and elafin concentration were considered together using a multiple regression model, each remained independent predictors of elafin status (current smoking ratio 1.81, 1.17–2.81, p=0.008, BMI 30–35 kg/m^2 ratio 1.34, 1.01 to 1.79, p=0.04).

5.3 Cathelicidin

5.3.1 Gestational profile

CVF cathelicidin concentrations did not change significantly according to gestation in high-risk women (Figure 5-3, multiplier per week 1.01, CI 0.99 to 1.03 p=0.12).

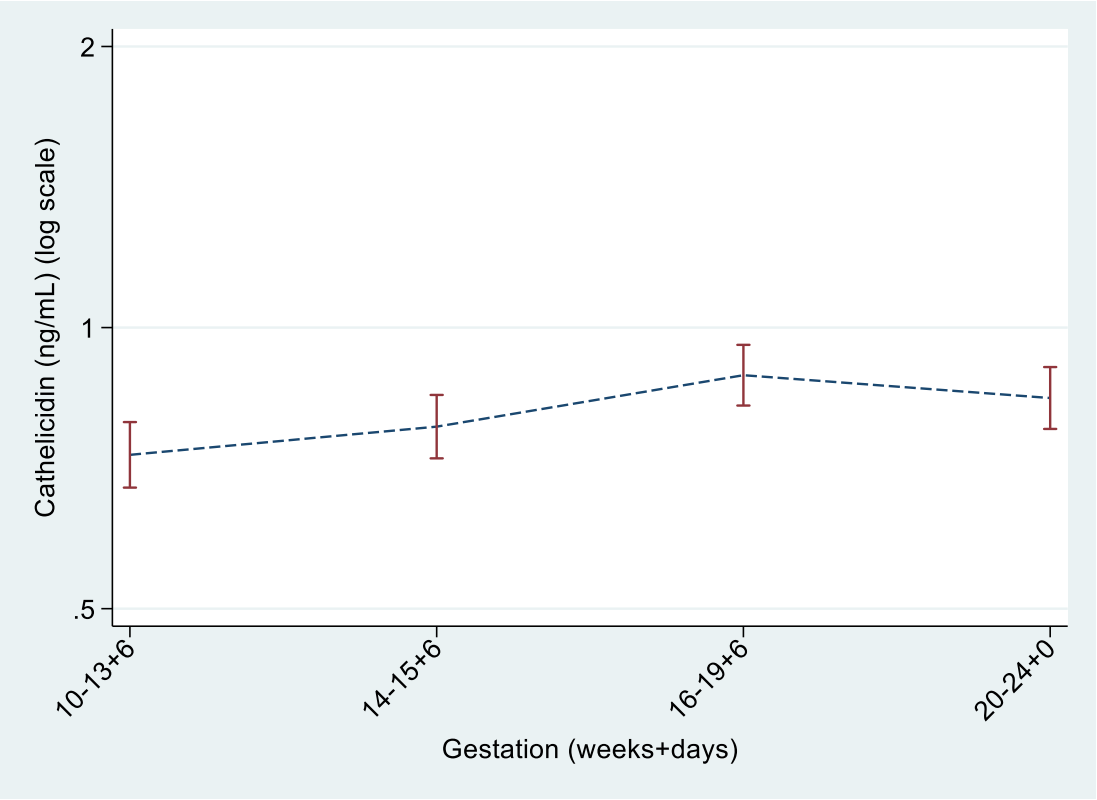

Figure 5-3. Longitudinal measurements of Cervicovaginal concentration of cathelicidin measured by enzyme linked immunosorbent assay during pregnancy in all high-risk women (n= 342).

Geometric means ± standard error bars.

5.3.2 Maternal BMI

There was no association between cathelicidin concentrations and maternal BMI (BMI <25 vs. BMI 30-35 ratio 0.97, 0.70 to 1.36, p=0.87; BMI <25 vs. BMI >35 1.29, 0.90 to 1.85, p=0.16).

5.3.3 Smoking status

There was no association between cathelicidin concentrations and smoking status (Current smoker vs. never smoked, multiplier 0.64, 0.38 to 1.08, p=0.10).

5.3.4 Maternal age

Association was noted between maternal age and CVF cathelicidin concentration; for every 10 years, CVF cathelicidin concentration reduced by 22% (37% to 4%, p=0.019).

5.3.5 Ethnicity

Cathelicidin concentrations were 32% higher in Black women compared to White women, after adjustment for gestation at visit (multiplier 1.03 to 1.67, p=0.026). Asian women had substantially higher cathelicidin levels, compared to White women (1.84, 1.15 to 2.036, p=0.01), illustrated graphically in Figure 5-4.

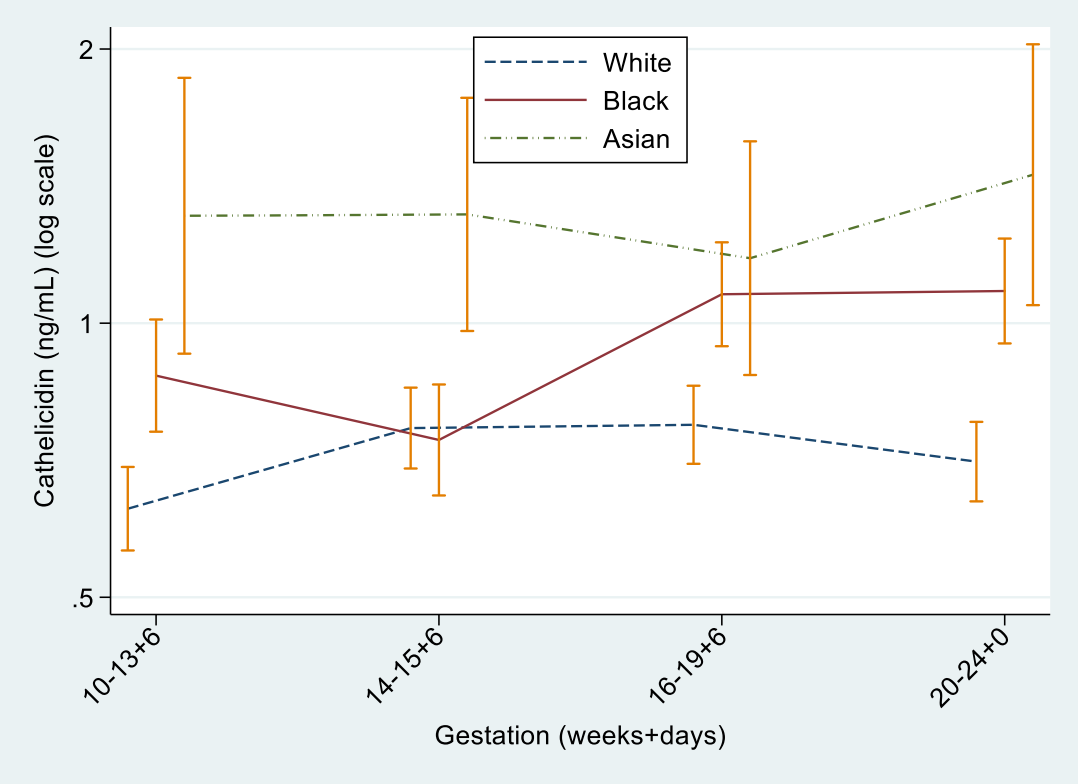

Figure 5-4. Longitudinal measurements of cervicovaginal concentrations of cathelicidin measured by enzyme linked immunosorbent assay during pregnancy according to ethnicity.

Geometric means ± standard error bars. Black women (n= 104, red solid line), White women (n=202 blue dashed line), Asian women (n=21 green dashed line).

5.4 Human neutrophil elastase

5.4.1 Gestational profile

CVF HNE concentration did not vary significantly across gestation (multiplier per week 1.01, 0.98 to 1.03, p=0.323).

5.4.2 Risk status at enrolment

CVF Concentrations of HNE were 33% higher in high-risk vs. low-risk women (Figure 5-5, 1.33, 1.01 to 1.76, p=0.04), most marked at early gestation.

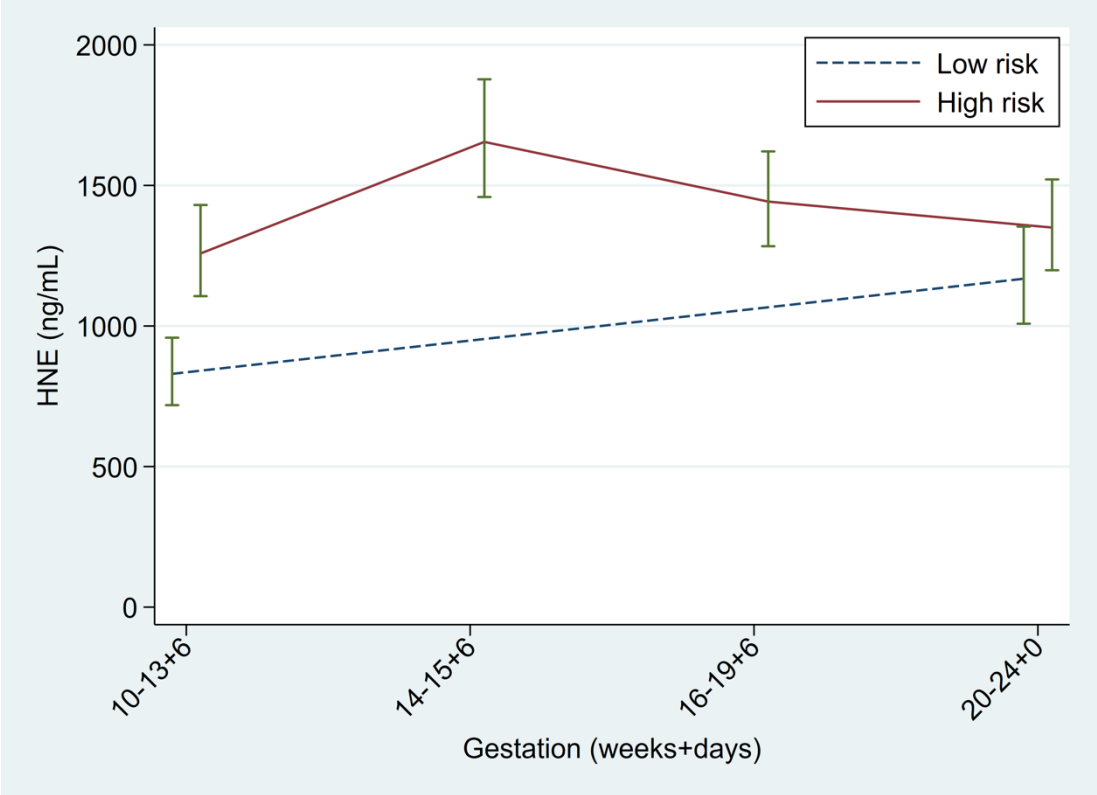

Figure 5-5. Longitudinal measurements of cervicovaginal human neutrophil elastase (HNE) measured by enzyme linked immunosorbent assay during pregnancy, according to risk status at booking.

Geometric means ± standard error bars. 3A. High-risk women (n= 358, red solid line), Low-risk women (n=205, blue dashed line).

5.4.3 Maternal BMI

There was no association between CVF HNE concentrations and maternal BMI (BMI <25 vs. BMI 30-35 ratio 0.92, 0.57 to 1.47, p=0.72; BMI <25 vs. BMI >35 1.24, 0.75 to 2.07, p=0.40).

5.4.4 Smoking status

There was no association between HNE concentrations and smoking status (Current smoker vs. never smoked; ratio 1.10, 0.53 to 2.28, p=0.80).

5.4.5 Maternal age

There was no statistically significant association between maternal age and CVF HNE concentration (<25 years vs. 25-29 years ratio 0.89, 0.44-1.80, p=0.74; <25 years vs. 35-39 years 1.20, 0.82-1.77, p=0.35; <25 vs. >40 years 1.25, 0.87 to 1.78, p=0.22).

5.4.6 Ethnicity

CVF HNE concentrations did not differ significantly between White and Black ethnicities but were significantly higher in Asian women; on average Asian women had concentrations 2.3 times that of White women (ratio 1.7 to 3.2, p=0.009), though this varied according to gestation (Figure 5-6).

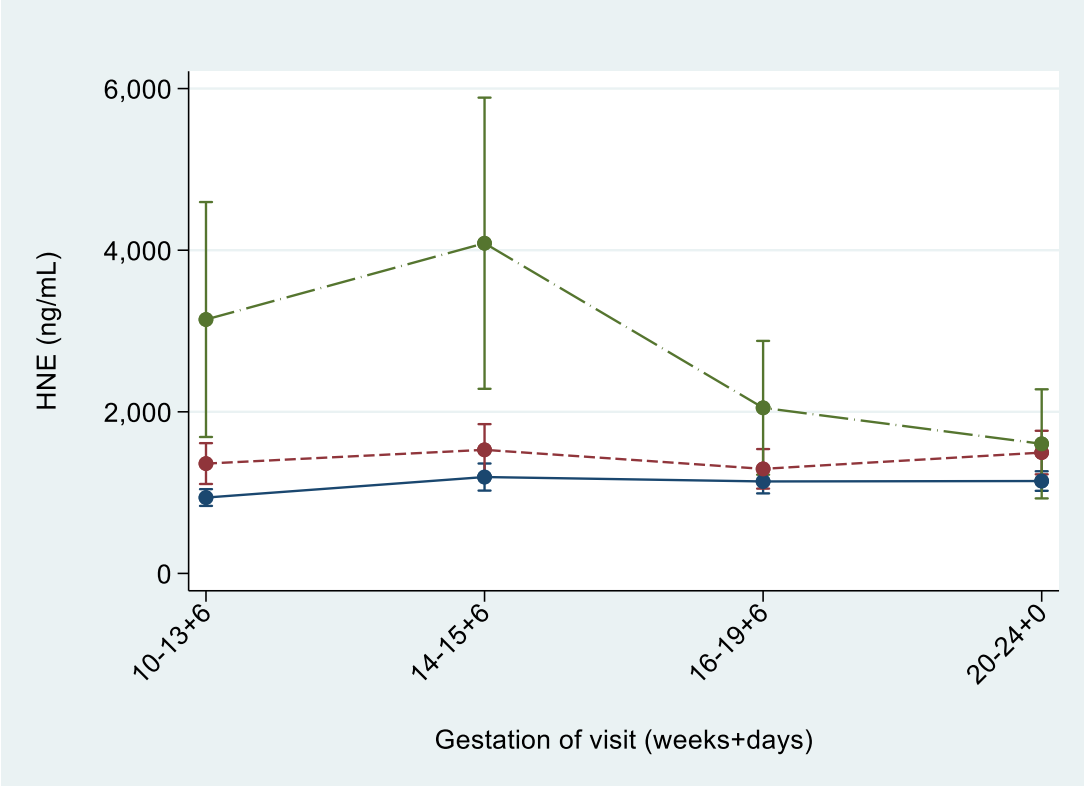

Figure 5-6. Longitudinal measurements of cervicovaginal human neutrophil elastase (HNE) measured by enzyme linked immunosorbent assay during pregnancy according to ethnicity.

Geometric means ±standard error bars. Black women (n= 134, red dash line), White women (n=369 blue solid line, Asian women (n=29 green dashed line).

5.5 Relationship between CVF elafin, cathelicidin, human neutrophil elastase and BV status

Table 5-1 describes the correlation between the CVF HDPs, as well as BV status, at each gestation. A positive correlation was detected between HNE and cathelicidin at each gestational sampling window. There was negative correlation between CVF elafin and cathelicidin concentration at early gestation (10 to 13^{+6} weeks' gestation) and a positive correlation between elafin and HNE by mid-trimester (20 to 24 weeks' gestation). Minimal statistically significant correlation between HDP and BV status was observed (further explored in Chapter 7).

Table 5-1.Correlation between host defence peptides (elafin, cathelicidin), human neutrophil elastase, and bacterial vaginosis in the whole cohort

Gestation at visit (+weeks)		Cathelicidin	HNE	BV
10 to 13^{+6}	Elafin	-0.11 (1.43)	0.08 (1.30)	-0.08 (0.13)
	Cathelicidin	-	**0.31 (<0.001)**	0.08 (0.28)
	HNE	-	-	-0.50 (0.30)
14 to 15^{+6}	Elafin	-0.16 (0.02)	0.02 (0.74)	-0.07 (0.27)
	Cathelicidin	-	**0.47 (<0.001)**	**0.14 (0.04)**
	HNE	-	-	0.04 (0.53)
16 to 19^{+6}	Elafin	-0.11 (0.17)	0.05 (0.50)	-0.06 (0.37)
	Cathelicidin	-	**0.47 (<0.001)**	0.08 (0.29)
	HNE	-	-	0.01 (0.92)
20 to 24^{+0}	Elafin	-0.09 (0.42)	**0.22 (<0.001)**	0.06 (0.33)
	Cathelicidin	-	0.2 (0.07)	0.06 (0.59)
	HNE	-	-	-0.02 (0.78)

Data are Spearman's Correlation Coefficient (p value). HNE=human neutrophil elastase. ***Statistically significant correlations are shown in bold***

5.6 Discussion

This chapter describes the longitudinal expression of HDPs of the innate immune system, together with HNE, in the early to mid-trimester of pregnancy, including how they relate to each other. It demonstrates that HDPs are modified by maternal demographics and characteristics.

Elafin was shown to be expressed most highly in early pregnancy, reducing as pregnancy progressed. This may well reflect the suppression of elafin expression by increasing circulating oestrogen (predominantly in the form of oestriol) in advancing pregnancy. There is strong evidence in the scientific literature that elafin concentration is affected by steroid sex hormones. Patel et al. (2013), studying cultured vaginal epithelial cells (collected by menstrual cup), demonstrated that

treatment with oestradiol (but not progesterone) dramatically reduced secretion of elafin, as well as human beta defensin-2 (measured in cell culture material by ELISA). Ghosh et al. (2010) found significantly higher levels of elafin protein in cervico-lavage samples, during the secretory phase of the menstrual cycle (low oestrogen) compared with the proliferative phase of the menstrual cycle. Kumar et al. (2011) showed that oestrogen-based hormone replacement therapy (HRT) supressed the expression of another whey acidic innate defence proteins SLPI in the vaginal and ectocervical epithelium from samples gained from post-menopausal women.

It is possible that these changes in elafin concentration are a result of direct effects of oestradiol on epithelial cells through oestrogen receptor α/β (Ghosh et al. 2010). There may also be specific oestrogen response elements in the elafin gene, though this has not been described. Alternatively, the effects may be indirect by hormone suppression of epithelial cell pattern-recognition receptors (particularly toll-like receptors) which regulate elafin production (Aflatoonian et al. 2007; Hirata et al. 2007). Cytokine and chemokine expression are also under hormonal influence; for example, oestradiol inhibits monocyte chemotactic protein 1 (MCP-1) expression in endometrial cells (Arici et al. 1999). It may therefore be that an oestrogen-mediated reduction in cytokines and chemokines, known to promote elafin production and activity, may contribute to a reduction in elafin concentration across pregnancy.

An alternative explanation for the reducing CVF elafin concentration as pregnancy progresses could also be related to the vaginal microbiome. Reduced oestrogen and glycogen in the menopausal vagina is associated with a microbiome shift to fewer lactobacilli and more anaerobic bacteria (Brotman, Shardell, et al. 2014), and dysbiosis is associated with reduced CVF elafin concentrations (Stock et al. 2009). Therefore, in a cohort such as ours which contains a number of high-risk women (particularly Black women with high sPTB rate), increasing vaginal dysbiosis (and associated risk of sPTB) as pregnancy progresses may be contributing to the

gestationally reduced elafin. Further investigation, with correlation between outcome and the vaginal environment is clearly warranted (Chapters 6 and 7).

How does this fit with the observation that, unlike elafin, cathelicidin (and HNE) did not significantly fluctuate between early and mid-gestation? Whilst immune cells in the endometrium are known to fluctuate throughout a woman's menstrual cycle (for example during the late secretory phase and menstruation when both oestrogen and progesterone levels are low, immune cell numbers increase) (King and Critchley 2010; Yeaman et al. 2001; Evans and Salamonsen 2012), sex hormone concentrations do not appear to affect immune cell populations (for example neutrophils from which cathelicidin and HNE originate) in the lower reproductive tract, which are maintained at a similar concentration throughout the reproductive cycle (Pudney, Quayle, and Anderson 2005). This may be why cathelicidin and HNE, originating from neutrophils, concentration in the CVF show minimal change here.

High elafin concentrations were independently associated with smoking and obesity. Given that elafin is released from epithelial cells as a protective response in response to inflammatory mediators (or directly induced by bacterial infection), this finding fits with the 'sterile inflammation' hypothesis, driven by increased adipose tissue and stimulated by components of cigarette smoke. Menon's research group used cigarette smoke extract to elegantly demonstrate the impact of sterile inflammation on an *in vitro* model of fetal membrane explants and primary amnion epithelial cells. They observed the generation of reactive oxygen species (ROS) (using immunofluorescence and inhibition with the antioxidant N-acetyl cysteine), in amnion cell cultures incubated with smoke extract, higher than those incubated with LPS (mimicking infective inflammation) (Behnia, Sheller, and Menon 2016). ROS cause cellular DNA damage (strand breaks observed using Comet silver staining) and stimulation of pro-inflammatory mediators, which likely result in increased elafin secretion. Excess adiposity has been associated with local and systemic low-grade inflammation, with infiltration of immune cells into adipose tissue and systemic

secretion of pro-inflammatory components (Neels and Olefsky 2006) and DAMPS (Romero, Miranda, et al. 2014).

Consistent with our findings, Pierson et al. (2013) observed that cigarette smoke extract upregulated gene expression of the antimicrobial peptides HBD 3, 5 and 9 in human lung alveolar cells, but that cathelicidin gene expression was not affected, though it was induced by IL1-ß treatment. So why should cathelicidin (and HNE) expression be unaffected by sterile inflammatory states, given that elafin and cathelicidin are both stimulated by the inflammatory cytokines and chemokines, produced in both sterile and infective inflammation? Focussing just on epithelial cells, *in vitro*, elafin and cathelicidin are differentially regulated by cytokines, endotoxins and vitamin D; elafin mRNA and protein are directly stimulated by pro-inflammatory cytokines and LPS, contrasting with cathelicidin, stimulated only by 1,25-(OH)2 (Chin-Smith et al. 2017). Cathelicidin *in vivo* is, however, correlated with CVF inflammatory cytokines (Abbott et al. 2014); but this relationship probably is driven by cytokines attracting neutrophils which in turn release cathelicidin. It may be that the cytokines involved in the sterile and infective pathway are different, or that the sterile inflammatory response is milder for cathelicidin and HNE compared with elafin. More speculatively, cathelicidin gene expression (in murine models) has been shown to be dependent on NF-kB and associated pathways (Li et al. 2009). Given that activation of NF-kB predominates in infective inflammatory pathways, but not sterile inflammatory pathways (Behnia, Sheller, and Menon 2016), it may be that that this accounts for the differing response to sterile inflammation between host defence peptides. In contrast, whilst binding sites for NF-kB do exist in the elafin gene (Clauss et al. 2006), and elafin can inhibit NF-kB, there is no evidence that elafin expression is dependent on NF-kB. It is also possible that the association of smoking with high elafin is mediated at least in part by the anti-oestrogenic effects of smoking (altered oestrogen metabolism and receptors) (Shearman et al. 2005), in a way which cathelicidin and HNE are not (as discussed above).

Despite no observed relationship in this cohort between cathelicidin and BMI, Zhang, Guerrero-Juarez, et al. (2015) demonstrated the expression of cathelicidin (and also HBD) mRNA from cultured adipocytes. They also observed serum cathelicidin to be elevated in mice fed a high fat diet vs normal diet, as well as in overweight human individuals (BMI >25) vs normal BMI (<25) (though only 10 human subjects in each group were examined). If this is the case *in vivo*, it may be that serum cathelicidin but not CVF cathelicidin is affected by BMI. More consistent with our findings, Hochberg et al. (2021) observed that whilst BMI was associated with increased cathelicidin mRNA expression in human visceral adipose tissue (resected during bariatric surgery), *in vivo*, serum concentrations of cathelicidin did not vary according to BMI (though all samples were within the high BMI range 31 to 60). They did also note that sex hormones did not affect CAMP expression from adipocytes, which correlates with the observations in our study of minimal change in cathelicidin concentration across gestation.

To our knowledge, this is the first report to describe ethnic variations in expression of elafin (as well as cathelicidin and HNE) throughout pregnancy. In this mixed cohort of high and low-risk women, elafin concentrations were overall 50% higher in Black women compared to White women, and cathelicidin 32% higher in Black women compared with White women. This was an unexpected finding. As previously discussed, women with BV (more prevalent in Black women than White women) have lower concentrations of CVF elafin than women without BV (seen in this cohort, Chapter 7), thus we expected overall CVF elafin to be lower in Black women compared with White women. However, the vaginal microbiota is more complex than just BV or no BV. Compared with White women, who tend to have a vaginal microbiome dominated by *Lactobacillus spp*., the most common vaginal environment (both pre-pregnancy and during pregnancy) in Black women is characterised by non-lactobacillus dominant mixed bacterial communities (Callahan et al. 2017; Zhou et al. 2010; Fettweis et al. 2019). It may be that such dysbiotic vaginal microbiota (perhaps on a continuum with BV) can generate local inflammation (Ma, Forney, and Ravel

2012) inducing HDPs such as elafin and cathelicidin. In contrast, higher cathelicidin concentrations are associated with BV (Frew et al. 2014) supporting the inflammatory hypothesis. The relationship with sPTB will be explored in Chapter 7.

We further explored these findings (Flaviani et al. 2021) in a sub-set of this Insight cohort by correlating CVF elafin, cathelicidin and HNE concentrations with the vaginal microbiota (16S pyrosequencing) and bacterial metabolites. CVF Cathelicidin concentrations were clearly affected by the principal components analysis (PCoA) group which dominated the vaginal microbiota; they were raised in PCoA C (*L. iners*) and D (diverse bacteria), both more common in Black women vs. White women, compared to PCoA A (*L. crispatus*). Elafin CVF concentrations, in contrast, were slightly raised in women with PCoA A (predominantly White women) compared with B (*L. gasseri*) and D. Furthermore, mean elafin concentration was similar in PCoA A and PCoA C. This correlates with the observation that CVF elafin is lower in women with BV as well as being consistent with the higher concentrations of CVF cathelicidin observed in Black women in our cohort. Whilst it does not explain the higher concentration of CVF elafin seen in Black women, many Black women in this cohort had microbiota characterised by PCoA C (*L. iners*), which was characterised by similar mean elafin concentration as PCoA A, thus mean elafin was not significantly depressed by BV/dysbiosis in this population of Black women. Furthermore, elafin was shown to positively correlate with CVF bacterial metabolites associated with bacterial health (lactate, aspartate, leucine). It may be that raised elafin in Black women compared with White women represents a way for the innate immune system to counteract the inflammatory effects of vaginal dysbiosis, and maintain vaginal health for most women, potentially avoiding negative reproductive outcomes. It is also probable that other ethnicity dependent host factors, as well as the microbial environment, are influencing the HDP response, including factors not measured in this study, e.g., we did not collect data on stress (reported or objectively measured using cortisol) which is frequently hypothesised to underlie ethnic

differences in pregnancy outcome (See Section 1.3.7), nor have we investigated the role of genetic factors.

Ethnic differences in biomarker expression in CVF in relation to sPTB have been previously highlighted; Menon et al. (2014) demonstrated racial disparity in the expression of maternal and cord plasma biomarkers (36 biomarkers using a protein microarray platform from 191 women) in African American and Caucasian women, with several markers associated with sPTB entirely unique to the racial group. More recently a case-control study of 107 sPTB and 407 term births found a similar ethnic variation in the expression of the host defence peptide human beta defensin 2 (HBD2), to that found with elafin in this study; CVF HBD2 at 16 to 20 weeks' gestation was low in African American women who delivered prematurely but not in non-African American women (Elovitz et al. 2019). This will be further explored in our cohort when relationship to pregnancy outcome is explored in Chapter 6.

With respect to HDP correlations with each other, expression of HNE and cathelicidin were positively correlated, particularly at 10-20 weeks. This was unsurprising, as cathelicidin is co-released with HNE from human neutrophils (Sørensen et al. 2001). Mechanistically, HNE is thought to be inhibited by elafin and vice versa (Fitch et al. 2006), and thus we anticipated a negative correlation between the two proteins. However, minimal relationship between the two was observed until after 20 weeks, when a positive correlation was observed. *In vitro,* pulmonary epithelial cell lines demonstrated increased gene expression of elafin mRNA when stimulated by HNE (a key effector of inflammation related lung injury), but downregulation of the secreted protein product itself (Reid et al. 1999). The authors of this paper consider whether this could be because elafin is synthesised for intracellular use, or whether a proportion of the secreted protein remains bound to the cell surface and is thus unmeasurable in secretions. This study, to our knowledge, is the first to correlate these peptides *in vivo* and it may be that inhibition of HNE by elafin (or vice versa) is concentration dependent and thus not easily discernible by simple correlation

coefficients. In this cohort, CVF cathelicidin and elafin concentrations have small, but significant, negative correlations with each other. This may be driven by the vaginal microbiota or other external factors as discussed above. This is explored further in Chapter 7.

5.7 Conclusions

Elafin, cathelicidin and HNE have distinct gestational profiles and relationships to each other. As pregnancy progresses, CVF elafin concentration reduces, and is associated with smoking and obesity, possibly via sterile inflammatory induction. Cathelicidin and HNE concentration remain more stable as pregnancy progresses and do not have an association with smoking or obesity. Cathelicidin and elafin had distinct ethnic profiles; Black women had higher concentrations than White women across gestation, which may be related to underlying inflammation, particularly vaginal dysbiosis; this will be explored further in relationship to pregnancy outcome and vaginal environment in forthcoming chapters.

6 Host response peptide expression; association with pregnancy outcome

6.1 Introduction

A primary aim of this study was, in an appropriately powered cohort, to validate the promising findings of the CLIC study; that concentration of CVF elafin (when measured in early pregnancy) predicted those women who would develop cervical shortening and subsequent sPTB.

Full methodology is detailed in Chapter 3. The following analysis was performed on the cohort of 619 women (405 high-risk, 214 low-risk), for elafin and HNE. Measurement of CVF cathelicidin was performed on high-risk women only). Primary outcome data was obtained for 611 of these women; 79 of these women experienced sPTB (75 high-risk women) prior to 37 weeks (45 cases from high-risk women were required to give the validation study adequate power). Given that most low-risk women only provided samples at 10 to 13^{+6} weeks of gestation and 20 to 24^{+0} weeks of gestation, gestational results for low-risk women and the whole cohort combined are only reported for these gestational categories.

Analysis was repeated in very-high-risk (previous sPTB, PPROM or late miscarriage) women only (at all four gestational categories), as the pathogenesis of sPTB is plausibly different in women with recurrent sPTB, and this also replicated the analysis performed in the pilot study. To assess the impact of intervention (cerclage, progesterone or Arabin pessary) on HDP concentrations, the prediction analysis was repeated with all samples taken after intervention excluded. This was particularly important since the pilot study excluded women from study entry who had intervention in place at study entry and randomised them to intervention only when cervical shortening was detected. In contrast, many of the women in the insight cohort had a prophylactic stitch *in situ* from early gestation or were using vaginal

progesterone pessaries. Given the noticeable effect of ethnicity on HDP concentration (Chapter 5), and the well-described ethnic disparity in sPTB (section 1.3.6) sub-group analysis was also performed according to ethnicity (Black vs. White ethnicity).

6.2 HDPs, sPTB, cervical length and adverse maternal/fetal outcomes

6.2.1 Elafin

6.2.1.1 *Whole cohort and adverse pregnancy outcomes*

In the whole cohort (high and low-risk women), mean CVF elafin concentrations were not significantly different in women who delivered prematurely, and those who delivered at term, neither when analysed crudely, nor after adjusting for maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled elafin concentration. Similarly, elafin concentrations were not associated with the pre-defined composite adverse maternal/fetal outcomes (Table 6-1). When stratified by gestation at sampling, elafin concentration was not different in women who delivered prematurely nor who experienced adverse fetal outcomes (Table 6-1), nor when stratified according to high-risk status at enrolment (Table 6-2). Furthermore, elafin CVF concentration was not predictive (ROC analysis) for sPTB before <37 or <34 weeks of gestation, nor composite maternal fetal/adverse outcomes (Table 6-3), nor was it predictive in high-risk women only (Table 6-4).

To closely mirror the cohort in the pilot study, prediction analysis was repeated only on women at the highest risk of sPTB (one or more of previous sPTB, PPROM or late miscarriage), excluding those with previous cervical surgery or Mullerian tract abnormalities who may have a different mechanism behind sPTB. In these women, CVF elafin concentration when taken at 20-24 weeks of gestation was related to outcome in later pregnancy; high CVF elafin was modestly predictive of PPROM

(n=200, AUC ROC 0.59, 95% CI 0.52 to 0.66) and objective infection (n=206, 0.63, 0.56-0.70) (Table 6-5).

6.2.1.2 *High-risk women and cervical shortening*

In contrast to our pilot study, mean elafin CVF concentrations in the whole cohort were not significantly different between high-risk women who developed a short cervix and high-risk controls with a normal CL, after adjusting for maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled elafin concentration (n= 361 ratio 0.97 95% CI 0.74 to 1.27, p=0.30), nor were they different at any of the gestational sampling timepoints. (Table 6-2). High CVF elafin was mildly predictive of cervical shortening in high-risk women when measured in early gestation (10-13^{+6} weeks) (n=232, AUC ROC 0.58, 95% CI 0.52-0.65). When analysis was repeated in only the women of the highest risk of sPTB, a similar AUC was achieved for prediction of cervical shortening using CVF elafin concentration (10-13^{+6} weeks), but this no longer reached statistical significance due to the reduced sample size (n=165, 0.58, 0.50 to 0.65) (Table 6-4). At 20-24 weeks of gestation, CVF elafin modestly predicted cervical shortening with an AUC of 0.59 (n=207, 95% CI 0.52 to 0.66) (Table 6-5).

Table 6-1. Ratio (95% confidence intervals) of the logged mean CVF elafin concentration in cases and controls overall, and stratified by gestation at sampling in the whole cohort (high-risk and low risk)

Outcome	Ratio (95% Confidence Interval) of elafin concentration cases:controls						
				Gestation category (weeks^{+days})			
	N (overall)	**Overall**		**10-13^{+6}**		**20-24^{+0}**	
		unadjusted	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**
sPTB <37	553	0.93 (0.72-1.20)	0.95 (0.74-1.22)	0.91 (0.64-1.28)	0.86 (0.61-1.22)	0.97 (0.69-1.37)	1.09 (0.77-1.54)
sPTB <34	562	1.12 (0.79-1.60)	1.05 (0.75-1.48)	1.28 (0.79-2.09)	1.14 (0.69-1.88)	1.03 (0.61-0.74)	1.10 (0.66-1.84)
PPROM	565	1.08 (1.78-1.50)	1.00 (0.73-1.37)	1.02 (0.66-1.55)	0.86 (0.57-1.31)	1.01 (0.64-1.59)	1.10 (0.70-1.73)
Objective infection	580	0.97 (0.74-1.28	0.94 (0.73-1.23)	0.93 (0.64-1.35)	0.86 (0.60-1.24)	1.17 (0.82-1.67)	1.18 (0.84-1.66)
Fetal adverse outcome	580	0.91 (0.70-1.19)	0.92 (0.72-1.20)	0.97 (0.67-1.41)	0.94 (0.65-1.37)	0.81 (0.57-1.15)	0.85 (0.60-1.20)

Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, body mass index, smoking and inter-plate pooled elafin concentration. Statistically significant ratios are shown in bold. CVF=cervicovaginal fluid; sPTB=spontaneous preterm birth; PPROM=premature prelabour rupture of membranes.

Table 6-2. Ratio (95% confidence intervals) of the logged mean CVF elafin concentration in cases and controls overall, and stratified by gestation at sampling in the high-risk cohort

Outcome		Ratio (95% Confidence Interval) of elafin concentration cases: controls									
		Gestation category (weeks^{+days})									
	N (overall)	**Overall**		**10-13^{+6}**		**14-15^{+6}**		**16-19^{+6}**		**20-24**	
		unadjusted	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**
Cervical shortening <25mm	361	1.14 (0.86-1.50)	0.97 (0.74-1.27)	1.31 (0.85-2.04)	1.09 (0.71-1.68)	0.83 (0.57-1.21)	0.74 (0.51-1.07)	0.93 (0.67-1.29)	0.80 (0.57-1.11)	1.15 (0.82-1.61)	0.97 (0.69-1.37)
sPTB <37	359	0.91 (0.69-1.21)	0.96 (0.74-1.27)	0.89 (0.59-1.35)	0.91 (0.61-1.36)	0.89 (0.60-1.31)	1.11 (0.76-1.63)	0.85 (0.60-1.20)	0.98 (0.70-1.37)	0.94 (0.65-1.35)	1.11 (0.77-1.58)
sPTB <34	367	1.13 (0.78-1.63)	1.07 (0.75-1.52)	1.32 (0.77-2.27)	1.14 (0.67-1.94)	1.15 (0.71-1.87)	1.33 (0.84-2.12)	0.98 (0.61-1.58)	1.03 (0.65-1.62)	1.02 (0.60-1.72)	1.15 (0.70-1.91)
PPROM	366	1.14 (0.80-1.64)	1.07 (0.76-1.51)	1.08 (0.65-1.81)	0.93 (0.58-1.50)	1.17 (0.70-1.95)	1.30 (0.79-2.15)	0.98 (0.61-1.56)	1.03 (0.66-1.62)	1.08 (0.66-1.78)	1.25 (0.77-2.02)
Objective infection	375	0.95 (0.68-1.33)	0.91 (0.66-1.24)	0.91 (0.55-1.50)	0.80 (0.50-1.28)	0.90 (0.57-1.43)	0.89 (0.58-1.37)	0.92 (0.60-1.42)	0.91 (0.61-1.37)	1.18 (0.77-1.81)	1.21 (0.80-1.82)
Fetal adverse outcome	375	0.95 (0.70-1.28)	1.02 (0.76-1.36)	1.08 (0.68-1.72)	1.12 (0.72-1.74)	0.90 (0.59-1.36)	1.07 (0.72-1.59)	0.88 (0.61-1.28)	0.94 (0.66-1.35)	0.83 (0.56-1.23)	0.96 (0.66-1.41)

Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, body mass index, smoking and inter-plate pooled elafin concentration. CVF=cervicovaginal fluid; sPTB=spontaneous preterm birth; PPROM=premature prelabour rupture of membranes. Statistically significant ratios are shown in bold.

Table 6-3. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome in all women, using CVF elafin concentration stratified by gestation

Outcome	**Elafin Receiver Operating Characteristic Area Under the Curve (95% Confidence Interval)**	
Gestation category (weeks^{+days})	**10-13^{+6}**	**20-24^{+0}**
sPTB <37	0.46 (0.42-0.51)	0.48 (0.44-0.53)
sPTB <34	0.54 (0.49-0.59)	0.50 (0.45-0.55)
PPROM	0.50 (0.45-0.55)	0.50 (0.45-0.54)
Objective infection	0.48 (0.43-0.53)	0.52 (0.47-0.57)
Fetal adverse outcomes	0.49(0.44 -0.54)	0.43 (0.38-0.47)

Statistically significant AUCs are shown in bold. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes

Table 6-4. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome in high-risk women, using CVF elafin concentration stratified by gestation

Outcome	**Elafin Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)**			
Gestation category (weeks^{+days})	**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24^{+0}**
Cervical shortening <25 mm	**0.58 (0.52-0.65)**	0.47 (0.41-0.53)	0.50 (0.44-0.56)	0.56 (0.50-0.62)
sPTB <37	0.46(0.39-0.53)	0.48(0.41-0.54)	0.48 (0.42-0.54)	0.48 (0.43-0.55)
sPTB <34	0.54(0.48-0.61)	0.55 (0.49-0.61)	0.51 (0.45-0.57)	0.52 (0.45-0.57)
PPROM	0.52(0.45-0.58)	0.52 (0.46-0.58)	0.49(0.43-0.55)	0.53 (0.47-0.59)
Objective infection	0.47 (0.40-0.53)	0.48(0.42-0.54)	0.47(0.41-0.53)	0.52 (0.46-0.58)
Fetal adverse outcomes	0.51(0.45-0.58)	0.47(0.40-0.53)	0.49(0.43-0.55)	0.44(0.38-0.50)

Statistically significant AUCs are shown in bold. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes.

Table 6-5. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome in very-high-risk women, using CVF elafin concentration stratified by gestation at sampling

Outcome	**Elafin Receiver Operating Characteristic Area Under the Curve (95% Confidence Interval)**			
Gestation category (weeks^{+days})	**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24^{+0}**
Cervical shortening <25 mm	0.58 (0.50-0.65)	0.45 (0.38-0.52)	0.50 (0.43-0.57)	**0.59 (0.52-0.66)**
sPTB <37	0.45 (0.38-0.54)	0.48 (0.40-0.55)	0.48 (0.41-0.55)	0.50 (0.43-0.57`)
sPTB <34	0.51 (0.43-0.59)	0.57 (0.49-0.64)	0.51 (0.45-0.59)	0.53 (0.46-0.60)
PPROM	0.56 (0.48-0.64)	0.56 (0.48- 0.63)	0.51 (0.44-0.59)	**0.59 (0.52- 0.66)**
Objective infection	0.56 (0.48-0.63)	0.53 (0.46-0.61)	0.55 (0.48-0.61)	**0.63 (0.56-0.70)**
Fetal adverse outcomes	0.53 (0.45-0.60)	0.48 (0.41-0.55)	0.51 (0.44-0.58)	0.45 (0.38-0.52)

Statistically significant AUCs are shown in bold. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes.

6.2.1.3 *Exclusion of samples taken after prophylactic intervention*

Due to the potential interaction effects with intervention to prevent sPTB, HDP concentrations and pregnancy outcome, analysis was repeated after exclusion of all samples taken after a woman received an intervention to prevent sPTB (cerclage, Arabin pessary or progesterone pessary).

Table 6-6 displays the unadjusted and adjusted elafin concentration case-control ratios, after exclusion of samples taken after intervention was received. Unadjusted elafin concentrations measured at 10-13^{+6} weeks' gestation were higher overall in women who delivered prior to 34 weeks' gestation (n=390, ratio 1.76, 95% CI 1.00 to 3.10, p=0.05) though this did not quite reach statistical significance, nor after adjustment (n=387, 1.59, 0.93-2.80). Among high-risk women, this pattern was similar (Table 6-7). Surprisingly, in the high-risk cohort the adjusted elafin concentration was lower when measured at 16-19^{+6} weeks of gestation in women who developed a short cervix (n=235, 0.64, 0.41 to 0.98, p=0.04) compared to women who did not develop a short cervix (Table 6-7).

Table 6-8 displays the ROC AUC for each outcome and gestation in all women using samples taken prior to intervention, and Table 6-9 shows ROC AUC for high-risk women only. In the whole cohort, CVF elafin was moderately predictive of sPTB <34 weeks (10 to 13^{+6} weeks of gestation n=386, AUC 0.64, 95% CI 0.59 to 0.69; 20 to 23^{+6} weeks of gestation n=387, 0.62, 0.57 to 0.67, Table 7-8). It was not predictive of sPTB <37 weeks, cervical shortening, or adverse fetal/maternal outcomes. In high-risk women (Table 6-9), high elafin concentration was moderately predictive of subsequent sPTB <34 weeks of gestation when sampled at 10 to 13^{+6} weeks of gestation (n=202, 0.63, 0.56 to 0.70), 14 to 15^{+6} weeks of gestation (n=211, 0.61, 0.54 to 0.67) and 20 to 23^{+6} weeks of gestation (0.64, 0.58 to 0.71). High elafin when measured at 10-13^{+6} weeks predicted cervical shortening with AUC ROC 0.60, 95% CI 0.53 to 0.66 (n=203) and adverse fetal outcome (n=207, AUC 0.60, 0.53 to 0.67). At 20 to 24 weeks of gestation, high elafin was also predictive of PPROM (n=205, 0.61,

0.53 to 0.67), but not at other gestational time points nor for other maternal/fetal outcomes (Table 6-9).

In women who had had a previous sPTB or previous late miscarriage (comprising the 'highest risk group' Table 6-10), high elafin was also moderately predictive of sPTB <34 weeks when taken at 10-13^{+6} weeks (n=140, 0.60, 0.51 to 0.68), 14-15^{+6} weeks of gestation (n=145, 0.56 to 0.72) and 20 to 24 weeks of gestation (n=131, 0.70, 0.62 to 0.78). It was also a good predictor of PPROM when taken at 20-24 weeks (n=131, 0.73, 0.64 to 0.81) and modest predictor of maternal/fetal objective infection when taken at 16 to 19^{+6} weeks (n=154, 0.62, 0.54 to 0.69), and 20 to 24 weeks (n=132, 0.69, 0.60-0.77). For prediction of cervical shortening, a slight improvement in prediction was seen for elafin concentration measured at 10 to 13^{+6} weeks of gestation, when samples taken after intervention had been commenced were removed (n=140, 0.60, 0.51 to 0.68).

