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# Mid-gestational changes in cervicovaginal fluid cytokine levels in asymptomatic pregnant women are predictive markers of inflammation-associated spontaneous preterm birth

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ABSTRACT

*Objectives:* Perturbation of the choriodecidual space before the onset of spontaneous preterm birth (sPTB) could lead to a concomitant rise in both cervicovaginal fluid (CVF) cytokine and fetal fibronectin (FFN), and assessing the concentrations of both markers could improve the prediction of sPTB (delivery before 37 completed weeks of gestation). Therefore, we prospectively determined mid-trimester changes in CVF cytokine and FFN concentrations, and their predictive capacity for sPTB in asymptomatic pregnant women.

*Study design:* CVF collected at $20^{+0}$–$22^{+6}$ weeks (n = 47: Preterm-delivered = 22, Term-delivered = 25) and $26^{+0}$–$28^{+6}$ weeks (n = 50: Preterm-delivered = 17, Term-delivered = 33) from 63 asymptomatic pregnant women at risk of sPTB were examined. Cytokine and FFN concentrations were determined by multiplexed bead-based immunoassay and 10Q Rapid analysis (Hologic, MA, USA) respectively. The $20^{+0}$–$22^{+6}$/$26^{+0}$–$28^{+6}$ weeks ratios of cytokines and FFN concentrations were compared between preterm- and term-delivered women using Receiver Operating Characteristics curves to predict sPTB. Also, bacterial 16S rDNA from 64 samples ($20^{+0}$–$22^{+6}$ weeks n = 36, $26^{+0}$–$28^{+6}$ weeks n = 28) was amplified by polymerase chain reaction to determine associations between vaginal microflora, cytokine and FFN concentrations.

*Results:* Changes in RANTES and IL-1β concentrations between $20^{+0}$–$22^{+6}$ and $26^{+0}$–$28^{+6}$ weeks, expressed as a ratios, were predictive of sPTB, RANTES (AUC = 0.82, CI = 0.62–0.94) more so than IL-1β (AUC = 0.71, CI = 0.53–0.85) and FFN (not predictive). Combining these markers (AUC = 0.83, CI = 0.63–0.95) showed similar predictive capacity as RANTES alone. FFN concentrations at $26^{+0}$–$28^{+6}$ weeks correlated with IL-1β ($r$ = 0.4, $P$ = 0.002) and RANTES ($r$ = 0.3, $P$ = 0.03). In addition, there was increased prevalence of vaginal anaerobes including *Bacteroides*, *Fusobacterium* and *Mobiluncus* between gestational time points in women who experienced sPTB compared to the term women ($P$ = 0.0006).

*Conclusions:* CVF RANTES and IL-1β in mid-trimester of pregnancy correlate with quantitative FFN. The levels of CVF RANTES and IL-1β decline significantly in women who deliver at term unlike women who deliver preterm. This observation suggests that sPTB may be characterised by sustained choriodecidual inflammation and may have clinical value in serial screening for sPTB if confirmed by larger studies.

## 1. Introduction

Ascending genital tract infection due to changes in the vaginal microbiota induces immune responses characterised by the release of inflammatory cytokines and chemokines capable of initiating preterm labour (PTL) and preterm birth (PTB) (Goldenberg et al., 2000; Huang et al., 2014; Jefferson, 2012). Intrauterine infection during gestation and the subsequent inflammatory processes that ensue can disrupt the maternal choriodecidual tissues and trigger matrix remodelling with concomitant leakage of fetal fibronectin (FFN) into the cervicovaginal space (Agrawal and Hirsch, 2012). Cervicovaginal fluid (CVF) quantitative fetal fibronectin (qFFN) is putatively the most widely employed