Table 6-6. Ratio (95% confidence intervals) of the logged mean CVF elafin concentration in cases and controls overall, and stratified by gestation at sampling in all women, after post intervention samples were excluded

Outcome	Ratio (95% Confidence Interval) of elafin concentration cases: controls**						
				Gestation category (weeks^{+days})			
	N (overall)	Overall		$10\text{-}13^{+6}$		$20\text{-}24^{+0}$	
		unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted
sPTB <37	517	1.09 (0.81-1.45)	1.07 (0.81-1.41)	1.02 (0.70-1.50)	0.96 (0.66-1.41)	1.09 (0.70-1.72)	1.24 (0.80-1.93)
sPTB <34	525	**1.63 (1.06- 2.50)**	1.40 (0.93-2.11)	1.76 (1.00-3.10)	1.59 (0.90-2.80)	1.68 (0.74-3.80)	1.72 (0.78-3.76)
PPROM	530	1.24 (0.85-1.82)	1.11 (0.77-1.60)	1.12 (0.71-1.80)	0.97 (0.61-1.54)	1.19 (0.64-2.21)	1.41 (0.77-2.58)
Objective infection	536	1.03 (0.76-1.39)	1.00 (0.75-1.34)	0.92 (0.62-1.37)	0.87 (0.59-1.26)	1.25 (0.82-1.89)	1.24 (0.82-1.86)
Fetal adverse outcome	536	1.05 (0.77-1.42)	1.00 (0.75-1.33)	1.26 (0.84-1.89)	1.21 (0.81-1.81)	1.05 (0.68-1.64)	1.04 (0.6-1.61)

Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled elafin concentration. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, BMI=Body Mass Index. Statistically significant ratios are shown in bold.

Table 6-7. Ratio (95% confidence intervals) of the logged mean CVF elafin concentration in cases and controls overall, and stratified by gestation at sampling in high-risk women, after post intervention samples were excluded

Outcome		Ratio (95% Confidence Interval) of elafin concentration cases: controls**									
				Gestation category (weeks^{+days})							
	N (overall)	**Overall**		**10-13^{+6}**		**14-15^{+6}**		**16-19^{+6}**		**20-24^{+0}**	
		unadjusted	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**
Cervical shortening <25mm	317	1.21 (0.87-1.68)	0.90 (0.65-1.23)	1.39 (0.86-2.24)	1.17 (0.73-1.87)	0.82 (0.53-1.27)	0.74 (0.48-1.13)	0.82 (0.53-1.27)	**0.64 (0.41-0.98)**	0.97 (0.53-1.75)	0.72 (0.41-1.28)
sPTB <37	319	1.08 (0.8-1.50)	1.11 (0.82-1.51)	1.02 (0.64-1.62)	1.03 (0.66-1.60)	0.96 (0.62-1.49)	1.24 (0.81-1.91)	0.79 (0.50-1.26)	0.93 (0.60-1.43)	1.10 (0.67-1.81)	1.30 (0.81-2.08)
sPTB <34	326	**1.63 (1.04-2.56)**	1.45 (0.95-2.20)	1.81 (0.97-3.39)	1.60 (0.87-2.92)	1.53 (0.82-2.84)	1.65 (0.93-2.94)	1.05 (0.50-2.23)	1.09 (0.54-2.19)	1.72 (0.75-3.94)	1.90 (0.88-4.10)
PPROM	328	1.37 (0.89-2.10)	1.25 (0.83-1.87)	1.25 (0.71-2.21)	1.10 (0.64-1.88)	1.15 (0.62-2.15)	1.47 (0.81-2.68)	0.94 (0.45-1.93)	1.02 (0.52-2.00)	1.49 (0.70-3.18)	1.98 (0.97-4.05)
Objective infection	334	1.02 (0.70-1.49)	0.98 (0.69-1.38)	0.92 (0.52-1.61)	0.82 (0.49-1.39)	1.01 (0.61-1.67)	1.01 (0.63-1.62)	0.99 (0.58-1.69)	1.00 (0.61-1.65)	1.33 (0.76-2.34)	1.30 (0.76-2.22)
Fetal adverse outcome	334	1.13 (0.79-1.62)	1.14 (0.82-1.59)	1.63 (0.96-2.74)	1.60 (0.96-2.64)	1.00 (0.62-1.63)	1.14 (0.72-1.79)	0.79 (0.48-1.32)	0.87 (0.53-1.42)	1.26 (0.73-2.16)	1.40 (0.83-2.33)

Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, body mass index, smoking and inter-plate pooled elafin concentration. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes. Statistically significant ratios are shown in bold.

Table 6-8. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome in all women, using CVF elafin concentration stratified by gestation at sampling in the whole cohort (high-risk and low-risk), after excluding samples taken once intervention (cerclage, pessary or progesterone) had been initiated.

Outcome	**Elafin Receiver Operating Characteristic Area Under the Curve (95% Confidence Interval)**	
Gestation category (weeks^{+days})	**10-13^{+6}**	**20-24^{+0}**
sPTB <37 w	0.50 (0.45-0.55)	0.51 (0.45-0.56)
sPTB <34 w	**0.64 (0.59-0.69)**	**0.62 (0.57-0.67)**
PPROM	0.52 (0.47-0.57)	0.53 (0.48-0.58)
Objective infection	0.49 (0.44-0.54)	0.53 (0.47-0.58)
Fetal adverse outcomes	0.55 (0.50-0.60)	0.48 (0.43-0.53)

Statistically significant AUCs are shown in bold, CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes

Table 6-9. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome, using CVF elafin concentration overall, and stratified by gestation at sampling in high-risk women, after excluding samples taken once intervention (cerclage, pessary or progesterone) had been initiated.

Outcome	**Elafin Receiver Operating Characteristic Area Under the Curve (95% Confidence Interval)**			
Gestation category (weeks^{+days})	**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24^{+0}**
Cervical shortening <25 mm	**0.60 (0.53-0.66)**	0.48 (0.42-0.55)	0.49 (0.43-0.56)	0.53 (0.46-0.60)
sPTB <37	0.49 (0.42-0.56)	0.48 (0.41-0.55)	0.48 (0.41-0.55)	0.52 (0.44-0.59)
sPTB <34	**0.63 (0.56-0.70)**	**0.61 (0.54-0.67)**	0.57 (0.50-0.63)	**0.64 (0.58-0.71)**
PPROM	0.54 (0.47-0.61)	0.52 (0.45-0.59)	0.49 (0.42-0.56)	**0.61 (0.53-0.67)**
Objective infection	0.49 (0.42-0.56)	0.49 (0.42-0.55)	0.50 (0.43-0.56)	0.53 (0.46-0.60)
Fetal adverse outcomes	**0.60 (0.53-0.67)**	0.50 (0.44-0.58)	0.48 (0.42-0.55)	0.52 (0.45-0.59)

Statistically significant AUCs are shown in bold. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes.

Table 6-10. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome in women with previous sPTB or late miscarriage, using CVF elafin concentration overall, and stratified by gestation at sampling, after excluding samples taken once intervention (cerclage, pessary or progesterone) had been initiated.

Outcome	Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)				
	Gestation category (weeks^{+days})	**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24^{+0}**
Cervical shortening <25 mm		0.60 (0.51-0.68)	0.45 (0.37-0.53)	0.50 (0.42-0.58)	0.59 (0.50-0.68)
sPTB <37		0.49 (0.40-0.58)	0.49 (0.40-0.57)	0.47 (0.38-0.55)	0.54 (0.45-0.63)
sPTB <34		**0.60 (0.51-0.68)**	**0.64 (0.56-0.72)**	0.58(0.49-0.65)	**0.70 (0.61-0.78)**
PPROM		**0.59 (0.51-0.68)**	0.57 (0.48-0.65)	0.52 (0.44-0.60)	**0.73 (0.65-0.81)**
Objective infection		0.58 (0.50-0.66)	0.55 (0.47-0.64)	**0.62 (0.54-0.69)**	**0.69 (0.60-0.77)**
Fetal adverse outcomes		**0.59 (0.51-0.68)**	0.51 (0.43-0.60)	0.49 (0.41-0.57)	0.56 (0.46-0.64)

Statistically significant AUCs are shown in bold. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes.

6.2.1.4 *Stratification by ethnicity*

Within the Insight cohort, 408/619 (66%) of women self-identified as White European ethnicity (White), 143/619 (23%) were Black African or Black Caribbean (Black), 32/619 (5%) of women were Asian, and 36/619 (6%) belonged to 'other' or 'unknown' ethnic groups. 246/405 (61%) of high-risk women were White and 114/405 (28%) Black, compared to 162/214 (75%) White and 29/214 (14%) Black in the low-risk category. The demographic characteristics of the cohort according to ethnicity (Black, White) at study entry, are shown in Table 6-11.

Black women in the whole cohort were overwhelmingly more likely to have experienced previous PTB (iatrogenic or spontaneous), late miscarriage or PPROM, as well as to have experienced cervical shortening, cerclage placement and premature birth/late miscarriage in their current pregnancy, likely because they made up a higher proportion of the high-risk group, compared with the low-risk group, as discussed in Chapter 4. A higher proportion of Black women were non-smokers (never or ex-smokers) compared to White women.

Table 6-11. Demographics of the INSIGHT cohort study population by ethnicity (Black vs White)

Characteristic	Sub-category	Black women N=143	White women N=408	Comparison (difference/risk ratio) (95% CI)*	P Value
Mean maternal age at booking, (Years ±SD)		32.1 (5.6)	32.8 (4.7)	-0.67 (-1.71-0.36)	0.20
Mean BMI at booking (±SD) Kg/m^2		29.81 (8.8)	24.56 (4.5)	**5.25 (3.73-6.76)**	<**0.001**
Risk factor N (%)	Low risk at booking	29(20.3)	162 (39.7)	**0.51 (0.36-0.72)**	**<0.001**
	Previous sPTB	98 (68.5)	60 (14.7)	**1.75 (1.35-2.26)**	**<0.001**
	Previous late miscarriage	72 (50.3)	46 (11.3)	**4.47 (3.25-6.13)**	**<0.001**
	Previous PPROM	38 (26.6)	63 (15.4)	**1.72 (1.21-2.45)**	**0.003**
	Previous cervical surgery	8 (5.6)	127 (31.1)	**0.18 (0.09-0.36)**	**<0.001**
	Incidental finding of a short cervix <25 mm	1 (0.6)	1 (0.2)	2.85 (0.18-45.32)	0.46
Smoking status N (%)	Never smoked	120 (83.9)	275 (67.4)	ref	0.004
	Current smoker	3 (2.1)	23 (5.6)	0.32 (0.10-1.03)	-
	Ex-smoker (gave up in current pregnancy)	7 (4.9)	33 (8.1)	0.51 (023-1.13)	-
	Ex (gave up before current pregnancy	13 (9.1)	76 (18.6)	**0.45 (0.26-0.79)**	-
	Unknown	0	1 (0.2)	-	-
History of domestic violence N (%)		9 (6.3)	11 (2.7)	2.31 (0.98-5.44)	0.05
History of recreational drug use N (%)		4 (2.8)	13 (3.2)	0.88 (0.29-2.64)	0.81
History of 2 or more UTI's in pregnancy		3 (2.1)	23 (5.6)	0.37 (0.11-1.22)	0.09
Past or current infection with BV (at booking)		23 (16.1)	33 (8.1)	**1.98 (1.21-3.26)**	**0.007**
Past or current GBS infection (at booking)		11 (7.7)	28 (6.9)	1.12 (0.57-2.19)	0.75

Chronic viral infection (HIV, Hepatitis, other)		4 (2.8)	2 (0.5)	**5.71 (1.06-30.82)**	**0.02**
Index of multiple deprivation quintile (5=least deprived)	1	48 (36.9)	40 (10.8)	**ref**	**<0.001**
	2	63 (48.5)	136 (36.7)	**0.73 (0.61-0. 88)**	-
	3	17 (13.1)	95 (25.6)	**0.37 (0.24-0.57)**	-
	4	1 (0.8)	54 (14.6)	**0.04 (0.01-0.25)**	-
	5	1 (0.8)	46 (12.4)	**0.04 (0.01-0.27)**	-
Pregnancy outcome N (%)	Term delivery >37 weeks	107 (74.8)	354 (86.8)	**0.56 (0.48-0.64)**	**<0.001**
	Iatrogenic PTB <37 weeks	12(8.4)	7(1.7)	**4.89 (1.96-12.18)**	**0.02**
	sPTB <37 weeks	37(25.9)	27 (6.7)	**3.91 (2.47-6.18)**	**0.001**
	sPTB <34 weeks	18 (12.2)	14 (3.4)	**3.67 (1.87-7.18)**	**<0.001**
	Late miscarriage	6 (4.2)	2 (0.5)	**8.56 (1.75-41.93)**	**<0.001**
	PPROM	21 (14.7)	18 (4.4)	**3.33 (1.83-6.07)**	**<0.001**
Cervical shortening <25 mm <24 weeks in current pregnancy N (%)		38 (26.6)	25 (6.1)	**4.34 (2.72-6.92)**	**<0.001**
Received prophylactic or emergency intervention to prevent sPTB N (%)	Abdominal or vaginal cerclage	57 (39.9)	24 (5.9)	**6.78 (4.38-10.49)**	**<0.001**
	Progesterone beyond 14 weeks' gestation	11 (7.7)	21 (5.1)	1.49 (0.74-3.02)	0.26
	Arabin pessary	2 (1.4)	4 (1.0)	1.43 (0.26-7.71)	0.68

*CVF=cervicovaginal fluid; sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, GBS=group B streptococcus, HIV=human acquired immunodeficiency virus, CI=confidence interval, SD=standard deviation, BMI=body mass index. *Quantitative variables analysed with t-test. Categorical variables analysed with X^2 test. Statistically significant differences are shown in bold.*

The variation in CVF elafin concentration observed across gestation and by ethnicity, prompted further gestational sub-analysis of HDP concentration in Black and White women.

6.2.1.4.1 Pregnancy outcome; stratification by ethnicity

Tables 6-12 and 6-13 display the ratios of CVF elafin concentration in cases and controls in all women (Table 6-12), and high-risk women only (Table 6-13), stratified by ethnicity and gestation at sampling. As seen in the whole mixed-ethnicity cohort, the mean elafin concentration across gestation (adjusted for maternal age, gestation at sampling, BMI, smoking and inter-plate pooled elafin concentration) was not significantly different in White women (333=term, 37=preterm <37 weeks) who delivered prematurely vs. those who delivered at term, nor in Black women (n=103 term, n=26 preterm) (Table 6-12). When stratified by gestation, high-risk White women who delivered prior to 34 weeks of gestation had more than 3-times (adjusted) increased CVF elafin concentration in early pregnancy (10-13^{+6} weeks) compared with women who delivered at term (ratio 3.37, 95% CI 1.17-9.78, p=0.03) (Table 6-13). Although a trend was seen, this was not statistically significant for other gestations or pregnancy outcomes, nor in high-risk Black women only (Table 6-13).

Table 6-12. Ratio (95% confidence intervals) of the logged mean CVF elafin concentration in cases and controls overall, and stratified by gestation at sampling, in all Black and White women

Outcome			Ratio (95% Confidence Interval) of elafin concentration cases: controls**					
	Ethnicity	**N (overall)**	**Overall**		**10-13^{+6}**		**20-24^{+0}**	
			unadjusted	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**
sPTB <37	White	367	0.77 (0.54-1.09)	0.87 (0.62-1.24)	0.79 (0.48-1.30)	0.85 (0.51-1.40)	0.70 (0.44-1.13)	0.83 (0.52-1.34)
	Black	129	0.94 (0.61-1.45)	0.93 (0.60-1.42)	0.82 (0.48-1.40)	0.85 (0.51-1.41)	0.91 (0.50-1.65)	0.84 (0.45-1.55)
sPTB <34	White	372	1.11 (0.65-1.90)	1.42 (0.82-2.44)	1.81 (0.76-4.29)	2.51 (1.00-6.27)	1.00 (0.50-2.00)	1.34 (0.66-2.71)
	Black	132	1.04 (0.62-1.75)	0.95 (0.57-1.57)	1.00 (0.53-1.89)	1.09 (0.60-1.98)	0.78 (0.32-1.87)	0.64 (0.26-1.59)
PPROM	White	373	1.02 (0.62-1.67)	1.13 (0.69-1.84)	0.85 (0.43-1.66)	0.92 (0.47-1.79)	0.94 (0.49-1.79)	1.17 (0.61-2.24)
	Black	133	0.89 (0.55-1.43)	0.85 (0.55-1.34)	0.88 (0.49-1.57)	0.88 (0.51-1.52)	0.73 (0.36-1.48)	0.66 (0.32-1.34)
Objective infection	White	378	0.94 (0.67-1.32)	0.96 (0.68-1.33)	0.95 (0.59-1.50)	0.92 (0.58-1.44)	1.08 (0.70-1.67)	1.18 (0.77-1.82)
	Black	136	0.90 (0.56-1.44)	0.89 (0.57-1.42)	0.64 (0.35-1.17)	0.64 (0.36-1.13)	1.13 (0.61-2.09)	1.02 (0.55-1.90)
Fetal adverse outcome	White	378	0.72 (0.50-1.02)	0.83 (0.58-1.17)	0.91 (0.54-1.54)	1.04 (0.61-1.77)	0.63 (0.40-1.00)	0.74 (0.47-1.18)
	Black	136	0.93 (0.61-1.43)	0.93 (0.62-1.41)	0.83 (0.48-1.45)	0.93 (0.56-1.57)	0.99 (0.55-1.80)	0.95 (0.53-1.72)

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled elafin concentration*

CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, BMI=body mass index.

Table 6-13. Ratio (95% confidence intervals) of the logged mean CVF elafin concentration in cases and controls overall, and stratified by gestation at sampling, in high-risk Black and White women

Outcome			Ratio (95% Confidence Interval) of elafin concentration cases: controls**									
	Ethnicity	N (overall)	Overall		10-13^{+6}		14-15^{+6}		16-19^{+6}		20-24^{+0}	
			unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted
Cervical shortening <25mm	White	210	1.34 (0.86-2.07)	1.25 (0.82-1.91)	2.11 (0.89-5.02)	1.36 (0.59-3.16)	1.00 (0.54-1.87)	1.06 (058-1.91)	0.88 (0.52-1.47)	0.79 (0.48-1.30)	1.45 (0.89-2.38)	1.37 (0.85-2.21)
	Black	110	0.69 (0.48-1.01)	**0.68 (0.47-0.99)**	0.92 (0.57-1.50)	0.91 (0.57-1.46)	**0.47 (0.27-0.82)**	**0.52 (0.31-0.87)**	**0.62 (0.39-0.99)**	**0.61 (0.37-0.99)**	0.64 (0.39-1.06)	0.63 (0.38-1.06)
sPTB <37	White	214	0.81 (0.55-1.19)	0.95 (0.65-1.39)	0.95 (0.51-1.78)	1.12 (0.60-2.10)	0.74 (0.45-1.36)	1.07 (0.62-1.84)	0.74 (0.46-1.20)	0.93 (0.58-1.48)	0.70 (0.44-1.13)	0.91 (0.57-1.46)
	Black	104	0.98 (0.61-1.55)	0.99 (0.63-1.56)	0.81 (0.49-1.36)	0.93 (0.57-1.53)	1.11 (0.55-2.53)	1.16 (0.60-2.23)	0.72 (0.40-1.31)	0.84 (0.45-1.56)	0.98 (0.50-1.92)	0.87 (0.44-1.83)
sPTB <34	White	219	1.19 (0.67-2.12)	1.55 (0.88-2.75)	2.32 (0.84-6.44)	**3.37 (1.17-9.78)**	1.23 (0.58-2.60)	1.85 (0.88-3.88)	1.07 (0.53-2.15)	1.30 (0.66-2.55)	1.06 (0.54-2.08)	1.56 (0.80-3.05)
	Black	107	1.01 (0.59-1.72)	0.94 (0.56-1.58)	0.88 (0.50-1.56)	1.02 (0.59-1.75)	1.39 (0.62-3.12)	1.33 (0.65-2.75)	0.60 (0.29-1.24)	0.64 (0.31-1.33)	0.78 (0.31-1.95)	0.62 (0.24-1.60)
PPROM	White	219	1.10 (0.63-1.99)	1.28 (0.74-2.21)	0.98 (0.40-2.32)	1.15 (0.50-2.66)	1.36 (0.58-3.22)	1.84 (0.79-4.26)	1.26 (0.60-2.62)	1.49 (0.73-3.04)	1.01 (0.52-1.99)	1.50 (0.77-2.92)
	Black	106	0.92 (0.56-1.54)	0.89 (0.55-1.46)	0.89 (0.52-1.52)	1.02 (0.62-1.68)	0.99 (0.44-2.20)	1.04 (0.51-2.13)	0.66 (0.34-1.28)	0.73 (0.37-1.42)	0.77 (0.34-1.76)	0.64 (0.28-1.45)
Objective infection	White	224	0.92 (0.59-1.42)	0.86 (0.57-1.32)	0.85 (0.41-1.75)	0.74 (0.37-1.47)	0.86 (0.46-1.61)	0.86 (0.47-1.55)	0.72 (0.41-1.29)	0.69 (0.40-1.21)	1.06 (0.62-1.81)	1.15 (0.68-1.93)
	Black	109	0.97 (0.57-1.65)	0.92 (0.56-1.53)	0.71 (0.39-1.30)	0.69 (0.40-1.20)	1.07 (0.49-2.35)	1.10 (0.53-2.29)	0.99 (0.50-1.98)	1.07 (0.54-2.13)	1.27 (0.60-2.66)	1.15 (0.54-2.42)
Fetal adverse outcome	White	224	0.79 (0.53-1.19)	0.93 (0.62-1.40)	1.24 (0.60-2.53)	1.53 (0.75-3.12)	0.74 (0.43-1.28)	0.92 (0.54-1.59)	0.68 (0.41-1.15)	0.80 (0.48-1.32)	0.70 (0.42-1.14)	0.89 (0.54-1.45)
	Black	109	1.02 (0.64-1.65)	0.97 (0.61-1.53)	0.90 (0.53-1.53)	0.87 (0.53-1.42)	0.93 (0.45-1.91)	0.89 (0.46-1.74)	0.91 (0.50-1.64)	0.93 (0.52-1.68)	1.19 (0.59-2.40)	1.24 (0.61-2.52)

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled elafin concentration. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, BMI=body mass index. Statistically significant ratios are shown in bold.*

Prediction analysis was performed using crude CVF elafin concentration for the whole cohort (Table 6-14) and high-risk cohort only (Table 6-15), stratified by ethnicity. In high-risk Black women, low CVF elafin when measured mid trimester at 16-19^{+6} weeks of gestation, was predictive of sPTB<37 weeks (n=89, AUC ROC 0.62, 95% CI 0.51 to 0.72), <34 weeks (n=90, 0.68, 0.57 to 0.77) (Figure 6-1) and PPROM with delivery <37 weeks (n=91, 0.65, 0.55 to 0.76). In comparison, for high-risk White women at this gestation (Table 6-15), the ROC curves were non-significant for most clinical outcomes, but high elafin at 10 to 13^{+6} weeks of gestation modestly predicted sPTB <34 weeks (n=135, 0.65, 0.57 to 0.73). High elafin at 16-19^{+6} weeks was also modestly predictive of PPROM (n=171, 0.58, 0.51-0.66).

Surprisingly, in the whole cohort of White women (Table 6-14), low elafin at 10-13^{+6} weeks was mildly predictive of sPTB <37 weeks (n=275, 0.57, 0.52 to 0.63). In addition, low elafin concentration in CVF samples taken from White women in later gestation at 20-24 weeks of gestation was mildly predictive of both sPTB <37 weeks (n=306, 0.61, 0.55 to 0.66) and fetal adverse outcome (n=313, 0.64, 0.58-0.69).

Table 6-14. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome in all women, using CVF elafin concentration by gestation at sampling in the whole cohort (high-risk and low risk), stratified by ethnicity

Outcome		**Elafin Receiver Operating Characteristic Area Under the Curve (95% Confidence Interval)**	
Gestation category (weeks^{+days})		**10-13^{+6}**	**20-24+^{0}**
sPTB <37	White	**0.43 (0.37-0.49)**	**0.39 (0.34-0.45)**
	Black	0.44 (0.33-0.54)	0.47 (0.38-0.57)
sPTB <34	White	**0.62 (0.56-0.68)**	0.49 (0.43-0.54)
	Black	0.50 (0.40-0.60)	0.45 (0.35-0.54)
PPROM	White	0.43 (0.37-0.50)	0.47 (0.41-0.53)
	Black	0.46 (0.35-0.56)	0.41 (0.32-0.51)
Objective infection	White	0.49 (0.43-0.55)	0.49 (0.44-0.55)
	Black	**0.39 (0.29-0.49)**	0.53 (0.43-0.62)
Fetal adverse outcomes	White	0.44 (0.38-0.50)	**0.37 (0.31-0.42)**
	Black	0.47 (0.37-0.57)	0.47 (0.40-0.57)

Statistically significant AUCs are shown in bold. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes

Table 6-15. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome, using CVF elafin concentration, by gestation at sampling in high-risk women, stratified by ethnicity

Outcome		**Elafin Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)**			
Gestation category (weeks^{+days})		**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24^{+0}**
Cervical shortening <25 mm	White	**0.64 (0.55-0.72)**	0.53 (0.44-0.61)	0.53 (0.44-0.60)	**0.61 (0.53-0.68)**
	Black	0.45 (0.33-0.57	**0.32 (0.22-0.44)**	**0.36 (0.26-0.46)**	0.40 (0.30-0.51)
sPTB <37	White	0.47 (0.38-0.55)	0.43 (0.34-0.51)	0.48 (0.40-0.55)	**0.41 (0.33-0.49)**
	Black	0.44 (0.33-0.57)	0.52 (0.41-0.64)	**0.38 (0.28-0.49)**	0.50 (0.39-0.61)
sPTB <34	White	**0.65 (0.56-0.73)**	0.53 (0.45-0.62)	0.57(0.49-0.65)	0.53 (0.45-0.61)
	Black	0.47(0.36-0.59)	0.60 (0.49-0.71)	**0.32 (0.23-0.43)**	0.45 (0.34-0.56)
PPROM	White	0.46 (0.37-0.54)	0.55 (0.46-0.63)	**0.58 (0.51-0.66)**	0.52 (0.44-0.60)
	Black	0.48 (0.36-0.60)	0.50 (0.39-0.62)	**0.35 (0.25-0.46)**	0.43 (0.32-0.54)
Objective infection	White	0.47 (0.38-0.56)	0.44 (0.35-0.52)	0.45 (0.37-0.52)	0.49 (0.41-0.57)
	Black	0.39 (0.28-0.51)	0.51 (0.40-0.63)	0.40 (0.30-0.51)	0.55 (0.44-0.66)
Fetal adverse outcomes	White	0.51 (0.42-0.59)	0.43 (0.35-0.52)	0.47 (0.39-0.54)	0.41 (0.33-0.48)
	Black	0.47 (0.35-0.58)	0.46 (0.35-0.58)	0.43 (0.33-0.54)	0.51 (0.40-0.61)

Statistically significant AUCs are shown in bold. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes

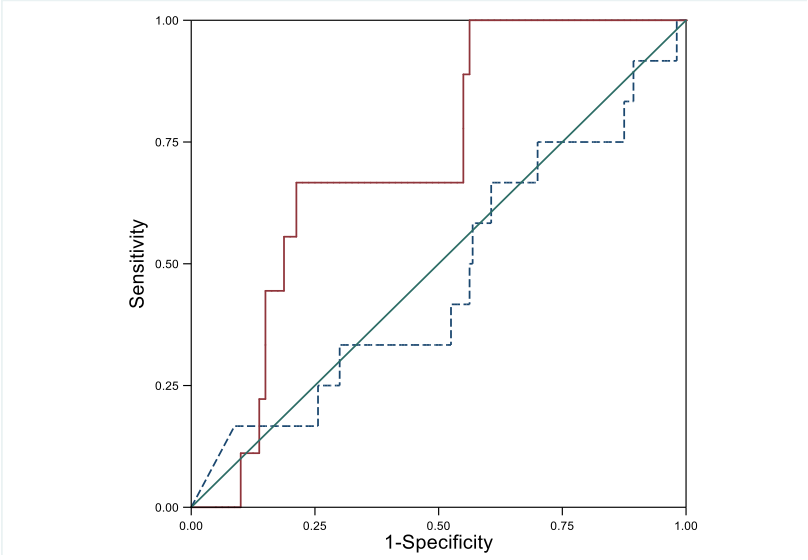

Figure 6-1. Receiver operating characteristic curves demonstrating the ability of low trappin2/elafin cervicovaginal fluid concentration taken at 16^{+0} to 19^{+6} weeks of gestation in high-risk Black women (red line, n = 90, AUC 0.68) and White women (blue line, n = 171, AUC 0.43) to predict spontaneous preterm birth prior to 34 weeks in pregnant women at high-risk of spontaneous preterm birth (X^2 =4.96, p=0.03).

6.2.1.4.2 Cervical shortening; stratified by ethnicity

Black women who developed a short cervix had lower overall mean CVF elafin (after adjusting for maternal age, BMI, smoking, gestation at sampling, and pooled elafin concentration) compared to Black high-risk controls with normal cervical length (Table 6-13, Figure 6-2a) (n= 110, ratio 0.68, 95% CI 0.47 to 0.99, p=0.04). When stratified by gestation, this was particularly evident 14 to 20 weeks of gestation; at 14 to 15^{+6} week of gestation, adjusted CVF elafin was 48% lower in cases vs controls (n=78, 0.52, 0.31-0.87, p=0.01) and 49% lower when measured at 16 to 19^{+6} weeks of gestation (n=97, 0.61, 0.37 to 0.99, p=0.04, Table 6-14). In White women, there was no significant difference in elafin concentrations in those who developed cervical shortening (Table 6-13 and Figure 6-2b, n= 210, 1.25, 0.82 to 0.9, p=0.25).

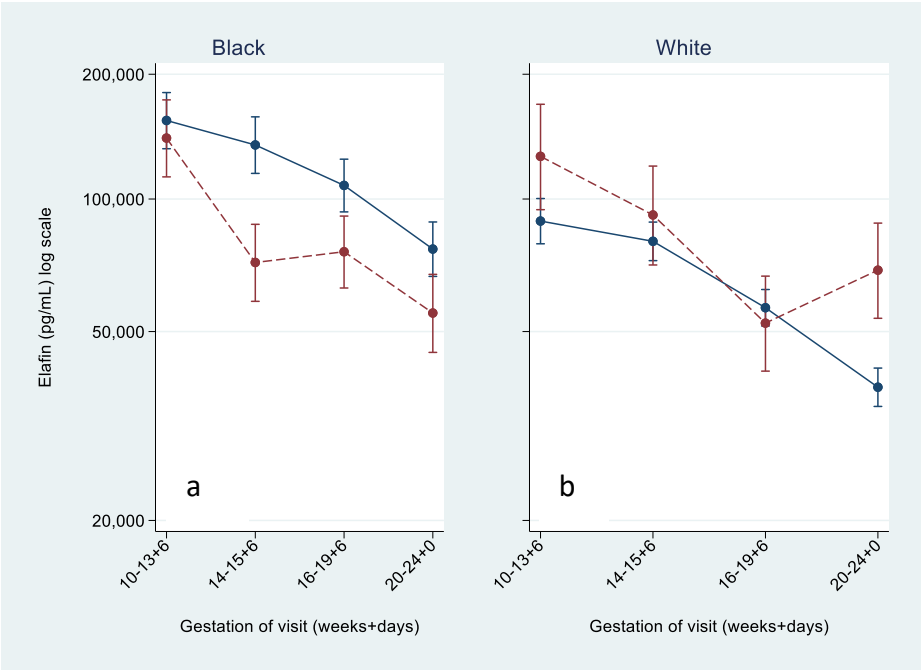

Figure 6-2. Longitudinal measurements of cervicovaginal elafin concentrations in women at high-risk of spontaneous preterm birth who subsequently develop a short cervix <25 mm prior to 24 weeks of gestation in Black women (6a n= 38 short, n= 65 not short) and White women (6b n=24 short, n= 174 not short). Geometric means and SE bars. The red hashed line represents measurements in women who developed a short cervix, and the blue solid line represents those who did not develop a short cervix.

Table 6-15 displays the prediction analysis for the high-risk cohort, stratified by ethnicity and gestation at sampling. In high-risk Black women low elafin was predictive of cervical shortening when measured at 14-15^{+6} weeks of gestation (n= 80, AUC ROC 0.68, 95% CI 0.56 to 0.78, figure 6.3) and at 16 to 19^{+6} weeks (n=92, 0.64, 0.54 to 0.74). In White women, high elafin was moderately predictive of development of a short cervix when measured at 10-13^{+6} weeks (n=133, 0.64, 0.55-0.72) and 20-24 weeks (n=172, 0.61, 0.53 to 0.68).

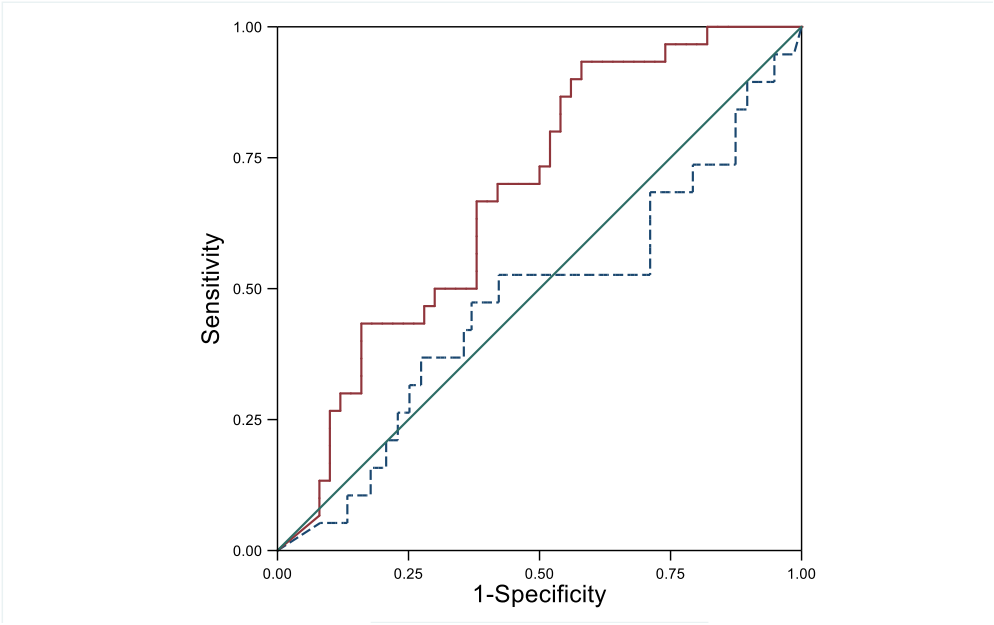

Figure 6-3. Receiver operating characteristic curves demonstrating the ability of low trappin2/elafin cervicovaginal fluid concentration taken at 14^{+0} to 15^{+6} weeks of gestation in high-risk Black women (red line, n=80, AUC 0.68) and White women (blue line, n=150, AUC 0.47) to predict cervical shortening <25 mm prior to 24 weeks of gestation (X^2=4.52, p=0.03).

6.2.1.4.3 Removal of post intervention samples

Explorative analysis was performed to evaluate whether removing samples taken once a prophylactic intervention was administered would affect outcome in the ethnically stratified groups, as it had done with the whole high-risk cohort (Tables 6-16 & 6-17). This did not appear to be the case; a similar pattern was seen to the analysis which did not exclude post intervention samples, though sample size became

small and confidence intervals were frequently too wide to make meaningful conclusions.

High-risk White women who delivered spontaneously prior to 34 weeks' gestation had nearly four times the concentration of CVF elafin in early gestation (10-13^{+6} weeks, n=104 ratio 3.80, 95% CI 1.23 to 11.71, p=0.02) after adjustment, compared with women who did not deliver prior to 34 weeks' gestation (Table 6-16). High-risk White women with adverse fetal outcomes had over two times CVF elafin at this early gestation (n=125, 2.71, 1.15-6.40, p=0.02) after adjustment, though crude values were not predictive of adverse fetal outcome when ROC analysis was performed (Table 6-17). In contrast, Black high-risk women who developed cervical shortening had less than half the concentration of CVF elafin at 14-15^{+6} weeks (n=55, 0.45, 0.25-0.82, p=0.01) compared with those who did not develop cervical shortening, but there was no statistically significant association between CVF elafin concentration and sPTB in these women (Table 6-16). Low CVF elafin expression in these women Black women taken between 14-15^{+6} weeks of gestation was still predictive of subsequent cervical shortening, now samples with prophylactic interventions (mainly cerclage) were removed (n=57, AUC ROC 0.69, 95% CI 0.55 to 0.83) compared White women at this gestation (n=134, 0.54, 0.37, 0.71, p=0.04, Table 6-17).

Conversely, in a small number of high-risk black women (n=45), women who experienced poor fetal outcome had far higher elafin CVF concentration at later gestation (20 to 24 weeks) than those who did not (ratio 21.34, 95% CI 2.89-157.35, p<0.001). High CVF elafin at this gestation of gestation was strongly predictive of adverse fetal outcome (AUC ROC 0.86, 95% CI 0.69 to 1.0) compared with White women (n=143, 0.47, 0.33-0.62, p<0.01) (Table 6-17).

Table 6-16. Ratio (95% confidence intervals) of the logged mean CVF elafin concentration in cases and controls overall, and stratified by gestation at sampling, in high-risk Black and White women, after removal of post intervention samples

Outcome			Ratio (95% Confidence Interval) of elafin concentration cases: controls**									
	Ethnicity	**N (overall)**	**Overall**		**10-13^{+6}**		**14-15^{+6}**		**16-19^{+6}**		**20-24^{+0}**	
			unadjusted	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**
Cervical shortening <25mm	White	193	1.15 (0.69-1.92)	1.00 (0.61-1.64)	2.15 (0.81-5.74)	1.24 (0.47-3.28)	1.00 (0.51-1.98)	1.15 (0.60-2.20)	0.73 (0.40-1.35)	0.64 (0.36-1.14)	1.19 (0.60-2.37)	1.09 (0.55-2.15)
	Black	90	0.79 (0.48-1.29)	0.69 (0.43-1.10)	1.01 (0.59-1.74)	1.04 (0.62-1.74)	**0.45 (0.24-0.87)**	**0.45 (0.25-0.82)**	0.59 (0.29-1.21)	0.52 (0.25-1.05)	0.38(0.11-1.32)	1.48 (0.04-0.48)
sPTB <37	White	198	0.90 (0.59-1.39)	1.03 (0.68-1.56)	1.08 (0.54-2.16)	1.22 (0.61-2.44)	0.76 (0.41-1.39)	0.94 (0.52-1.67)	0.71 (0.42-1.21)	0.87 (0.53-1.44)	0.81 (0.45-1.44)	0.90 (0.52-1.56)
	Black	86	1.10 (0.60-2.00)	1.14 (0.64-2.02)	0.84 (0.47-1.50)	0.90 (051-1.59)	1.18 (0.49-2.83)	1.43 (0.63-3.24)	0.79 (0.26-2.35)	1.02 (0.32-3.30)	1.69 (0.40-7.13)	1.40 (0.30-6.42)
sPTB <34	White	202	1.45 (0.76-2.77)	1.70 (0.90-3.19)	2.99 (0.98-9.14)	**3.97 (1.23-11.71)**	1.37 (0.56-3.36)	1.65 (0.69-3.92)	0.95 (0.42-2.19)	1.02 (0.46-2.24)	1.44 (0.60-3.49)	1.48 (0.64-3.45)
	Black	89	1.32 (0.65-2.71)	1.30 (0.64-2.59)	0.97 (0.50-1.89)	1.11 (0.58-2.13)	1.51 (0.52-4.37)	1.78 (0.67-4.71)	0.74 (0.11-5.13)	0.84 (0.13-5.59)	4.17 (0.24-73.24)	8.60 (0.58-128.20)
PPROM	White	204	1.26 (0.66-2.41)	1.36 (0.73-2.54)	1.06 (0.40-2.84)	1.23 (0.48-3.12)	1.51 (0.56-4.09)	1.73 (0.66-4.48)	1.19 (0.52-2.73)	1.31 (0.60-2.89)	1.27 (0.53-3.07)	1.52 (0.65-3.53)
	Black	89	1.10 (0.56-2.13)	1.11 (0.59-2.10)	0.99 (0.54-1.81)	1.10 (0.63-1.93)	0.84 (0.30-2.34)	1.23 (0.49-3.11)	0.69 (0.14-3.42)	0.79 (0.15-4.04)	1.99 (0.25-15.73)	2.58 (0.39-17.26)
Objective infection	White	207	0.90 (0.56-1.44)	0.84 (0.53-1.32)	1.03 (0.47-2.24)	0.80 (0.38-1.71)	0.84 (0.44-1.62)	0.86 (0.46-1.61)	0.70 (0.38-1.29)	0.70 (0.39-1.26)	1.08 (0.58-1.01)	1.14 (0.62-2.09)
	Black	91	1.13 (0.56-2.26)	1.07 (0.56-2.04)	0.72 (0.35-1.49)	0.62 (0.33-1.18)	1.20 (0.46-3.13)	1.26 (0.52-3.07)	2.06 (0.69-6.19)	2.67 (0.86-8.27)	1.85 (0.48-7.13)	1.51 (0.37-6.25)
Fetal adverse outcome	White	207	0.87 (0.55-1.39)	0.99 (0.63-1.57)	2.22 (0.94-5.22)	**2.71 (1.15-6.40)**	0.76 (0.41-1.42)	0.88 (0.48-1.59)	0.63 (0.35-1.12)	0.70 (0.40-1.22)	0.96 (0.53-1.75)	1.06 (0.60-1.89)
	Black	91	1.53 (0.82-2.85)	1.19 (0.66-2.15)	1.07 (0.60-1.94)	0.98 (0.58-1.67)	1.27 (0.51-3.16)	1.19 (052-2.73)	1.52 (0.43-5.42)	1.20 (0.27-4.47)	**7.86 (1.69-36.62)**	**21.34 (2.89-157.35)**

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled elafin concentration. Statistically significant ratios in bold. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, BMI=body mass index,*

Table 6-17. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome, using CVF elafin concentration, by gestation at sampling in high-risk women, stratified by ethnicity, after removal of post intervention samples

Outcome		**Elafin Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)**			
Gestation category (weeks^{+days})		**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24^{+0}**
Cervical shortening <25 mm	White	0.65 (0.45-0.84)	0.54 (0.37-0.71)	0.51 (0.35-0.66)	0.54 (0.40-0.68)
	Black	0.48 (0.34-0.63)	**0.31(0.17-0.45)**	0.36 (0.21-0.51)	0.37 (0.12-0.62)
sPTB <37	White	0.50 (0.36-0.64)	0.42 (0.28-0.57)	0.48 (0.34-0.62)	0.44 (0.33-0.56)
	Black	0.45 (0.28-0.61)	0.56 (0.37-0.75)	0.39 (0.21-0.55)	0.64 (0.44-0.84)
sPTB <34	White	0.71 (0.49-0.92)	0.57 (0.35-0.78)	0.57 (0.32-0.80)	0.61 (047-0.75)
	Black	0.50 (0.29-0.71)	0.65 (0.41-0.90)	0.36 (0.00-0.82)	-
PPROM	White	0.48 (0.29-0.66)	0.58 (0.35-0.81)	0.57 (0.38-0.77)	0.57 (0.39-0.75)
	Black	0.51 (0.32-0.70)	0.47 (0.22-0.72)	0.34 (0.06-0.61)	0.69 (0.34-1.00)
Objective infection	White	0.50 (0.32-0.69)	0.43 (0.28-0.58)	0.43 (0.28-0.60)	0.48 (0.34-0.62)
	Black	0.39 (0.21-0.58)	0.54 (0.32-0.77)	0.57 (0.27-0.88)	0.63 (0.27-0.98)
Fetal adverse outcomes	White	0.63 (0.46-0.81)	0.45 (0.29-0.60)	0.46 (0.30-0.61)	0.47 (0.33-0.62)
	Black	0.52 (9.36-0.69)	0.55 (0.34-0.76)	0.49 (0.10-0.87)	**0.86 (0.70-1.00)**

Statistically significant ratios in bold CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes

6.2.2 Cathelicidin

6.2.2.1 *High-risk cohort and pregnancy outcomes*

Overall, after adjustment, compared to women who delivered at term, CVF cathelicidin concentration was numerically 32% higher in high-risk women who delivered prematurely <37 weeks (p=0.04), and 17% higher in women who delivered <34 weeks though this did not reach statistical significance (p=0.38) (Table 6-18). When stratified by gestation, adjusted cathelicidin concentration in CVF samples taken at 14 to 15^{+6} weeks of gestation was 86% higher in women who delivered spontaneously prior to 37 weeks (p=0.008) and nearly double in women who subsequently experienced PPROM (p=0.03), compared to women who delivered at term (Table 6-18). Figure 6-4 illustrates the longitudinal profile of cathelicidin in women who gave birth prematurely vs. those who delivered at term.

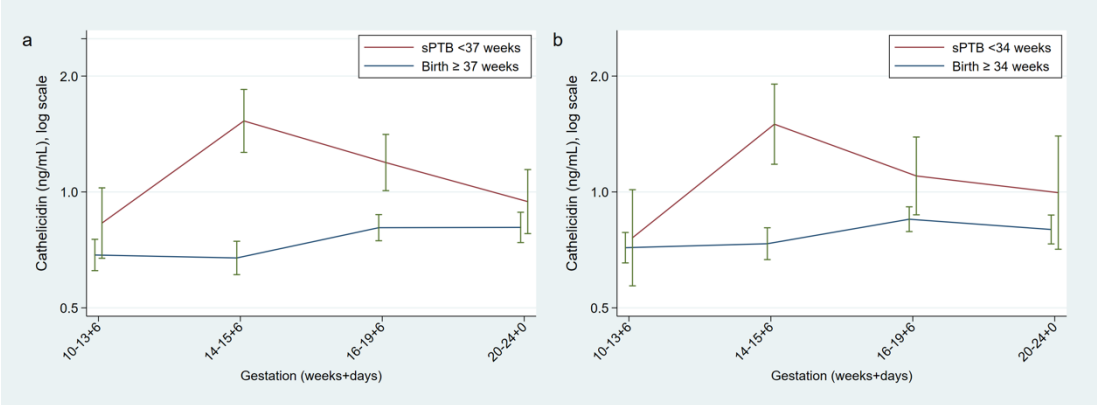

Figure 6-4. Longitudinal measurements of cervicovaginal cathelicidin concentrations in high-risk women who deliver spontaneously prior to 34 weeks of gestation (sPTB) (6-6a) and prior to 37 weeks of gestation (6-6b) Geometric means and SE bars: The red solid line represents measurements in women who delivered spontaneously prematurely before 37 weeks (n=271 term, n= 68 preterm) or 34 weeks of gestation (n=303 >34 weeks, n =36 <34 weeks) and the blue line represents those who did not deliver prematurely.

At 14-15^{+6} weeks, CVF Cathelicidin was moderately predictive of sPTB <37 weeks (n=224, AUC ROC 0.67, 95% CI 0.61 to 0.74), <34 weeks (n=229, 0.63, 0.56 to 0.69) and PPROM (n=225, 0.64, 0.58 to 0.71), as well as objective infection (n=232, 0.59,

0.53-0.66) (Table 7-19). Cathelicidin samples taken later at 16-19^{+6} weeks were also mildly predictive of objective infection (n=255, 0.57, 0.51-0.63), with a similar AUC ROC values at 20-24^{+0} weeks, although this did not reach statistical significance (Table 6-19).

6.2.2.2 *High-risk cohort and cervical shortening*

Although unadjusted mean cathelicidin CVF concentration overall was significantly higher in women who developed a short cervix (ratio 1.35, 95% CI 1.03-1.75, p=0.03), after adjustment this did not reach significance (1.26, 0.96-1.65, p=0.09, Table 6-18). When stratified by gestation, cathelicidin concentrations were weakly predictive of developing a short cervix when sampled at 14-15^{+6} weeks (n=232, AUC ROC 0.58, 95% CI 0.52 to 0.64), 16^{+0} to 19^{+6} weeks (n=255, 0.59, 0.53 to 0.65), and 20 to 24^{+0} weeks (n=245, 0.58, 0.52-0.64) (Table 6-19).

Table 6-18 Ratio (95% confidence intervals) of the logged mean CVF cathelicidin concentration in cases and controls overall, and stratified by gestation at sampling, amongst high-risk women

Outcome		Ratio (95% Confidence Interval) of cathelicidin concentration cases: controls**									
				Gestation category (weeks^{+days})							
	N (overall)	**Overall**		**10-13^{+6}**		**14-15^{+6}**		**16-19^{+6}**		**20-24**	
		unadjusted	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**
Cervical shortening <25 mm	321	**1.35 (1.03-1.75)**	1.26 (0.96-1.65)	1.23 (0.79-1.92)	1.28 (0.82-1.99)	1.42 (0.94-2.14)	1.42 (0.91-2.21)	1.36 (1.00-1.84)	1.24 (0.91-1.70)	**1.52 (1.05-2.20)**	1.26 (0.85-1.87)
sPTB <37	338	**1.40 (1.06-1.83)**	**1.32 (1.01-1.72)**	1.26 (0.84-1.88)	1.26(0.85-1.88)	**2.11 (1.37-3.28)**	**1.86 (1.18-2.92)**	1.36 (0.97-1.89)	1.30 (0.94-1.80)	1.16 (0.76-1.76)	1.14 (0.75-1.72)
sPTB <34	338	1.28 (0.90-1.83)	1.17 (0.83-1.65)	1.20 (0.71-2.02)	1.10 (0.65-1.84)	**1.81 (1.03-3.18)**	1.43 (0.81-2.51)	1.25 (0.81-1.92)	1.19 (0.78-1.80)	1.14 (0.63-1.06)	1.09 (0.61-1.93)
PPROM	331	1.35 (0.95-1.92)	1.26 (0.90-1.78)	1.06 (0.63-1.76)	1.01 (0.61-1.65)	**2.00 (1.10-3.64)**	**1.94 (1.06-3.54)**	1.42 (0.93-2.17)	1.35 (0.89-2.03)	1.43 (0.82-2.50)	1.32 (0.76-2.28)
Objective infection	338	1.14 (0.82-1.57)	1.11 (0.82-1.51)	0.91 (0.55-1.50)	1.03 (0.64-1.67)	1.15 (0.68-1.97)	1.07(0.64-1.79)	1.24 (0.84-1.84)	1.29 (0.89-1.88)	1.35 (0.83-2.18)	1.28 (0.80-2.05)
Fetal adverse outcome	338	0.99 (0.73-1.34)	0.94 (0.71-1.26)	0.76 (0.48-1.20)	0.78 (0.50-1.23)	1.30 (0.80-2.10)	1.14 (0.71-1.83)	1.20 (0.84-1.70)	1.15 (0.82-1.62)	0.84 (0.53-1.33)	0.83 (0.53-1.29)

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled cathelicidin concentration. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, BMI=body mass index. Statistically significant ratios in bold.*

Table 6-19. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome in high-risk women, using CVF cathelicidin concentration stratified by gestation at sampling

Outcome	Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)			
Gestation category (weeks^{+days})	**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24^{+0}**
Cervical shortening <25 mm	0.56 (0.49-0.63)	**0.58 (0.51-0.64)**	**0.59 (0.53-0.65)**	**0.58 (0.52-0.64)**
sPTB <37	0.57 (0.50-0.64)	**0.67 (0.61-0.74)**	0.57 (0.50-0.63)	0.53 (0.46-0.59)
sPTB <34	0.54 (0.47-0.61)	**0.63 (0.56-0.69)**	0.53 (0.47-0.60)	0.54 (0.48-0.61)
PPROM	0.52 (0.45-0.59)	**0.64 (0.58-0.71)**	**0.61 (0.55-0.67)**	**0.60 (0.53-0.66**)
Objective infection	0.48 (0.41-0.55)	**0.59 (0.53-0.66)**	**0.57 (0.51-0.63)**	0.57 (0.50-0.63)
Fetal adverse outcomes	0.47 (0.40-0.54)	0.56 (0.49-0.63)	0.54 (0.48-0.60)	0.49 (0.43-0.56)

Statistically significant area under the curve in bold. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes,

6.2.2.3 *Exclusion of samples taken after prophylactic intervention*

After excluding all samples taken in women who had a prophylactic intervention to reduce risk of sPTB prior to enrolment in study, or after cervical shortening had been detected, associations were strengthened; women delivering prior to 37 weeks of gestation had more than 2.5 times the CVF concentration of cathelicidin compared to controls at 14-15^{+6} weeks of gestation (n=189, ratio 2.67, 95% CI 1.59 to 4.47, p<0.001), and more than double in women with PPROM (p=0.03) (Table 6-20). CVF cathelicidin was predictive of sPTB <37 weeks when sampled at all gestational windows between 10 and 20 weeks and sPTB <34 weeks between 10 and 15^{+6} weeks. When sampled at 14-15^{+6} weeks, CVF cathelicidin predicted sPTB <37 weeks with ROC AUC of 0.75 (n=184, 95% CI 0.68 to 0.81) (Table 6-21).

Table 6-20. Ratio (95% confidence intervals) of the logged mean CVF cathelicidin concentration in cases and controls overall, and stratified by gestation at sampling, amongst high-risk women, after exclusion of samples taken after intervention had been employed

Outcome		Ratio (95% Confidence Interval) of cathelicidin concentration cases: controls**									
				Gestation category (weeks+days)							
	N (overall)	Overall		10-13+6		14-15+6		16-19+6		20-24+0	
		unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted
Cervical shortening <25mm	280	1.30 (0.96-1.77)	1.28 (0.93-1.77)	1.28 (0.81-2.01)	1.36 (0.87-2.13)	1.39 (0.82.22)	1.45 (0.89-2.38)	1.29 (0.88-1.88)	1.23 (0.82-1.82)	1.29 (0.66-2.52)	1.43(0.71-2.90)
sPTB <37	299	**1.78 (1.31-2.42)**	**1.70 (1.26-2.30)**	**1.65 (1.08-2.52)**	1.51 (0.99-2.30)	**2.96 (1.79-4.90)**	**2.67 (1.59-4.47)**	**1.63 (1.07-2.49)**	**1.57(1.04-2.38)**	1.50 (0.83-2.70)	1.53 (0.86-2.74)
sPTB <34	299	**1.45 (0.93-2.25)**	1.31 (0.85-2.02)	1.38 (0.77-2.47)	1.14 (0.64-2.09)	2.14 (0.99-4.63)	1.88 (0.89-3.98)	1.51 (0.79-2.89)	1.44 (0.77-2.67)	1.34 (0.49-3.64)	1.35 (0.50-3.59)
PPROM	294	1.52 (0.99-2.32)	1.42 (0.93-2.16)	1.38 (0.79-2.40)	1.15 (0.67-1.97)	**2.34 (1.09-1.97)**	**2.35 (1.09-5.09)**	1.55 (0.81-2.94)	1.40 (0.76-2.60)	2.01 (0.79-5.10)	1.87 (0.73-4.80)
Objective infection	299	1.19 (0.82-1.71)	1.16 (0.82-1.64)	1.32 (0.77-2.28)	1.40 (0.84-2.35)	1.20 (0.66-2.19)	1.11 (0.62-1.99)	1.03 (0.65-1.64)	1.13 (0.72-1.77)	1.26 (0.66-2.39)	1.27 (0.66-2.42)
Fetal adverse outcome	299	0.98 (0.69-1.38)	0.93 (0.66-1.30)	0.94 (0.57-1.55)	0.91 (0.55-1.49)	1.25 (0.70-2.25)	1.14 (0.65-2.02)	1.18 (0.74-1.90)	1.11 (0.70-1.76)	0.54 (0.28-1.04)	0.54 (0.28-1.05)

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled cathelicidin concentration. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, BMI=body mass index. Statistically significant ratios in bold.*

Table 6-21 Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome in high-risk women, using CVF cathelicidin concentration stratified by gestation at sampling, after exclusion of samples taken once prophylactic intervention had been initiated

Outcome	Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)			
Gestation category (weeks^{+days})	**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24**
Cervical shortening <25 mm	**0.58 (0.51-0.66)**	0.58 (0.50-0.65)	**0.58 (0.51-0.65)**	0.49 (0.41-0.57)
sPTB <37	**0.64 (0.56-0.71)**	**0.75 (0.68-0.81)**	**0.61 (0.53-0.68)**	0.58 (0.50-0.65)
sPTB <34	**0.58 (0.51-0.66)**	**0.68 (0.61-0.75)**	0.57 (0.44-0.62)	0.53 (0.46-0.61)
PPROM	0.58 (0.50-0.65)	**0.67 (0.61-0.78)**	**0.65 (0.58-0.72)**	**0.66 (0.58-0.73)**
Objective infection	0.56 (0.49-0.63)	**0.62 (0.54-0.69)**	0.53 (0.46-0.60)	0.53 (0.45-0.60)
Fetal adverse outcomes	0.53 (0.45-0.60)	0.56 (0.49-0.63)	0.54 (0.47-0.61)	0.40 (0.33-0.48)

Statistically significant AUCs in bold. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes.

6.2.2.4 *Stratification by ethnicity*

Table 6-22 displays the ratio of cathelicidin in high-risk cases and controls when stratified by ethnicity (Black/White women). CVF cathelicidin overall was 50% higher in White women who delivered spontaneously prior to 37 weeks after adjustment (n=202, ratio 1.53, 95% CI 1.03 to 2.27, p=0.04) but not Black women. When stratified by gestation at testing, this was most apparent at 14-15^{+6} weeks of gestation where concentration of CVF cathelicidin was nearly three times that in women who delivered prior to 37 weeks, and two and a half times in those women who delivered prior to 34 weeks (<37 weeks, n=133, 2.93, 1.57 to 5.50, p=0.001, <34 weeks n=133, 2.52, 1.05 to 6.01, p=0.04). This pattern was not seen in Black women. In Black women, unadjusted CVF cathelicidin concentration at 16-19^{+6} weeks was higher in women who experienced adverse fetal outcomes compared to those who did not, but this was not statistically significant when adjusted for age, gestation at sampling, BMI, smoking and pooled cathelicidin concentration (Table 6-22).

Prediction of outcome using CVF cathelicidin concentration remained was strong for White women but not Black women (Table 6-23). High cathelicidin concentrations at 14 to 15^{+6} weeks of gestation predicted sPTB in White women <34 weeks (n= 132, AUC ROC 0.72, 95% CI 0.63 to 0.79), <37 weeks (n=129, 0.77, 0.68 to 0.84), but was not predictive of these outcomes in Black women only. Figure 6-5 illustrates the ROC curve for Black and White women, for prediction of sPTB <37 women using CVF cathelicidin measured at 14-15^{+6} weeks of gestation. However, low cathelicidin at early gestation (10-13^{+6} weeks) seemed to be predictive of adverse fetal outcome (but not sPTB) in White women (n=121, ROC 0.64, 0.55 to 0.73) and high cathelicidin was related to both objective infection and adverse outcomes in Black women at later gestations. High cathelicidin concentration in early pregnancy (10-13^{+6} weeks) was weakly predictive of developing a short cervix (n=118, 0.60, 0.51 to 0.69) in high-risk White women compared with Black women (n=68, 0.49, 0.36 to 0.61) but the difference in the AUC was not significant (p=0.35).

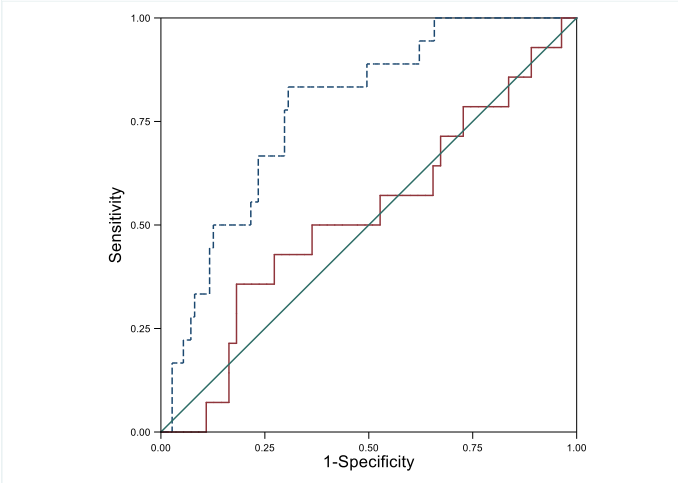

Figure 6-5. Receiver operating characteristic curves demonstrating the ability of cathelicidin cervicovaginal fluid concentration taken at 14 to 15^{+6} weeks of gestation in high-risk White women (blue line, n = 129, AUC= 0.77) and Black women (red line, n =69, AUC= 0.52) to predict spontaneous preterm birth prior to 37 weeks (X^2 =5.42, p=0.02)

Table 6-22. Ratio (95% confidence intervals) of the logged mean CVF cathelicidin concentration in cases and controls overall, and stratified by gestation at sampling, in high-risk Black and White women

Outcome		Ratio (95% Confidence Interval) of cathelicidin concentration cases: controls**										
		Gestational category (weeks^{+days})										
	Ethnicity	**N (overall)**	**Overall**		**10-13^{+6}**		**14-15^{+6}**		**16-19^{+6}**		**20-24^{+0}**	
			unadjusted	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**
Cervical shortening <25mm	White	184	1.28 (0.81-2.02)	1.15 (0.73-1.80)	1.54 (0.59-4.06)	1.44 (0.52-4.00)	1.34 (0.70-2.54)	1.08 (0.57-2.05)	1.17 (0.69-1.99)	1.04 (0.61-1.77)	1.62 (0.86-3.04)	1.40 (0.73-2.69)
	Black	102	1.24 (0.87-1.78)	1.30 (0.93-1.84)	0.95 (0.57-2.58)	1.24 (0.77-2.01)	1.47 (0.77-2.80)	1.51 (0.80-2.84)	1.32 (0.86-2.04)	1.54 (1.01-2.33)	1.13 (0.69-1.87)	1.20 (0.72-1.99)
sPTB <37	White	202	1.49 (1.0-2.23)	**1.53 (1.03-2.27)**	1.37 (0.75-2.51)	1.55 (0.85-2.83)	**3.01 (1.57-5.78)**	**2.94 (1.57-5.50)**	1.49 (0.88-2.50)	1.37 (0.81-2.32)	1.03 (0.55-1.93)	1.05 (0.56-1.99)
	Black	101	1.11 (0.73-1.69)	1.06 (0.72-1.58)	1.20 (0.68-2.09)	1.23 (0.73-2.07)	1.11 (0.50-2.47)	0.80 (0.36-1.78)	1.14 (0.69-1.90)	1.03 (0.63-1.69)	1.06 (0.57-1.96)	1.02 (0.55-1.90)
sPTB <34	White	202	1.34 (0.74-2.43)	1.42 (0.79-2.54)	1.20 (0.45-3.23)	1.56 (0.56-4.30)	**2.71 (1.12-6.58)**	**2.52 (1.50-6.01)**	1.49 (0.72-3.09)	1.42 (0.70-2.88)	0.83 (0.35-1.99)	0.90 (0.37-2.20)
	Black	101	1.13 (0.69-1.85)	1.17 (0.74-1.86)	0.98 (0.53-1.82)	0.98 (0.55-1.75)	1.27 (0.49-3.29)	1.11 (0.44-2.77)	1.16 (0.63-2.14)	1.15 (0.64-2.05)	1.39 (0.61-3.18)	1.30 (0.57-2.96)
PPROM	White	198	1.05 (0.57-1.92)	1.01 (0.56-1.81)	0.76 (0.31-1.87)	0.79 (0.33-1.89)	2.23 (0.75-6.27)	2.03 (0.73-5.67)	1.36 (0.61-3.04)	1.14 (0.53-2.45)	0.79 (0.31-1.98)	0.83 (0.33-2.11)
	Black	98	1.22 (0.76-1.94)	1.29 (0.84-1.99)	1.03 (0.56-1.89)	1.16 (0.87-1.54)	1.38 (0.55-2.21)	1.29 (0.53-3.13)	1.19 (0.68-2.08)	1.19 (0.70-2.02)	1.58 (0.77-3.25)	1.55 (0.77-3.15)
Objective infection	White	202	0.96 (0.61-1.53)	0.92 (0.59-1.43)	0.92 (0.43-1.99)	1.01 (0.48-2.13)	0.96 (0.46-2.02)	0.84 (0.42-1.68)	0.84 (0.48-1.50)	0.80 (0.45-1.40)	1.06 (0.54-2.12)	1.03 (0.51-2.08)
	Black	101	1.37 (0.85-2.22)	1.42 (0.92-2.20)	0.94 (0.50-1.79)	1.07 (0.60-1.91)	1.23 (0.49-3.07)	1.07 (0.60-1.91)	**1.83 (1.03-3.08)**	1.63 (0.95-2.82)	1.72 (0.86-3.41)	1.75 (0.88-3.46)
Fetal adverse outcome	White	202	0.78 (0.51-1.19)	0.75 (0.49-1.15)	**0.42 (0.21-0.84)**	0.49 (0.24-1.01)	1.21 (0.60-2.42)	1.02 (0.53-1.98)	1.04 (0.61-1.78)	0.97 (0.57-1.63)	0.58 (0.30-1.13)	0.59 (0.30-1.17)
	Black	101	1.35 (0.87-2.09)	1.38 (0.92-2.06)	1.22 (0.67-2.23)	1.39 (0.79-2.40)	1.64 (0.74-6.31)	1.42 (0.65-3.13)	1.54 (0.92-2.60)	1.61 (0.99-2.61)	1.07 (0.56-2.07)	1.04 (0.54-2.00)

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled cathelicidin concentration. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, BMI=body mass index. Statistically significant ratios in bold*

Table 6-23. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome, using CVF cathelicidin concentration, by gestation at sampling in high-risk women, stratified by ethnicity

Outcome		**Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)**			
Gestation category (weeks^{+days})		**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24^{+0}**
Cervical shortening <25 mm	White	**0.60 (0.51-0.69)**	0.55 (0.46-0.64)	0.55 (0.46-0.64)	0.58 (0.49-0.66)
	Black	0.49 (0.36-0.61)	0.58 (0.46-0.70)	0.58 (0.46-0.68)	0.51 (0.40-0.62)
sPTB <37	White	0.58 (0.49-0.68)	**0.77 (0.68-0.84)**	0.57 (0.49-0.66)	0.49 (0.40-0.57)
	Black	0.56 (0.43-0.69	0.52 (0.39-0.64)	0.56 (0.44-0.67)	0.50 (0.38-0.62)
sPTB <34	White	0.52 (0.42-0.61)	**0.72 (0.63-0.79)**	0.52 (0.43-0.60)	0.44 (0.35-0.53)
	Black	0.48 (0.36-0.61)	0.53 (0.41-0.65)	0.54 (0.43-0.66)	0.60 (0.48-0.70)
PPROM	White	0.44 (0.35-0.54)	**0.68 (0.59-0.76)**	**0.61 (0.52-0.68)**	0.42 (0.33-0.51)
	Black	0.51 (0.38-0.63)	0.56 (0.43-0.68)	0.56 (0.45-0.68)	0.61 (0.50-0.72)
Objective infection	White	0.49 (0.40-0.59)	0.56 (0.46-0.64)	0.48 (0.39-0.56)	0.48 (0.39-0.56)
	Black	0.49 (0.38-0.62)	0.60 (0.48-0.72)	**0.68 (0.57-0.78)**	**0.67 (0.55-0.77)**
Fetal adverse outcomes	White	**0.36 (0.27-0.45)**	0.56 (0.49-0.65)	0.50 (0.42-0.59)	0.40 (0.32-0.50)
	Black	0.57 (0.43-0.68)	0.60 (0.48-0.72)	**0.63 (0.52-0.73)**	0.57(0.45-0.68)

CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes. Statistically significant AUCs in bold

6.2.2.5 *Removal of post-intervention samples*

When post intervention samples were removed, patterns were largely similar, though small numbers and wide confidence intervals make it difficult to reach conclusions (Table 6-24 & 6-25). Interestingly, the relationship between sPTB <37 weeks and cathelicidin concentration in White women was strengthened at gestations 10 to 19^{+6} weeks. In particular, CVF cathelicidin at 14-15^{+6} weeks was strongly predictive of sPTB <37 weeks with AUC 0.82 (n=113), compared to Black women (n=51, AUC 0.54) and the difference between these ROC curves was significant at a p value of 0.02. It was similarly predictive of sPTB <34 weeks (White women n= 115, AUC 0.79 vs. Black women n=52, AUC 0.40, p=0.01). In White women, CVF cathelicidin was also moderately predictive of PPROM (n= 116, AUC 0.69) and fetal adverse outcome (n=118, AUC 0.60) though the difference prediction in White and Black women was not statistically significant (p=0.73).

Although there were small numbers of high-risk Black women in each group once samples obtained post-intervention were removed (Table 6-24) it is notable that high cathelicidin at later gestations (16 to 24^{+0} weeks of gestation) were predictive of objective infection in Black women (16 to 19^{+6} weeks n=54, AUC ROC 0.71, 0.56 to 0.82; 20 to 24 weeks n=38, ROC 0.79, 0.62 to 0.95), and this was statistically different to prediction in White women (p=<0.001). Furthermore, at 20 to 24^{+0} weeks of gestation, high cathelicidin also predicted sPTB <34 weeks in Black women (n=38, 0.78, 0.63 to 0.90). In contrast, at this late gestation, low cathelicidin predicted fetal adverse outcome in White women (n=115, 0.62, 0.52 to 0.71) as well as Black women (n=38, 0.62, 0.43 to 0.76) though this did not reach statistical significance in Black women, likely due to the small sample size.

Table 6-24. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome, using CVF cathelicidin concentration, by gestation at sampling in high-risk women, stratified by ethnicity, after exclusion of post-intervention samples

Outcome		**Cathelicidin Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)**			
Gestation category (weeks^{+days})		**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24**
Cervical shortening <25 mm	White	**0.62 (0.52-0.71)**	0.54 (0.44-0.63)	0.51 (0.42-0.60)	0.57 (0.47-0.66)
	Black	0.52 (0.38-0.65)	0.58 (0.44-0.72)	0.58 (0.43-0.71)	0.33 (0.20-0.51)
sPTB <37	White	**0.65 (0.55-0.74)**	**0.82 (0.73-0.88)**	**0.61 (0.51-0.69)**	0.54 (0.36-0.72)
	Black	0.66 (0.49-0.84)	0.54 (0.34-0.75)	0.60 (0.45-0.73)	0.67 (0.47-0.86)
sPTB <34	White	0.55 (0.46-0.65)	**0.79 (0.71-0.86)**	0.53 (0.24-0.82)	0.49 (0.39-0.59)
	Black	0.55 (0.35-0.75)	0.40 (0.18-0.62)	0.55 (0.00-1.00)	**0.78 (0.63-0.90)**
PPROM	White	0.52 (0.42-0.61)	**0.69 (0.61-0.78)**	**0.65 (0.56-0.73)**	0.45 (0.36-0.55)
	Black	0.57 (0.43-0.70)	0.49 (0.34-0.63)	0.49 (0.35-0.63)	**0.81 (0.66-0.92)**
Objective infection	White	0.54 (0.44-0.64)	0.53 (0.44-0.63)	0.46 (0.30-0.61)	0.45 (0.36-0.55)
	Black	0.57 (0.34-0.70)	0.63 (0.47-0.75)	**0.71 (0.56-0.82)**	**0.79 (0.62-0.95)**
Fetal adverse outcomes	White	0.45 (0.35-0.55)	**0.60 (0.51-0.69)**	0.53 (0.44-0.62)	**0.38 (0.29-0.48)**
	Black	0.59 (0.39-0.78)	0.55 (0.41-0.70)	0.58(0.43-0.71)	0.38 (0.24-0.57)

CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes. Statistically significant AUCs in bold

6.2.3 Human neutrophil elastase

6.2.3.1 *HNE and adverse pregnancy outcomes*

Overall, CVF HNE concentration was not higher in women who delivered prematurely compared to women who delivered at term (Table 6-25) after adjustment for age, BMI, ethnicity, smoking, gestation at visit, and inter-assay plate variation (<37 weeks ratio 1.27, 95% CI 0.83 to 1.95 p=0.27, <34 weeks 1.05, 0.59 to 1.89, p=0.86), nor when stratified according to risk status at enrolment (Table 6-26). When stratified according to gestation, at 20-24 weeks gestation, women who subsequently experienced sPTB <34 weeks, and who experienced composite adverse neonatal outcome, had lower adjusted CVF HNE concentration than those women who did not

(Table 6-25 and 6-26). Low HNE at this gestation was moderately predictive of sPTB <34 weeks (n=444, AUC ROC 0.57, 95% CI 0.53-0.62) adverse fetal outcomes (n=446, 0.58, 0.53-0.62), and objective infection (n=446, 0.56, 0.52-0.61) (Table 6-27). In contrast, amongst the high-risk cohort only (Table 6-28), early gestation (14 to 15^{+6} weeks) women who delivered early with sPTB <34 and 37 weeks of gestation had higher HNE concentration (Figure 6.6a) compared with controls, though this did not reach statistical significance after adjustment. Nonetheless crude HNE concentration at 14^{+0} and 15^{+6} weeks of gestation was modestly predictive of sPTB <37 weeks (n=236, AUC ROC 0.63, 95% CI 0.56 to 0.69), <34 weeks (n=241, 0.61, 0.55-0.68), PPROM (n=241, 0.65, 0.59 to 0.71), objective infection (n=249, 0.60, 0.53-0.66) and adverse fetal outcomes (n=249, 0.60, 0.54-0.66). In contrast, a similar pattern of low HNE at a later gestation (20 to 24^{+0} weeks) predicted sPTB <34 weeks and adverse fetal outcomes, as per the total cohort (Table 6-28).

Table 6-25 Ratio (95% confidence intervals) of the logged mean CVF HNE concentration in cases and controls overall, and stratified by gestation at sampling in the whole cohort (high-risk and low-risk)

Outcome		Ratio (95% Confidence Interval) of HNE concentration cases: controls**					
		Gestation category (weeks^{+days})					
	N (overall)	**Overall**		**10-13^{+6}**		**20-24^{+0}**	
		unadjusted	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**
sPTB <37	552	1.39 (0.91-2.12)	1.27 (0.83-1.95)	1.62 (0.86-3.06)	1.55 (0.80-3.00)	0.69 (0.39-1.23)	0.69 (0.38-1.25)
sPTB <34	552	1.13 (0.64-2.03)	1.05 (0.59-1.89)	1.32 (0.54-3.24)	1.31 (0.51-3.33)	**0.31 (0.13-0.73)**	**0.32 (0.14-0.76)**
PPROM	545	1.63 (0.96-2.76)	1.46 (0.85-2.50)	1.53 (0.70-3.35)	1.45 (0.65-3.20)	0.97 (0.45-2.08)	0.98 (0.45-2.13)
Objective infection	552	0.90 (0.57-1.41)	0.90 (0.57-1.41)	0.64 (0.33-1.23)	0.65 (0.34-1.26)	0.67 (0.38-1.20)	0.70 (0.39 1.24)
Fetal adverse outcome	552	0.88 (0.57-1.39)	0.84 (0.54-1.32)	0.65 (0.33-1.29)	0.65 (0.32-1.32)	**0.45 (0.25-0.81)**	**0.44 (0.25-0.80)**

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled HNE concentration. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, BMI=body mass index, HNE=human neutrophil elastase. Statistically significant ratios in bold*

Table 6-26. Ratio (95% confidence intervals) of the logged mean CVF HNE concentration in cases and controls overall, and stratified by gestation at sampling in high-risk women

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled HNE concentration. CVF=cervicovaginal fluid;*

Outcome		Ratio (95% Confidence Interval) of HNE concentration cases: controls**									
		Gestation category (weeks^{+days})									
	N (overall)	**Overall**		**10-13^{+6}**		**14-15^{+6}**		**16-19^{+6}**		**20-24^{+0}**	
		unadjusted	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**
Cervical shortening <25 mm	347	**1.58 (1.03-2.44)**	1.57 (1.00-2.46)	0.91 (0.48-1.72)	1.15 (0.60-2.23)	**2.43 (1.28-4.61)**	**2.95 (1.52-5.75)**	1.34 (0.75-2.40)	1.50 (0.82-2.76)	1.66 (0.89-3.08)	1.42 (0.75-2.68)
sPTB <37	352	1.24 (0.78-1.95)	1.10 (0.70-1.73)	1.37 (0.69-2.71)	1.31 (0.66-2.60)	**2.15 (1.11-4.14)**	**1.83 (1.91-3.66)**	1.22 (0.65-2.29)	1.03 (0.54-1.97)	0.64 (0.34-1.22)	0.60 (0.31-1.15)
sPTB <34	352	0.97 (0.53-1.77)	0.86 (0.48-1.57)	1.02 (0.42-2.50)	1.03 (0.42-2.54)	2.18 (0.95-5.00)	1.89 (0.81-4.38)	0.69 (0.29-1.64)	0.53 (0.22-1.25)	**0.28 (0.12-1.69)**	**0.27 (0.11-0.66)**
PPROM	345	1.56 (0.87-2.78)	1.38 (0.78-2.46)	1.18 (0.50-2.76)	1.11 (0.49-2.54)	**2.82 (1.18-6.79)**	2.38 (0.98-5.80)	1.33 (0.58-3.02)	1.07 (0.46-2.47)	1.14 (0.48-2.75)	1.05 (0.43-2.55)
Objective infection	352	1.09 (0.64-1.87)	1.09 (0.64-1.84)	0.60 (0.26-1.36)	0.69 (0.31-1.52)	**2.22 (1.01-4.91)**	2.20 (1.00-4.84)	0.98 (0.46-2.08)	0.96 (0.46-2.01)	1.05 (0.49-2.22)	1.09 (0.52-2.26)
Fetal adverse outcome	352	0.93 (0.56-1.54)	0.88 (0.53-1.45)	0.88 (0.40-1.94)	1.01 (0.46-2.21)	1.88 (0.92-3.84)	1.88 (0.92-3.84)	0.86 (0.44-1.69)	0.74 (0.38-1.45)	**0.41 (0.21-0.82)**	**0.36 (0.18-0.72)**

sPTB=spontaneous preterm birth,PPROM=premature prelabour rupture of membranes, BMI=body mass index, HNE=human neutrophil elastase. Statistically significant ratios in bold

Table 6-27. Receiver Operating Characteristic Area under the Curve (95% confidence intervals for prediction of outcome in all women, using CVF HNE concentration stratified by gestation at sampling

Outcome	Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)	
Gestation category (weeks^{+days})	**10-13^{+6}**	**20-24^{+0}**
sPTB <37	**0.57 (0.52-0.62)**	0.47 (0.42-0.51)
sPTB <34	0.55 (0.50-0.60)	**0.43 (0.38-0.47)**
PPROM	**0.57 (0.53-0.62)**	0.49 (0.44-0.54)
Objective infection	**0.44 (0.39-0.49)**	**0.44 (0.39-0.48)**
Fetal adverse outcomes	0.46(0.41-0.51)	**0.42 (0.38-0.47)**

CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes. Statistically significant AUCs in bold

Table 6-28. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome in high-risk women, using CVF human neutrophil elastase (HNE) concentration stratified by gestation at sampling

Outcome	Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)			
Gestation category (weeks^{+days})	**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24^{+0}**
Cervical shortening <25 mm	0.53 (0.46-0.60)	**0.65 (0.59-0.71)**	**0.58 (0.52-0.64)**	**0.64 (0.58-0.69)**
sPTB <37	0.56 (0.49-0.63)	**0.63 (0.56-0.69)**	0.56 (0.50-0.62)	0.47 (0.40-0.53)
sPTB <34	0.52 (0.45-0.59)	**0.61 (0.55-0.68)**	0.50 (0.44-0.56)	**0.42 (0.36-0.48)**
PPROM	0.55 (0.48-0.62)	**0.65 (0.59-0.71)**	0.57 (0.50-0.63)	0.52 (0.46-0.58)
Objective infection	**0.41 (0.35-0.48)**	**0.60 (0.53-0.66)**	0.50 (0.44-0.56)	0.48 (0.42-0.54)
Fetal adverse outcomes	0.50 (0.43-0.56)	**0.60 (0.54-0.66)**	0.51 (0.45-0.57)	**0.42 (0.36-0.48)**

CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes. Statistically significant AUCs in bold.

6.2.3.2 *High-risk women and cervical shortening*

Figure 6-6b shows the longitudinal expression of HNE in cases (short cervix <25mm by 24 weeks of gestation) vs. controls (normal cervix at 24 weeks of gestation). In high-risk women, both crude (ratio 2.43, 1.28-4.61, p=0.007) and adjusted (n=222, ratio 2.95, 1.52-5.75, p=0.001) CVF HNE concentrations were higher in women who later developed a short cervix by 24 weeks of gestation, when sampled at 14-15^{+6} weeks of gestation.

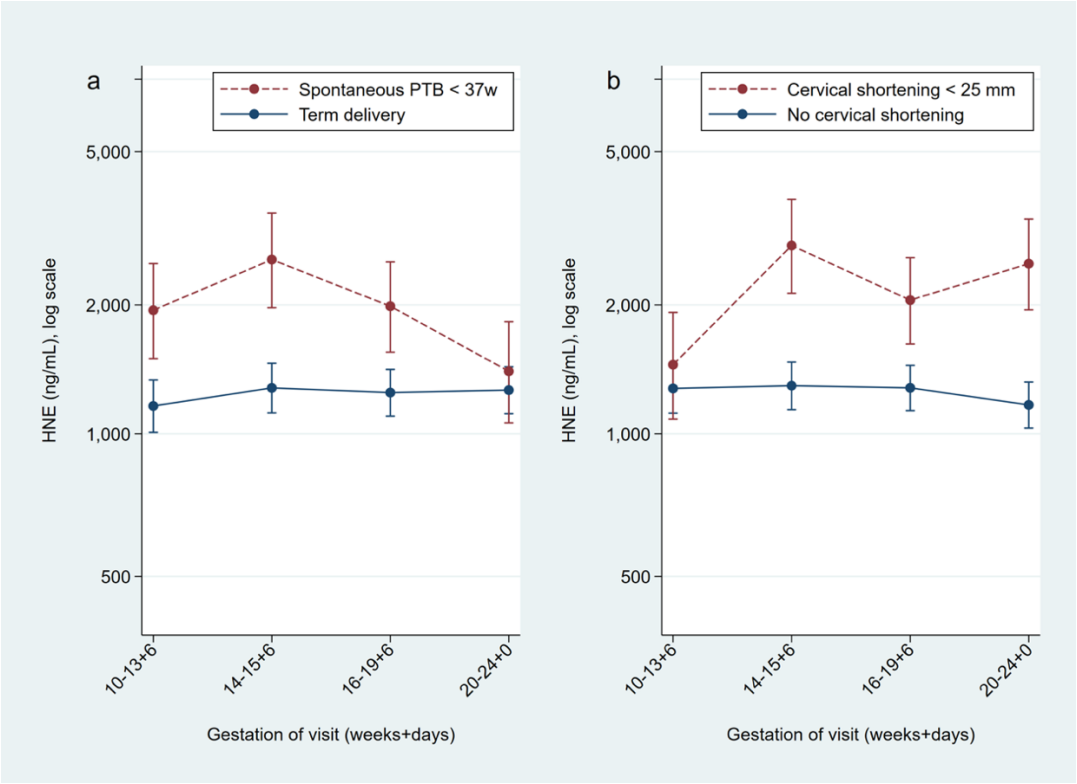

Figure 6-6. Longitudinal measurements of cervicovaginal human neutrophil elastase (HNE) concentrations in high-risk women who deliver spontaneously prior to 37 weeks of gestation(sPTB) (6-6a, n=281 term, n= 64 preterm) and women who subsequently develop a short cervix (6-6b n=62 short, n=198 not short). Geometric means and SE bars.

High CVF HNE was moderately predictive of cervical shortening when sampled at all gestational categories between 14 and 24 weeks of gestation (Table 6-28).

6.2.3.3 *Removal of post intervention samples*

Once samples taken after intervention (cerclage, progesterone or Arabin pessary) had been initiated, the relationship between low CVF HNE concentration at 20 to 24^{+0} weeks of gestation (the gestation at which many women typically receive intervention) was more pronounced in all women, and high-risk women only (Tables 6-29 and 6-30). Women in the whole cohort who delivered spontaneously prior to 34 weeks of gestation had far lower CVF HNE concentration (n=386 ratio 0.14, 95% CI 0.04-0.45, p=0.001) at 20 to 24^{+0} weeks, after adjustment by maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled HNE concentration, as did women with adverse fetal outcomes (n=391, 0.46, 0.23 to 0.91, p=0.03) and evidence of objective infection (n= 391, 0.47, 0.25 to 0.88, p=0.02). Low CVF HNE at 16 to 19^{+6} weeks was also associated with sPTB <34 weeks in high-risk women (n=242, 0.28, 0.09-0.89, p=0.03). This was reflected in the prediction analysis (Tables 6-31 and 6-32); low HNE concentration at 16 to 20 weeks, and 20 to 24 weeks predicted sPTB prior to 34 weeks AUCs 0.63 and 0.65 respectively in the whole cohort, performing similarly in high-risk women only.

In contrast, high CVF HNE concentrations in the whole cohort at early gestations (10 to 13^{+6} weeks) were weakly predictive of sPTB prior to 37 and 34 weeks of gestation, as well as PPROM (Table 6-31). Adjusted CVF HNE at 14 to 15^{+6} weeks of gestation was high in high-risk women who developed a short cervix (n=179, ratio 2.28, 95% CI 1.76 to 4.43, p=0.02), with AUC ROC for crude concentration 0.61 (95 % CI 0.54-0.67).

Table 6-29. Ratio (95% confidence intervals) of the logged mean CVF HNE concentration in cases and controls overall, and stratified by gestation at sampling in the whole cohort (high-risk and low-risk) after post intervention samples were excluded

Outcome	Ratio (95% Confidence Interval) of HNE concentration cases: controls**						
				Gestation category (weeks^{+days})			
	N (overall)	**Overall**		**16-19^{+6}**		**20-24^{+0}**	
		unadjusted	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**
sPTB <37	515	1.45 (0.91-2.30)	1.39 (0.87-2.21)	1.58 (0.79-3.19)	1.40 (0.68-2.88)	0.76 (0.36-1.52)	0.75 (0.37-1.54)
sPTB <34	515	0.78 (0.40-1.53)	0.76 (0.39-1.51)	0.41 (0.13-1.25)	**0.32 (0.10-0.97)**	**0.13 (0.04-0.44)**	**0.14 (0.04-0.45)**
PPROM	510	1.50 (0.82-2.73)	1.35 (0.73-2.48)	1.55 (0.53-4.56)	1.30 (0.44-3.85)	1.09 (0.41-2.93)	1.10 (0.40-3.02)
Objective infection	515	0.77 (0.48-1.22)	0.78 (0.49-1.25)	1.01 (0.46-2.25)	1.12 (0.51-2.48)	0.44(0.24-0.83)	0.47 (0.25-0.88)
Fetal adverse outcome	515	0.84 (0.51-1.36)	0.84 (0.52-1.37)	0.86 (0.40-1.88)	0.76 (0.35-1.67)	**0.44 (0.23-0.87)**	**0.46 (0.23-0.91)**

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled HNE concentration. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, BMI=body mass index, HNE=human neutrophil elastase. Statistically significant ratios in bold*

Table 6-30. Ratio (95% confidence intervals) of the logged mean CVF HNE concentration in cases and controls in high-risk women, and stratified by gestation at sampling after post intervention samples were excluded

Outcome		Ratio (95% Confidence Interval) of HNE concentration cases: controls**									
				Gestation category (weeks^{+days})							
	N (overall)	**Overall**		**10-13^{+6}**		**14-15^{+6}**		**16-19^{+6}**		**20-24^{+0}**	
		unadjusted	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**
Cervical shortening <25mm	308	1.11 (0.69-1.76)	1.12 (0.69-1.83)	0.79 (0.40-1.57)	0.97 (0.48-1.97)	1.58 (0.84-2.98)	**2.28 (1.18-4.43)**	1.00 (0.52-1.91)	1.24 (0.62-2.49)	0.76 (0.31-1.86)	0.72 (0.29-1.80)
sPTB <37	315	1.37 (0.84-2.25)	1.23 (0.75-2.02)	1.83 (0.84-3.95)	1.76 (0.80-3.89)	1.91 (0.97-3.75)	1.80 (0.88-3.65)	1.51 (0.73-3.12)	1.29 (0.61-2.72)	0.80 (0.37-1.76)	0.70 (0.31-1.58)
sPTB <34	315	0.71 (0.36-1.41)	0.63 (0.32-1.25)	1.16 (0.0-3.32)	1.14 (0.40-3.28)	0.82 (0.33-2.05)	0.76 (0.31-1.87)	0.38 (1.12-1.21)	**0.28 (0.09-0.89)**	**0.13 (0.04-0.46)**	**0.14 (0.04-0.45)**
PPROM	310	1.50 (0.77-2.91)	1.27 (0.65-2.47)	1.98 (0.73-5.35)	1.66 (0.62-4.42)	1.17 (0.46-2.97)	0.97 (0.38-2.51)	1.48 (0.49-4.48)	1.17 (0.38-3.61)	1.80 (0.52-6.20)	1.54 (0.44-5.40)
Objective infection	315	0.93 (0.53-1.65)	0.96 (0.55-1.67)	0.79 (0.31-2.00)	0.89 (0.36-2.19)	1.74 (0.80-3.79)	1.81 (0.84-3.91)	0.96 (0.42-2.18)	1.05 (0.46-2.38)	0.62 (0.26-1.48)	0.71 (0.30-1.67)
Fetal adverse outcome	315	0.95 (0.55-1.66)	0.94 (0.54-1.62)	1.38 (0.55-3.43)	1.57 (0.63-3.90)	1.34 (0.64-2.80)	1.49 (0.72-3.07)	0.81 (0.37-1.81)	0.71 (0.32-1.59)	**0.43 (0.19-0.97)**	**0.41 (0.18-0.91)**

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled HNE concentration. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, BMI=body mass index, HNE=human neutrophil elastase. Statistically significant ratios in bold*

Table 6-31. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome in all women, using CVF HNE concentration stratified by gestation at sampling, after removal of post intervention samples

Outcome	Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)	
Gestation category (weeks^{+days})	**10-13^{+6}**	**20-24^{+0}**
sPTB <37	**0.60 (0.55-0.65)**	0.50 (0.45-0.55)
sPTB <34	**0.58 (0.53-0.63)**	**0.35 (0.30-0.40)**
PPROM	**0.63 (0.58-0.68)**	0.49 (0.41-0.53)
Objective infection	0.46 (0.41-0.51)	**0.38 (0.33-0.43)**
Fetal adverse outcomes	0.49 (0.44-0.54)	**0.42 (0.37-0.47)**

CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=Premature prelabour rupture of membranes. Statistically significant AUCs in bold.

Table 6-32. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome in high-risk women, using CVF HNE concentration stratified by gestation at sampling, after post intervention samples were removed

Outcome	**Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)**			
Gestation category (weeks^{+days})	**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24**
Cervical shortening <25mm	0.51 (0.43-0.58)	**0.61 (0.54-0.67)**	0.54 (0.47-0.61)	0.56 (0.48-0.62)
sPTB <37	0.59 (0.52-0.66)	**0.60 (0.53-0.67)**	0.55 (0.48-0.62)	0.52 (0.45-0.59)
sPTB <34	0.55 (0.47-0.62)	0.48 (0.41-0.55)	**0.36 (0.30-0.43)**	**0.36 (0.29-0.42)**
PPROM	**0.62 (0.55-0.69)**	0.54 (0.47-0.61)	0.54 (0.48-0.61)	0.57 (0.50-0.64)
Objective infection	0.45 (0.38-0.52)	0.57 (0.50-0.64)	0.46 (0.39-0.53)	**0.39 (0.32-0.46)**
Fetal adverse outcomes	0.55 (0.48-0.62)	0.57 (0.50-0.64)	0.49 (0.42-0.56)	**0.41 (0.35-0.49)**

CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=Premature prelabour rupture of membranes. Statistically significant AUCs in bo

6.2.3.4 *Stratification by ethnicity*

When stratified by ethnicity (Table 6-33 and 6-34) there were no statistically significant differences in pattern of HNE expression related to outcome in Black and White women. The previously observed pattern of low CVF HNE concentration at 20 to 24 weeks of gestation and association with sPTB <34 weeks and adverse fetal outcome was statistically significant in White women but not Black women, likely due to smaller numbers in the cohort of Black women.

Unlike the whole mixed ethnicity cohort, high-risk White women who delivered prior to 37 weeks had significantly higher CVF HNE concentration after adjustment at 14-15^{+6} weeks (sPTB <37 weeks, n=134, ratio 2.71, 95% CI 1.11 to 6.61, p=0.03). This was reflected in the ROC curve for prediction of sPTB <37 weeks (Table 6-35) using high HNE in White women only (AUC ROC 0.65, 95% CI 0.56-0.73). Furthermore, high-risk White women (Table 6-36) who developed a short cervix had more than double the CVF HNE concentration overall, compared with women who did not develop cervical shortening (n=202, adjusted ratio 2.17, 95% CI 1.08 to 4.39, p=0.03). This was most apparent at 14-15^{+6} weeks, a more marked increase in White women, than seen in the cohort overall (n=116, 5.54, 2.04 to 14.16, p=0.001), with the equivalent AUC ROC 0.73 (95% CI 0.65-0.80).

Table 6-33. Ratio (95% confidence intervals) of the logged mean CVF HNE concentration in cases and controls overall, and stratified by gestation at sampling, in Black and White women (whole cohort)

Outcome			Ratio (95% Confidence Interval) of HNE concentration cases: controls**					
			Gestation category (weeks^{+days})					
	Ethnicity	N (overall)	Overall		10-13^{+6}		20-24^{+0}	
			unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted
sPTB <37	White	363	1.34 (0.74-2.40)	1.39 (0.77-2.51)	1.66 (0.65-4.25)	1.83 (0.69-4.84)	0.59 (0.27-1.26)	0.59 (0.27-1.30)
	Black	131	0.67 (0.32-1.41)	0.66 (0.31-1.39)	1.05 (0.42-2.65)	1.30 (0.49-3.41)	0.45 (1.16-1.24)	0.45 (0.16-1.32)
sPTB <34	White	363	0.79 (0.33-1.94)	0.88 (0.35-1.18)	1.88 (0.37-9.69)	2.79 (0.49-15.71)	**0.22 (0.07-0.65)**	**0.22 (0.07-0.68)**
	Black	131	0.83 (0.34-2.03)	0.89 (0.37-2.16)	0.55 (0.19-1.61)	0.83 (0.28-2.42)	0.56 (0.13-2.32)	0.57 (0.14-2.36)
PPROM	White	359	1.53 (0.67-3.49)	1.63 (0.71-3.71)	1.08 (0.31-3.81)	1.39 (0.40-4.90)	1.14 (0.38-3.40)	1.23 (0.41-3.68)
	Black	128	0.92 (0.41-2.06)	1.07 (0.48-2.37)	1.04 (0.40-2.71)	1.59 (0.60-4.20)	0.44 (0.14-1.40)	0.52 (0.17-1.63)
Objective infection	White	363	0.82 (0.46-1.44)	0.85 (0.48-1.49)	0.58 (0.25-1.38)	0.58 (0.25-1.37)	0.74 (0.37-1.50)	0.77 (0.38-1.54)
	Black	131	0.94 (0.42-2.09)	0.94 (0.4-2.10)	0.79 (0.30-2.06)	0.82 (0.32-2.12)	0.55 (0.19-1.58)	0.64 (0.23-1.77)
Fetal adverse outcome	White	363	0.64 (0.35-1.18)	0.63 (0.34-1.17)	0.55 (0.20-1.54)	0.55 (1.19-1.54)	**0.32 (0.15-0.68)**	**0.31 (0.14-0.67**)
	Black	131	1.09 (0.51-2.34)	1.01 (0.48-2.16)	0.66 (0.26-1.67)	0.60 (0.24-1.52)	0.59 (0.22-1.62)	0.62 (0.24-1.64)

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled HNE concentration. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, BMI=body mass index, HNE=human neutrophil elastase. Statistically significant ratios in bold.*

Table 6-34. Ratio (95% confidence intervals) of the logged mean CVF HNE concentration in cases and controls overall, and stratified by gestation at sampling, in high-risk Black and White women

Outcome			Ratio (95% Confidence Interval) of HNE concentration cases: controls**									
			Gestation (weeks $^{+days}$)									
	Ethnicity	**N (overall)**	**Overall**		**10-13^{+6}**		**14-15^{+6}**		**16-19^{+6}**		**20-24^{+0}**	
			unadjusted	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**
Cervical shortening <25mm	White	202	**2.10 (1.06-4.20)**	**2.17 (1.08-4.39)**	1.41 (0.34-5.84)	1.18 (0.28-5.06)	**4.72 (1.83-12.16)**	**5.54 (2.12-14.49)**	1.38 (0.55-3.47)	1.69 (0.67-4.29)	1.93 (0.73-5.05)	1.67 (0.62-4.47)
	Black	107	1.27 (0.65-2.48)	1.47 (0.71-2.91)	0.85 (0.45-1.61)	1.12 (0.56-2.26)	2.11 (0.65-6.83)	2.34 (0.67-7.45)	1.28 (0.48-3.46)	1.38 (0.51-3.78)	2.07 (0.85-5.04)	**2.57 (1.01-6.53)**
sPTB <37	White	210	1.24 (0.66-2.32)	1.25 (0.67-2.34)	1.56 (0.58-4.25)	1.57 (0.57-4.32)	**2.43 (1.04-5.70)**	**2.71 (1.11-6.61)**	1.51 (0.63-3.58)	1.60 (0.64-3.96)	0.57 (0.24-1.35)	0.57 (0.24-1.37)
	Black	105	0.59 (0.26-1.33)	0.54 (0.24-1.24)	0.84 (0.31-2.33)	0.97 (0.32-2.89)	0.87 (0.21-3.62)	0.91 (0.21-3.88)	0.38 (0.12-1.22)	0.32 (0.10-1.04)	0.41 (0.13-1.33)	0.32 (0.09-1.09)
sPTB <34	White	210	0.74 (0.29-1.87)	0.82 (0.32-2.08)	1.66 (0.32-8.59)	2.71 (0.50-14.92)	1.77 (0.54-5.80)	1.99 (0.58-6.82)	0.60 (0.16-2.21)	0.57 (0.15-2.15)	**0.23 (0.07-0.75)**	**0.23 (0.07-0.77)**
	Black	105	0.72 (0.28-1.85)	0.74 (0.29-1.92)	0.44 (0.15-1.29)	0.57 (0.18-1.77)	1.99 (0.41-9.65)	2.64 (0.55-12.67)	0.44 (0.10-1.92)	0.46 (0.11-2.00)	0.48 (0.10-2.20)	0.37 (0.08-1.66)
PPROM	White	206	1.60 (0.65-3.95)	1.62 (0.66-3.95)	1.01(0.25-4.11)	1.19 (030-4.68)	2.56 (0.63-10.35)	2.63 (0.65-10.71)	1.82 (0.51-6.52)	1.56 (0.43-5.66)	1.43 (0.40-5.10)	1.43 (0.40-5.15)
	Black	102	0.86 (0.36-2.08)	0.96 (0.39-2.34)	0.84 (0.29-2.42)	1.20 (0.39-3.65)	2.29 (0.48-10.92)	2.46 (0.53-11.32)	0.53 (1.34-2.01)	0.66 (1.17-2.52)	0.40 (0.10-1.52)	0.33 (0.08-1.25)
Objective infection	White	210	1.05 (0.51-2.14)	1.07 (0.53-2.16)	0.54 (0.17-1.76)	0.58 (0.19-1.77)	1.59 (0.58-4.38)	1.74 (0.62-4.85)	0.83 (0.30-2.26)	0.97 (0.36-2.64)	1.38 (0.52-3.65)	1.28 (0.48-3.39)
	Black	105	1.21 (0.48-3.07)	1.17 (0.47-2.93)	0.86 (0.28-2.67)	1.14 (0.37-3.53)	4.06 (0.85-19.51)	3.22 (0.66-15.64)	1.20 (0.30-4.88)	1.11 (0.28-4.44)	0.95 (0.25-3.54)	1.03 (0.29-3.71)
Fetal adverse outcome	White	210	0.78 (0.39-1.55)	0.76 (0.38-1.51)	1.10 (0.32-3.76)	1.16 (0.34-3.96)	1.50 (0.60-3.70)	1.58 (0.63-3.97)	0.54 (0.21-1.36)	0.48 (0.19-1.22)	**0.33 (0.13-0.84)**	**0.30 (0.12-0.76)**
	Black	105	1.01 (0.43-2.41)	0.89 (0.38-2.10)	0.55 (0.19-1.55)	0.56 (0.20-1.61)	3.39 (0.78-14.82)	2.78 (0.64-12.07)	0.94 (0.27-3.24)	0.79 (0.23-2.70)	0.56 (0.17-1.87)	0.48 (1.15-1.58)

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI, smoking and inter-plate pooled HNE concentration. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, BMI=body mass index, HNE=human neutrophil elastase. Statistically significant ratios in bold.*

Table 6-35. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome in all women, using CVF Human Neutrophil Elastase concentration by gestation at sampling in the whole cohort (high-risk and low risk), stratified by ethnicity

Outcome	**Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)**		
Gestation category (weeks^{+days})		**10-13^{+6}**	**20-24^{+0}**
sPTB <37	White	0.55 (0.49-0.61)	0.46 (0.40-0.51)
	Black	0.56 (0.46-0.67)	0.42 (0.32-0.52)
sPTB <34	White	0.56 (0.50-0.60)	**0.36 (0.31-0.42)**
	Black	0.46 (0.35-0.56)	0.54 (0.44-0.64)
PPROM	White	0.50 (0.44-0.57)	0.50 (0.44-0.56)
	Black	0.56 (0.46-0.67)	0.44 (0.35-0.55)
Objective infection	White	0.45 (0.38-0.51)	0.46 (0.40-0.51)
	Black	0.42 (0.32-0.53)	0.41 (0.32-0.51)
Fetal adverse outcomes	White	0.43 (0.37-0.50)	**0.37 (0.32-0.43)**
	Black	0.46 (0.36-0.57)	0.48 (0.38-0.58)

CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes. Statistically significant AUCs in bold.

Table 6-36. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome, using CVF Human Neutrophil Elastase concentration, by gestation at sampling in high-risk women, stratified by ethnicity

Outcome		**HNE Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)**			
Gestation category (weeks^{+days})		**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24^{+0}**
Cervical shortening <25 mm	White	**0.63 (0.54-0.71)**	**0.73 (0.65-0.80)**	0.57 (0.48-0.64)	**0.67 (0.59-0.75)**
	Black	0.46 (0.35-0.59)	0.59 (0.47-0.70)	0.57 (0.46-0.68)	**0.65 (0.53-0.75)**
sPTB <37	White	0.55 (0.46-0.64)	**0.65 (0.56-0.73)**	0.55 (0.48-0.63)	0.46 (0.38-0.54)
	Black	0.55 (0.42-0.67)	0.52 (0.39-0.63)	0.44 (0.33-0.56)	0.42 (0.31-0.54)
sPTB <34	White	0.55 (0.46-0.64)	**0.62 (0.53-0.70)**	0.44 (0.36-0.52)	**0.38 (0.30-0.46)**
	Black	0.43 (0.31-0.55)	0.56 (0.45-0.68)	0.47 (0.36-0.59)	0.52 (0.42-0.64)
PPROM	White	0.51 (0.42-0.60)	**0.69 (0.60-0.76)**	0.56 (0.48-0.64)	0.52 (0.44-0.60)
	Black	0.55 (0.43-0.67)	0.58 (0.47-0.70)	0.45 (0.34-0.56)	0.45 (0.34-0.57)
Objective infection	White	**0.40 (0.32-0.49)**	0.57 (0.48-0.65)	0.43 (0.36-0.52)	0.50 (0.42-0.58)
	Black	0.45 (0.33-0.57)	**0.65 (0.53-0.75)**	0.59 (0.48-0.70)	0.51 (0.39-0.62)
Fetal adverse outcomes	White	0.49 (0.40-0.58)	**0.60 (0.51-0.68)**	0.46 (0.38-0.53)	**0.38 (0.31-0.47)**
	Black	0.46 (0.34-0.58)	**0.63 (0.51-0.74)**	0.51 (0.41-0.63)	0.48 (0.37-0.60)

CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM; premature prelabour rupture of membranes. Statistically significant AUCs in bold.

6.2.4 Cathelicidin to elafin ratio

Given the relationship observed between low CVF elafin in some populations (e.g., Black high-risk women), and high cathelicidin concentration, with adverse pregnancy outcome, we explored whether combining CVF cathelicidin and elafin concentration in a ratio would yield superior prediction of sPTB in high-risk women.

6.2.4.1 *Cathelicidin:elafin (CE) ratio, pregnancy outcome and cervical shortening*

Whereas neither CVF elafin nor cathelicidin concentration alone predicted cervical shortening in the high-risk cohort, a high CE ratio at 14-15^{+6} weeks was associated with cervical shortening <25mm (n=204, ratio 2.22, 95% CI 1.26 to 3.91, p=0.006), after adjustment for ethnicity, maternal age, BMI and smoking (Table 6-37). When prediction analysis was applied (Table 6-41), this equated to AUC ROC of 0.57 (95% CI 0.51 to 0.64). Crude CE ratio when measured at 14 to 15^{+6} weeks of gestation was predictive of sPTB <37 weeks (AUC ROC 0.64, 95% CI 0.58-0.71), sPTB < 34 weeks (0.58, 0.51-0.64) and PPROM (0.59, 0.53-0.66), Table 6-41.

As expected, this was most pronounced in Black women, in whom low elafin and high cathelicidin was found to be associated with cervical shortening (albeit with wide confidence intervals due to small sample size) (Table 6-38). Black high-risk women who developed a short cervix <25mm prior to 24 weeks had an adjusted CE ratio at 14-15^{+6} weeks of nearly three and a half times that of women who did not develop a short cervix (n=69, ratio 3.46, 95% CI 1.29 to 9.26, p=0.01), also raised at 16 to 19^{+6} weeks (n=84, 2.92, 1.43-5.97, p=0.004). In addition, it was raised later in gestation (20-24^{+0} weeks), in high-risk Black women who had PPROM with premature delivery (n=76, ratio 2.87, 1.17 to 7.08). This supports a role of these HDPs in the biological response, or even effectors of cervical shortening. The potential utility as a predictive biomarker was also examined and this provided crude concentrations AUC ROC of 0.65 (95% CI 0.53 to 0.76) at 14-15^{+6} weeks, 0.65 (054 to 0.76) at 16-19^{+6} weeks to predict cervical shortening, 0.68 (0.55 to 0.77) to predict PPROM at 20-24 weeks of

gestation and 0.66 (0.55-0.76) at 16-19^{+6} weeks to predict objective infection, Table 6-41.

Interestingly, the CE ratio at 14 to 15^{+6} weeks of gestation was also significantly raised in white women who delivered prior to 37 weeks (n=125, ratio 3.55, 95% CI 1.50 to 8.43, p=0.004), with crude concentrations equating to AUC ROC of 0.74 (95% CI 0.65 to 0.81). Yet at early gestations (10-13^{+6} weeks of gestation), a low CE ratio in high-risk White women, was associated with adverse fetal outcome (n=121, ratio 0.24, 95% CI 0.07 to 0.78, p=0.02). This equated to AUC ROC of 0.66 (95% CI 0.57 to 0.74) for a low CE ratio. The CE ratio was also lower in women who had sPTB <37 weeks and cervical shortening when calculated at this early gestation, although these did not reach statistical significance (Table 6-38).

6.2.4.1.1 Removal of post intervention samples

Once samples taken post-intervention were removed, the CE ratio at 14-15^{+6} weeks of gestation was nearly two and a half times higher in women who delivered spontaneously and prematurely before 37 weeks of gestation (n=180, ratio 2.42, 95% CI 1.22 to 4.82, p=0.01, Table 6-41). This is represented by an AUC ROC of 0.69 (95% CI 0.62-0.76, Table 6-42). This association was particularly pronounced in White women (Table 6-42); when calculated at 14 to 15^{+6} of gestation, a high CE ratio predicted sPTB <37 weeks (AUC ROC 0.76) and sPTB <34 weeks (AUC ROC 0.68), though earlier in pregnancy (12-13^{+6} weeks), a high CE ratio was moderately predictive of sPTB <37 in Black women (AUC ROC 0.65). Unexpectedly, a low CE ratio was a good predictor of adverse fetal outcome (AUC ROC 0.75, 95% CI 0.57-0.87) in Black women at 20 to 24 weeks gestation, albeit in a low sample size (n=38), despite not showing prediction for sPTB or infective outcomes at this gestation. For the outcome cervical shortening, the results were relatively unchanged to those calculated prior to removal of post-intervention samples (Tables 6-39 and 6-42).

Table 6-37 Ratio (95% confidence intervals) of the CE ratio in cases and controls overall, and stratified by gestation at sampling, in high-risk women

Outcome		Ratio (95% Confidence Interval) of CE ratio cases: controls									
				Gestation category (weeks^{+days})							
	N (overall)	**Overall**		**10-13^{+6}**		**14-15^{+6}**		**16-19^{+6}**		**20-24^{+0}**	
		unadjusted	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**
Cervical shortening <25mm	315	1.23 (0.83-1.82)	1.43 (0.95-2.15)	0.95 (0.49-1.82)	1.27 (0.61-2.63)	**1.78 (1.04-3.04)**	**2.22 (1.26-3.91)**	1.34 (0.86-2.08)	1.48 (0.90-2.43)	1.28 (0.78-2.10)	1.33 90.73-2.43)
sPTB <37	330	1.45 (0.98-2.16)	1.35 (0.91-2.00)	1.25 (0.70-2.32)	1.05 (0.58-1.89)	**2.34 (1.35-4.07)**	1.78 (0.96-3.28)	1.38 (0.79-2.42)	1.19 (0.66-2.17)	1.09 (0.67-1.76)	1.00 (0.61-1.63)
sPTB <34	330	1.15 (0.68-1.94)	1.14 (0.68-1.92)	1.01 (0.45-2.25)	0.91 (0.42-2.01)	1.59 (0.85-3.00)	1.22 (0.62-2.39)	1.20 (0.53-2.76)	1.10 (0.46-2.62)	1.23 (0.67-2.28)	1.20 (0.62-2.31)
PPROM	322	1.21 (0.72-2.03)	1.25 (0.75-2.10)	0.88 (0.43-1.79)	0.95 (0.45-1.98)	1.87 (0.89-3.93)	1.59 (0.74-3.43)	1.29 (0.73-2.27)	1.17 (0.65-2.10)	1.30 (0.81-2.07)	1.18 (0.77-2.04)
Objective infection	330	1.22 (0.76-1.95)	1.29 (0.82-2.05)	1.12 (0.57-2.20)	1.46 (0.72-2.94)	1.15 (0.52-2.52)	1.18 (0.57-2.44)	1.24 (0.67-2.28)	1.30 (0.71-2.36)	1.21 (0.70-2.11)	1.30 (0.73-2.30)
Fetal adverse outcome	330	0.95 (0.61-1.47)	0.90 (0.58-1.38)	0.61 (0.29-1.30)	0.59 (0.28-1.23)	1.45 (0.76-2.77)	1.19 (0.59-2.40)	1.05 (0.59-1.86)	0.99 *0.56-1.73)	0.90 (0.39-2.09)	0.87 (0.36-2.08)

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI and smoking. CVF=cervicovaginal fluid; sPTB=spontaneous preterm birth; PPROM=premature prelabour rupture of membranes, BMI=body mass index, CE=cathelicidin:elafin. Statistically significant ratios in bold.*

Table 6-38 Ratio (95% confidence intervals) of the CE ratio in cases and controls overall, and stratified by gestation at sampling, in White and Black women

Outcome			Ratio (95% Confidence Interval) of CE ratio cases: controls									
					Gestation category (weeks^{+days})							
	Ethnicity	N (overall)	Overall		10-13^{+6}		14-15^{+6}		16-19^{+6}		20-24^{+0}	
			unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted
Cervical shortening <25mm	White	180	0.91 (0.48-1.72)	1.00 (0.53-1.91)	0.64 (0.16-2.55)	0.85 (0.14-5.12)	1.28 (0.49-3.34)	1.25 (0.50-3.15)	0.92 (0.41-2.04)	1.06 (0.48-2.34)	1.07 (0.48-2.36)	1.06 (0.47-2.41)
	Black	100	**1.94 (1.09-3.44)**	**2.33 (1.32-4.12)**	1.16 (0.53-2.56)	1.81 (0.73-4.53)	**3.14 (1.24-7.93)**	**3.46 (1.29-9.26)**	**2.47 (1.28-4.77)**	**2.92 (1.43-5.97)**	1.73 (0.78-3.86)	2.02 (0.75-5.44)
sPTB <37	White	196	1.66 (0.95-2.91)	1.62 (0.93-2.83)	1.10 (0.45-2.68)	0.94 (0.36-2.44)	**4.02 (1.83-8.85)**	**3.55 (1.50-8.43)**	1.65 (0.65-4.19)	1.41 (0.56-3.58)	1.32 (0.69-2.52)	1.30 (0.60-2.78)
	Black	99	1.20 (0.60-2.41)	1.10 (0.54-2.20)	1.46 (0.63-3.36)	1.34 (0.49-3.68)	0.95 (0.34-2.68)	0.77 (0.22-2.67)	1.61 (0.73-3.61)	1.15 (0.47-2.81)	1.04 (0.51-2.15)	1.12 (0.44-2.88)
sPTB <34	White	196	1.14 (0.50-2.59)	1.04 (0.45-2.38)	0.58 (0.11-3.10)	0.54 (0.08-3.51)	2.28 (0.78-6.71)	1.88 (0.60-5.84)	1.36 (0.25-7.44)	1.23 (0.24-6.32)	0.90 (0.35-2.28)	0.90 (0.29-2.80)
	Black	99	1.18 (0.52-2.69)	1.31 (0.57-2.99)	1.11 (0.43-2.87)	1.02 (0.35-2.92)	0.84 (0.28-2.54)	1.06 (0.34-3.29)	1.89 (0.75-4.79)	1.72 (0.68-4.33)	**2.08 (1.07-4.06)**	2.83 (0.93-8.58)
PPROM	White	192	0.91 (0.40-2.11)	0.89 (0.38-2.05)	0.67 (0.19-2.36)	0.62 (0.16-2.48)	1.52 (0.35-6.65)	1.33 (0.32-5.55)	0.73 (0.33-1.66)	0.67 (0.29-1.58)	0.80 (0.37-1.71)	0.75 (0.28-2.04)
	Black	96	1.40 (0.64-3.05)	1.51 (0.69-3.29)	1.07 (0.43-2.68)	1.05 (0.36-3.11)	1.44 (0.50-4.12)	1.73 (0.59-5.05)	1.82 (0.83-3.97)	1.60 (0.72-3.55)	**2.25 (1.22-4.16)**	**2.87 (1.17-7.08)**
Objective infection	White	196	1.13 (0.60-2.13)	1.23 (0.66-2.30)	1.30 (0.50-3.42)	1.73(0.52-5.81)	1.06 (0.37-3.07)	1.06 (0.36-3.09)	0.97 (0.43-2.17)	1.08 (0.48-2.41)	1.03 (0.46-2.29)	1.15 (0.49-2.70)
	Black	100	1.48 (0.66-3.31)	1.69 (0.78-3.68)	1.26 (0.42-3.77)	1.49 (0.55-4.08)	0.95 (0.19-4.87)	1.04 (0.22-4.92)	2.37 (0.88-6.38)	1.89 (0.76-4.68)	1.73 (0.82-3.67)	2.28 (0.86-6.01)
Fetal adverse outcome	White	196	0.80 (0.44-1.46)	0.76 (0.42-1.39)	**0.25 (0.08-0.83)**	**0.24 (0.07-0.78)**	1.64 (0.65-4.13)	1.44 (0.51-4.05)	1.01 (0.43-2.37)	1.00 (0.44-2.31)	0.85 (0.31-2.34)	0.87 (0.29-2.54)
	Black	100	1.40 (0.68-2.91)	1.56 (0.76-3.17)	1.49 (0.56-3.94)	1.77 (0.69-4.52)	1.78 (0.52-6.12)	2.03 (0.59-6.94)	1.60 (0.60-4.25)	1.60 (0.72-3.57)	0.92 (0.16-5.20)	0.91 (0.15-5.54)

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI and smoking. CVF=cervicovaginal fluid; sPTB=spontaneous preterm birth; PPROM=premature prelabour rupture of membranes, BMI=body mass index; CE=cathelicidin:elafin. Statistically significant ratios in bold.*

Table 6-39. Ratio (95% confidence intervals) of the CE ratio in cases and controls overall, and stratified by gestation at sampling, in high-risk women, after exclusion of post intervention samples

Outcome		Ratio (95% Confidence Interval) of CE ratio cases: controls									
				Gestation category (weeks^{+days})							
	N (overall)	**Overall**		**10-13^{+6}**		**14-15^{+6}**		**16-19^{+6}**		**20-24^{+0}**	
		unadjusted	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**	**unadjusted**	**adjusted**
Cervical shortening <25 mm	268	1.26 (0.92-1.73)	1.23 (0.88-1.72)	0.98 (0.49-1.94)	1.31 (0.60-2.90)	1.83 (0.99-3.40)	**2.23 (1.11-4.49)**	1.38 (0.79-2.42)	1.75 (0.94-3.25)	0.79 (0.23-2.68)	0.84 (0.23-3.04)
sPTB <37	272	**1.72 (1.27-2.35)**	**1.61 (1.18-2.19)**	1.38 (0.76-2.48)	1.10 (0.58-2.07)	**3.48 (1.79-6.75)**	**2.42 (1.22-4.82)**	1.80 (0.80-4.08)	1.62 (0.72-3.64)	1.15 (0.57-2.32)	1.08 (0.53-2.18)
sPTB <34	278	1.45 (0.92-2.28)	1.30 (0.83-2.04)	0.78 (0.32-1.90)	0.66 (0.26-1.67)	1.65 (0.59-4.57)	1.37 (0.57-3.31)	1.46 (0.25-8.47)	1.42 (0.27-7.56)	1.09 (0.47-2.51)	1.55 (0.60-3.98)
PPROM	280	1.46 (0.95-2.25)	1.30 (0.84-2.01)	0.99 (0.52-1.69)	0.93 (0.45-1.91)	2.34 (0.78-7.06)	1.77 (0.63-4.97)	1.43 (0.49-4.13)	1.40 (0.47-4.11)	1.17 (0.50-2.76)	0.89 (0.39-2.02)
Objective infection	285	1.16 (0.81-1.67)	1.16 (0.81-1.65)	1.57 (0.85-2.92)	1.92 (1.00-3.69)	1.13 (0.44-2.89)	1.06 (0.45-2.52)	0.98 (0.46-2.07)	1.05 (0.50-2.20)	1.03 (0.47-2.25)	1.16 (0.53-2.53)
Fetal adverse outcome	285	1.00 (0.71-1.44)	0.96 (0.68-1.37)	0.49 (0.21-1.15)	0.46 (020-1.09)	1.23 (0.54-2.80)	1.12 (0.47-2.66)	1.03 (0.42-2.53)	1.02 (0.45-2.29)	0.36 (0.09-1.39)	0.40 (0.10-1.58)

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI and smoking. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, BMI=body mass index, CE=cathelicidin:elafin. Statistically significant ratios in bold.*

Table 6-40. Ratio (95% confidence intervals) of the CE ratio in cases and controls overall, and stratified by gestation at sampling, in high-risk Black and White women, after exclusion of post intervention samples

Outcome			Ratio (95% Confidence Interval) of CE ratio cases: controls									
			Gestation category (weeks^{+days})									
	Ethnicity	N (overall)	Overall		10-13^{+6}		14-15^{+6}		16-19^{+6}		20-24^{+0}	
			unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted
Cervical shortening <25 mm	White	164	1.16 (0.68-1.98)	1.19 (0.69-2.04)	0.73 (0.17-3.22)	1.24 (0.18-8.53)	1.20 (0.41-3.47)	1.04 (0.36-3.04)	0.88 (0.35-2.20)	1.07 (0.42.59)	1.45 (0.49-4.32)	1.61 (0.49-5.32)
	Black	76	1.07 (0.71-1.60)	1.24 (0.81-1.91)	1.19 (0.51-2.81)	1.67 (0.58-4.78)	**3.50 (1.20-10.15)**	**4.26 (1.46-12.38)**	**2.80 (1.21-6.51)**	**3.61 (1.51-8.63)**	1.00 (0.24-4.16)	3.56 (0.31-40.60)
sPTB <37	White	172	**1.75 (1.12-2.73)**	**1.82 (1.17-2.83)**	1.30 (0.51-3.31)	1.10 (0.41-2.94)	**4.96 (2.03-12.07)**	**4.63 (1.82-11.75)**	2.00 (0.67-6.00)	1.66 (0.57-4.81)	1.44 (0.59-3.50)	1.47 (0.57-3.76)
	Black	71	1.47 (0.90-2.41)	1.29 (0.76-2.18)	1.98 (0.88-4.45)	1.66 (0.54-5.10)	1.22 (0.34-4.33)	0.91 (0.32-2.62)	2.09 (0.62-7.11)	1.14 (0.22-5.96)	1.37 (0.58-3.24)	1.58 (0.15-16.13)
sPTB <34	White	175	1.53 (0.77-3.05)	1.71 (0.87-3.39)	0.48 (0.07-3.23)	0.49 (0.07-3.59)	2.67 (0.61-11.63)	2.57 (0.63-10.44)	1.89 (0.19-18.36)	1.77 (0.22-14.27)	0.90(0.17-4.74)	1.13 (0.18-6.94)
	Black	74	1.09 (0.56-2.09)	0.99 (0.49-1.98)	1.29 (0.53-3.16)	0.89 (0.28-2.84)	0.36 (0.10-1.27)	0.63 (0.16-2.43)	1.60 (0.09-27.09)	1.30 (0.06-28.78)	0.93 (0.46-1.85)	0.31 (018-5.52)
PPROM	White	177	1.05 (0.52-2.13)	1.06 (0.53-2.14)	0.98 (0.31-3.09)	0.91(0.27-3.16)	1.27 (0.17-9.52)	1.33 (0.23-7.63)	0.80 (0.31-2.10)	0.74 (0.29-1.86)	0.68 (0.18-2.65)	0.75 (0.22-2.61)
	Black	74	1.20 (0.66-2.20)	1.18 (0.62-2.25)	1.20 (0.51-2.83)	0.99 (0.33-2.98)	1.44 (0.31-6.81)	1.70 (0.41-7.09)	1.53 (0.22-10.55)	1.17 (0.10-13.47)	2.04 (0.55-7.55)	0.87 (0.14-5.35)
Objective infection	White	180	1.01 (0.62-1.64)	1.04 (0.65-1.68)	1.68 (0.77-3.66)	2.61 (0.85-8.03)	0.96 (0.32-2.92)	0.91 (0.31-2.73)	0.94 (0.40-2.22)	0.99 (0.42-2.33)	0.94 (0.36-2.46)	1.03 (0.40-2.61)
	Black	76	1.40 (0.77-2.54)	1.29 (0.70-2.38)	1.61 (0.51-5.09)	1.58 (0.55-4.54)	0.70 (0.06-8.94)	0.87 (0.09-8.53)	1.49 (0.27-8.24)	0.89 (0.14-5.89)	2.18 (0.47-10.13)	4.19 (0.70-24.96)
Fetal adverse outcome	White	180	0.78 (0.48-1.27)	0.82 (0.51-1.33)	**0.17 (0.04-0.76)**	**0.17(0.04-0.69)**	1.65 (0.51-5.34)	1.61 (0.46-5.63)	1.19 (0.42-3.36)	1.24 (0.46-3.30)	0.62(0.15-2.59)	0.75 (0.19-3.01)
	Black	76	1.63 (0.94-2.84)	1.38 (0.76-2.50)	1.36 (0.47-3.96)	1.61 (0.60-4.36)	0.99 (0.21-4.83)	1.30 (0.35-4.93)	0.81 (0.06-11.75)	1.08 (0.16-7.29)	0.06 (0.00-1.95)	0.02 (0.00-2.50)

***Ratios reported after adjustment by maternal age, gestation at sampling, ethnicity, BMI and smoking. CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, BMI=body mass index, CE=cathelicidin:elafin. Statistically significant ratios in bold*

Table 6-41. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome, using CVF cathelicidin:elafin ratio by gestation at sampling in the high-risk cohort, stratified by ethnicity

Outcome	**CE ratio Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)**				
Gestation category (weeks^{+days})		**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24**
Cervical shortening <25 mm	All	0.49 (0.42-0.56)	**0.57 (0.51-0.64)**	0.55 (0.49-0.61)	0.52 (0.46-0.59)
	White	0.46 (0.36-0.55)	0.51 (0.42-0.60)	0.47 (0.39-0.56)	0.49 (0.41-0.58)
	Black	0.52 (0.39-0.64)	**0.64 (0.53-0.76)**	**0.65 (0.54-0.76)**	0.58 (0.46-0.68)
sPTB <37	All	0.55 (0.47-0.62)	**0.64 (0.57-0.71)**	0.54 (0.48-0.61)	0.52 (0.45-0.59)
	White	0.54 (0.44-0.63)	**0.74 (0.65-0.81)**	0.57 (0.48-0.65)	0.55 (0.47-0.63)
	Black	0.57 (0.43-0.69)	0.49 (0.36-0.61)	0.59 (0.47-0.70)	0.52 (0.40-0.63)
sPTB <34	All	0.49 (0.42-0.56)	**0.58 (0.51-0.64)**	0.49 (0.43-0.55)	0.53 (0.47-0.60)
	White	0.41 (0.32-0.50)	**0.65 (0.56-0.73)**	0.45 (0.37-0.54)	0.47 (0.39-0.56)
	Black	0.51 (0.39-0.64)	0.46 (0.34-0.58)	0.61 (0.49-0.72)	**0.64 (0.53-0.75)**
PPROM	All	0.48 (0.41-0.55)	**0.59 (0.53-0.66)**	0.55 (0.48-0.61)	0.54 (0.48-0.61)
	White	0.45 (0.36-0.55)	0.57 (0.48-0.66)	0.47 (0.39-0.56)	0.45 (0.37-0.54)
	Black	0.51 (0.38-0.63)	0.54 (0.41-0.66)	0.60 (0.48-0.71)	0.68 (0.55-0.77)
Objective infection	All	0.53 (0.46-0.60)	0.55 (0.48-0.62)	0.53 (0.47-0.60)	0.52 (0.45-0.58)
	White	0.56 (0.47-0.65)	0.52 (0.43-0.60)	0.49 (0.40-0.57)	0.50 (0.42-0.59)
	Black	0.54 (0.40-0.65)	0.54 (0.42-0.66)	**0.66 (0.55-0.76)**	0.56 (0.44-0.67)
Fetal adverse outcomes	All	0.45 (0.38-0.52)	0.55 (0.48-0.62)	0.51 (0.45-0.58)	0.54 (0.48-0.61)
	White	**0.34 (0.26-0.44)**	0.57 (0.48-0.65)	0.49 (0.40-0.57)	0.50 (0.42-0.59)
	Black	0.58 (0.44-0.70)	0.57 (0.45-0.69)	0.62 (0.50-0.72)	0.61 (0.49-0.72)

CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, CE=cathelicidin:elafin. Statistically significant AUCs in bold.

Table 6-42. Receiver Operating Characteristic Area under the Curve (95% confidence intervals) for prediction of outcome, using CVF cathelicidin: elafin ratio by gestation at sampling in the high-risk cohort, stratified by ethnicity, after exclusion of samples taken post intervention

Outcome	**CE ratio Receiver Operating Characteristic Area Under the Curve (95% Confidence interval)**				
Gestation category (weeks^{+days})		**10-13^{+6}**	**14-15^{+6}**	**16-19^{+6}**	**20-24**
Cervical shortening <25 mm	All	0.50 (0.43-0.58)	0.57 (0.50-0.64)	0.54 (0.47-0.61)	0.49 (0.41-0.57)
	White	0.50 (0.40-0.60)	0.50 (0.40-0.59)	0.46 (0.37-0.55)	0.54 (0.44-0.63)
	Black	0.53 (0.40-0.67)	**0.66 (0.51-0.78)**	**0.66 (0.52-0.78)**	0.47 (0.31-0.64)
sPTB <37	All	0.57 (0.49-0.64)	**0.69 (0.62-0.76)**	0.58 (0.50-0.65)	0.52 (0.45-0.60)
	White	0.56 (0.46-0.66)	**0.76 (0.67-0.83)**	0.60 (0.50-0.68)	0.55 (0.45-0.64)
	Black	**0.65 (0.51-0.78)**	0.52 (0.37-0.66)	0.62 (0.48-0.76)	0.55 (0.37-0.71)
sPTB <34	All	0.46 (0.39-0.54)	0.58 (0.50-0.65)	0.46 (0.39-0.53)	0.43 (0.35-0.50)
	White	0.39 (0.29-0.50)	**0.68 (0.58-0.76)**	0.47 (0.38-0.56)	0.46 (0.36-0.55)
	Black	0.56 (0.41-0.69)	**0.32 (0.19-0.46)**	0.57 (0.43-0.71)	0.40 (0.24-0.57)
PPROM	All	0.50 (0.42-0.58)	**0.62 (0.54-0.69)**	0.55 (0.48-0.62)	0.50 (0.43-0.58)
	White	0.49 (0.39-0.58)	0.54 (0.44-0.63)	0.49 (0.40-0.58)	0.41-0.32-.52)
	Black	0.54 (0.40-0.67)	0.54 (0.40-0.69)	0.56 (0.41-0.70)	0.64 (0.46-0.78)
Objective infection	All	**0.58 (0.51-0.66)**	0.55 (0.47-0.62)	0.49 (0.42-0.56)	0.49 (0.41-0.57)
	White	0.59 (0.49-0.68)	0.50 (0.41-0.60)	0.48 (0.40-0.57)	0.49 (0.39-0.58)
	Black	0.60 (0.48-0.74)	0.53 (0.38-0.67)	0.56 (0.42-0.70)	0.56 (0.38-0.71)
Fetal adverse outcomes	All	0.43 (0.36-0.51)	0.52 (0.44-0.59)	0.52 (0.4-0.59)	0.42 (0.34-0.50)
	White	**0.30 (0.21-0.40)**	0.56 (0.47-0.66)	0.52 (0.43-0.61)	0.47 (0.37-0.57)
	Black	0.58 (0.43-0.70)	0.49 (0.35-0.63)	0.56 (0.42-0.70)	**0.25 (0.13-0.43)**

CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM=premature prelabour rupture of membranes, CE=cathelicidin:elafin. Statistically significant AUCs in bold

6.3 Discussion

This chapter sought to validate the association between CVF elafin and cathelicidin expression in early to mid- pregnancy, with cervical shortening and subsequent sPTB, as well as undertake a preliminary investigation into the expression of HNE, in a large cohort of low and high-risk pregnancies, within which 80 women delivered spontaneously and prematurely, work which has now been published (Hezelgrave et al. 2020).

Here, we demonstrate that CVF concentrations of cathelicidin and HNE were higher in women who developed cervical shortening and who delivered prematurely, but that, contrary to the hypothesis derived from our pilot study, high CVF elafin concentrations in early pregnancy were not associated with cervical shortening or sPTB in the large cohort of women (neither whole cohort nor when stratified by risk status at enrolment). However, when samples taken from women after prophylactic treatment had been commenced (usually those with a short cervix, or those deemed to be at the highest risk of sPTB early in pregnancy), high elafin in early pregnancy was modestly predictive of subsequent cervical shortening <25 mm prior to 24 weeks, and sPTB prior to 34 weeks of gestation. Calculation of CE ratio improved this prediction slightly.

This relates back to our original hypothesis; where infection or inflammation is overwhelming (or indeed if the innate immune system is hyper-responsive) this may result in high levels of HDP secretion (high cathelicidin and elafin in the CVF) and accompanying high proteases (e.g., HNE) resulting in tissue damage (cervical shortening), ascending infection, and in some women, sPTB. Furthermore, when our analysis was stratified by ethnicity, key differences between Black and White women emerged with respect to HDP expression and relationship with pregnancy outcome, as elaborated below.

6.3.1 Elafin

Unlike the pilot study (n=74), in this cohort of women (high and low-risk), mean CVF elafin (10 to 24 weeks of gestation) was not higher in women who experienced sPTB, adverse birth outcomes, nor cervical shortening. Given that the pilot study included only women with previous poor pregnancy outcome, and our larger cohort broadened the definition of high-risk to include other known sPTB risk pathology (previous cervical surgery, uterine abnormalities and incidental finding of short cervix in pregnancy) the analysis was repeated in women with only a history of sPTB or late miscarriage, given that is plausible that they may have a fundamentally different pathogenesis of sPTB (for example, pregnancy mechanical uterine distension exerting force upon a structurally weak cervix/abnormally shaped uterus, rather than primary infection and inflammation). However, elafin did not prove to be a useful predictor in these women either.

The original pilot study was conducted as part of an exploratory RCT of treatment for a short cervix; women who developed a short cervix were randomised to intervention (either vaginal progesterone or cervical cerclage). An exclusion criterion for pilot study entry was therefore existing intervention *in situ*. However, as per UK clinical guidelines (National Collaborating Centre for and Children's 2015), women in the larger Insight study (observational only) who had a previous sPTB or late miscarriage and/or who developed a short cervix were offered prophylactic treatment such as cervical cerclage, vaginal progesterone or Arabin Pessary (routine clinical practice, or part of associated RCT SUPPORT) (Hezelgrave, Watson, et al. 2016). In addition, women may also have received early prophylactic intervention in early pregnancy. It is thus plausible that intervention may affect CVF protein expression, resulting in an intervention mediated biomarker shift. We therefore performed a repeat analysis, excluding samples taken once an intervention had been started/was in situ. A modest, but statistically significant association between high elafin in early pregnancy and subsequent cervical shortening <25 mm prior to 24 weeks of gestation was demonstrated, as well as an association between high elafin and sPTB prior to 34

weeks of gestation. It is possible that women destined to deliver preterm (with corresponding biomarker expression), may have had their delivery delayed by intervention, weakening the apparent predictive capacity of any HDP biomarkers.

The more modest association seen here between CVF elafin and cervical shortening/sPTB may be potentially due to a number of reasons. It may be that the pilot study was subject to Type 1 error by virtue of numbers (n = 74), and this validation acts as a reminder of the need to validate small studies in large, powered cohorts. Furthermore, whilst sample acquisition, preparation and protein measurement were performed using the same methodology, allowance for the dilutional effect of sample buffer on measured elafin protein concentration was not considered in the pilot (see methods section Chapter 3); control samples were diluted to 1:10, and cases to 1:50 or 1:100 to ensure positioning on the standard curve. This may have led to some overestimation of true protein concentration in cases whereby dilution of samples has encouraged protein release from the cell matrix, or underestimation of control sample concentration. By correcting for the dilution effect here, we have presented a more accurate representation of protein concentration within the limitation of ELISA methodology.

The relationship between elafin and pregnancy outcome likely reflects the complex both pro-and anti-inflammatory function that plays in the reproductive tract; high elafin could be protective against cervical shortening and sPTB, limiting damage to host tissues by neutrophil activity by inhibiting neutrophil elastase and proteases (Motta et al. 2011), counteracting ascending infection and the associated cervical degradation and inflammation. Thus, many women with appropriately high elafin will not deliver prematurely. However, elafin may also cause tissue damage through its immunomodulatory actions via neutrophil recruitment and release of further neutrophil elastase (Williams et al. 2006) (high HNE correlated with sPTB in this study), potentiating the inflammatory process and leading to increased risk of cervical shortening and sPTB. In this scenario, for some women, high elafin may represent a

biomarker for subsequent sPTB. Yet, the clinical question remains; how to identify those women in whom high elafin may indicate potential sPTB? Part of the answer to this question may lie in exploration of the vaginal microbiota, particularly in light of the finding that elafin is lower in women with BV (Stock et al. 2009), and that HDPs are induced by some bacterial species (e.g., *P. aeruginosa*) (Williams et al. 2006) and supressed by others (e.g. *E. coli, C. trachomatis*) (Mukura et al. 2017; Cole and Nizet 2016). This is further investigated and discussed in Chapter 7.

We have also here demonstrated that biomarker assessment and validation may not be appropriate in an ethnically diverse cohort. Given the well described ethnic disparity in rates of PTB (Manuck 2017), and the ethnic variation in vaginal microbiota (Kenyon, Colebunders, and Crucitti 2013; Callahan et al. 2017) it is not surprising that we observed ethnic variation in elafin expression (and other HDPs), and that this was related to birth outcome. Across gestation, Black women expressed CVF elafin in higher concentrations than white women (Chapter 5). Yet Black women who had low elafin concentration were more likely to develop a short cervix; this was true for mean elafin CVF concentrations across gestation, particularly so for samples taken between 14 to 15^{+6} weeks, and 20 to 24^{+0} weeks of gestation. In contrast the overall mean elafin concentration was no different for White women who developed a short cervix, but high elafin was moderately predictive of cervical shortening when measured at an early gestation (10-13^{+6} weeks). The relationship with pregnancy outcomes followed a similar pattern; low CVF elafin at 16 to 19^{+6} weeks predicted sPTB <34 and 37 weeks, as well as PPROM in Black women in the high-risk cohort. Conversely, White high-risk women who subsequently delivered spontaneously prior to 34 weeks had over three times adjusted CVF elafin at an earlier gestation (10-13^{+6} weeks), and crude values were predictive of sPTB<34 weeks and PPROM.

When samples obtained prior to intervention were excluded, these differences were even more pronounced; high-risk White women who delivered prior to 34 weeks had more than four times adjusted elafin concentration than their term counterparts and

babies who experienced adverse fetal outcome had more than double the elafin concentration at the earliest testing gestation (10-13^{+6} weeks). This is in stark contrast to Black high-risk women who developed cervical shortening, who had less than half the CVF elafin concentration at 14 to 15^{+6} weeks gestation compared to women who developed a short cervix.

The ethnic differences in elafin expression, and relationship with pregnancy outcome may be due to underlying genetics or the vaginal microbiome. We hypothesise that a higher prevalence of vaginal dysbiosis (including BV) and associated suppressed innate immune response (low elafin) could contribute to the higher rate of cervical shortening and sPTB in Black women. It may be that constitutive low elafin encourages overgrowth of BV associated bacteria, or that low elafin is a consequence of dysbiosis initiating a state of local immunosuppression, increasing vulnerability to cervical degradation and microbial ascent associated with sPTB. This inference is supported by Valore, Wiley, and Ganz (2006) who observed that reduced concentrations of the HDPs HBD 1 & 2, SLPI and human neutrophil peptide (HNP) in women with BV, could be normalised by antibiotic treatment. Nonetheless, relationships between vaginal microflora and host defence peptides are complex and ethnic differences in the microbiome may explain differences in the host response or indeed vice versa.

We did postulate that the differences in the ethnicity profile in the pilot study vs. main study may have contributed to the vastly different results seen in the two studies; the case-control pilot study (not matched for ethnicity) consisted of 72% Black women, vs 22% White women, unlike the main Insight cohort study (high-risk cohort; 61% White, 27% Black). When just the highest risk population was considered in the cohort study to match that of the pilot study, this was still slightly over-represented by White women (49% White women, 38% Black women). This therefore this does not explain our findings that White women had a stronger association with elafin and cervical shortening/sPTB as we would have expected to find superior

predictive capacity in the main study vs. pilot with a higher proportion of White women.

Some results obtained are even more difficult to explain. In the high-risk cohort (all ethnicities) adjusted elafin concentration was lower at 16-19^{+6} weeks of gestation in women who developed a short cervix compared to women who did not develop a short cervix (once samples taken post intervention were removed). This may represent the contribution of Black women to the cohort (in whom low elafin was consistently associated with cervical shortening), whereas at other gestations this did not reach statistical significance. In addition, albeit in a small number of high-risk Black women (n=45), after removal of post-intervention samples, high elafin was associated with adverse fetal outcome (ROC 0.86) when measured at 20-24 weeks of gestation, despite low elafin being consistently associated with cervical shortening and sPTB in these women. We suspect that this may be a spurious result based on small numbers. However, given that adverse fetal outcome may be defined by contributors other than gestational age at birth, it is not impossible that higher elafin concentrations are alerting to other processes leading to adverse fetal outcome.

6.3.2 Cathelicidin

Cathelicidin was found in higher concentrations in the CVF of women who subsequently delivered spontaneously and prematurely <37 weeks, particularly when measured at 14-15^{+6} weeks of gestation, where cathelicidin expression was 86% higher in women who delivered spontaneously and nearly double in women who subsequently experienced PPROM. When measured at 16 to 19^{+6} weeks, cathelicidin concentration was modestly predictive of subsequent fetal and/or maternal infection. Once samples obtained after intervention were excluded, as with elafin, associations were strengthened. In particular, cathelicidin concentration at 14 to 15^{+6} weeks was a good predictor of subsequent sPTB in these women (ROC 0.75). Mean cathelicidin concentration was also higher in women who developed a short cervix prior to 24 weeks of gestation and had mild predictive value for this outcome at all

gestational sampling points between 14 to 24 weeks, improved when post-intervention samples were removed.

These results differ slightly from those seen in the smaller pilot study, where cathelicidin concentrations were similar in women who developed a short cervix until 18 weeks of gestation, and subsequently rose in those women who developed a short cervix (around 19 weeks' gestation) but were not found to be predictive of cervical shortening or sPTB (Abbott et al. 2014). Our larger powered cohort has demonstrated that cathelicidin concentrations in high-risk women do indeed rise in many women prior to cervical shortening and may be useful to predict sPTB.

The cleaved form of cathelicidin, LL37, is stored and secreted by neutrophils and epithelial cells (expressed from the endometrium, cervix and vagina), but is also inducible from inflammatory cells such as natural killer cells, B cells, monocytes and mast cells (Yarborough et al. 2015). Not only can it be induced by a range of inflammatory stimuli (e.g., Vitamin D, bacterial products, TNF-$\propto$ etc) it can also act like a chemokine itself, modulating immune cells (Vandamme et al. 2012). Given that cervical ripening is associated with a neutrophil influx and inflammation mediated by chemokines and cytokines (Kelly 2002), and that our group previously demonstrated strong association between CVF cathelicidin and cytokine concentration with cervical shortening (Abbott et al. 2014), the rise in CVF cathelicidin (and HNE) concentration prior to cervical shortening (median 18^{+3} weeks) was not unexpected. Cathelicidin has been shown to mediate the pro-inflammatory response in mouse models of term and sPTB with cathelicidin-deficient knock-out mice less susceptible to LPS induced sPTB (Boeckel et al. 2019). That our knowledge about the mechanism behind cathelicidin, cervical shortening and preterm labour is limited to mouse models such as these, does make pathogenesis hard to elucidate. Nonetheless, we postulate that cathelicidin has a responsive role to tissue damage around the time of cervical shortening, stimulating angiogenesis (Koczulla et al. 2003), re-epithelialisation and promoting wound healing (Ramos et al. 2011) and immune cell infiltration, or may be

instrumental in the process of cervical shortening itself. That a more substantive relationship between cathelicidin and sPTB was not found may be related to the fact that we as clinicians intervene in cases of cervical shortening (for example with cervical cerclage) which may delay or prevent a number of births destined for prematurity.

Like elafin, cathelicidin expression had a distinct profile according to ethnicity (higher in Black and Asian women compared to White women across gestation, see Chapter 5), and similarly had a stronger relationship to pregnancy outcome (sPTB and cervical shortening) in White women vs. Black women; for example at 14-15^{+6} weeks, cathelicidin was nearly three times higher in women who delivered prematurely before 37 weeks compared to those who delivered at term whereas this pattern was not seen in Black women. Similarly, prediction of sPTB at 14-15^{+6} weeks of gestation using CVF cathelicidin was strong for White women but not Black women. High-risk White women (n=246) did form a larger cohort than high-risk Black women (n=114) and neither group when sub-analysed was adequately powered for meaningful prediction. Thus, it is possible that the cohort of Black women in particular is too small to detect statistically and clinically significant differences in cathelicidin concentration. However, given the differences in ethnic profiles of cathelicidin, and similar ethnic variations seen with elafin, it is not unlikely that expression of cathelicidin and relationship with pregnancy outcome is different according to ethnicity.

There have been no other studies exploring the differences in cathelicidin expression, ethnicity and relationship with pregnancy outcome. Frew et al. (2014) described higher expression of CVF cathelicidin in individuals with concurrent BV (more common in Black women), though they did not specify the ethnicity of the subjects (n=116 Scottish pregnant participants) in this paper. However, ethnic differences in other CVF biomarker expression in relation to sPTB have certainly been previously highlighted (see Chapter 5 discussion).

Like elafin, some cathelicidin-outcome associations were unexpected; high concentration of cathelicidin at later gestations (20 to 24 weeks) was a good predictor of sPTB <34 weeks (AUC 0.78), PPROM (AUC 0.81) and maternal and/or fetal infection (AUC 0.79) in Black women, but not White women, once post-intervention samples had been excluded. Similar prediction was seen for high elafin in White women for sPTB and PPROM at earlier gestations (14 to 15^{+6} weeks). Why associations vary according to gestation (and ethnicity), particularly with a biomarker such as cathelicidin which showed little gestational variation, warrants further investigation.

6.3.3 Human neutrophil elastase

CVF HNE expression (whose expression correlated with cathelicidin in this study) were also one and a half times higher overall in high-risk women who developed cervical shortening (though this association was lost after adjustment). At 14 to 15^{+6} weeks however, adjusted HNE was three times higher in women who subsequently developed cervical shortening, and also higher in those who delivered prematurely <37 weeks. Crude concentrations at this gestation were modesty predictive of cervical shortening, sPTB PPROM, adverse fetal outcome and objective infection. These associations were strengthened slightly when post-intervention samples were removed. HNE not only targets bacteria, but has been shown to initiate proteolysis of collagen-IV and elastin found in cervical tissue, (Pipoly and Crouch 1987). Thus, like cathelicidin, it may well be key in the process of cervical damage and shortening. As seen for cathelicidin, at 14 to 15^{+6} weeks of gestation, the relationship between high HNE and cervical shortening and sPTB <37 weeks was strong in white women but not Black women, with a ROC AUC of 0.73 for cervical shortening at this gestation.

6.3.4 CE ratio

The calculated CE ratio had the strongest association with sPTB and cervical shortening, particularly when stratified by ethnicity and when potentially misleading post-intervention samples were removed from the analysis. For all women, a high CE

ratio at 14 to 15^{+6} weeks gestation was associated with sPTB and cervical shortening, particularly strong for White women (ROC 0.76 for sPTB <37 weeks). Thus, for many women, particularly those of White ethnicity, when cathelicidin is high and elafin low, they are more likely to develop a short cervix and deliver prematurely. We previously hypothesised that some individuals (possibly due to their dysbiotic vaginal microbial environment) may not be able to mount an effective (and perhaps protective) epithelial cell driven innate immune response (elafin remains low). Yet if their neutrophil response to infection and inflammation, signalled by high cathelicidin, with associated release of tissue damaging neutrophil elastase and other inflammatory mediators, then their susceptibility to sPTB is highest.

In contrast, a low CE ratio at 10-13^{+6} weeks in White women was predictive of composite adverse fetal outcome (but not cervical shortening or sPTB) once post intervention samples had been removed. It is possible that sPTB is a protective mechanism against remaining in an inflammatory environment (reflected by higher elafin levels). Infants who remained in this inflammatory environment may have poorer composite neonatal outcomes despite a longer gestational time period. Alternatively, variation in the cathelicidin and elafin concentrations may reflect other pathology which are related to adverse outcomes, unrelated to sPTB.

6.4 Conclusions

In this chapter, we have demonstrated evidence supporting the contribution of HDPs to the mechanisms leading to some sPTB. Further investigation of this complex innate immune response is warranted to further understand their role in mediating risk of sPTB, and the potential to harness this information to develop more sophisticated prediction and prevention techniques than currently clinically utilised. In particular, the relationship between elafin and cathelicidin, ethnicity and specific sPTB phenotype subgroups, as well as correlation with the vaginal environment (i.e., BV and the vaginal microbiota) could provide additional insight (Chapter 7). Of the three

CVF HDPs/proteins measured here, mid-trimester cathelicidin and CE ratio has promising biomarker potential particularly for White women, and combination with other inflammatory markers, and/or other HDPs (Chapter 8) may prove clinically useful in future.

7 Bacterial vaginosis, host defence peptide concentration and ethnicity

7.1 Introduction

Racial disparity in rates of SPTB are well described, though inadequately explained. Black women are more likely to experience sPTB than White women even after correction for known sPTB risk factors such as smoking, maternal education and socioeconomic status, as well as biological risk factors such as the higher incidence of urogenital infection in black women (particularly BV). A diagnosis of BV (polymicrobial infection of the lower genital tract with mixed anaerobic bacteria species such as *Gardnerella, Prevotella* and *Atopobium* and deficiency of the normal population of lactobacillus), is associated with two to five times increase in risk of sPTB (Gravett, Hummel et al. 1986, Hill 1998, et al. 2005), and is associated with PPROM and chorioamnionitis. Whilst it may present with typically malodorous vaginal discharge, in the majority of women it is asymptomatic.

In this thesis, the data have so far demonstrated that CVF concentration of HDPs is affected by ethnicity, and that the relationship between HDP expression in pregnancy and pregnancy outcome (particularly cervical shortening and sPTB) is influenced by ethnicity (Chapter 6). In this chapter, the relationship between BV and CVF HDP expression in our cohort of mixed ethnicity women (1505 gram-stained slides with paired HDP measurements from n=600 women) was examined. I hypothesised that the vaginal BV status influences expression of HDPs and this may vary according to ethnicity. Chapter 3 contains full methods used for the following analysis.

7.2 Results

7.2.1 Characteristics of study population

One or more BV swab results were determined from 600/619 women in the Insight cohort. The incidence of BV (diagnosed at one or more sampling gestations) was 103/600 (17.2%). 127/600 (21.2%) of women had one or more intermediate swab results, but never had BV diagnosed at any of their visits. 370/600 (61.7%) women had normal flora at every sampling visit. The characteristics of the groups classified according to BV result are shown in Table 7-1.

Table 7-1. Characteristics of women from whom cervicovaginal samples for bacterial vaginosis testing were obtained

	Normal flora	Intermediate flora	Bacterial vaginosis	P value
n	370	127	103	-
Low risk for sPTB at enrolment	39.2 (34.2-44.2)	32.3 (24.0-40.5)	27.2 (18.4-35.9)	0.05
Age (years)	33.0 (4.8)	32.7 (5.1)	31.2 (4.8)	**0.003**
Caucasian	72.2 (67.2-76.6)	60.6 (51.5-69.1)	50.5 (40.5-60.4)	**<0.001**
Non-smokers	76.0 (70.2-80.2)	75.4 (66.8-82.4)	64.1 (54.0-73.1)	**0.04**
BMI (Kg/m^2)	25.4 (5.1)	26.1 (9.0)	27.4 (5.9)	**0.02**
IMD 1 (most deprived)	14.4	17.1	27.7	**0.01**

Age: mean +/- SD and BMI +- SD analysed by 1-way analysis of variance (ANOVA)

Risk, race, smoking- data expressed as percentage (95% confidence interval) and analysed by chi2 test

BMI=Body Mass Index, IMD=index of multiple deprivation, sPTB=Spontaneous preterm birth.

Statistically significant p values in bold

Women with normal flora were more likely to be older in age (p=0.003). Compared with women age <20 years, the OR for BV in women between 20 and 30 was 0.51 (0.32-0.81, p=0.004) and 0.20 (0.06-0.68, p=0.01) for women aged >40. Women with normal flora were likely to be in the low-risk category (though this did not quite reach statistical significance; numerically more high-risk women were found to have BV (75/386, 19.4%) than low risk women (28/214, 13.1%), which may have been partially

or completely due to increased visit and therefore testing frequency in the high-risk women. Women with BV were more likely to be current smokers than women with intermediate or normal swab results (8.7% BV, 5.5% intermediate, 2.7% normal, p=0.02). Indeed, BV was associated with current smoking with an OR of 2.70, 1.19 to 6.12, p=0.016 compared with non-smokers. A higher proportion of women with obesity (BMI >30 kg/m^2) were found amongst women with BV (28.4%) vs. normal (17.6%) and intermediate (18.9%) swab results, though this did not quite reach statistical significance (p=0.06). However, when regression analysis was performed with BMI as a continuous variable, high BMI was associated with BV diagnosis (p=0.02). Women with normal flora were less likely to be in the most deprived IMD category (p=0.01). This equated to an OR of 2.28 (1.32-3.39) for risk of BV for women in the most deprived quintile compared to the other deprivation quintiles (p=0.003).

Women with normal flora were more likely to be Caucasian (p<0.001). Table 7-2 shows the distribution of normal, intermediate and BV swabs according to ethnicity. BV was more common in Black women; 43/143 (30.1%) of Black women had BV diagnosed on one of their visits, compared to 52/408 (12.7%) of White women (p<0.001); 8/68 (11.8%) of Asian or other ethnicities had BV diagnosed at one or more visits during the study period.

Table 7-2. Distribution of bacterial vaginosis results according to ethnicity in the whole insight cohort (low and high-risk)

	BV category (worst measured during pregnancy)				
Ethnicity	Normal N (%)	Intermediate N (%)	BV N (%)	Missing data N (%)	Total (%)
White	267 (65.4)	77 (18.9)	52 (12.7)	12 (2.9)	408 (100)
Black	60 (42.0)	35(24.5)	43 (30.1)	5 (3.5)	143 (100)
Asian	22 (68.8)	8 (25.0)	2 (6.3)	0 (0)	32 (100)
Other	21 (58.3)	7 (19.4)	6 (16.7	2 (5.6)	36 (100)
Total	370 (59.8)	127 (20.5)	103 (16.6)	19 (3.1)	619 (100)

BV=bacterial vaginosis

When these variables were considered together using multiple logistic regression analysis, only Black ethnicity and current smoking remained significant independent predictors of BV in this cohort of women (Table 7-3).

Table 7-3. regression model; Odds ratios and 95% confidence intervals obtained by multivariate logistic regression analysis (adjusted for all predictors in the table) for bacterial vaginosis

Independent variables	**Sub-category**	**Multivariate analysis**		
		OR	**95% CI**	**P value**
Maternal age		0.96	0.93-1.00	0.06
Maternal BMI		1.02	0.99-1.05	0.17
Ethnicity	White	ref	ref	ref
	Black	**2.92**	**1.84-4.563**	**<0.001**
	Asian	0.87	0.38-1.96	0.74
Smoking status	Never smoked	ref	ref	ref
	Current smoker	**2.73**	**1.13-6.561**	**0.03**
	Gave up in pregnancy	1.58	0.97-2.58	0.07
	Gave up before pregnancy	1.09	0.50-2.41	0.853
IMD quintile		0.96	0.81-1.13	0.61

BMI=Body Mass Index, IMD=index of multiple deprivation, OR=Odds Ratio. ***Significant p values in bold***

7.2.2 BV and pregnancy outcome

The relationship between BV status and pregnancy outcome (sPTB <37 weeks and sPTB <34 weeks) are shown in Table 7-4 and 7-5. In the whole cohort (mixed risk and ethnicity), there was no association between BV status and risk of sPTB <37 (normal vs intermediate, p=0.44, normal vs BV p=0.81) or sPTB <34 weeks (normal vs intermediate, p=0.74, normal vs BV p=0.86).

Table 7-4. Association between worst BV status and sPTB <37 weeks in the whole cohort

	Worst BV category			Total*
sPTB <37 weeks	Normal N (%)	Intermediate N (%)	BV (%)	N (%)
No	307 (84.3)	101 (80.8)	87 (84.5)	495 (86.5)
Yes	46 (12.6)	19 (15.2)	12 (11.7)	77 (13.5)
Total	364 (100)	125 (100)	103 (100)	572 (100)
Odds ratio (95% CI)	ref	1.26 (0.70-2.24)	0.92 (0.47-1.81)	-
P value	ref	0.44	0.81	-

PTB=preterm birth, BV=bacterial vaginosis, CI=confidence interval, sPTB=spontaneous preterm birth

**Iatrogenic deliveries excluded from analysis*

Table 7-5. Association between worst BV status and sPTB <34 weeks in the whole cohort (low and high-risk)

	Worst BV category			Total*
sPTB <34 weeks	Normal N (%)	Intermediate N (%)	BV (%)	N (%)
No	334 (93.6)	114 (92.7)	95 (94.1)	543 (93.5)
Yes	23 (6.4)	9 (7.3)	6 (5.9)	38 (6.5)
Total	357 (100)	123 (100)	101(100)	581 (100)
Odds ratio (95% CI)	ref	1.15 (0.52-2.55)	0.92 (0.36-2.32)	-
P value	ref	0.74	0.86	-

PTB=preterm birth, BV=bacterial vaginosis, CI=confidence interval, sPTB=spontaneous preterm birth

**Iatrogenic deliveries excluded from analysis*

When sub-group analysis by ethnicity (Black/White) was performed, again there was no association between BV and sPTB <37 or <34 weeks of gestation (Tables 7-6 to 7-9).

Table 7-6. Association between worst BV status and sPTB <37weeks in Black women

	Worst BV category			Total
sPTB <37 weeks	Normal N (%)	Intermediate N (%)	BV (%)	N (%)
No	47 (82.5)	23 (71.9)	35 (87.5)	105 (81.4)
Yes	10 (17.5)	9 (28.1)	5 (12.5)	24 (18.6)
Total	57 (100)	32 (100)	40 (100)	129 (100)
Odds ratio (95% CI)	ref	1.84 (0.66-5.15)	0.67 (0.21-0.14)	-
P value	ref	0.25	0.50	-

PTB=preterm birth, BV=bacterial vaginosis, CI=confidence interval, sPTB=spontaneous preterm birth

**Iatrogenic deliveries excluded from analysis*

Table 7-7. Association between worst BV status and sPTB <34 weeks in Black women

	Worst BV category			Total
sPTB <34 weeks	Normal N (%)	Intermediate N (%)	BV (%)	N (%)
No	51 (87.9)	27 (81.8)	39 (95.1)	117 (88.6)
Yes	7 (12.1)	6 (18.2)	2 (4.9)	15 (11.4)
Total	58 (100)	33 (100)	41 (100)	132
Odds ratio (95% CI)	ref	1.62 (0.49-5.30)	0.37 (0.07-1.90)	-
P value	ref	0.43	0.24	-

PTB=preterm birth, BV=bacterial vaginosis, CI=confidence interval, sPTB=spontaneous preterm birth

**Iatrogenic deliveries excluded from analysis*

Table 7-8. Association between worst BV status and sPTB <37 weeks in White women

	Worst BV category			Total
sPTB <34weeks	Normal N (%)	Intermediate N (%)	BV (%)	N (%)
No	248 (96.5)	74 (97.4)	49 (94.2)	371 (96.4)
Yes	9 (3.5)	2 (2.6)	3 (5.8)	14 (3.6)
Total	257 (100)	76 (100)	52 (100)	385 (100)
Odds ratio (95% CI)	ref	1.01 (0.42-2.44)	1.28 (0.50-3.32)	-
P value	ref	0.99	0.61	-

PTB=preterm birth, BV=bacterial vaginosis, CI=confidence interval, sPTB=spontaneous preterm birth

**Iatrogenic deliveries excluded from analysis*

Table 7-9. Association between worst BV status and sPTB <34weeks in White women

	Worst BV category			Total
sPTB <37weeks	Normal N (%)	Intermediate N (%)	BV (%)	N (%)
No	231 (90.6)	67 (90.5)	45 (88.2)	343 (90.3)
Yes	24 (9.4)	7 (9.5)	6 (11.8)	37 (9.7)
Total	255 (100)	74 (100)	51 (100)	380 (100)
Odds ratio (95% CI)	ref	0.74 (0.16-3.52)	1.69 (0.44-6.46)	-
P value	ref	0.71	0.45	-

PTB=preterm birth, BV=bacterial vaginosis, CI=confidence interval, sPTB=spontaneous preterm birth

**Iatrogenic deliveries excluded from analysis*

7.2.3 Relationship between CVF elafin and BV

A total of 1423 CVF samples from 578 women had a paired BV analysis and a valid elafin concentration. Elafin CVF concentrations were 20% lower (ratio 0.80, 95% CI 0.69 to 0.94, p=0.007) in women with BV *vs* normal samples (adjusted for repeated tests and gestation at testing). Elafin concentrations were 8% lower in intermediate *vs* normal samples, but this result did not reach statistical significance (0.92, 0.80 to 1.05, p=0.22). Elafin concentrations were 13% lower in BV compared with intermediate samples, though not significantly so (0.87, 0.74 to 1.04, p=0.122, see Figure 7-1). When adjusted for known confounders (ethnicity, smoking, maternal age and BMI) this relationship was maintained (normal vs BV 0.77, 0.65-0.91, p=002; normal vs intermediate 0.92, 0.80-0.91, p=0.22).

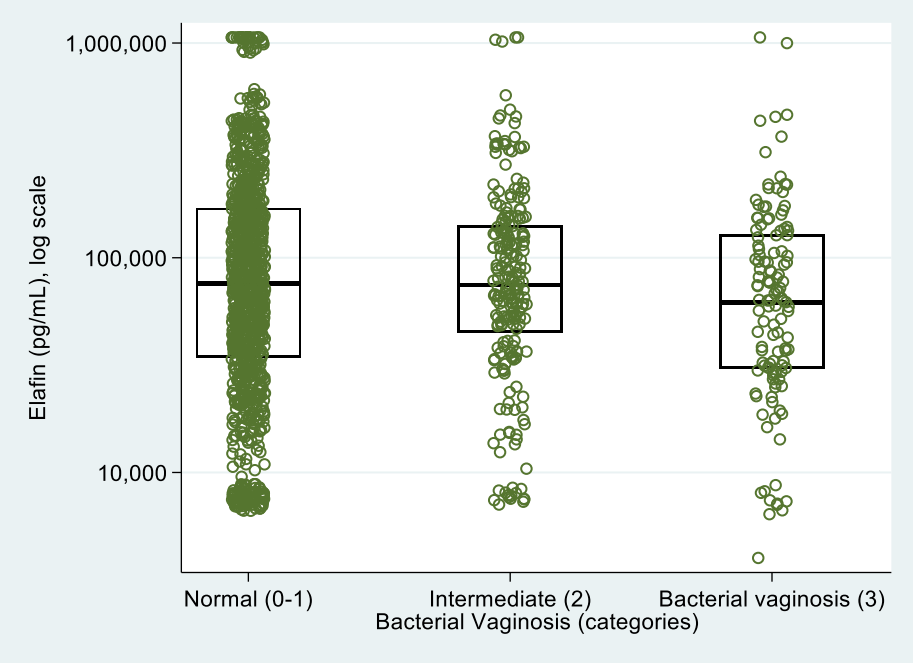

Figure 7-1. Box plot demonstrating the median (IQR) cervicovaginal concentration of elafin in women with positive bacterial vaginosis (BV) smear results compared to those with intermediate and no BV ('normal') gradings

When sub-group analysis by ethnicity was performed, the fall in elafin concentration according to BV disease was more pronounced in Black women than White women (Figure 7-2). In White women, elafin concentrations were 17% lower (ratio 0.83, 95% CI 0.65 to 1.05, p=0.13) in BV presence compared with normal smears. In Black women, this was 24% (0.76, 0.60 to 0.96, p=0.02), though the difference in the relationship between BV and elafin concentration in white women and black women did not reach significance (interaction test, p=0.473). Figure 7-3 demonstrates the longitudinal gestational profile of CVF elafin in women with BV vs normal CVF samples, stratified by ethnicity.

Figure 7-2. Box plot demonstrating the median (IQR) cervicovaginal concentration of elafin in women with positive bacterial vaginosis (BV) smear results compared to those with intermediate and no BV ('normal') gradings in White and Black women

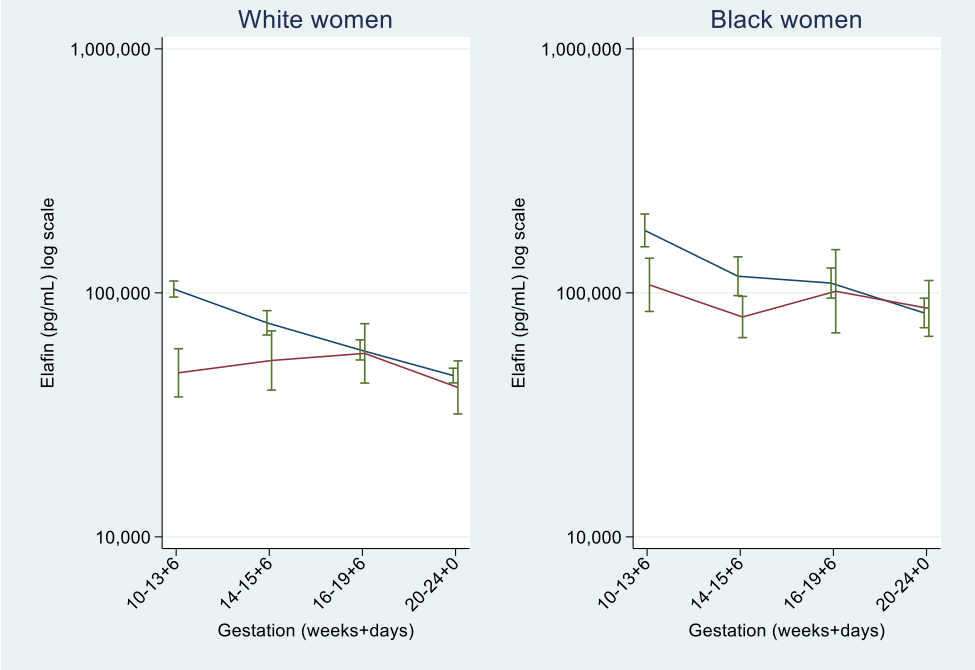

Figure 7-3. Longitudinal measurements of cervicovaginal elafin concentrations in White women) with bacterial vaginosis (red solid line) and normal smears (Blue line) and Black women with bacterial vaginosis and normal smears. Geometric means and SE bars.

7.2.4 Relationship between CVF Cathelicidin and BV

A total of 899 samples from 335 high-risk women provided both valid CVF cathelicidin concentrations and BV smear results. In the whole high-risk cohort, when adjusted for repeated tests and gestation at testing, there was a trend towards higher cathelicidin concentrations in women with intermediate and BV smears compared with normal smears, although this only reached statistical significance comparing intermediate and normal smears (BV vs normal ratio 1.32, 95% CI 0.99 to 1.77 p=0.06; intermediate vs. normal 1.30, 1.04 to 1.60 p=0.02; BV vs. intermediate 1.02, 0.76 to 1.38 p=0.89). This relationship was maintained when adjusted for potential confounders BMI, maternal ag, ethnicity and smoking status (BV vs. normal 1.26, 0.94-1.70, p=0.13; intermediate vs normal 1.27, 1.02-1.60, p=0.03). When stratified by ethnicity, differences in cathelicidin concentration according to BV status was only seen in Black women (Figure 7-4, BV vs. normal 1.5, 1.12 to 2.24, p=0.009). Figure 7-5 displays the profile of CVF cathelicidin across gestation, according to BV status and ethnicity.

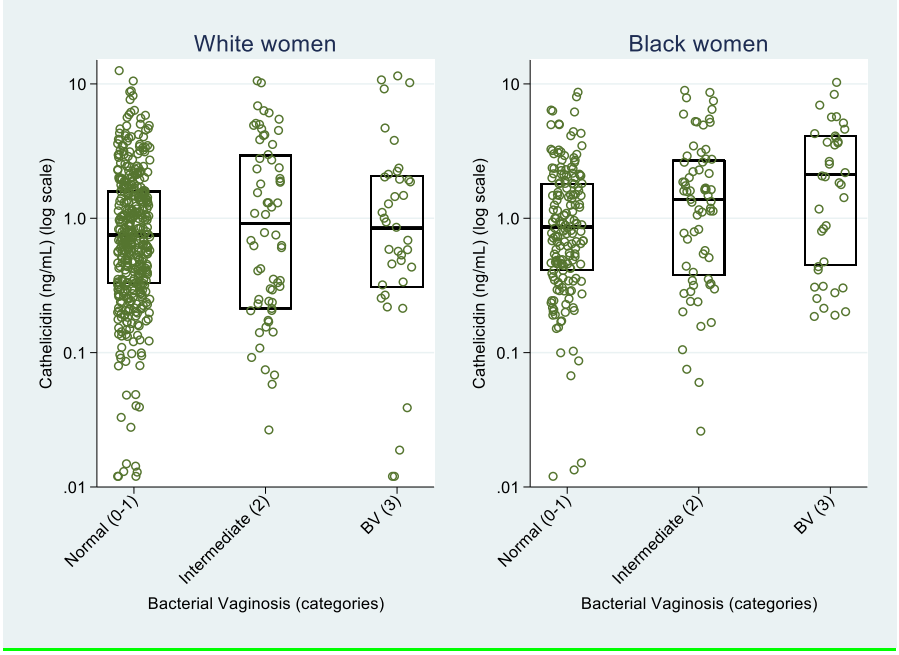

Figure 7-4. Box plot demonstrating the median (IQR) cervicovaginal concentration of cathelicidin in women with positive bacterial vaginosis (BV) smear results compared to those with intermediate normal gradings, split by ethnicity (White vs. Black women).

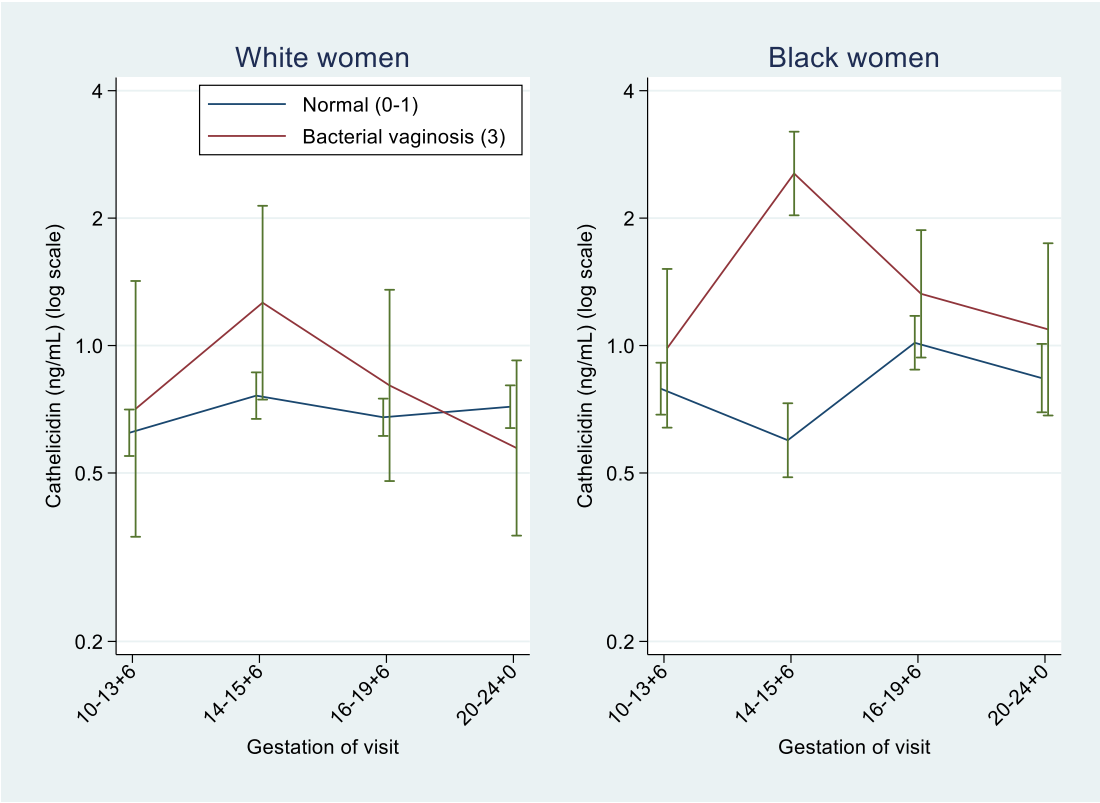

Figure 7-5. Longitudinal measurements of cervicovaginal cathelicidin concentrations in White women with bacterial vaginosis (n=30, red solid line) and normal smear grading (n=133, Blue solid line) and Black women with bacterial vaginosis (n= 32) and normal smear grading (n= 42).

Geometric means and SE bars

7.2.5 Relationship between CVF HNE and BV

A total of 1154 samples from 556 women (high and low risk) had paired HNE CVF concentration and BV smear results. In the whole high-risk cohort, when adjusted for repeated tests and gestation at testing, there was no difference in HNE concentrations in women with intermediate and BV smears compared with normal smears (BV vs. normal ratio 0.97, 95% CI 0.64 to 1.45, p=0.87; intermediate vs. normal 1.04, 0.77 to 1.41, p=0.80; BV vs intermediate 0.92, 0.58 to 1.50, p=0.76). When adjusted for potential confounders (maternal age, BMI, smoking and ethnicity) this relationship was maintained (BV vs normal 0.98, 0.77 to 1.26, p=0.90; intermediate vs. normal 1.06, 0.87 to 1.30, p=0.56). When stratified by ethnicity, no statistically significant differences in HNE concentration according to BV status were noted (Figure 7-6, White women BV vs. normal 1.04, 0.59 to 1.83, p=0.89; Black women BV

vs. normal 0.81 ,0.40 to 1.64, p=0.56; intermediate vs. normal 0.81, 0.46 to 1.41, p=0.45; BV vs. Intermediate 1.00, 0.45 to 2.23, p=0.99). Figure 7-7 displays the profile of CVF HNE across gestation, according to BV status and ethnicity.

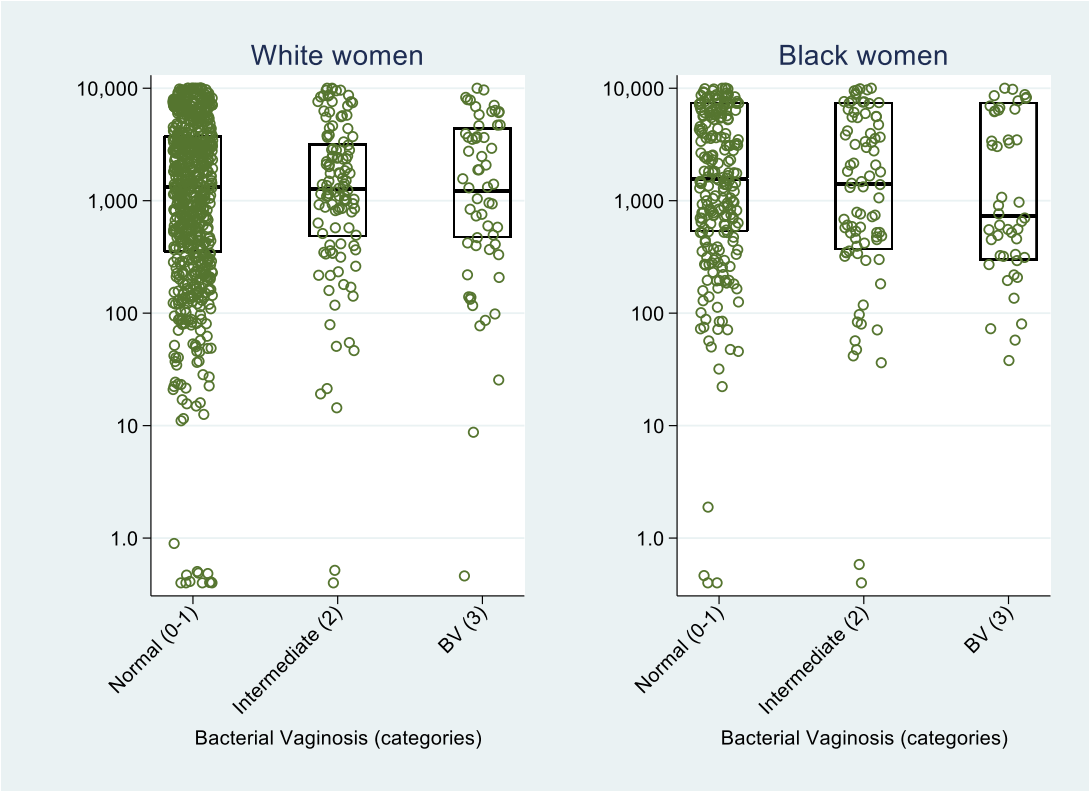

Figure 7-6. Box plot demonstrating the median (IQR) cervicovaginal concentration of human neutrophil elatase (HNE) in women with positive bacterial vaginosis (BV) smear results compared to those with intermediate normal gradings, split by ethnicity (White vs. Black women).

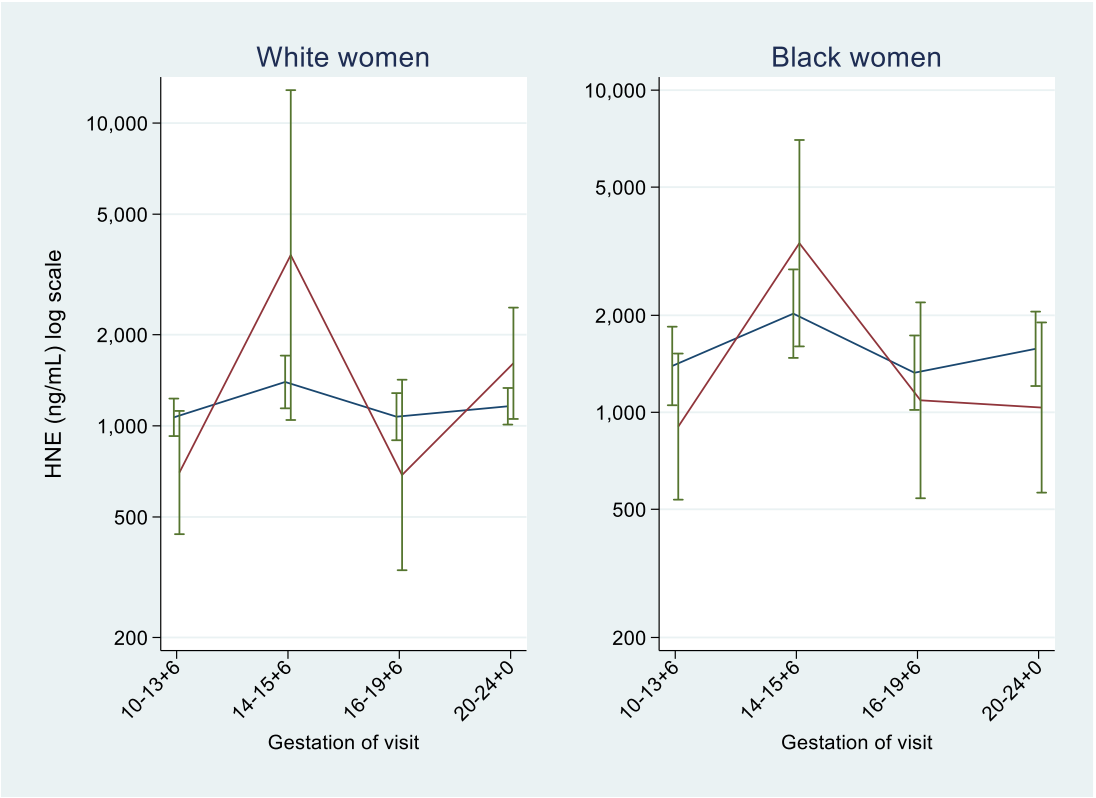

Figure 7-7. Longitudinal measurements of cervicovaginal human neutrophil elastase (HNE) concentrations in White women with bacterial vaginosis (red solid line) and normal smear grading (blue solid line) and Black women with bacterial vaginosis (red solid line) and normal smear grading (blue solid line) Geometric means and SE bars

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7.3 Discussion

This sub-study of BV, HDPs and pregnancy outcome confirmed that in this cohort of women, BV is associated with a number of sociodemographic variables (maternal age, Black ethnicity, smoking, BMI and social deprivation), of which smoking and ethnicity were independent predictors. In this cohort, however, BV diagnosed at any point in pregnancy between 10 and 24^{+0} weeks of gestation was not associated with risk of sPTB. When correlations with HDPs were explored, levels of CVF elafin were slightly reduced in pregnant women with BV compared to women with normal flora (more pronounced in Black women) whereas cathelicidin concentrations were higher in women with BV (also mainly in Black women). No significant relationship was seen between BV and HNE CVF concentration.

Consistent with our data, both ethnicity and smoking have been consistently shown to be associated with BV, both inside and outside of pregnancy (Kenyon, Colebunders, and Crucitti 2013; Ravel et al. 2011; Fiscella and Klebanoff 2004; Hay et al. 1994; Larsson et al. 2007; Ness et al. 2003; Hellberg, Nilsson, and Mårdh 2000; Thorsen et al. 2006). Often exhibiting a dose-dependent relationship with risk of BV (after controlling for known cofounders such as sexual behaviour and alcohol) (Hellberg, Nilsson, and Mårdh 2000; Bagaitkar, Demuth, and Scott 2008), smoking increases risk of BV by via a number of postulated mechanisms. Nicotine and its metabolites can be detected in the CVF of smokers (Hellberg et al. 1988; Sasson et al. 1985) and it has been hypothesised that the build-up of amines, together with the anti-oestrogenic effects of smoking via altered oestrogen metabolism/receptors (Shearman et al. 2005) may predispose to BV. Given that the vaginal microbiome is also influenced by oestrogen (low relative abundance of *Lactobacillus spp*. in post-menopausal women, particularly those with vaginal atrophy) (Brotman et al. 2018), this hypothesis seems plausible. Furthermore, in a small cross-sectional study (40 non pregnant female smokers and non-smokers), smokers were found to have a lower proportion of vaginal lactobacilli (though the smokers were more likely to have African American origin and results were not adjusted for ethnicity), and smoking cessation was observed to shift microbial community states towards a lactobacillus dominated profile (particularly *L. iners*) (Brotman, He, et al. 2014). Black women are more likely to have BV and a more diverse (less lactobacillus dominated) vaginal microbiome, compared to White women, which may be related to behavioural, sociodemographic, environmental or genetic factors (Ness et al. 2003).

It was surprising that no relationship between BV and sPTB was elucidated in this Insight cohort, given the well-established relationship between BV and sPTB in the literature (section 1.4.1.2), as consistently seen in larger cohorts (Gravett, Hummel et al. 1986, Hill 1998, et al. 2005; Klebanoff et al. 2005). However, given that most women who have BV do not deliver prematurely, it is likely that our cohort was not large enough to detect a significant association. Furthermore, we only tested for BV

until 24 weeks of gestation (women may have developed BV later in pregnancy). Finally, our Insight cohort was comprised of majority high-risk women, whose other risk factors may have overwhelmed any contribution that BV may make to risk of sPTB.

As has been found elsewhere (Stock et al. 2009), CVF concentrations of elafin were reduced in women with BV. Mechanistically BV colonisation is known to affect vaginal epithelial cell integrity and the genital immune cell populations in non-pregnant women (Mirmonsef et al. 2012; Thurman and Doncel 2011). Elafin production could be functionally reduced by destruction of epithelial cells. Additionally, factors secreted by the BV microflora may inhibit may be inhibitive. Valore et al. (2006) measured HDP concentration in vaginal lavage samples from 64 healthy non-pregnant women of mixed ethnicity and found reduced concentrations of SLPI, HBD1 & 2 and alpha defensins (human neutrophil peptides, HNP), which largely normalised after successful treatment of BV. That many of these HDPs originate from different sites and cell types, led the authors to suppose that BV may lead to inhibition of multiple innate immune pathways. They suggest that factors secreted by the BV microbial flora may inhibit cytokine and HDP production; indeed they observed low concentrations of IL-8 (the main chemotactic factor for neutrophils) in women with BV. They also propose that 'healthy' lactobacillus species may induce some HDPs and their chemotactic factors, concurring with our research groups observation that a vaginal microbial environment rich in *L. crispatus* had high CVF elafin (Flaviani et al. 2021), and/or that BV associated bacteria may actively supress inflammation (Valore, Wiley, and Ganz 2006). This may also explain why women with BV seem to be more susceptible to other genital tract infections, including HIV (Moodley, Connolly, and Sturm 2002; Sewankambo et al. 1997). Given that low oestrogen is associated with BV (Wilson et al. 2007) but high elafin, the observed relationship between BV and low elafin is not driven by, and may even be despite of, oestrogen status.

In contrast with elafin, CVF concentration of cathelicidin was increased in women with BV. This is consistent with work by Frew et al. (2014) who demonstrated higher cathelicidin in CVF samples of women in their first trimester with BV. As with elafin, it is unclear whether changes in cathelicidin concentration occur as a protective response to bacterial overgrowth and/or lack of lactobacilli, or whether high cathelicidin predisposes to BV, or even whether the relationship is mediated by vitamin D (thought to regulate cathelicidin, deficiency of which may be associated with BV) (Bodnar, Krohn, and Simhan 2009), though vitamin D status (albeit in a largely vitamin deplete population) did not correlate with cathelicidin concentration in our pilot study (Abbott et al. 2014) nor in the Frew study. That gene and protein expression of another HDP HBD-2 has been shown to be increased after culture with BV associated bacteria does suggest that a similar microbial mechanism could explain the rise in cathelicidin seen in our study in women with BV. Eade et al. (2012) developed an *in vitro* endocervical, ectocervical and vaginal epithelial cell model, and observed the response of cytokines and human beta defensin-2 to culture with commensal lactobacilli and BV associated bacteria. They reported that culture with most *Lactobacilli* spp. did not increase expression of the beta defensin HBD-2, but that *L. iners* (thought to be associated with sPTB) and *L. vaginalis*, as well as other BV associated bacteria (*G. vaginalis, and A. vaginae)* did induce HBD2 (and cytokine) expression. Similarly, Doerflinger et al. (2014), in a 3D vaginal cell model, demonstrated induction of HBD-2 gene expression by L. iners.

Our recent analysis of the relationship between elafin, cathelicidin and the vaginal microbiome in a sub-set of women from the Insight cohort demonstrated that elafin and cathelicidin concentrations were clearly affected by the composition of the resident bacterial community (as defined by PCoA groups and Operational Taxonomic Units, OTUs) (Flaviani et al. 2021), but more strongly correlated with bacterial metabolites (such as lactate, aspartate and leucine). It may be that expression of host defence peptides is also regulated by bacterial metabolites, as well as inflammatory mediators (Delgado-Diaz et al. 2019). Whilst elafin production by epithelial cells can

be supressed by bacteria or their metabolites, cathelicidin production from neutrophils appeared to be induced by dysbiosis and its related environment.

When stratified by ethnicity, alterations in CVF concentration of both elafin and cathelicidin remained statistically significant in Black women, rather than White women. This may be because the proportion of Black women with BV was higher, despite being a smaller cohort overall, or may point to some other genetic or sociodemographic element which influences innate immunity and microbial response. Previous studies noting association between elafin with BV were overwhelmingly dominated (>95%) by White women, though had a higher proportion of BV diagnosis within this group (24% vs. 12% in our White sub-group) (Stock et al. 2009). Likewise, the cathelicidin/BV cohort described by Frew et al. (2014) were also 95% Caucasian, with a higher incidence of BV infection (21%).

7.4 Conclusions

In summary, we have demonstrated that CVF concentration of elafin and cathelicidin (but not HNE) are altered in BV, indicating potential involvement in the pathogenesis of the polymicrobial infection, or a consequence of it. How these relate mechanistically to the in-depth sequenced microbiome and host hormone status (particularly oestrogen) warrant exploration. Further work is needed to elucidate whether the relationship between HDPs and BV is more significant in Black women, and whether this is related to underlying genetic or socio-demographic factors which may drive both risk of BV and innate immune response.

8 Fetal fibronectin and host defence peptides

8.1 Introduction

Currently, the most widely used biochemical marker for prediction of sPTB is qfFN (see section 1.3.9.2). fFN is an extracellular glycoprotein found in placental tissue, amniotic fluid and between the chorion and decidua, normally detectable in the cervicovaginal fluid (CVF) in pregnancy before the fusion of the decidua and fetal membranes. Once fusion has occurred, CVF fFN concentrations drop and may become undetectable from approximately 18 weeks of gestation, after which time, release of fFN into the CVF by presumed inflammatory, infective or mechanical disruption to the chorionic-decidual interface is associated with an increased risk of sPTB. More commonly used in women with symptoms of PTL, its utility to predict sPTB in high-risk asymptomatic women is increasingly recognised and clinically utilised from 18 weeks of gestation onwards, though thought to be slightly more accurate after 22 weeks of gestation, particularly for prediction of early sPTB <34 weeks of gestation (Hezelgrave, Abbott, et al. 2016).

This chapter evaluates the relationship between qfFN and expression of HDPs to determine whether there is predictive utility in combining them as a biomarker test. A full description of methods is detailed in Chapter 3.

8.2 Results

In total, 541 qfFN tests were performed on 275 high-risk women (18 to 24^{+0} weeks gestation) in the Insight cohort. As per the manufacturer's recommendation, 19 tests were excluded due to blood staining and 23 tests were excluded due to a history of sexual intercourse in the past 48 hours prior to test, leaving 499 qfFN tests performed on 267 women. The demographics of this cohort are described in Table 8-1. When split by gestation for analysis purposes (after exclusions), 189 qfFN tests were

performed on 177 individual high-risk women between 18^{+0} to 19^{+6} weeks of gestation and 310 qfFN tests were performed on 235 high-risk women between 20 and 24^{+0} weeks of gestation.

Table 8-1 Demographics and obstetric history of high-risk women tested for cervicovaginal fluid fetal fibronectin concentration (n=267)

Characteristic	Sub-category	Mean ± or N (%)
Age (years)		33.33 ± 5.06
BMI (Kg/m^2)		26.81 ±- 7.56
Ethnicity	White	144 (53.9)
	Black	92 (34.5)
	Asian	16 (6.0)
	Other	15 (5.6)
Previous sPTB		111 (41.6)
Previous late miscarriage		110 (41.2)
Previous PPROM		68 (25.5)
Previous cervical surgery		87 (32.6)
Uterine abnormality		7 (2.6)
Smoking history	Never smoked	196 (73.4)
	Ex-smoker (gave up prior to pregnancy)	50 (18.7)
	Ex-smoker (gave up in pregnancy)	12 (4.5)
	Current smoker	9 (3.4)
History of domestic violence		9 (3.4)
History of recreational drug use		5 (1.9)
History of GBS		24 (9.3)
History of recurrent UTI		11 (4.1)
History of BV		32 (12.0)

BMI=body mass index, GBS= group B streptococcus, UTI=urinary tract infection, PPROM=premature prelabour rupture of membranes; sPTB=spontaneous preterm birth

Appendix 3 details the rates of sPTB observed according to qfFN category, the OR for sPTB using qfFN alone, and assessment of qfFN as a predictor for sPTB (AUC ROC), including when stratified for ethnicity.

8.2.1 qfFN, HDPs and sPTB

Table 8-2 presents the Spearman's rank correlations for qfFN with HDPs, for simultaneously measured biomarkers (first test taken between 18-19^{+6} weeks of gestation, and first test taken between 20 to 24 weeks of gestation). Cathelicidin correlates positively with qfFN at both gestational timepoints. As in the previous analysis (section 5.5), cathelicidin maintains positive correlation with HNE concentration, and a negative correlation with elafin when measured at 16 to 19^{+6} weeks of gestation.

Table 8-2 Correlation between HDPs (elafin, cathelicidin), human neutrophil elastase, and quantitative fetal fibronectin in high-risk women, at two gestational timepoints

Correlation coefficient (p-value)				
18 to 19^{+6} weeks of gestation				
Biomarker	**qfFN**	**Elafin**	**Cathelicidin**	**HNE**
qfFN	1.0	-	-	-
Elafin	-0.15 (0.09)	1.0	-	-
Cathelicidin	**0.29 (0.003)**	**-0.14 (0.03)**	1.0	-
HNE	0.10 (0.29)	0.02 (0.75)	**0.46 (<0.001)**	1.0
20 to 24^{+0} weeks of gestation				
	qfFN	**Elafin**	**Cathelicidin**	**HNE**
qfFN	1.0	-	-	-
Elafin	-0.10 (0.14)	1.0	-	-
Cathelicidin	**0.33 (<0.001)**	-0.06 (0.37)	1.0	-
HNE	0.09 (0.19)	**0.17 (<0.001)**	**0.4 (<0.001)**	1.0

HNE= human neutrophil elastase, qfFN= quantitative fetal fibronectin. Significant values in bold.

This was further explored by looking at how CVF qfFN concentration correlated with HDP concentrations taken at an earlier gestation (focussing on timepoints when previous analysis has suggested that HDPs may have association with outcome). qfFN

concentration at 18-19^{+6} weeks gestation had a negative correlation with elafin at 14 to 15^{+6} weeks gestation (n=112, coefficient -0.20, p=0.04) but not at other gestational timepoints. No significant correlation between qfFN and HNE at any gestational sampling point was found (data not shown). Cathelicidin taken at each gestational timepoint positively correlated with qfFN measured at both 18 to 19^{+6} weeks, and 20 to 24^{+0} weeks of gestation (Table 8-3).

Table 8-3 Correlation between cervicovaginal quantitative fetal fibronectin (18 to 24^{+0} weeks of gestation) and cathelicidin (10 to 24^{+0} weeks of gestation) in high-risk women

Biomarker (gestation)	**qfFN (18 to 19^{+6} weeks)**		**qfFN (20-24^{+0} weeks)**	
	N	Correlation coefficient (p-value)	**N**	Correlation coefficient (p-value)
Cathelicidin (10-13^{+6} weeks)	72	**0.28 (0.02)**	125	**0.20 (0.02)**
Cathelicidin (14-15^{+6} weeks)	95	**0.22 (0.03)**	149	**0.19 (0.02)**
Cathelicidin (16-19^{+6} weeks)	107	**0.29 (0.003)**	171	**0.30 (<0.001)**
Cathelicidin (20-24^{+0} weeks)	98	0.20 (0.05)	181	**0.33(<0.001)**

Significant values in bold

8.2.1.1 *Prediction of sPTB using cathelicidin and qfFN*

A total of 177 high-risk women had both CVF cathelicidin concentration measured at 14-15^{+6} weeks, and qfFN measured at 18 to 24^{+0} weeks of gestation. Table 8-4 shows that combination of CVF cathelicidin concentration combined with the qfFN concentration enhanced the prediction of sPTB <37 weeks (AUC ROC=0.74), compared to fFN alone (AUC ROC=0.69), but comparison of these did not reach statistical significance (p=0.10). This was also the case for prediction of sPTB <34 weeks of gestation (p=0.40). qfFN predicted cervical shortening (AUC ROC=0.67), with no improvement in prediction when the earlier cathelicidin result was considered (p=0.34).

Table 8-4 ROC curve areas for performance of quantitative fetal fibronectin (18-24 weeks of gestation) and cathelicidin (14-15^{+6} weeks of gestation), separately and combined, to predict sPTB and cervical shortening in high-risk women.

Biomarker	N	AUC	95% confidence interval	P value
		Prediction of sPTB <37 weeks of gestation		
qfFN	177	**0.69**	**0.59-0.78**	
Cathelicidin	177	**0.67**	**0.57-0.77**	**<0.001**
qfFN & Cathelicidin	177	**0.74**	**0.65-0.83**	
		Prediction of sPTB <34 weeks of gestation		
qfFN	177	**0.73**	**0.61-0.84**	
Cathelicidin	177	**0.64**	**0.51-0.77**	**0.002**
qfFN & Cathelicidin	177	**0.75**	**0.64-0.87**	
		Prediction of cervical shortening prior to 24 weeks of gestation		
qfFN	177	**0.67**	**0.58-0.76**	
cathelicidin	177	0.58	0.49-0.67	**<0.001**
qfFN & cathelicidin	177	**0.69**	**0.60-0.77**	

Preterm iatrogenic deliveries prior to the outcome of interest have been excluded. sPTB=spontaneous preterm birth, AUC=area under the receiver operating characteristic curve; qfFN=quantitative fetal fibronectin. Significant results in bold.

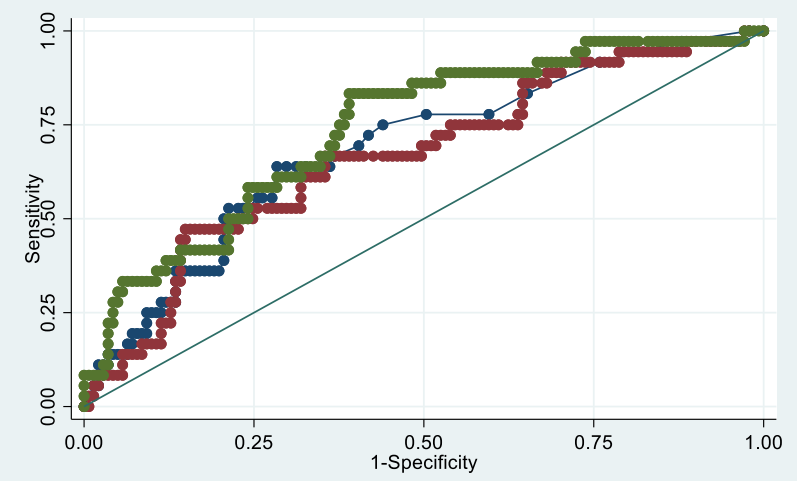

Figure 8-1. Receiver operating characteristic curves demonstrating the ability of cathelicidin cervicovaginal fluid concentration taken at 14 to 15^{+6} weeks of gestation (red line, n=177, AUC= 0.67) and quantitative fetal fibronectin taken between 18^{+0} and 24^{+0} weeks of gestation (blue line, n=177, AUC 0 .69), and the two markers combined (green line, n=177, AUC 0.69), in high-risk women to predict sPTB prior to 37 weeks.

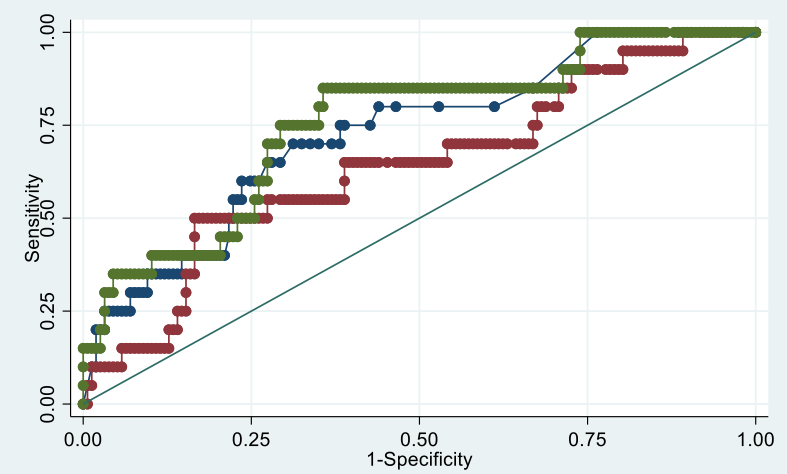

Figure 8-2. Receiver operating characteristic curves demonstrating the ability of cervicovaginal fluid cathelicidin concentrations taken at 14^{+0} to 15^{+6} weeks of gestation (red line, n=177, AUC= 0.64) and quantitative fetal fibronectin taken between 18^{+0} and 24^{+0} weeks of gestation (blue line, n=177, AUC 0 .73), and the two markers combined (green line, n=177, AUC 0.75), in high-risk women to predict sPTB prior to 34 weeks.

When the high-risk cohort was stratified by ethnicity, CVF cathelicidin concentration measured at 14 to15^{+6} weeks of gestation was a superior predictor of sPTB <37 weeks (n=88, AUC ROC 0.76, 95% CI 0.62 to 0.89) compared with qfFN after 18 weeks (n=88, 0.66, 0.49 to 0.82), although the differences between these ROC curves did not reach statistical significance, likely due to small numbers (p=0.32). The combination of the two CVF markers in White high-risk women had the best prediction (n=88, 0.78, 0.65 to 0.92), though again this was not statistically different to cathelicidin alone (p=0.40) or qfFN alone (p=0.11). Table 8-5 and Figure 8-3 illustrate the prediction of sPTB using individual and combined markers in White high-risk women. This pattern was similar for prediction of sPTB <34 weeks, though the low numbers of events (sPTB) in this sub-stratified cohort were small.

Table 8-5 ROC curve areas for performance of quantitative fetal fibronectin (18-24 weeks of gestation) and cathelicidin (14-15^{+6} weeks of gestation), separately and combined, to predict sPTB and cervical shortening in White high-risk women.

Biomarker	N	AUC	95% confidence interval	P value
		Prediction of sPTB <37 weeks of gestation		
qfFN	88	0.66	0.49-0.82	
Cathelicidin	88	**0.76**	**0.62-0.89**	**0.01**
qfFN & Cathelicidin	88	**0.78**	**0.65-0.92**	
		Prediction of sPTB <34 weeks of gestation		
qfFN	88	0.70	0.49-0.91	
Cathelicidin	88	0.72	0.50-0.95	0.32
qfFN & Cathelicidin	88	**0.76**	**0.55-0.99**	
		Prediction of cervical shortening prior to 24 weeks of gestation		
qfFN	88	0.60	0.43-0.77	
Cathelicidin	88	0.54	0.37-0.72	0.38
qfFN & Cathelicidin	88	0.61	0.43-0.79	

Preterm iatrogenic deliveries prior to the outcome of interest have been excluded. sPTB=spontaneous preterm birth, AUC=area under the receiver operating characteristic curve; qfFN=quantitative fetal fibronectin. Significant results in bold

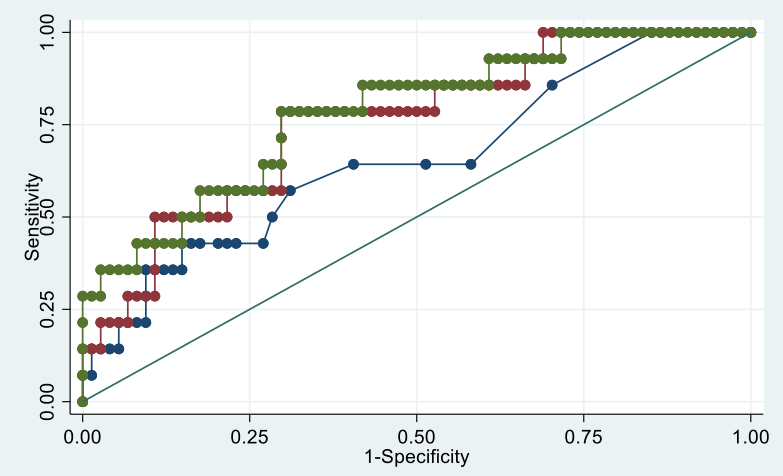

Figure 8-3. Receiver operating characteristic curves demonstrating the ability of cathelicidin cervicovaginal fluid concentration taken at 14^{+0} to 15^{+6} weeks of gestation (red line, n=88, AUC=0.76) and quantitative fetal fibronectin taken between 18^{+0} and 24^{+0} weeks of gestation (blue line, n=88, AUC=0.66), and the two markers combined (green line, n=88, AUC 0.78), in White high-risk women, to predict sPTB prior to 37 weeks.

As seen in Chapter 6, CVF cathelicidin performed less well as a predictor of sPTB in Black high-risk women, and no statistically significant difference was seen between prediction using qfFN, CVF cathelicidin or a combination of the markers in this small cohort of women (Table 8-6), though qfFN appeared to be the superior predictor in this group. Numbers were too small to detect significant differences in biomarker prediction of a short cervix in White or Black women, though ROC AUCs were similar to the whole cohort.

Table 8-6 ROC curve areas for performance of quantitative fetal fibronectin (18-24 weeks of gestation) and cathelicidin (14-15^{+6} weeks of gestation), separately and combined, to predict sPTB and cervical shortening in Black high-risk women.

Biomarker	N	AUC	95% confidence interval	P value
		Prediction of sPTB <37 weeks of gestation		
qfFN	68	0.64	0.48-0.81	
Cathelicidin	68	0.53	0.35-0.72	0.49
qfFN & Cathelicidin	68	0.65	0.49-0.81	
		Prediction of sPTB <34 weeks of gestation		
qfFN	68	**0.70**	**0.52-0.88**	
Cathelicidin	68	0.55	0.32-0.78	0.32
qfFN & Cathelicidin	68	**0.69**	**0.51-0.88**	
		Prediction of cervical shortening prior to 24 weeks of gestation		
qfFN	68	0.63	0.49-0.76	
cathelicidin	68	0.56	0.42-0.70	0.66
qfFN & cathelicidin	68	0.62	0.49-0.76	

Preterm iatrogenic deliveries have been excluded. sPTB=spontaneous preterm birth, AUC=area under the receiver operating characteristic curve; qfFN=quantitative fetal fibronectin. Significant results in bold

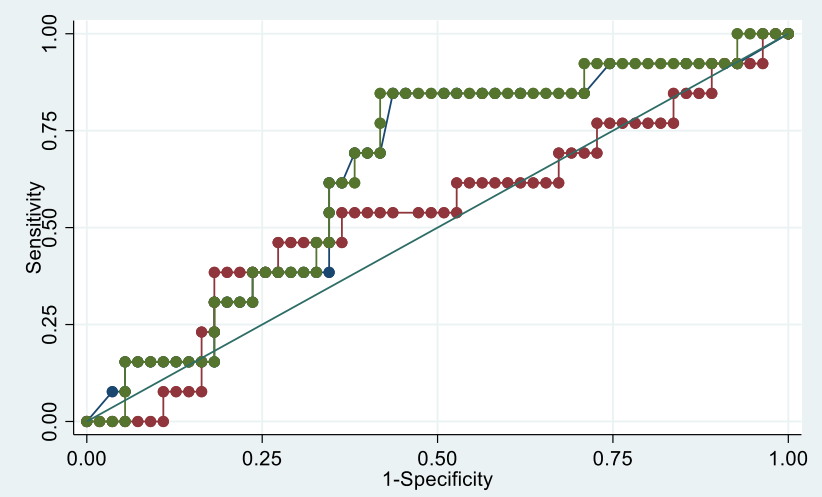

Figure 8-4. Receiver operating characteristic curves demonstrating the ability of cathelicidin cervicovaginal fluid concentration taken at 14^{+0} to 15^{+6} weeks of gestation (red line, n=68, AUC=0.53) and quantitative fetal fibronectin taken between 18^{+0} and 24^{+0} weeks of gestation (blue line, n=68, AUC 0.64), and the two markers combined (green line, n=68, AUC 0.65), in Black high-risk women, to predict sPTB prior to 37 weeks.

8.2.1.2 *Prediction of sPTB combining HNE and qfFN*

A total of 190 high-risk women had both CVF HNE concentration measured at 14-15^{+6} weeks, and qfFN measured at 18 to 24^{+0} weeks of gestation (after exclusion of preterm iatrogenic deliveries <37 weeks). Table 8-7 displays the AUC for the biomarkers, both individually and combined. Addition of HNE to qfFN did not improve prediction of sPTB<37 or <34 weeks' gestation. qfFN appeared to be the better predictor of cervical shortening compared to HNE (though not statistically significantly so, p=0.26), which was not improved by the combination of the two markers (p=0.37).

Table 8-7. ROC curve areas for performance of quantitative fetal fibronectin (18-24 weeks of gestation) and human neutrophil elastase (14-15^{+6} weeks of gestation), separately and combined, to predict sPTB and cervical shortening in high-risk women.

Biomarker	N	AUC	95% confidence interval	P value
		Prediction of sPTB <37 weeks of gestation		
qfFN	190	**0.68**	**0.59-0.78**	
HNE	190	0.61	0.50-0.72	0.07
qfFN & HNE	190	**0.69**	**0.60-0.79**	
		Prediction of sPTB <34 weeks of gestation		
qfFN	190	**0.75**	**0.64-0.86**	
HNE	190	0.60	0.46-0.73	0.07
qfFN & HNE	190	**0.74**	**0.62-0.87**	
		Prediction of cervical shortening prior to 24 weeks of gestation		
qfFN	190	**0.69**	**0.60-0.77**	
HNE	190	**0.61**	**0.52-0.73**	**0.02**
qfFN & HNE	190	**0.70**	**0.61-0.79**	

Preterm iatrogenic deliveries have been excluded. sPTB=spontaneous preterm birth, AUC=area under the receiver operating characteristic curve; qfFN=quantitative fetal fibronectin, HNE= Human Neutrophil Elastase. Significant results in bold.

When stratified by ethnicity small numbers limited interpretation. However, in White women (Table 8-8), addition of HNE concentration may have slightly improved prediction of sPTB <37 and <34 weeks, compared with fFN alone, but not statistically significantly so (<34 weeks p=0.77; <37 weeks p=0.37). The addition of HNE concentration to qfFN improved prediction of cervical shortening, but this was of borderline statistical significance (p=0.05, Figure 8.5). In Black women, the addition of HNE did not improve prediction of sPTB nor cervical shortening using qfFN (Table 8-9).

Table 8-8. ROC curve areas for performance of quantitative fetal fibronectin (18-24^{+0} weeks of gestation) and human neutrophil elastase (14-15^{+6} weeks of gestation), separately and combined, to predict sPTB and cervical shortening in White high-risk women.

Biomarker	N	AUC	95% confidence interval	P value
		Prediction of sPTB <37 weeks of gestation		
qfFN	94	0.65	0.50-0.80	
HNE	94	0.64	0.49-0.79	0.21
qfFN & HNE	94	**0.69**	**0.53-0.85**	
		Prediction of sPTB <34 weeks of gestation		
qfFN	94	0.68	0.47-0.90	
HNE	94	0.58	0.37-0.78	0.28
qfFN & HNE	94	0.70	0.48-0.91	
		Prediction of cervical shortening prior to 24 weeks of gestation		
qfFN	94	0.61	0.44-0.78	
HNE	94	**0.69**	**0.54-0.85**	**0.002**
qfFN & HNE	94	**0.74**	**0.58-0.90**	

Preterm iatrogenic deliveries have been excluded. sPTB=spontaneous preterm birth, AUC=area under the receiver operating characteristic curve, qfFN=quantitative fetal fibronectin, HNE= Human Neutrophil Elastase. Significant results in bold

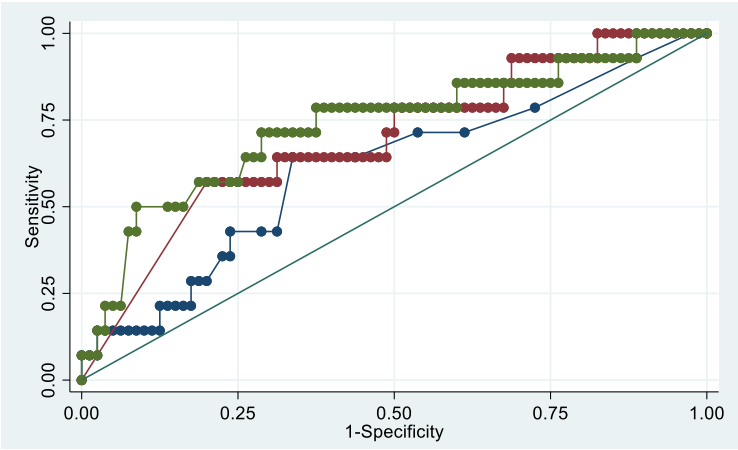

Figure 8-5. Receiver operating characteristic curves demonstrating the ability of cervicovaginal fluid human neutrophil elastase concentration, measured at 14^{+0} to 15^{+6} weeks of gestation (red line, n = 94, AUC=0.69), and quantitative fetal fibronectin values between 18^{+0} and 24^{+0} weeks of gestation (blue line, n=94, AUC 0.61), and the two markers combined (green line, n=94, AUC 0.74), in White high-risk women, to predict cervical shortening prior to 24 weeks of gestation

Table 8-9. ROC curve areas for performance of quantitative fetal fibronectin (18-24^{+0} weeks of gestation) and Human neutrophil elastase (14-15^{+6} weeks of gestation), separately and combined, to predict sPTB in Black high-risk women.

Biomarker	N	AUC	95% confidence interval	P value
		Prediction of sPTB <37 weeks of gestation		
qfFN	71	**0.68**	**0.52-0.85**	
HNE	71	0.54	0.33-0.75	0.46
qfFN & HNE	71	**0.70**	**0.57-0.84**	
		Prediction of sPTB <34 weeks of gestation		
qfFN	71	**0.74**	**0.58-0.89**	
HNE	71	0.60	0.38-0.83	0.47
qfFN & HNE	71	**0.74**	**0.55-0.92**	
		Prediction of cervical shortening prior to 24 weeks of gestation		
qfFN	71	**0.65**	**0.52-0.78**	
HNE	71	0.58	0.44-0.73	0.65
qfFN & HNE	71	**0.65**	**0.51-0.78**	

Preterm iatrogenic deliveries have been excluded. sPTB=spontaneous preterm birth, AUC=area under the receiver operating characteristic curve; qfFN=quantitative fetal fibronectin, HNE= human neutrophil elastase. Significant results in bold

8.2.1.3 *Prediction of sPTB with elafin and qfFN*

A number of high-risk women (n=221) had both CVF elafin concentration measured at 16-19^{+6} weeks of gestation, and a qfFN measured after 18 weeks of gestation. Addition of elafin concentration did not improve prediction of sPTB <37 (p=0.99) or <34 weeks of gestation (p=0.49) compared with qfFN alone (Table 8-10, Figure 8-6 and 8-7).

Table 8-10 ROC curve areas for performance of quantitative fetal fibronectin (18-24 weeks of gestation) and CVF elafin (16-19^{+6} weeks of gestation), separately and combined, to predict sPTB in high-risk women

Biomarker	N	AUC	95% confidence interval	P value
		Prediction of sPTB <37 weeks of gestation		
qfFN	221	**0.64**	**0.55-0.74**	
elafin	221	0.48	0.38-0.57	**0.02**
qfFN & elafin	221	**0.64**	**0.55-0.74**	
		Prediction of sPTB <34 weeks of gestation		
qfFN	221	**0.70**	**0.58-0.81**	
elafin	221	0.54	0.42-0.66	**0.01**
qfFN & elafin	221	**0.71**	**0.59-0.83**	
		Prediction of cervical shortening prior to 24 weeks of gestation		
qfFN	221	**0.68**	**0.59-0.76**	
elafin	221	0.48	0.38-0.57	**0.007**
qfFN & elafin	221	**0.67**	**0.58-0.76**	

Preterm iatrogenic deliveries have been excluded. sPTB=spontaneous preterm birth, AUC=area under the receiver operating characteristic curve; qfFN=quantitative fetal fibronectin. Significant results in bold

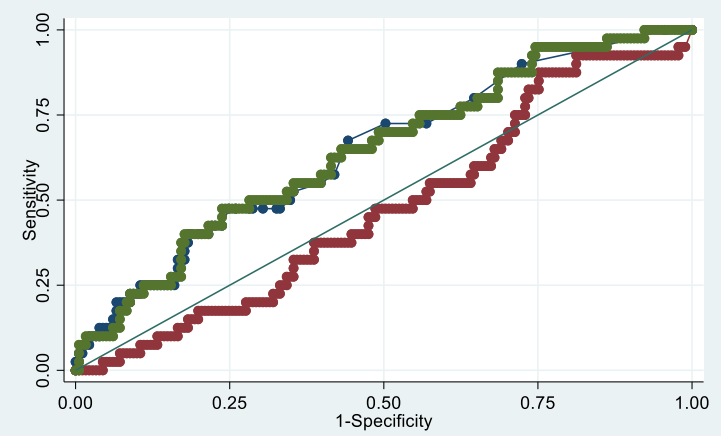

Figure 8-6. Receiver operating characteristic curves demonstrating the ability of elafin cervicovaginal fluid concentration taken at 16^{+0} to 19^{+6} weeks of gestation (red line, n=221, AUC=0.48) and quantitative fetal fibronectin taken between 18^{+0} and 24^{+0} weeks of gestation (blue line, n=221, AUC=0.64), and the two markers combined (green line, n=221, AUC=0.64), in high-risk women, to predict sPTB prior to 37 weeks.

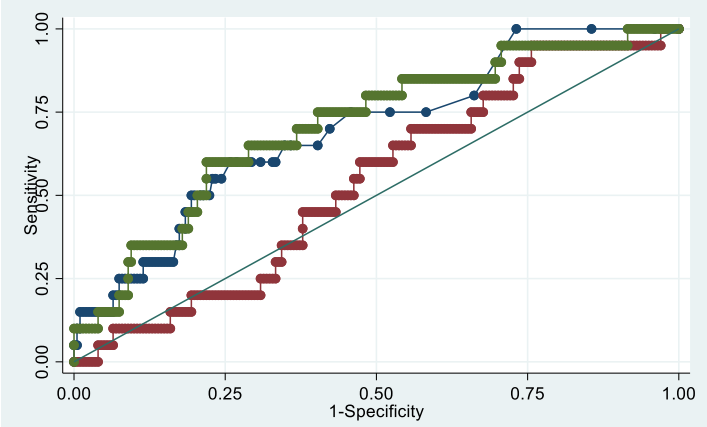

Figure 8-7. Receiver operating characteristic curves demonstrating the ability of elafin cervicovaginal fluid concentration taken at 16^{+0} to 19^{+6} weeks (red line, n = 221, AUC=0.54) and quantitative fetal fibronectin taken between 18^{+0} and 24^{+0} weeks (blue line, n=221, AUC=0.70), and the two markers combined (green line, n=221, AUC=0.71), in high-risk women, to predict sPTB prior to 34 weeks

In White women, neither elafin nor qfFN could be shown to predict outcome with statistical confidence, though numbers were small (Table 8-11, Figure 8-8). As seen in chapter 6, however, low elafin in Black women was predictive of a short cervix (and trended towards being predictive of sPTB). However, this result did not add predictive accuracy to using high qfFN alone (Table 8-12, Figure 8.9).

Table 8-11 ROC curve areas for performance of quantitative fetal fibronectin (18-24^{+0} weeks of gestation) and CVF elafin (16-19^{+6} weeks of gestation), separately and combined, to predict sPTB and cervical shortening weeks in high-risk White women

Biomarker	N	AUC	95% confidence interval	P value
		Prediction of sPTB <37 weeks of gestation		
qfFN	115	0.57	0.44-0.70	
elafin	115	0.47	0.32-0.62	0.54
qfFN & elafin	115	0.58	0.44-0.72	
		Prediction of sPTB <34 weeks of gestation		
qfFN	115	0.60	0.42-0.79	
elafin	115	0.63	0.43-0.82	0.40
qfFN & elafin	115	0.66	0.46-0.87	
		Prediction of cervical shortening prior to 24 weeks of gestation		
qfFN	115	0.57	0.41-0.72	
elafin	115	0.50	0.33-0.67	0.85
qfFN & elafin	115	0.58	0.43-0.73	

Preterm iatrogenic deliveries have been excluded. sPTB=spontaneous preterm birth, AUC=area under the receiver operating characteristic curve; qfFN=quantitative fetal fibronectin

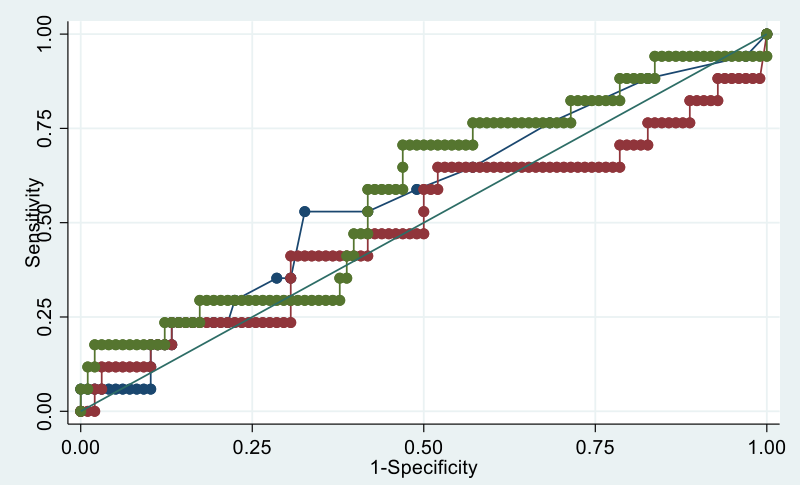

Figure 8-8. Receiver operating characteristic curves demonstrating the ability of elafin cervicovaginal fluid concentration taken at 16^{+0} to 19^{+6} weeks of gestation (red line, n=115, AUC=0.50) and quantitative fetal fibronectin taken between 18^{+0} and 24^{+0} weeks of gestation (blue line, n=115, AUC=0.57), and the two markers (low elafin and high qfFN) combined (green line, n=115, AUC=0.58), in high-risk White women, to predict cervical shortening prior to 24 weeks of gestation

Table 8-12. ROC curve areas for performance of quantitative fetal fibronectin (18-24 weeks of gestation) and CVF elafin (16-19^{+6} weeks of gestation), separately and combined, to predict sPTB in high-risk Black women

Biomarker	N	AUC	95% confidence interval	P value
		Prediction of sPTB <37 weeks of gestation		
qfFN	78	**0.68**	**0.53-0.84**	
elafin	78	0.38	0.24-0.52	**0.05**
qfFN & elafin	78	**0.68**	**0.52-0.84**	
		Prediction of sPTB <34 weeks of gestation		
qfFN	78	**0.71**	**0.56-0.87**	
elafin	78	0.34	0.17-0.50	**0.008**
qfFN & elafin	78	**0.71**	**0.55-0.88**	
		Prediction of cervical shortening prior to 24 weeks of gestation		
qfFN	78	**0.67**	**0.55-0.81**	
elafin	78	**0.36**	**0.23-0.48**	**0.005**
qfFN & elafin	78	**0.68**	**0.55-0.81**	

Preterm iatrogenic deliveries have been excluded. sPTB=spontaneous preterm birth, AUC=area under the receiver operating characteristic curve, qfFN=quantitative fetal fibronectin. Significant results in bold

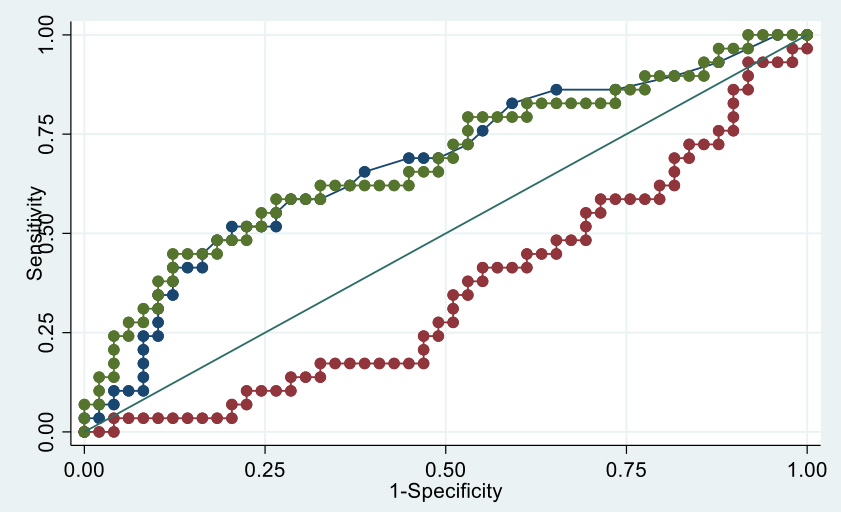

Figure 8-9. Receiver operating characteristic curves demonstrating the ability of elafin cervicovaginal fluid concentration taken at 16^{+0} to 19^{+6} weeks of gestation (red line, n = 78, AUC=0.36) and quantitative fetal fibronectin taken between 18^{+0} and 24^{+0} weeks of gestation (blue line, n=78, AUC=0.67), and the two markers (low elafin and high fFN) combined (green line, n=78, AUC=0.68), to predict cervical shortening prior to 24 weeks of gestation, in high-risk Black women.

8.2.2 Combining biomarkers to create a model to predict sPTB

Based on our previous results, we used multiple predictor logistic regression to investigate whether a predictive model could be created using a combination of the above biomarkers (including CE ratio) measured at different gestational timepoints, as well as sociodemographic factors as explored in Chapter 4.

When combining HDPs with qfFN for ROC prediction analysis and model generation, the gestation at which the HDP had proved most predictive during single marker analysis was used; for elafin this was 10-13^{+6} weeks of gestation and 16-19^{+6} weeks, cathelicidin and HNE 14 to 15^{+6} weeks of gestation. This was combined with the first qfFN concentration taken after 18 weeks of gestation to generate a combined ROC AUC (see methods section 3.10.5 for full explanation).

8.2.2.1 *Model 1: incorporating all socio-demographic and biomarker data available by 24 weeks of gestation*

A total of 215 high-risk women had biomarker data available for all selected biomarker parameters (qfFN, elafin, cathelicidin and HNE), as well as socioeconomic information and pregnancy outcome. For prediction of sPTB <37 weeks of gestation, CVF cathelicidin and qfFN concentration, CE ratio, history of recurrent UTI, worst risk factor and ethnicity were significant predictors of sPTB in the single predictor analysis (at $p<0.05$). After stepwise logistic regression modelling, only a history of recurrent UTI's and qfFN remained significant predictors. Logistic regression was then performed on the whole high-risk cohort who had data regarding these two parameters (N=266) and the final model (ORs, 95% CI) is reported in Table 8-13. This predicted sPTB with an AUC ROC 0.70 (95% CI 0.62 to 0.78).

For prediction of sPTB <34 weeks of gestation, 208 high-risk women had full biomarker profiles and complete sociodemographic and outcome data. For single predictor (univariate) analysis, qfFN, a history of late miscarriage and ethnicity were identified as independent predictors of outcome. After stepwise logistic regression

modelling only qfFN remained a reliably predicted sPTB <34 weeks (Table 8-13). 272 women had qfFN concentration between 18 and 24 weeks of gestation, which predicted sPTB <34 weeks with an AUC ROC of 0.73 (95% CI 0.63 to 0.83).

For prediction of cervical shortening, qfFN, CVF HNE concentration, BMI, smoking, history of a late miscarriage and Black ethnicity were significant univariate predictors of outcome. After stepwise logistic regression modelling, a history of a previous late miscarriage and qfFN remained significant risk factors for high-risk women (n=214). In the 272 women who had both measurements, they predicted cervical shortening with AUC 0.73 (0.63 to 0.83).

Table 8-13. Final regression model for prediction of sPTB before 37 and 34 weeks and cervical shortening <25 mm prior to 24 weeks of gestation in women at high-risk of sPTB

	Multivariate analysis			
	Prediction of sPTB <37 weeks of gestation			
Independent variables	**Coefficient**	**OR**	**95% CI**	**P value**
qfFN* (first between 18 to 24 weeks of gestation)	**1.83**	**1.43**	**1.19-1.72**	**<0.001**
History of recurrent UTI's	**0.36**	**6.24**	**1.84-21.12**	**<0.001**
Constant	**-2.33**	**-**	**-**	**-**
	Prediction of sPTB <34 weeks of gestation			
qfFN* (first between 18 to 24 weeks of gestation)	**0.50**	**1.64**	**1.29-2.08**	**<0.001**
Constant	**-3.58**	**-**	**-**	**-**
	Prediction of cervical shortening			
qfFN* (first between 18 to 24 weeks of gestation)	**0.32**	**1.38**	**1.15-1.64**	**<0.001**
History of previous late miscarriage	**1.10**	**3.00**	**1.64-5.47**	**<0.001**
Constant	**-2.52**	**-**	**-**	**-**

*UTI=Urinary tract infection; qfFN= quantitative fetal fibronectin; OR=Odds Ratio; CI=Confidence interval; sPTB=spontaneous preterm birth. Statistically significant risk differences/ratios are shown in bold. *natural log of qfFN used for modelling purposes.*

Given the notably different expression of HDPs according to ethnicity, analysis was repeated in high-risk White and Black women, to evaluate whether a predictive model may vary in these two distinct groups. In White high-risk women (n=106), for prediction of sPTB <37 weeks of gestation, cathelicidin between 10 and 15^{+6} weeks of gestation, qfFN between 18 and 24 weeks of gestation and past obstetric history were identified as significant univariate predictors. When stepwise logistic multifactor regression was performed, as with the whole cohort, only recurrent UTI and qfFN concentration remained significant predictors. When these were included in a multi-factor logistic regression model (Table 8-14) in all women who had these tests performed (n=142), recurrent UTI no longer reached statistical significance (p=0.07), and the AUC for prediction of sPTB was 0.66 (95% CI 0.55 to 0.77). For prediction of sPTB <34 weeks of gestation, only recurrent UTI and qfFN concentration were significant multivariate predictors in the 105 women with all biomarker and data available (Table 8-14). This equated to AUC of 0.77 (0.62 to 0.92) in all women who had data for these two parameters (n=145). For prediction of cervical shortening, only a history of a late miscarriage remained a significant predictor in White high-risk women (n=106) (Table 8-14), equating to AUC of 0.59 (0.46 to 0.72), not reaching statistical significance in the 241 who had cervical length measurements.

Table 8-14. Final regression model for prediction of sPTB before 37 and 34 weeks of gestation and cervical shortening <25 mm prior to 24 weeks of gestation in White high-risk women

	Prediction of sPTB <37 weeks of gestation			
		Multivariate analysis		
Independent variables	**Coefficient**	**OR**	**95% CI**	**P value**
qfFN* (first between 18 to 24 weeks of gestation)	**0.30**	**1.35**	**1.01-1.80**	**0.04**
History of recurrent UTI's	1.39	4.00	0.92-17.45	0.07
Constant	**-2.06**	**-**	**-2.82- -1.30**	**<0.001**
	Prediction of sPTB <34 weeks of gestation			
qfFN* (first between 18 to 24 weeks of gestation)	**0.66**	**1.94**	**1.23-3.08**	**0.005**
History of recurrent UTI's	**2.33**	**10.29**	**1.73-61.23**	**0.01**
Constant	**-3.98**	**-**	**-**	**-**
	Prediction of cervical shortening			
History of previous late miscarriage	**1.20**	**3.33**	**1.18-9.44**	**0.02**
Constant	**-2.08**	**-**	**-**	**-**

UTI=urinary tract infection, qfFN=quantitative fetal fibronectin, OR=odds ratio, CI=confidence interval, sPTB=spontaneous preterm birth. Statistically significant risk differences/ratios are shown in bold.

**natural log of qfFN used for modelling purposes.*

For high-risk Black women analysis was limited by small numbers. For prediction of sPTB <37 weeks of gestation, chronic viral infection, recurrent UTI, smoking status and worse risk factor were identified as significant univariate predictors. Stepwise multivariate logistic regression (n=93) revealed only qfFN and worst risk factor (history of late miscarriage) as statistically significant independent predictors of outcome (Table 8-15), with an AUC ROC (n=93) of 0.75 (0.65 to 0.85).

For prediction of sPTB <34 weeks, only qfFN concentration was independently associated with outcome (Table 8-15), with AUC ROC of 0.73 (0.58 to 0.88, n=96). This was also the case for prediction of cervical shortening (Table 8-15), though did not quite reach statistical significance on the whole cohort (p=0.06), though predicted cervical shortening with AUC ROC (n=96) of 0.73 (0.58 to 0.88).

Table 8-15. Final regression model for prediction of sPTB before 37 and 34 weeks and cervical shortening <25 mm prior to 24 weeks of gestation in Black high-risk women

	Multivariate analysis			
	Prediction of sPTB <37 weeks of gestation			
Independent variables	**Coefficient**	**OR**	**95% CI**	**P value**
qfFN* (first between 18 to 24 weeks of gestation)	**0.37**	**1.45**	**1.06-1.98**	**0.02**
History of previous late miscarriage	**1.41**	**4.08**	**1.04-15.95**	**0.04**
Constant	**-2.56**	**-**	**-**	**-**
	Prediction of sPTB <34 weeks of gestation			
qfFN* (first between 18 to 24 weeks of gestation)	**0.43**	**1.54**	**1.06-2.24**	**0.02**
Constant	**-3.47**	**-**	**-**	**-**
	Prediction of cervical shortening			
qfFN* (first between 18 to 24 weeks of gestation)	**0.23**	**1.26**	**0.99-1.60**	**0.06**
Constant	**-1.19**	**-**	**-**	**-**

qfFN= quantitative fetal fibronectin, OR=Odds Ratio, CI=Confidence interval, sPTB=spontaneous preterm birth
Statistically significant risk differences/ratios are shown in bold
**natural log of qfFN used for modelling purposes.*

8.2.2.2 *Model 2: incorporating all socio-demographic and biomarker data available by 16 weeks of gestation*

The above results suggest that the addition of CVF HDP concentrations to existing biomarker tests performed at a later gestation do not add predictive value for sPTB nor cervical shortening. However, we have seen in earlier chapters that HDPs may have some value used alone as biomarkers for prediction of cervical shortening and sPTB and can be performed earlier in pregnancy than qfFN (and before most cases of cervical shortening have been detected by ultrasound). For this reason, a predictive model using earlier performed tests could potentially have value; arguably if risk of sPTB can be detected earlier in gestation, then monitoring and/or preventative intervention could be initiated at an earlier gestation, which may improve success. The following models evaluate the combined predictive value of HDP biomarkers (elafin, cathelicidin, HNE and CE ratio) taken prior to 16 weeks of gestation plus

sociodemographic information (as per methods section Chapter 3), for prediction of sPTB and cervical shortening.

A total of 269 high-risk women had biomarker data available for all selected parameters (qfFN, elafin, cathelicidin and HNE), as well as socioeconomic information and pregnancy outcome. For prediction of sPTB before 37 weeks of gestation, CVF cathelicidin, CE ratio, worst risk factor, recurrent UTI and ethnicity were significant predictors of sPTB in the single predictor analysis (at $p<0.05$). After stepwise logistic regression modelling, CVF cathelicidin concentration, recurrent UTIs and history of previous late miscarriage remained significant predictors. Using these predictors, logistic regression on the whole high-risk cohort (n=300) produced the final predictive model (Table 8-16), which predicted sPTB <37 weeks with AUC ROC of 0.70 (95% CI 0.63 to 0.78).

For prediction of sPTB <34 weeks, only a history of late miscarriage and ethnicity were significant univariate predictors, and only late miscarriage remained after stepwise multivariate regression (Table 8-16). When applied to the whole cohort (n=394) this predicted sPTB<34 weeks with AUC ROC of 0.65 (0.57 to 0.73). For prediction of cervical shortening, CVF HNE concentration, BMI, smoking, history of a late miscarriage and Black ethnicity were independent univariate predictors, and after stepwise regression modelling, only CVF HNE concentration, history of a late miscarriage and Black ethnicity remained independent predictors of outcome (n=280). However, when this model was applied to the whole cohort (with full data on these predictors, n=329), the contribution of CVF HNE to this model did not reach statistical significance. Table 8-16 shows the final regression model for prediction of cervical shortening (n=388), using a history of a previous miscarriage, and Black ethnicity, which has an AUC ROC for prediction of 0.70 (0.64 to 0.77).

Table 8-16. Final regression model for prediction of sPTB before 37 and 34 weeks and cervical shortening <25 mm prior to 24 weeks of gestation in high-risk women, using socio-demographic and biochemical data available prior to 16 weeks of gestation.

	Multivariate analysis			
	Prediction of sPTB <37 weeks of gestation			
Independent variables	**Coefficient**	**OR**	**95% CI**	**P value**
CVF cathelicidin concentration 10 to 15^{+6} weeks gestation	**0.29**	**1.34**	**1.05-1.71**	**0.02**
History of recurrent UTI	**1.22**	**3.38**	**1.35-8.46**	**0.009**
History of previous late miscarriage	**0.93**	**2.53**	**1.40-4.56**	**0.002**
Constant	**-1.83**	**-**	**-**	**-**
	Prediction of sPTB <34 weeks of gestation			
History of previous late miscarriage	**1.22**	**3.37**	**1.72-6.60**	**<0.001**
Constant	**-2.73**	**-**	**-**	**-**
	Prediction of cervical shortening			
History of previous late miscarriage	**0.88**	**2.40**	**1.36-4.27**	**0.003**
Black ethnicity	**1.00**	**2.71**	**1.53-4.80**	**0.001**
Constant	**-2.26**	**-**	**-**	**-**

OR=Odds Ratio, CI=confidence interval, UTI= urinary tract infection, CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth. Statistically significant risk differences/ratios are shown in bold.

This analysis was repeated for Black and White women. For prediction of sPTB <37 weeks of gestation, 158 high-risk White women had biomarker data available for all selected parameters. Univariate analysis revealed only CVF cathelicidin concentration, a history of previous sPTB, and a history of late miscarriage to be significant risk factors, and these persisted to be independently associated with outcome after stepwise regression analysis, as shown in Table 8-17 (n=176 White women with data on both parameters). They predicted sPTB with AUC ROC of 0.70 (0.59 to 0.81). For prediction of sPTB <34 weeks of gestation, 155 women had a full set of data available for stepwise regression, which revealed only a history of UTI to be a significant predictor of sPTB in this analysis. When applied to the 241 high-risk White women with this information available, the model is described in Table 8-17,

and the AUC ROC for prediction (0.61, 95% CI 0.48 to 0.73) was not statistically significant. For prediction of cervical shortening, 155 White women had all data parameters available, and only a history of previous late miscarriage emerged as a significant predictor during stepwise logistic regression. When the model was applied to the 232 women with this data available (Table 8-17), the AUC ROC for prediction was 0.64 (0.53 to 0.74).

Table 8-17. Final regression model for prediction of sPTB before 37 and 34 weeks and cervical shortening <25 mm prior to 24 weeks of gestation in high-risk White women, using socio-demographic data and biochemical data available prior to 16 weeks of gestation

Independent variables	Multivariate analysis			
	Prediction of sPTB <37 weeks of gestation			
	Coefficient	**OR**	**95% CI**	**P value**
CVF cathelicidin concentration 10 to 15^{+6} weeks gestation	**0.36**	**1.43**	**1.01-2.02**	**0.04**
History of previous sPTB or PPROM	**1.25**	**3.49**	**1.16-10.45**	**0.03**
History of late miscarriage	**1.28**	**3.60**	**1.10-11.81**	**0.03**
Constant	**-2.45**	**-**	**-**	**-**
	Prediction of sPTB <34 weeks of gestation			
History of recurrent UTI	**1.60**	**4.94**	**1.40-17.43**	**0.01**
Constant	**0.05**	**-**	**-**	**-**
	Prediction of cervical shortening			
History of previous late miscarriage	**1.35**	**3.86**	**1.62**	**9.21**
Constant	**-2.50**	**-**	**-**	**-**

OR=Odds Ratio, CI=confidence interval, UTI= urinary tract infection, CVF=cervicovaginal fluid, sPTB=spontaneous preterm birth, PPROM =premature prelabour rupture of membranes. Statistically significant risk differences/ratios are shown in bold.

For prediction of sPTB <37 weeks in Black women, 102 women had all data parameters available. Univariate analysis (n=72) revealed only a history of a previous late miscarriage as a significant predictor for this small cohort of high-risk women; when applied to the whole available cohort of 107 women, the predictive model is

shown in Table 8-18, and predictive ability of using only this risk factor was mild (AUC ROC 0.63, 0.54 to 0.73). The Black high-risk cohort event rate (n=17 out of 78 women who had data for all required parameters) was too small to create a logistic regression model for prediction of sPTB <34 weeks; no predictive component was identified as statistically valid. For prediction of cervical shortening, the CE ratio was the only predictor identified in the univariate analysis (n=85). When applied to the high-risk cohort (n=93) the model is shown in Table 8-18, and the ROC AUC for prediction was moderate (0.61, 0.50 to 0.73).

Table 8-18. Final regression model for prediction of sPTB before 37 and 34 weeks and cervical shortening <25 mm prior to 24 weeks of gestation in high-risk Black women, using socio-demographic data and biochemical data available prior to 16 weeks of gestation

	Multivariate analysis			
	Prediction of sPTB <37 weeks of gestation			
Independent variables	**Coefficient**	**OR**	**95% CI**	**P value**
History of late miscarriage	**1.34**	**3.83**	**1.20-12.19**	**0.02**
Constant	**0.11**	**-**	**-**	**-**
	Prediction of cervical shortening			
CVF C:E ratio 10 to 15^{+6} weeks gestation	**0.25**	**1.29**	**1.01- 1.64**	**0.04**
Constant	**4.07-**	**-**	**-**	**-**

OR=Odds Ratio, CI=confidence interval, CVF=cervicovaginal fluid, CE=cathelicidin to elafin. Statistically significant risk differences/ratios are shown in bold.

8.3 Discussion

In this chapter we have demonstrated that in asymptomatic high-risk women, CVF concentrations of cathelicidin and qfFN are positively correlated with each other when assessed at multiple gestational timepoints. In contrast, no correlation was observed between CVF HNE and qfFN concentration, and qfFN was negatively correlated with CVF elafin, but only when measured between 14-16 weeks of gestation. Furthermore, while knowledge of cathelicidin concentration did not add

predictive value to qfFN, in this cohort of women, it appears to be as good a predictor of sPTB <37 weeks as qfFN and can be measured earlier in gestation.

Secretion of fFN into the CVF is generally considered a downstream event, after an initial pathological insult leading to disruption of fetal membranes through mechanical, inflammatory and/or infective processes, potentially resulting in sPTB (Genc and Ford 2010); the more considerable this disruption, the greater the released fFN into the CVF, and higher the risk of premature delivery. As has been shown in other high-risk cohorts (Abbott et al. 2015; Tran et al. 2020), in this Insight cohort, higher concentrations of CVF qfFN in asymptomatic high-risk women, from 18 weeks of gestation were associated with increased risk of sPTB, particularly delivery <34 weeks of gestation, with greatest risk in women with CVF concentration over 200 ng/ml. However, the strong correlation between qfFN and cathelicidin, an inflammatory response protein does suggest that fFN may be related to the inflammatory cascade, possibly driven by inflammation and cytokine release, with concurrent cathelicidin release from neutrophils associated with the inflammatory response. Given that fFN is released from fetal membranes, it also suggests that the cervical inflammatory process is reflected in the fetal membranes. This is supported by other evidence. *In vitro*, Sibille et al. (1986) noted that fFN was released from the extracellular membrane of a fetal amniotic membrane model when exposed to activated neutrophils, likely due to elastase activity. Mogami et al. (2013) observed *in vitro* fFN was induced from amnion epithelial cells in culture by LPS and pro-inflammatory cytokines. They demonstrated fFN-induced matrix metalloproteinase (MMP) mRNA and enzyme activity (leading to membrane degradation), COX-2 mRNA synthesis and prostaglandin E2 synthesis (cervical ripening and uterine contractions) in mesenchymal cells, mediated by TLR-4. Furthermore, intermembrane inoculation of pregnant mice with fFN resulted in PTB, demonstrating both the response of fFN to simulated infection/inflammation, and its potential pathological consequence. *In vivo*, our research group found a strong relationship between placental inflammation (evaluated at post-mortem after sPTB) and earlier high fFN concentration vs. those

sPTBs with non-inflammatory placental pathology (van der Krogt et al. 2017). Whilst this may represent a fFN response to inflammation, it may also be that an inflammatory pathology causes greater disruption to the materno-fetal interface and release of fFN, compared to vascular or maternal pathology for example. As discussed in earlier chapters, *in vitro* cathelicidin has not been demonstrated to be regulated by cytokines (Chin-Smith et al. 2017). However, in our Insight pilot study we observed cathelicidin to be correlated with cytokines *in vivo* (e.g., a doubling of CVF IL1-β concentrations was associated with an 88% increase in cathelicidin) (Abbott et al. 2014). We suspect that *in vivo*, the contribution of cathelicidin release from neutrophils as part of the inflammatory response is responsible for the relationship between cytokines and cathelicidin, and that fFN is also driven by this inflammatory response. It would be interesting to evaluate whether cathelicidin could directly stimulate fFN from fetal membranes or vice versa, or whether the relationship is merely associative.

Given that qfFN is the leading clinically available predictor of sPTB, we therefore compared it to, and combined it with our experimental HDP biomarkers, to assess whether they could improve upon fFN as a clinical predictive tool. When fFN and earlier measured HDP concentrations were compared in this cohort of high-risk women, cathelicidin measured at 14-15^{+6} weeks was equivalent in predictive capacity for sPTB <37 weeks (AUC 0.67) as qfFN (taken 18 to 24^{+0} weeks of gestation) (AUC 0.69), though qfFN was a superior predictor of early sPTB <34 weeks. There also appeared to be some merit in combining the markers for prediction of sPTB <34 (AUC 0.75) and <37 (AUC 0.74) weeks of gestation, though in this small cohort, we were unable to demonstrate a statistically significant improvement. Given that cathelicidin is measurable and useful a number of weeks before qfFN can be relied upon, further investigation is warranted to evaluate whether this may be useful to guide earlier sPTB prophylactic intervention (which may be more successful than later initiation of therapy guided by CL as is current practice), or at least more intensive cervical surveillance for these women. If internally and externally validated, appropriate cut-

off values would need to be established to aid clinical utility, based on the sensitivity and specificity of the test at different thresholds.

Despite itself being a predictor of outcome (sPTB), cervical shortening <25 mm prior to 24 weeks of gestation was included as an outcome in this analysis. Even though not all women with a short cervix will deliver prematurely, a short cervix is currently used in clinical practice to identify which women may benefit from intervention (usually cerclage or progesterone) to prevent early delivery, and thus prediction of cervical shortening may have clinical utility, particularly if cervical scanning is not available. In this cohort, however, neither cathelicidin (14 to 15^{+6} weeks of gestation) nor elafin (16 to 19^{+6} weeks of gestation) added value to the use of qfFN to identify which women may have or develop a short cervix. Nor was qfFN an accurate predictor of cervical shortening itself (AUC 0.67). Only in White women, did the knowledge of an earlier CVF HNE concentration possibly improve detection of a short cervix (ROC 0.74 vs 0.61) but this was of borderline statistical significance in a small stratified sub-cohort (n=94). Given that the usual gestation of cervical shortening is around the same gestation that qfFN can be useful, this combined predictive test would only be useful to potentially guide intervention if cervical length ultrasound scanning was not available. However, it is still possible that the predictive ability of HNE alone to predict cervical shortening, performed at 14 to 15^{+6} weeks, particularly in White women (ROC 0.73) could be used to guide earlier intervention in women destined to develop cervical shortening; this would need validation followed by randomised evaluation.

When all available biochemical and sociodemographic data were analysed together using stepwise logistic regression to create a multi-data predictive model, we saw that qfFN remained the best predictor of sPTB <37 and <34 weeks of gestation in all women, superseding most other demographic risk-factors (including ethnicity). A history of recurrent UTI enhanced prediction of sPTB <37 weeks, and a history of late miscarriage was a significant additional predictor of a short cervix.

Assuming that earlier risk-stratification could increase the window of opportunity for surveillance and intervention, thus improving outcomes, this model was repeated only for data available prior to 16 weeks of gestation (excluding fetal fibronectin). Here, CVF cathelicidin (10 to 16 weeks of gestation), and history of UTI and late miscarriage were good predictors of sPTB <37 weeks (AUC ROC 0.70) but for prediction of earlier sPTB<34 weeks and cervical shortening, only sPTB risk factor (history of late miscarriage) and ethnicity had predictive value. For Black women only, however, CE ratio taken early in pregnancy was the optimal predictor for cervical shortening.

The limitations of building and relying upon stepwise regression models such as these, whereby after each variable is added to the model, all other candidate markers are assessed to see if their significance level falls below a predefined (but arbitrary) tolerance level ($p<0.05$), in which case they are thrown out of the model, must be acknowledged. Firstly, the model can only be created using women with all data points available. The nature of the Insight study (opportunistic testing from multiple sites some with limited capacity to process certain biomarkers, and funding restrictions allowing certain HDPs (e.g., cathelicidin) to be measured only in a sub-group of high-risk women) meant that numbers were restricted, reducing statistical power and increasing the chance of 'throwing out' important data. To maximise the power of the model, we applied the final selected variables to the whole cohort with data points relating to those variables but risked losing significant predictors during the process. When similar models were evaluated using different model selection algorithms (e.g., forward vs. backward regression), inconsistencies in predictor selection illustrated the poor reliability of such models created using a limited dataset. Yet the models are useful as an adjunct to the previous work that has been done on the whole cohort of women; to identify potential clinically useful predictors of sPTB that may subsequently require full internal and external validation in more appropriately powered and ethnically stratified datasets and assess whether they could add value to currently used clinical risk predictive models.

As described previously, the commonly used predictive algorithm, Quipp, currently exists to assess an individual's risk of sPTB, incorporating a woman's obstetric history, symptoms, qfFN concentration and/or cervical length measurement to assess risk of early delivery (Watson et al. 2019). It would now be interesting to assess whether addition of CVF HDP concentration (particularly early pregnancy cathelicidin) could improve the predictive algorithm. Furthermore, the role of a woman's history of recurrent UTI's needs to be more closely evaluated, as this is often overlooked. Whilst this was a self-reported measure (and may reflect an element of recall or treatment bias), it certainly requires consideration for inclusion. In addition, the concept of 'worst risk factor' is novel to this analysis and could be considered for use in existing models.

8.4 Conclusions

The correlation of qfFN with CVF cathelicidin suggests that fFN may be also driven by inflammation and cytokine release. CVF cathelicidin concentration measured in early pregnancy (14 to 16 weeks of gestation) has potential to be a clinically useful predictor of sPTB, with ROC curves obtained similar to those achieved by qfFN, the most accurate currently used biomarker predictor of sPTB for asymptomatic high-risk women. Use of this biomarker, once validated in a larger dataset, with identification of clinically appropriate thresholds, could enable earlier surveillance or intervention for high-risk women.

General discussion and conclusions

This PhD thesis has addressed the aims set out in Chapter 2; to describe the longitudinal expression of elafin, cathelicidin and associated protein HNE in pregnancy, as well as investigating the influence of host factors (socio-demographics and the vaginal environment) in their expression and pregnancy outcome, and finally their potential utility as early-pregnancy predictors of sPTB. In this final chapter, the outcome of this investigation performed in a prospectively collected cohort of 619 women, is summarised and discussed, both in terms of pathophysiological insight, and potential clinical implications.

8.5 Summary of key findings

8.5.1 Host defence peptides have distinct patterns of expression through pregnancy

Chapter 5 described the longitudinal expression elafin, cathelicidin, and related enzyme HNE through the mid-trimester pregnancy (10 to 24 weeks of gestation). Whilst CVF elafin steadily declined in pregnancy, cathelicidin and HNE remained stable. It is possible that the reduction in elafin (but not cathelicidin nor HNE) across gestation is related to the rise in serum oestradiol as pregnancy advances (Patel et al. 2013; Ghosh et al. 2010). Oestradiol could have a direct effect on epithelial cells via oestrogen receptors (ERα/β) (Ghosh et al. 2010) and/or specific oestrogen response elements in the gene encoding elafin, or indirectly via oestrogen reactive pattern recognition receptors (Aflatoonian et al. 2007; Hirata et al. 2007) and/or cytokine expression (Arici et al. 1999). It may also be related to a shifting vaginal microbiome across gestation, which is also under hormonal influence (Brotman, Shardell, et al. 2014). The reported observation that neutrophils in the lower reproductive tract do not appear to fluctuate with hormone concentrations (Pudney, Quayle, and Anderson 2005) (King and Critchley 2010; Yeaman et al. 2001; Evans and Salamonsen

2012) may explain why a similar trend in cathelicidin and HNE (predominantly released by neutrophils) was not observed.

8.5.2 CVF elafin, but not cathelicidin nor HNE, is associated with host BMI and smoking status

Unlike cathelicidin, CVF concentrations of elafin were higher in individuals who smoked, and also those with higher BMI. Just as elafin is increased in circumstances of infective inflammation, we suspect that it also responds to an obesity or smoking-related sterile inflammatory environment. Figure 8-1 illustrates the mechanistic pathways of non-sterile (LPS) and sterile (cigarette-smoke extract) inflammation proposed by Behnia, Sheller, and Menon (2016) and how this may lead to PPROM and/or sPTB. Whilst infection and generation of reactive oxygen species initiate inflammation largely via the NF-kB pathway without causing DNA damage, exposure to chronic sterile inflammatory factors such as obesity and cigarette smoke is thought to lead to oxidative stress-induced DNA damage, premature cellular aging and cell death (Behnia, Sheller, and Menon 2016; Bredeson et al. 2014).

The differences in the response of the HDPs to conditions associated with sterile inflammatory environment is likely reflect their different origins, functional roles and their respective regulatory mechanisms.

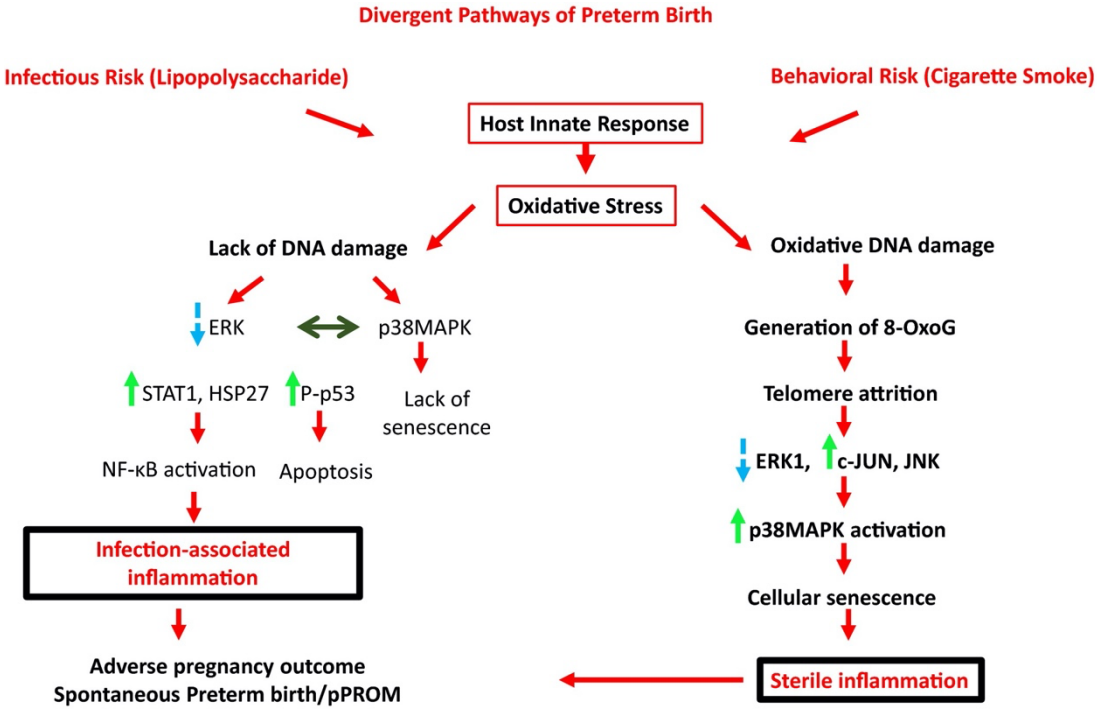

Figure 0-1. Two different mechanistic pathways of inflammation proposed to lead to spontaneous preterm birth and preterm prelabour rupture of membranes (pPROM); infection-associated inflammation and sterile inflammation (Behnia, Sheller, and Menon 2016).

Cathelicidin releasing neutrophils are key inflammatory cells in the pathways of both sterile and non-sterile inflammation, particularly recruited by IL-1α, a major DAMP in the initiation of both sterile inflammation (Chen and Nuñez 2010) and pathogen-associated inflammation (Di Paolo and Shayakhmetov 2016). However, it is possible that the magnitude of sterile inflammation (compared to pathogen-initiated inflammation) is insufficient to activate neutrophils (a multi-step process required for cathelicidin release) and/or the chronic nature of the sterile inflammatory stimuli vs. the acute infective insult fails to activate neutrophils sufficiently for cathelicidin release. We also hypothesised in Chapter 5 that the reliance on NF-kB (predominant in the infective but not sterile inflammatory pathway) for cathelicidin expression (Li et al. 2009) but not elafin, may restrict the cathelicidin (and thus HNE) response to sterile inflammation.

Obesity and smoking may also affect concentrations of HDPs via effects on the vaginal microbiome; both obesity and smoking have been shown in to be associated with increased diversity and reduced *Lactobacillus* predominance (particularly in Black women), with BV-associated taxa more common in overweight and obese women (Allen et al. 2021; Nelson et al. 2018). In the case of elafin, this is contradictory to our findings (see section 8.5.4) that a 'healthy' *L. crispatus* dominant vaginal microbiome is associated with high elafin. Multiple and complex mechanisms to drive elafin production must therefore be at play, particularly the role of inflammation as described above, as well as potential effects of bacterial load or other drivers, such as viruses.

8.5.3 Host defence peptides concentrations are ethnicity dependent

Both elafin and cathelicidin were expressed in higher concentration in Black women compared to White women. Large population-based studies have shown that there are distinct ethnic differences in sPTB phenotypes, as well as ethnic differences in CVF biomarker expression in relation to sPTB (Menon et al. 2014; Elovitz et al. 2019), and thus it was no surprise to see ethnic disparity in expression of elafin and cathelicidin. However, this is the first report to describe ethnic differences in the constitutive expression of these components of the innate immune system.

The reasons for the disparity are likely to be complex and could be related to a variety of factors including BMI (although associations persisted after adjusting for potential confounders), genetics, the vaginal microbiome, and/or socio-economic and cultural factors. I have begun preliminary work exploring potential genetic variation in elafin concentration (Appendix 4), using extracted DNA from serum blood samples taken from recruits to this Insight study. After identifying from the literature 4 known polymorphisms of elafin (rs17333103, rs1983649, rs6032040 and rs2664581), I investigated the relationship between the polymorphisms and CVF elafin concentration at each of the gestational categories using linear regression. I found that the variant allele (C) of rs2664581 SNP on the short arm of chromosome 20, was

associated with increased expression of elafin in the CVF, a similar finding to Tejera et al., (2014) who investigated secretion of elafin in the respiratory tract and association with genetic variants (see Section 1.6.2). However, given that the frequency of the C allele in an African population is 0.1 vs 0.9 for the A allele is slightly lower than the frequency in a European population (0.2 vs 0.8, https://www.ncbi.nlm.nih.gov/snp/rs2664581#frequency_tab), this observation is unlikely to explain the ethnic disparity in elafin concentration reported here. Furthermore, no association was seen between any of the SNPS and sPTB either in the whole cohort, or when stratified by ethnicity. However, this was only an exploratory analysis of a small ethnically mixed cohort, whereas robust genetic studies of this nature require large ethnically stratified databases.

Given the known ethnic differences in the vaginal microbial environment, we further hypothesised that this ethnic disparity in HDP concentration could be driven by the vaginal microbiome. Although full investigation of this hypothesis required correlation with sequencing of the vaginal microbiota using 16S RNA sequencing (discussed in 8.5.4; published [Flaviani et al. 2021] and ongoing research by our group using biological samples from this cohort), I also explored this in Chapter 7, focusing on the relationship between BV detection (dysbiosis of the vaginal environment known to be more common in Black women and associated with sPTB) and HDPs, as discussed below.

8.5.4 Concentration of HDP in CVF is associated with host BV status

CVF cathelicidin concentrations were slightly higher in women with BV, an observation that persisted even after adjustment for ethnicity and other confounders. This observation is consistent with other studies (Frew et al. 2014) and strengthens the inference that a dysbiotic vaginal microbiome influences release of this particular HDP. CVF elafin concentrations were, in contrast, lower in women with BV, an observation shared with other research groups e.g. (Stock et al. 2009; Jespers et al. 2017). BV cannot therefore explain the observed relationship between higher -

elafin concentrations and Black ethnicity (Chapter 5), suggesting that other drivers of sterile inflammation may be more important in this context.

The association between BV or vaginal dysbiosis and the vaginal innate immunity profile (HDPs) has been reported previously (Fichorova et al. 2020; Jespers et al. 2017; Stock et al. 2009; Wira et al. 2011; Yarbrough, Winkle, and Herbst-Kralovetz 2014). What remains unanswered, however, is by what mechanism does BV-induced disturbance to the vaginal milieu (metabolite balance, pH, inflammation etc.) affect expression of HDPs in pregnancy? As described in Chapter 7, low CVF elafin in BV infection may be related to inhibition of cytokine and HDP production by factors secreted by BV microbial flora (Valore, Wiley, and Ganz 2006) and/or be related to direct suppression of elafin by micro-organisms. These are likely to be bacteria specific. A number of microorganisms have been shown to downregulate HDP expression. After co-culturing a colon-derived epithelial cell line with the labile toxin of enterotoxigenic *E. coli* Chakraborty et al. (2008) demonstrated suppression of host cell expression of HBD and cathelicidin. *N. gonnorhoea* downregulates the expression of cathelicidin gene and protein expression (Bergman et al. 2005) from cervical epithelial cell lines. *L. jensenii* (abundant in women without BV) can induce HBD-2 from vaginal epithelial cell cultures (Valore et al. 2006) and cultured BV related bacteria *A. vaginae and P. bivia* can upregulate HBD2 but not SLPI (elafin and cathelicidin expression not assessed in this 3D vaginal epithelial cell model) (Doerflinger, Throop, and Herbst-Kralovetz 2014). Similarly, HBD2 can be upregulated in epithelial cells in response to BV associated bacterial inoculation (*G. vaginalis*, and *A. vaginae*) but not in response to other commensal *Lactobacillus spp (*Eade et al. 2012). Therefore, the question whether BV associated bacteria (or their metabolites) can downregulate elafin warrants investigation.

The inflammatory effects of BV may also affect HDP expression; whilst women with BV have been shown to have higher concentrations of pro-inflammatory cytokines IL-1B, IL-6, TNF-α (Hedges et al. 2006; Cauci and Culhane 2007; Cauci et al. 2002; Valore,

Wiley, and Ganz 2006; Eade et al. 2012; Jespers et al. 2017), Valore et al. (2006) observed lower concentration of IL-8 (a cytokine induced by HNE and chemotactic for neutrophils) in the CVF lavage samples from women with BV, and speculate that microbes such as *G. vaginalis* may actively supress inflammation, which could lead to low elafin. Puzzlingly, the neutrophil-specific cathelicidin is not suppressed by BV despite suppression of the chemotactic IL-8. Nonetheless, it is still plausible that the association of BV and low elafin and high cathelicidin could depend on the complex vaginal cytokine milieu.

Indeed, such alterations in the vaginal inflammatory and immune environment are thought to at least partly explain why BV is associated with increased risk of HIV acquisition (Royce et al. 1999; Cohen et al. 1995; Sewankambo et al. 1997), as well as other STDs such as *N. gonorrhoea, C. trachomatis*, *Trichomonas vaginalis* and Herpes simplex, even after controlling for sexual behaviour and other potential behavioural or lifestyle confounders (Coudray and Madhivanan 2020; Thurman and Doncel 2011). It is thought that alterations in the inflammatory vaginal environment resulting from BV create a permissive environment for infections such as HIV, by disrupting and weakening the epithelial cell barrier, activating promoter regions of HIV via NF-kB (Osborn, Kunkel, and Nabel 1989) and potentially by modulating the immune response (reduced CVF concentration of innate immune components such as elafin and SLPI). Correspondingly Ghosh et al. (2010) observed reduced CVF elafin in HIV positive vs. HIV negative women, consistent with observations that elafin has anti-HIV activity (Drannik et al. 2012). This reduction in elafin associated with BV could underlie the increased susceptibility to HIV, though it is possible that the viral infection could directly supress elafin. This local inflammation and permissive route for infection may also underlie the relationship between BV infection and increased risk of sPTB.

Closer interrogation of the microbiome is clearly required to further explore these associations. This work (to which I am contributing) is ongoing using a sub-group of

the Insight study recruits. By way of an example, Figure 8.2 illustrates the relationship between elafin, cathelicidin and microbiota communities based on PCoA groups in early pregnancy (10-15^{+6} weeks) in women of mixed ethnicity. CVF elafin concentrations were significantly increased in women with PCoA group A (dominated by *L. crispatus)* compared to B (*L. gasseri*) and E (predominance of both *L. crispatus* and *L. gasseri*), suggesting that elafin may be modulated by bacteria (or their metabolites), and/or that the reduction in elafin associated with BV may be due to a deficiency in 'healthy' lactobacillus (e.g., *L. crispatus*). Inverse patterns were observed for cathelicidin which was lower in the PCoA A group compared to C (*L. iners*) and D (a range of diverse bacteria). When this was evaluated using individual operational taxonomic units (OTUs) and metabolites, whilst elafin did not significantly correlate with specific OTUs, it showed inverse correlations with regard to metabolites most associated with OTUs linked to dysbiosis. Cathelicidin had negative correlation with *L. crispatus* (thought to be protective against sPTB), and positive correlation with metabolites associated with diverse bacteria (PCoA D) Ca^{2+}, formate, betaine, methionine and acetate. These relationships provide further evidence of the different regulatory mechanisms of various HDPs, in relation to the microbiota.

The majority of Black women in this study had vaginal microbiota characterised by PCoA groups C or D, compared with White women (PCoA). This supports our observation in the whole Insight cohort that White women had lower baseline CVF cathelicidin concentration than Black women. Moreover, OTU-1 (*L. crispatus*), which was associated with low cathelicidin, was protective against sPTB in this sub-study, more strongly for White women than Black women, which correlates with our findings (discussed below, Section 9.1.6). What is still unclear is why White women have lower elafin concentrations compared to Black women (given that PCoA A, most commonly seen in White women was associated with higher elafin in this sub-study), though in the microbiome study, these associations were not adjusted for confounders such as BMI or smoking which may influence the findings, nor was the

PCoA analysis sub-analysed by ethnicity specifically (largely due to limited sample sizes after ethnic stratification). However, the observation that few Black women who delivered prematurely had a microbiome characterised by *L. crispatus* (associated with high elafin) does fit with the hypothesis that reduced elafin may be associated with dysbiosis and sPTB (discussed below). Furthermore, the majority of Black women in the cohort had microbiota characterised by *L. iners*. Perhaps elafin concentration fell as their microbiome became more dysbiotic, and these were the women at higher risk of cervical shortening and sPTB. Clearly it doesn't explain all sPTBs; not all Black women with low elafin had cervical shortening or sPTB, and some Black women with *L. crispatus* dominance delivered prematurely. Further research is needed to elucidate other drivers beyond the microbiota which may cause ethnicity related differences in the innate immune system response.

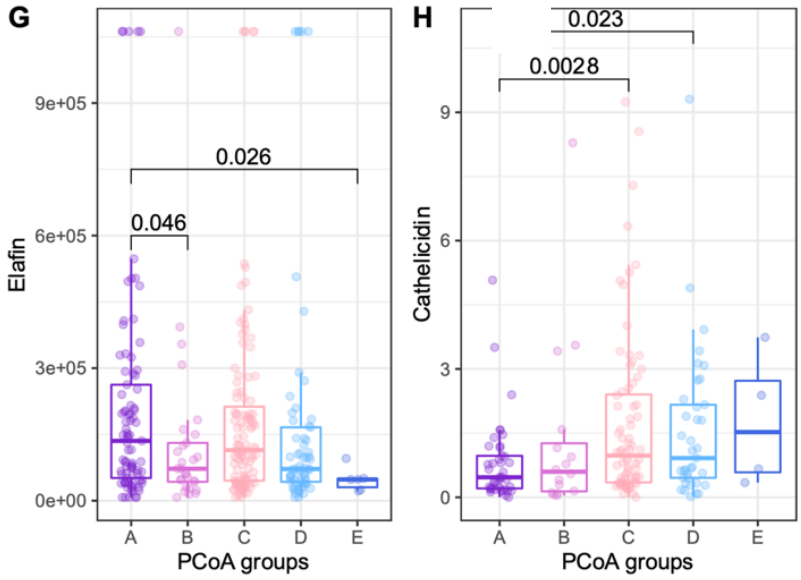

Figure 8.2. Relationship between host defence peptides elafin and cathelicidin and bacterial composition based on Principal Components Analyses (PCoA) groups in early pregnancy (10-15^{+6} weeks). Figure taken from Flaviani et al. 2021). Used elafin and cathelicidin data measured as part of this PhD.

8.5.5 CVF elafin concentration does not reliably predict sPTB in an ethnically mixed high (or low) risk cohort of women

Given the original pilot study results indicating that high elafin was associated with sPTB, and that Black women in both this cohort, and in larger cohorts, are known to have increased risk of sPTB, it seemed reasonable to hypothesise that this association could underlie the observed association between ethnicity and elafin concentration; i.e. that inflammation and infection related preterm birth may be more common in Black women (for a host of known, speculated and unknown reasons) and that elafin (and other HDPs) are elevated in response. This generated anticipation that CVF elafin concentrations could be translated into a clinically useful biomarker to identify those at most risk, and target surveillance and intervention to delay or prevent sPTB.

Yet, when we undertook a powered validation of the prediction of sPTB using elafin (as well as cathelicidin and HNE) as an early pregnancy predictor of cervical shortening and sPTB (Chapter 6) this was not found to be the case. Both in the whole cohort (some of whom received prophylactic intervention to delay or prevent sPTB prior to sample acquisition, unlike the pilot study population), and when stratified according to risk-status to more closely mimic the pilot study population, elafin did not prove to be a useful predictor of cervical shortening, sPTB nor adverse maternal or fetal outcomes. Once samples which were taken after intervention (cervical cerclage, vaginal progesterone or Arabin pessary) had been initiated, high elafin in early pregnancy was only modestly predictive of cervical shortening and sPTB prior to 34 weeks of gestation. While it was reasonable to exclude these samples (any vaginal intervention, foreign body or hormonal, may interfere with the local microbial or inflammatory environment potentially causing an intervention mediated biomarker shift), elafin was still not the ‘perfect’ early pregnancy predictor as expected following the Pilot study (AUC ROC 1.0 for prediction of cervical shortening).

Of interest, however, was the opposing direction of association between elafin and sPTB/cervical shortening in Black and White women. Black women who had low

elafin CVF concentration were more likely to develop a short cervix, whereas high elafin at early gestation was moderately predictive of cervical shortening in White women, with the relationship with pregnancy outcomes following a similar pattern. This serves as a reminder that evaluation of predictive tests may be more complex in an ethnically diverse cohort, and that ethnicity must always be considered.

Why the observed difference in host defence response and pregnancy outcome according to ethnicity? Whilst we hypothesised that this may be related to the vaginal microbial environment, in this thesis I was unable to provide evidence that dysbiosis (measured here as microscopic diagnosis of BV) was related to the ethnically different responses. Whilst BV was not associated with sPTB in this cohort, and thus did not modulate the relationship between HDP expression and sPTB (Chapter 7), the vaginal microbiome may still be involved in shifting the HDP profile and altering risk of sPTB. Ethnicity may also be a surrogate for other factors not considered here.

As described earlier (Section 8.5.4), in our parallel microbiome sub-study using this cohort of women we demonstrated elafin to be higher in women with a 'healthy' vaginal environment; PCoA group A (dominated by *L. crispatus),* compared to other PCoA groups associated with dysbiosis. Yet how this observation fits with the inverse relationship between elafin and sPTB in Black women, and the opposite for White women is unclear. We suggest that this may be both related to differing pathophysiology of sPTB in women of different ethnicity, and/or differing immune responses in Black and White women to microbial challenge and infection related preterm birth. The composition of microbial communities within any 'dysbiotic' community state may also differ between different groups of women.

Women with dysbiotic vaginal environments (and lower CVF concentrations of elafin) may be unable to mount an effective immune response against pathogenic insult or have specific bacteria existing withing their vaginal microenvironment which supress elafin, the result of which leads to cervical shortening possibly with microbial invasion

of the amniotic cavity and sPTB. Not all women with dysbiosis deliver prematurely, but the lower elafin concentration in those Black women (and also potentially some White women) who go on to deliver early may indicate compromised immune defences. White women, however, who are more likely to have 'healthy' lactobacillus-dominated vaginal environments, are able to mount an elafin mediated immune response to any emerging microbial insult. In these women, a high elafin in early pregnancy may represent a persistent infective or inflammatory insult, rendering them susceptible to subsequent late miscarriage and sPTB.

8.5.6 High CVF cathelicidin concentration was associated with sPTB

A more promising and consistent predictor of sPTB was revealed of cathelicidin, found in higher concentrations in the CVF of women who subsequently delivered spontaneously and prematurely. When measured at 14-15^{+6} weeks of gestation cathelicidin expression was 86% higher in high-risk women who delivered spontaneously and prematurely, with strengthened association seen when samples taken after intervention were excluded (AUC ROC for prediction of sPTB <37 weeks 0.75), as well as being moderately predictive for cervical shortening prior to 24 weeks of gestation. These associations were stronger in White women than Black women, though it is not clear whether this is due to smaller numbers in the Black women group, or whether, as hypothesised with elafin, the innate immune response to stimuli is fundamentally different between ethnicities.

It is likely that cathelicidin response is driven by infection and inflammation, induced by a range of inflammatory stimuli (microbial products and cytokines), and is higher in the presence of a dysbiotic environment (BV) as evidenced in Chapter 7. Indeed, in the parallel microbiome sub-study, we demonstrated a positive correlation between high cathelicidin and metabolites Ca^{2+}, formate, betaine, methionine and acetate, corresponding to PCoA group D (diverse bacteria), and a negative correlation with *L. crispatus*, a composition of a healthy vaginal environment (and associated with reduced risk of sPTB).

8.5.7 Host defence peptide concentrations add little value to clinically available biomarkers but may be useful for earlier prediction

As discussed above, CVF cathelicidin concentration measured in early pregnancy has potential to be a clinically useful predictor of sPTB, with ROC curves obtained similar to those achieved by qfFN, the most accurate clinically used biomarker predictor of sPTB for asymptomatic high-risk (whose use is restricted to later pregnancy). If found to be valuable, this may enable earlier surveillance or intervention for high-risk women, with the potential to improve outcome; this would need not only validation in a larger cohort which allows greater stratification by ethnicity, but also interrogation of this larger dataset to see whether clear cut-offs could be used to identify those at lower vs. higher risk (which did not emerge in this smaller cohort), and ideally a randomised controlled trial of early intervention to improve outcome based on biomarker results.

Yet when a multi-predictor model was created using all biomarker and demographic information, measurement of HDPs did not greatly improve on prediction of sPTB using qfFN (taken after 18 weeks of gestation). Model creation such as this relies on each participant to have a full set of data; thus we broadened the gestation in which cathelicidin (and other natural HDPs) were measured to generate adequate numbers for analysis. Given that we have previously demonstrated that cathelicidin (and other HDPs) only predict sPTB in specific narrow gestational windows, this may be why it was not carried forward into the main model. When the regression model was run using biochemical and sociodemographic data available prior to 16 weeks of gestation (thus excluding qfFN), CVF cathelicidin concentration improved prediction of sPTB <37 weeks amongst already high-risk women, but it did not add value to predict earlier sPTB <34 weeks; rather a history of having had a late miscarriage was the strongest predictor here.

8.5.8 Consideration of recurrent urinary tract infection, and re-thinking hierarchy of risk factors for high-risk women may improve existing sPTB risk stratification tools

The concept of 'worst risk factor' amongst already high-risk women emerged as a useful predictor of outcome in both Chapters 4 and 8. In many studies, previous late miscarriage and previous sPTB are frequently considered together as one risk-factor. That many 'high-risk' women have had both sPTB and late miscarriage (and may have other risk factors) is rarely disentangled. By first evaluating the relative contribution of risk factors to risk of sPTB in women with only one risk factor (after establishing that number of risk factors did not affect risk of sPTB) and establishing a risk-hierarchy (late miscarriage; sPTB; cervical surgery), we were able to improve risk stratification. Women with previous late miscarriage, regardless of other risk factors, were at higher risk of subsequent sPTB than their high-risk counterparts who had not. This was then applied to the whole cohort and included in predictive modelling (Chapter 8). It may be worth evaluating 'worst risk factor' for inclusion in models such as the widely used (UK) 'QUIPP' app (Kuhrt et al. 2016, updated by Watson et al. 2020), which originally excluded late miscarriage (in favour of previous sPTB) as a predictor.

The role of recurrent UTI's, which also consistently emerged as a risk predictor for sPTB, needs further investigation. Whilst this was a self-reported measure, and thus subject to recall or detection bias, there is good evidence that UTI, particularly if untreated and progressing to pyelonephritis (Kass 1960; Wing, Fassett, and Getahun 2014), is associated with sPTB (Sheiner, Mazor-Drey, and Levy 2009). This may be related to local or systemic inflammation, and/or the microbiome (*E. coli* UTI being more common in women with vaginal dysbiosis [Gupta et al. 1998]), with potential for *E. coli* to suppress elafin (Cole and Nizet 2016). UTIs may also be an indirect/surrogate result of generalised immune suppression in some women.

8.6 Strengths and limitations of the work

This study is a robust evaluation of elafin as a predictive biomarker for sPTB, in a large and powered prospectively collected cohort of women (requiring at least 80 sPTBs), with reliably collected, ethnically stratified demographic and outcome data, reflecting three years of consistent recruitment over four geographically distinct sites in the UK. Furthermore, it has provided insight into the pathophysiology behind infection and inflammation related PTB and revealed interesting and contrasting patterns in antimicrobial expression according to host factors, particularly ethnicity.

In the absence of genotyping, the ethnic stratification was limited by being a self-reported measure, and we cannot rule out that ethnicity may wholly or partly be a surrogate for other influencing variables such as the environment, diet and social stressors. Whilst we did partly control for socio-economic status using IMD score, data on other social, economic and cultural variables would have improved the study.

Elafin did not prove to be an accurate or clinically useful predictor of cervical shortening, sPTB or adverse maternal or fetal outcome. This may reflect Type-1 error within the pilot study, by virtue of small numbers (N=74), and/or methodology differences; although sample attainment, processing and ELISA protein measurement was consistent in the pilot and validation study, the wide range of elafin concentrations in CVF meant that samples required measuring at two dilutions. It was apparent only in the validation study that the sample buffer had a significant dilutional effect on matrix release of elafin i.e., over or underestimation of true protein content, requiring statistical correction (see Chapter 3).

By sub-analysing the data according to ethnicity, as well as removing samples taken after intervention, we revealed interesting relationships with birth outcome according to ethnicity; however considerable reduction in our sample size after stratification means that further validation (internal and external) is required,

particularly for patterns in the smaller cohort of Black women. Given the characteristic gestational profile of the biomarkers, and multiple visits of each participant, our power to detect significant differences/predictive capacity was further reduced by analysis according to gestational age at sampling in relatively narrow gestational sampling windows; not all women attended or consented to sampling at each visit, and different sites had differing capacity for sample processing; thus the number of samples obtained at each gestational window were lower than anticipated. Validation of the promising cathelicidin (and CE ratio) for prediction of cervical shortening and sPTB will also requires a larger number of women stratified by ethnicity; should our promising results be confirmed, the data requires interrogation to find clinically useful thresholds at which diagnostic test accuracy is optimal, which were not clear in this smaller cohort.

Although our primary clinical endpoint (sPTB <37 weeks) is one used commonly in prediction studies such as these, arguably the most clinically important outcome is fetal or maternal wellbeing (including long term neuro-developmental follow up beyond infancy). This is particularly the case in trials of treatment, where prolonging pregnancy duration may not be associated with improved clinical outcomes for mother or baby, particularly in the presence of infection. To power a study such as this, we would need greater numbers; in this cohort no consistent associations between HDP expression and maternal or fetal outcomes were seen.

8.7 Questions remaining

We originally hypothesised that the natural antimicrobial defences of cervicovaginal environment (contributing to an individual woman's sensitivity to ascending infection) would be modified by maternal characteristics and demographics, and we have indeed shown they have distinct gestational patterns of expression and can be affected by maternal demographics such as BMI and smoking status, as well as the vaginal environment (BV). We demonstrated that concentration of cathelicidin is

raised in some women who subsequently develop a short cervix and deliver prematurely, an observation which strengthens our hypothesis that a healthy vaginal environment and constitutive immune response (low level secretion of HDPS including elafin and cathelicidin) would defend the cervix and intrauterine environment from infection and inflammation, and an inflammatory environment may compromise cervicovaginal defence, leading to cervical shortening and (potentially predictable) sPTB in some women. In contrast, we also hypothesised that an individual's inability to mount an innate immune response (hypo-responsiveness) may also lead to adverse pregnancy outcome, and that this may relate to vaginal dysbiosis. This has been supported by a demonstrated reduction in elafin associated with BV, as well as sPTB in Black women. These observations do point to a role for immune hypo-responsiveness in the pathogenesis of some sPTB, which may be ethnically determined.

The Insight study has generated as many questions as have been addressed. Fortunately, in setting up the study, we now have a large biobank of biological samples (including cervicovaginal brushings, CVF blood plasma and extracted DNA) matched to high-quality data including sociodemographic information and pregnancy outcomes that can be used for future work and collaborations, ideally leading to clinical tool development. This amounts to stored longitudinal samples through pregnancy from over 2000 women, at high and low risk of sPTB. Furthermore, the parallel SuPPoRT study (trial of cerclage, progesterone, or Arabin Pessary) (Hezelgrave et al. 2016), which I designed and managed (wrote and published the protocol, obtained ethical approval, and set-up recruitment in over 10 sites across the UK) under the guidance of Principal Investigators Professor Shennan and Tribe is currently ongoing (currently n=385, target, 510).

From a pathophysiological perspective, we need to further understand the mechanism behind elafin and cathelicidin expression (and other HDPs) in normal

circumstances, and in settings of dysbiosis, infection and inflammation in the female reproductive tract.

1. *What is the relationship between dysbiosis and HDP expression and how are these affected by the vaginal environment?*

In vitro, endocervical cells or vaginal epithelial cells from pregnant women, with or without neutrophil co-culture as a potential source of cathelicidin, could be used to assess elafin or cathelicidin gene and protein expression in response to particular strains of bacteria associated with dysbiosis to further understand the relationship between bacterial colonisation and anti-microbial peptide production.

Use of siRNA knockdown of elafin or cathelicidin in vaginal epithelial cell lines, and subsequent microbial or inflammatory challenge would also be useful in elucidating the role of the HDPs under different conditions (for example altered pH). Next-generation 16S sequencing of the vaginal microbiome, NMR determination of the vaginal metabolome and paired CVF HDP concentrations will provide further insight into their inter-relationship, as well as measuring other candidate HDPs (e.g. SLPI, HBD2) and exploring additional markers of inflammation or dysbiosis e.g. cytokines profiles, vitamin D, sialidase (produced by anaerobic bacteria) and lactic acid.

High-performance mass spectrometry could be employed to determine the ratio of elafin and trappin-2 in CVF, the differentiation of which may improve upon the quantification of active molecule and improve the prediction of sPTB. Furthermore, creation of synthetic peptides corresponding to full length elafin or cathelicidin may allow further elucidation of their roles, particularly in challenging bacteria under different environmental circumstances (e.g., pH, vitamin D, LPS and cytokine challenges).

Whilst this thesis, and related microbiome work, has concentrated on the role of the vaginal bacterial environment, the contribution of viral infection to HDP expression and pregnancy outcome must also be considered. For example, we know that elafin can inhibit HIV replication and has anti-HIV activity (Ghosh et al. 2010). qPCR for a panel of viruses could be performed on remaining CVF samples and correlated with HDP profiles and pregnancy outcome. Viruses of interest include HPV, adenovirus, cytomegalovirus and enterovirus. 18S rRNA screen for fungal infections, which also affect the inflammatory vaginal environment could also be assessed. Care must be taken to stratify all analyses by ethnicity due to the stark ethnic differences in immune response highlighted by this study.

2. *Does sterile inflammation influence HDP concentration?*

Taking inspiration from Menon's work on sterile inflammation (Menon 2014), the effect of oxidative stress-induced cellular senescence on HDP expression by vaginal epithelial cells could be investigated. The influence of exposure of VECs to cigarette smoke extract on elafin and cathelicidin gene and protein expression (and measurement of generation of reactive oxygen species as a marker of oxidative stress) would further elucidate their differential response to sterile inflammation.

3. *Why is the HDP response different according to ethnicity?*

Genetic variants in elafin or cathelicidin genes may influence both constitutive CVF protein concentration and/or response to inflammation and infection. We have started to evaluate this from DNA extracted from this research cohort (See Appendix 4) however genetic studies of this nature require larger ethnically stratified GWAS to confidently evaluate meaningful associations with pregnancy outcome. Environmental exposure factors must also be considered, including ethnically stratified microbiome profiling (as described above) as well as more comprehensive inclusion of markers of stress (self-reported or objectively measured such as serum cortisol), diet, socioeconomic data and behavioural practices such as douching.

4. Is cathelicidin a clinically useful early predictor of sPTB?

We plan to perform a full powered validation (using full training and validation set) of CVF cathelicidin as an early pregnancy predictor of sPTB using additional recruits within the Insight cohort, as well as other populations of women, (for example the Precise cohort, https://precisenetwork.org) with early consideration given to ethnic stratification. The potential clinical utility of CE ratio, particularly in Black women must be assessed, to establish whether it may prove a clinically useful early pregnancy biomarker, and guide for intensive monitoring. A question of particular interest would be whether prediction is really confined to a narrow gestational window (or was this observation related to lack of power), and if so why?

The relationship between biomarker expression in women and success of randomised intervention in the SuPPoRT study (Hezelgrave et al. 2016), also needs exploring (the majority of recruits have stored biological samples available for analysis taken both before and after the intervention). Differential biomarker expression may provide insight into the pathophysiology of sPTB, which may predict which interventions to prevent sPTB are effective in prolonging gestation and which are not, a first step towards more personalised treatment for women with a short cervix at high-risk of sPTB. Using an ethnically-stratified multiple panel of biomarkers, measured on a multiplex/Luminex assay to optimise sample use, may be more appropriate to identify candidates for a predictive test. Combination with other candidate biomarkers (discovered via gene expression data and bioinformatics) recently identified such as human interleukin-1 receptor antagonist protein (IL-1RA), g-glutamyl hydrolase (GGH), extracellular matrix protein 1 (ECM1), vitamin D-binding protein (VDBP), metalloproteinase inhibitor 1 (TIMP-1), human laminin subunit gamma-2 (LAMC2) and pigment epithelium-derived factor (PEDF), which together had a combined AUC ROC for prediction of sPTB <37 week of 0.86, N=257) should be given consideration (Leow et al. 2020). This may also involve more deeply

phenotypically categorising sPTBs e.g., according to placental histology, clinical characteristics (such as pregnancy-related vaginal bleeding or PPROM) or fetal growth restriction, as well as maternal health factors and stratification by sociodemographics; the combination of phenotypic clusters (identified using large complex datasets) and bioinformatic data would be an innovative way to explore sPTB prediction (Souza et al. 2019).

8.8 Future directions

Could elafin or cathelicidin hold any therapeutic potential in preventing sPTB, as it is hoped in other inflammatory conditions such as ARDS? Adenoviral gene vectors containing the elafin gene have been used in experimental models for delivering human elafin to in vitro cell models, and murine disease models. In a cell model of ARDS, Simpson, Wallace et al. (2001) demonstrated that adenovirus encoding elafin cDNA protected epithelial cells derived from bronchioloalveolar cell carcinoma, against the actions of neutrophil elastase and whole human neutrophils *in vitro*, as well as protecting murine lungs against injury from *P. aeruginosa in vivo*. They also observed that pulmonary epithelial cells transfected with the recombinant elafin adenovirus significantly upregulated expression of elafin compared to controls in response to LPS (Simpson, Cunningham, et al. 2001). This could be replicated in vaginal cells or animal models of sPTB. Firstly, it would be interesting to explore whether elafin and cathelicidin expression affects bacterial ascent from vagina into uterine cavity, as has been seen with HBD3 mouse model. Suff et al. (2020) administered an intravaginal adeno-associated virus vector containing the HBD3 gene or control, and introducing ascending infection using bioluminescent *E. coli.* They observed a higher proportion of inflammatory ascent and mouse pups born prematurely to the *E.coli* infected mice compared to the control population, but reduced bacterial ascent and an increased proportion of living pups born to the HBD3 treated mice. It would be interesting to see whether similar response is seen using the elafin or cathelicidin gene. If so, the question of whether elafin or cathelicidin

could be used to augment reproductive tract innate immunity and protect against ascending infection must be considered, and whether the therapeutic provision of synthetic peptides could have a future role in prevention of sPTB.

8.9 Conclusions

Patterns of host response markers elafin, cathelicidin and HNE in pregnancy have provided insight into the cervicovaginal inflammatory environment during pregnancy and support the contribution of HDPs to the mechanisms resulting in some sPTB. Furthermore, whilst ethnic differences could be a surrogate reflecting differences in environmental and social variables, the marked racial disparity in the expression of these peptides must refute the universality of a sPTB pathway and potential for 'one size fits all' predictive tests, and indeed treatment to prevent sPTB. Thus, both in this thesis, and in the decades of PTB research preceding this, no single biomarker has proved effective to predict sPTB. Continued investigation into biological causes and plausibility, with united collaborative research effort to generate sufficient sample sizes to is required to move the field forward and develop more sophisticated prediction and prevention techniques than currently in clinical practice.

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Appendix 1. Patient information leaflet Insight Study

Guy's and St Thomas' NHS
NHS Foundation Trust

Biomarkers in Preterm Birth
Predictive Markers for Preterm Labour

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and family if you wish. Please ask if anything is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Background

Babies born very early are more likely to have health problems than babies born at the right time. Although many do survive, being born early may affect them throughout their life.

Doctors have done a lot of work to try and stop babies being born too soon, but they still cannot accurately predict which women will have their baby early. We carried out a small study to measure a biomarker of inflammation called 'elafin' in vaginal fluid. We found in women who had their babies early (less than 37 weeks) there was more elafin in the fluid than we would expect.

We would like to investigate this further to make sure this test works. We need to look at this substance in vaginal fluid in a much larger group of pregnant women. We also want to measure other natural substances in vaginal fluid, cells and blood, for example, markers of infection or inflammation, the presence of infection and

the mother's cell response to it. All will give important insight into prediction and mechanisms of preterm birth.

Why have I been chosen?

You have been asked to take part because you are pregnant and have either delivered early before or had an operation on your cervix (neck of the womb). This may mean that you are at higher than average risk of a preterm delivery.

What do I have to do if I take part?

You will be seen by a research midwife who will answer any questions you may have. Once you have agreed to take part you will be asked to sign a consent form and given a copy of this to keep. The midwife will ask you about your past medical & obstetric history and collect relevant information from your clinical record/notes.

We would like to collect vaginal swab samples via a speculum exam (as used for a smear test) and a blood test from your arm during pregnancy (early pregnancy between 10-24 weeks and later in pregnancy) and at the time of labour.

At recruitment, you will be asked to give have an initial blood sample and vaginal swabs (with up to 6 swabs taken at the same time). If you need a routine fetal fibronectin (FFN test and internal ultrasound scan to look at the length of the cervix, we will take the other swabs for research at the same time so you will not need to have another speculum examination.

If you are willing, and if you have further appointments to monitor your risk of early birth, you will be asked to provide other blood, and swab samples on each occasion (usually 2-4 visits). This may include an additional FFN swab and you will know the result within 30 minutes and which the clinical team will discuss this with you. Your usual antenatal care will continue, and you will be seen by your normal doctors and midwives. We would also like to take these samples later in the pregnancy after 24 weeks, as well as when you go into labour.

You will also be asked if you are willing to provide a hair sample from the head to see if we can determine stress levels in women who deliver early. You will also be asked if you agree for us to take samples when you come into hospital in labour,

as well as cord blood, placenta and neonatal blood samples when your baby is born to give us more information about labour and delivery.

We will use all of these research samples to measure infection, markers of infection and inflammation, and look at how your cells (blood or vaginal) respond to the pregnancy environment. We would look at how your genetic background may protect or predispose you to preterm birth by studying your DNA and RNA. All of these measurements may help us understand why some women have a preterm birth and to predict preterm birth in the future. Some analyses will be undertaken by KCL researchers, commercial partners and other academic collaborators.

Who will have access to my information?

King's College London and Guy's and St Thomas' Foundation trust co-sponsor this UK-based study. We will be using information from you and from your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally identifiable information possible. You can find out more about how we use your information by contacting us below.

For research purposes, we will record what happened to you and your baby through the hospital medical records and your hand-held notes. You will also be asked if you are willing for us to use your NHS number and your baby's, to link what we have discovered in our study to you and your child's (up to 16 years old) future health, social and educational data. Access to this information will only be held by specific study staff.

What will happen to my information?

King's College London/Guy's and St Thomas' Foundation trust will keep your name, date of birth and contact details confidential. King's College London/Guy's and St Thomas' Foundation trust will only use this information as needed, to contact you about the research study, and make sure that relevant information about the study

is recorded for your care, and to oversee the quality of the study. The people who analyse the information will not be able to identify you and will not be able to find out your name, date of birth or contact details. King's College London/Guy's and St Thomas' Foundation trust will keep identifiable information about you from this study for 25 years after the study has finished.

Do I have to take part?

Whether you decide to take part or not is entirely up to you. Your decision will not affect the care you receive in any way. If you agree to take part, you are free to withdraw at a later stage, without giving a reason, although you may be asked if you mind us collecting details about your delivery from your medical notes. Again, it is entirely up to you if you agree to this.

What are the benefits of taking part?

You may not benefit personally from taking part, but you may help us develop a screening test that helps women in the future. The extra information the doctors looking after you get from the FFN test may change your antenatal care but the results from our research will not affect your antenatal care.

What are the side-effects of taking part?

Some women can find high vaginal swabs uncomfortable. Taking part may increase the time you need to spend in the clinic. Blood samples will be taken that are not related to your routine care. They are associated with momentary discomfort and occasionally bruising. The dental examination may be associated with very mild discomfort.

What happens if anything goes wrong?

In the unlikely event that you are harmed by taking part in this study no special insurance applies. However, if you are harmed due to negligence normal NHS indemnity may apply, but you may have to pay for this action. Regardless of this, should you wish to complain about any aspect of the way you have been approached or treated during the course of this study the normal NHS complaints mechanism is available to you.

What drug is being tested?

There is no drug being tested.

What will happen to my sample?

Samples will be frozen and stored in freezers at St Thomas' Hospital, London. We will measure substances in the samples to pick up differences between women who have their babies early and women who do not. No one will be able to identify you from the samples.

Fetal fibronectin samples are analysed immediately and then discarded (they are not stored).

What will happen to the results of the study?

The results will be published in medical journals. You will not be identified in any report/publication.

Who is paying for this research?

Tommy's Charity, the National Institute for Health Research (NIHR) and the Rosetree Trust has funded this study.

Who has reviewed this study?

NRES Committee London-City & East reviewed and agreed this study.

What do I do if I have further questions or want to take part?

For further information please contact:

Research Midwives tel: 020 7188 3634

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (as above). If you remain unhappy and wish to complain formally, you can do this through the Guy's and St Thomas' Patients Advice and Liaison Service (PALS) on 020 7188 8801, pals@gstt.nhs.uk. The PALS team are based in the main entrance on the ground floor at St Thomas' Hospital and on the ground floor at Guy's Hospital in the Tower Wing.

In the event that something does go wrong and you are harmed during the research you may have grounds for legal action for compensation against Guy's and St Thomas' NHS Foundation Trust and/or King's College London but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

Thank you for taking time to read this leaflet and considering taking part in this study. You will be given a copy of this leaflet and signed consent form to keep if you choose to participate

Student Name: Natasha Lee Hezelgrave

Student Number: 0325246

Appendix 2: Consent form Insight study

Guy's and St Thomas' NHS
NHS Foundation Trust

Centre number:
Study number:

Consent Form

Title of Project: Insight: A study of biomarkers in women at risk of preterm labour

Name of Researchers: Professor A Shennan, Dr R Tribe & Dr N Hezelgrave

To be completed by the participant: If you agree to the following statements please confirm by initialling boxes below: eg SG

1. I confirm that I have read and understand the patient information sheet entitled "Participation Information Leaflet: Insight" Dated 14th June 2018 (Version 5.0) for the above study and have had the opportunity to ask questions. ☐

2. I understand my participation is voluntary and I am free to withdraw at any time, without giving any reason without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from Kings College London/St Thomas' Hospital, from regulatory authorities or from the NHS organisation, where it is relevant to my taking part in this research study. I give permission for these individuals to have access to my records. ☐

4. I agree to give (for analysis):
   - Blood ☐
   - Cervico-vaginal secretions ☐
   - Cervicovaginal brushings ☐
   - Hair clippings ☐

5. I agree to have a transvaginal ultrasound scan ☐

6. I agree to take part in the above study. ☐

7. I agree that my samples, taken as part of the above named study, can be used for genetic analysis ☐

8. I agree for my NHS hospital number to be used to link my current data with my future health, social and educational data ☐

9. I understand my samples and data may be analysed in other institutions/for future studies/ and/or
in commercial collaborations with appropriate research ethical approval ☐

Participant's signature:..... Date:.....

PRINT NAME:.....

Signature of person taking consent:..... Date:.....

PRINT NAME:.....

Witness (where appropriate)..... Date.....

PRINT NAME:.....

Appendix 3: Fetal fibronectin and sPTB

Rates of sPTB <37 and <34 weeks of gestation according to fFN category are shown in Table 1. The higher the quantitative fetal fibronectin, the greater the risk of spontaneous preterm birth, particularly at concentrations >10 ng/ml, and >200 ng/ml.

Table 1. Number and rate (%) of spontaneous preterm birth according to quantitative fetal fibronectin category

N= total in category, n (%)=total and % of sPTB births in category after exclusion of iatrogenic deliveries prior to

	N	sPTB <34 weeks n (%)	sPTB <37 weeks n (%)
Gestation 18-19^{+6} weeks			
fFN category			
< 10 ng/ml	102	5 (4.9)	14 (13.7)
10-49 ng/ml	40	7 (17.9)	13 (32.5)
50-200 ng/ml	21	3 (14.2)	8 (38.1)
>200 g/ml	10	5 (50.0)	5 (50.0)
Total	173	20 (11.6)	40 (23.1)
Gestation 20-24^{+0} weeks			
<10 ng/ml	148	6 (4.1)	20 (13.5)
10-49 ng/ml	56	4 (7.3)	11 (19.6)
50-200 ng/ml	21	2 (10.0)	4 (20.0)
>200 g/ml	10	5 (50.0)	5 (50.0)
Total	235	17 (7.3)	40 (17.1)

specific gestation of interest. sPTB; spontaneous preterm birth, fFN; fetal fibronectin

First test taken within gestational timeframe used for analysis purposes

Table 2 displays the ORs for sPTB according to qfFN category. As expected, as the fFN concentration rises, so too does the odds of sPTB. Again, this was particularly marked for CVF fFN concentrations >200 ng/ml (though the confidence interval is wide due to the small numbers of tests falling into this category)

Table 2. Odds ratio for spontaneous preterm birth at according to quantitative fetal fibronectin category

	Odds ratio* (95% Confidence Interval)	
qfFN category	**sPTB <34 weeks**	**sPTB <37 weeks**
Gestation 18-19^{+6} weeks		
10-49 ng/ml	**3.25 (1.02-10.29)**	**2.73 (1.15-6.47)**
50-200 ng/ml	2.15 (0.50-9.20)	**3.07 (1.08-8.78)**
>200 g/ml	**14.31 (3.37-60.64)**	**6.64 (1.63- 27.07)**
Gestation 20-24 weeks		
10-49 ng/ml	1.71 (0.47-6.30)	1.64 (0.74-3.63)
50-200 ng/ml	3.79 (0.87-16.42)	**3.68 (1.25-10.81)**
>200 g/ml	**13.71 (3.14-59.95)**	**8.06 (1.77-36.63)**

*N= total in category, n (%= total of sPTB births in category and % after exclusion of iatrogenic deliveries prior to specific gestation. sPTB; spontaneous preterm birth, qfFN; quantitative fetal fibronectin. *relative to fFN category 0-9 ng/ml. Significant results in bold*

Table 3 displays the prediction analysis (AUC ROC) for sPTB <37 weeks using qfFN at the two gestational timepoints, for all high-risk women, and when stratified by ethnicity. AUC ROC for prediction of sPTB <37 weeks for the tests taken between 18 to 19^{+6} weeks was 0.66 (N=188, 95% CI 0.57-0.76) and 0.65 (n=237, 0.55 to 0.74) for tests taken between 20 and 23^{+6} weeks. When sub-analysis was performed according to ethnicity, no differences in prediction between White and Black women were seen (comparison between ROC areas for 18-19^{+6} week test <37 weeks p=0.07; 20-24^{+0} week test <37 weeks p=0.79; 18-19^{+6} week test <34 weeks p= 0.62, 20-24^{+0} week test <34 weeks p=0.96). Diagnostic test performance for qfFN was not explored for this cohort, as it has been described elsewhere in a much larger (and demographically similar) cohort (Abbott et al. 2015).

Table 3. ROC areas for performance of quantitative fetal fibronectin to predict spontaneous preterm birth in all women, and stratified by ethnicity

	sPTB <37 weeks			sPTB <34 weeks		
	N	**AUC**	**95% Confidence interval**	**N**	**AUC**	**95% Confidence interval**
Gestation 18 to 19^{+6} weeks						
All women	188	**0.66**	**0.57- 0.76**	191	**0.71**	**0.60-0.83**
White women	97	**0.64**	**0.52-0.80**	98	0.68	0.46-0.89
Black women	70	0.64	0.49-0.80	72	0.66	0.49-0.84
Gestation 20 to 24^{+0} weeks						
All women	237	**0.65**	**0.56-0.74**	243	**0.74**	**0.61-0.86**
White women	128	**0.69**	**0.57-0.81**	131	**0.75**	**0.59-0.91**
Black women	81	0.64	0.38-0.91	84	**0.78**	**0.57-1.00**

Preterm Iatrogenic deliveries prior to gestation of interest have been excluded. sPTB=spontaneous preterm birth; ROC=receiver operating characteristic Significant results in bold

Appendix 4 Cervicovaginal fluid elafin concentrations and relation to genetic polymorphisms of Elafin

Introduction: The gene for elafin, a member of the Trappin gene family (Schalkwijk, Wiedow, and Hirose 1999), has been mapped to the chromosome 20q12-13.1, a locus containing 14 genes expressing protease inhibitor domains (Clauss, Lilja, and Lundwall 2002). The gene is polymorphic, with 23 identified SNPs (Chowdhury et al.. 2006) (Figure 6.1). 11 of these have been found in the promoter region of the gene, sites of transcription factor binding. We hypothesised that the polymorphisms in the gene may be associated with the function or expression of elafin, and pregnancy outcome.

Methods: Of the 619 women enrolled into the Insight study, whole blood samples were available for 586 women, from which buffy coats were extracted (see Methods section 2.2.2). Most (n=365 samples) of the genomic DNA was prepared from 200µl of buffy coats using the QIAmp® DNA mini kit (Qiagen, Germany), according to manufacturer's instructions, described above in section 3.10.1, Pilot study). The remaining genomic DNA (n=221 samples) was prepared from buffy coats using Kleargene blood DNA extraction kits (LGC genomics, Germany), the resulting DNA samples stored on 96 wells at -20°C.

Genotyping was undertaken using a competitive allele-specific PCR SNP genotyping system utilizing a fluroescence resonance energy transfer (FRET) quencher cassette oligonucleotides (KASPTM, LGC Genomics) with DNA concentrations adjusted as appropriate. Plates were initially read on a BMG PHERAStar plate reader, having been visually inspected by a member of the genotyping team to assess the progress of the PCR reaction. They were then recycled (3 cycles per recycle step) and read after each

recycle step the PCR reached its endpoint. Genotypes were then identified using Kraken software.

Allelic frequencies, genotype distribution and adjusted odds ratios (OR) with 95% confidence intervals (CI) were calculated using STATA 14.0. STATA 'genhwcci' software was used to test deviation from the Hardy-Weinberg equilibrium. Since profiles of CVF elafin had a skewed distribution, log transformed data was used in the analysis. Multivariate logistic regression was used to estimate the genotype specific Odds Ratios (95% CI, crude and adjusted for age, smoking and BMI, and ethnicity) for cervical shortening and sPTB risk, and results the stratified by ethnicity (White/Black). The relationship between the 4 identified elafin polymorphisms, and CVF elafin concentration at each of the gestational categories was investigated (crude and adjusted) using linear regression. Analysis was then repeated according to stratification by ethnicity (White, Black) to make allowances for polymorphism frequencies according to ethnicity.

Results: No significant differences were found in minor allele frequency of any of the four SNPs between cases and controls. X^2 analysis of genotype frequencies (homozygous vs. homozygous) revealed no association between cases (sPTB <37w) and control population for rs2664581 (X^2 0.42, p=0.81), rs17333103 (X2 0.98, p=0.61), rs1983649 (X^2 0.76, p=0.69) or rs6032040 (X^2 0.39, p=0.82), nor for sPTB <34 weeks; rs2664581 (X^2 0.64, p=0.73), rs17333103 (X^2 1.04, p=0.59), rs1983649 (X^2 1.73, p=0.42) or rs6032040 (X^2 0.16, p=0.92), nor was association found when stratified by ethnicity

rs2664581

Women with the C (variant) allele had significantly higher CVF elafin concentrations when sampled at 10-13^{+6} weeks, 14 to 15^{+6} weeks, and 20 to 24^{+0} weeks of gestation, compared with women homozygous for A allele (wild type allele). This also yielded

true at each gestational category for both crude and adjusted (ethnicity, maternal age, BMI and smoking status) linear regression analysis.

rs1983649

Women with the A allele (wild-type allele) had significantly higher CVF elafin concentrations when sampled at 10-12^{+6} weeks, 16 to 19^{+6} weeks, and 20 to 24^{+0} weeks of gestation, compared with women homozygous for T allele (variant allele), when analysed by two sample t-test, as well as general linear model analysis, both crude and adjusted (Table 9-14).

rs6032040

No association between genotype and elafin concentration was found (data not shown), nor when stratified by ethnicity

rs17333103

Women with the T allele (variant allele) had significantly higher CVF elafin concentrations when sampled at 10-12^{+6} weeks and 20 to 24^{+0} weeks of gestation (with borderline significant results for the other 2 gestational categories), compared with women homozygous for C allele (wild-type allele), when analysed by two sample t-test (Table 9-17).

Appendix 5 Published papers related to this thesis

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Cervicovaginal natural antimicrobial expression in pregnancy and association with spontaneous preterm birth

Natasha L. Hezelgrave✉, Paul T. Seed, Evonne C. Chin-Smith, Alexandra E. Ridout, Andrew H. Shennan & Rachel M. Tribe

There is much interest in the role of innate immune system proteins (antimicrobial peptides) in the inflammatory process associated with spontaneous preterm birth (sPTB). After promising pilot work, we aimed to validate the association between the antimicrobial peptides/proteins elafin and cathelicidin and sPTB. An observational cohort study of 405 women at high-risk, and 214 women at low-risk of sPTB. Protein concentrations of elafin and cathelicidin, and the enzyme human neutrophil elastase (HNE) were measured in over 1,000 cervicovaginal fluid (CVF) samples (10 to 24 weeks' gestation). Adjusted CVF cathelicidin and HNE concentrations (but not elafin) were raised in high-risk women who developed cervical shortening and who delivered prematurely and were predictive of sPTB < 37 weeks, with an area under the curve (AUC) of 0.75 (95% CI 0.68 to 0.81) for cathelicidin concentration at 14 to 15+6 weeks. Elafin concentrations were affected by gestation, body mass index and smoking. CVF elafin in early pregnancy was modestly predictive of sPTB < 34 weeks (AUC 0.63, 0.56–0.70). Alterations in innate immune response proteins in early pregnancy are predictive of sPTB. Further investigation is warranted to understand the drivers for this, and their potential to contribute towards clinically useful prediction techniques.

Spontaneous preterm birth (sPTB), defined as spontaneous birth prior to 37 weeks of gestation, is a leading cause of neonatal morbidity and mortality. Pathophysiologically, it may be initiated by a number of different and overlapping causative factors including intra-amniotic infection, decidual haemorrhage or senescence, cervical weakness, breakdown of maternal–fetal tolerance, declining progesterone activity[1] and sterile inflammation[2]. The culmination of these pathological processes is a process of systemic inflammation, likely similar to the inflammatory pathway associated with early and established term labour (uterine and cervical neutrophil infiltration and release of cytokines, prostaglandins and matrix metalloproteins)[3–5]. Thus, increasing attention has been given to the potential use of inflammatory biomarkers to identify women at high risk of subsequent sPTB. These may have potential to guide prevention and/or management strategies, particularly if measurable in early pregnancy and linked to adverse outcome.

Natural antimicrobial peptides/proteins (NAPs), the body's rapid 'first defence' response to foreign antigens, are small cationic host defence molecules functioning within the innate immune system. Expressed throughout the female genital tract at epithelial surfaces, their anti-protease action counteract the effect of protease mediated inflammation and tissue destruction[6]. NAPs exhibit antibacterial, antiviral and antifungal activity, largely attributed to disruption of microbial membranes[7]. Trappin2/elafin (also known as peptidase inhibitor 3) or skin-derived anti-proteinase (SKALP), secretory leucocyte protease inhibitor (SLPI), human β-defensins 1–3 and cathelicidin antimicrobial peptide (LL37/cathelicidin) are amongst the NAPs identified in the reproductive tract. Trappin2/elafin (referred to hereafter as elafin) expression has been reported to be raised in fetal membranes from women with preterm prelabour rupture of membranes (PPROM)[8] and reduced in cervicovaginal fluid (CVF) from women with bacterial vaginosis (BV)[9]. Both cathelicidin and elafin have a relationship with human neutrophil elastase (HNE), a serine protease; cathelicidin is co-released with HNE from human neutrophils[10] and HNE is inhibited by elafin[11].

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BMC Pregnancy and Childbirth

STUDY PROTOCOL Open Access

Rationale and design of SuPPoRT: a multi-centre randomised controlled trial to compare three treatments: cervical cerclage, cervical pessary and vaginal progesterone, for the prevention of preterm birth in women who develop a short cervix

Natasha L. Hezelgrave, Helena A. Watson, Alexandra Ridout, Falak Diab, Paul T Seed, Evonne Chin-Smith, Rachel M. Tribe* and Andrew H. Shennan

Abstract

Background: Clinically, once a woman has been identified as being at risk of spontaneous preterm birth (sPTB) due to a short cervical length, a decision regarding prophylactic treatment must be made. Three interventions have the potential to improve outcomes: cervical cerclage (stitch), vaginal progesterone and cervical pessary. Each has been shown to have similar benefit in reduction of sPTB, but there have been no randomised control trials (RCTs) to compare them.

Methods: This open label multi-centre UK RCT trial, will evaluate whether the three interventions are equally efficacious to prevent premature birth in women who develop a short cervix (<25 mm on transvaginal ultrasound). Participants will be asymptomatic and between 14^{+0} and 23^{+6} weeks' gestation in singleton pregnancies. Eligible women will be randomised to cervical cerclage, Arabin pessary or vaginal progesterone (200 mg once daily) ($n = 170$ women per group).
The obstetric endpoints are premature birth rate <37 weeks' of gestation (primary), 34 weeks and 30 weeks (secondary outcomes) and short-term neonatal outcomes (a composite of death and major morbidity). It will also explore whether intervention success can be predicted by pre-intervention biomarker status.

Discussion: Preterm birth is the leading cause of perinatal morbidity and mortality and a short cervix is a useful way of identifying those most at risk. However, best management of these women has presented a clinical conundrum for decades.
Given the promise offered by cerclage, Arabin pessary and vaginal progesterone for prevention of preterm birth in individual trials, direct comparison of these prophylactic interventions is now essential to establish whether one treatment is superior. If, as we hypothesise, the three interventions are equally efficacious, this study will empower women to make a choice of treatments based on personal preference and quality of life issues also explored by the study.
(Continued on next page)

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Original Article

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Host Defense Peptide Expression in Human Cervical Cells and Regulation by 1,25-Dihydroxyvitamin D3 in the Presence of Cytokines and Bacterial Endotoxin

Evonne C. Chin-Smith, BSc, MSc, PhD[1], Natasha L. Hezelgrave, BSc, MBBS[1], and Rachel M. Tribe, BSc, PhD[1]

Abstract

Host defense peptides (HDPs) in the pregnant female reproductive tract provide protection against infection. The relationship between HDPs and infection/inflammation is poorly understood. Therefore, we investigated the regulation of HDPs by 1α, 25-dihydroxyvitamin D3 (1,25-(OH)2) in the presence/absence of infectious/inflammatory agents. Endocervical epithelial cells (END1/E6E7, n = 6) were exposed to 1,25-(OH)2, calcipotriol, interleukin 1β (IL-1β), granulate-macrophage colony-stimulating factor (GM-GSF), and lipopolysaccharide (LPS). Elafin, human beta defensin (hBD2), cathelicidin, secretory leucocyte protease inhibitor, interleukin 8, 1,25-(OH)2 receptor, and toll-like receptor 4 (TLR4) expression was determined using quantitative polymerase chain reaction and/or enzyme-linked immunosorbent assay. Host defense peptide gene and protein expression was assessed in cervicovaginal cells/fluid, respectively, from first trimester pregnant women (n = 8-12). Interleukin 1β induced elafin and hBD2. The 1,25-(OH)2 induced cathelicidin expression in the presence of IL-1β and LPS. The 1,25-(OH)2 also attenuated IL-1β-induced IL-8 expression and LPS enhancement of TLR4. Host defense peptides and TLR4 profiles in cervicovaginal cells and fluid samples from pregnant women were similar to END1/E6E7 cells. In conclusion, HDPs are differentially regulated in END1/E6E7 cells. The 1,25-(OH)2 induction of cathelicidin and suppression of IL-8 highlights a mechanism by which 1,25-(OH)2 supplementation could enhance the pregnant innate immune defenses.

Keywords

host defense peptide, human cervix, inflammation, infection, 1,25-(OH)2

Introduction

Spontaneous preterm birth (sPTB, particularly at gestations <34 weeks of pregnancy) is often associated with reproductive tract inflammation and ascending infection.[1-3] Several pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor alpha, have been detected in cervicovaginal fluid in women at high risk of sPTB and women in threatened preterm labor.[3-5]

There is growing literature demonstrating the expression of host defense peptides (HDPs) in the human female reproductive tract, including the cervical mucus plug..[6-8] Moreover, emerging evidence suggests that the reproductive tract production of elafin is altered in bacterial vaginosis (BV),[7] chorioamnionitis,[9] and in women at high risk of preterm labor.[8] Regulation of reproductive tract HDPs expression in pregnancies associated with sPTB is less well described.

Evidence from in vitro cell studies and the nonpregnant literature on HIV indicates that HDPs are produced from epithelial cells and leucocytes in response to pathogens and damage associated molecular patterns.[10,11] Pro-inflammatory cytokines, neutrophil proteases, microbial endotoxins,[7,12] steroids,[13,14] and vitamin D[12,15-17] have been shown to upregulate a variety of HDPs in vitro.

The potential interaction between vitamin D and HDPs is of interest, as vitamin D serum status has been shown to have an impact on female reproductive health[18,19] and deficiencies have been linked with preterm birth.[20,21] Indeed in a recent study, the majority of women at high risk of sPTB in our population had reduced or deficient plasma vitamin D concentrations.[8]

The impact of vitamin D on cervical epithelial cell HDPs responses in the presence of inflammation has not been

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Email: rachel.tribe@kcl.ac.uk

Student Name: Natasha Lee Hezelgrave

Student Number: 0325246

JCI insight

Cervicovaginal microbiota and metabolome predict preterm birth risk in an ethnically diverse cohort

Flavia Flaviani, … , Andrew James Mason, Rachel M. Tribe

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