*Abbreviations:* PTB, preterm birth; sPTB, spontaneous preterm birth; PTL, preterm labour; CVF, cervicovaginal fluid; FFN, fetal fibronectin; qFFN, quantitative fetal fibronectin; CL, ultrasound cervical length; RANTES, regulated on activation normal T cell expressed and secreted; BMI, body mass index; GTP, gestational time point; CBA, cytometric bead array; PCR, polymerase chain reaction; ROC, receiver operating characteristics curve; rDNA, ribosomal deoxyribonucleic acid; MMP, matrix metalloproteinase; PG, prostaglandin

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clinical test for predicting PTB in asymptomatic and symptomatic women. However, it is most often employed in assessing women presenting with symptoms of PTL (Abbott et al., 2015; Heng et al., 2015; Stock et al., 2015) as a rule-out test, largely due to its high negative predictive value (Abbott et al., 2015).

Altered cytokine concentrations between gestations may reflect inflammatory processes associated with spontaneous preterm birth (sPTB). Several inflammatory mediators of PTB in asymptomatic and symptomatic pregnant women, including Interleukin (IL) -1, 2, 4, 6, 8, 10, 12 and 17, tumor necrosis factor-alpha (TNF-α), Interferon gamma (INF-γ), RANTES (regulated on activation, normal T cell expressed and secreted), C-reactive protein (CRP), have been investigated in CVF, amniotic fluid and blood (Agrawal and Hirsch, 2012; Chan, 2014; Chandiramani et al., 2012, Goldenberg et al., 2005; Hee, 2011; Vogel et al., 2007; Vrachnis et al., 2012; Witkin et al., 2011). However, none of these markers has been noted to attain clinically applicable predictive utility for PTB in asymptomatic (low and high risk) (Conde-Agudelo et al., 2011; Hee, 2011) as well as symptomatic pregnant women (Hee, 2011); and especially in asymptomatic women at mid-gestation (Gervasi et al., 2012). Regarding these studies, differences in clinical settings and experimental designs (e.g. sample sizes, inclusion/exclusion criteria, gestational age at sampling etc.) have militated against the identification of consistently accurate predictive biomarkers of PTB (Heng et al., 2015). Improving study designs and methodologies, and exploring multiple promising biochemical diagnostic tests may therefore improve the prediction of PTB (Chan, 2014; Georgiou et al., 2015; Heng et al., 2015).

Furthermore, false positive CVF FFN test results contribute to the relatively low sensitivity and positive predictive value of the test, often resulting from factors such as unprotected vaginal intercourse, digital examination, bleeding or contamination with amniotic fluid following ruptured membranes (Heng et al., 2015). Hitherto, no studies have explored combining the predictive utility for sPTB of CVF cytokine concentrations with qFFN in high-risk asymptomatic women.

We hypothesised that perturbation of the choriodecidual space before the onset of sPTB would lead to a concomitant rise in both CVF cytokine and qFFN, and that assessing the concentrations of both markers could improve the prediction of sPTB (spontaneous delivery before 37 completed weeks of gestation). This study explores this hypothesis by investigating the changes in CVF cytokine and FFN concentrations across mid-trimester in asymptomatic high-risk women who subsequently delivered prematurely and those who delivered at term. We determined whether these changes in cytokine concentrations are predictive of sPTB, either alone or in combination with qFFN.

## 2. Materials and methods

### 2.1. Study design

This is a predefined pilot case-control study that was reviewed and approved by the Yorkshire & Humber (Sheffield) Committee of the UK National Research Ethics Service (REC Number 13/YH/0167). All samples were obtained from participants following written informed consent.

#### 2.1.1. Study participants and sample collection

CVF samples were obtained by high-vaginal swabs (HVS) from 63 asymptomatic high-risk pregnant women at 2 mid-gestational time points (GTP): $20^{+0}$–$22^{+6}$ weeks (GTP1, n = 47: Preterm-delivered = 22, Term-delivered = 25) and $26^{+0}$–$28^{+6}$ weeks (GTP2, n = 50: Preterm-delivered = 17, Term-delivered = 33). These GTPs, which fall within the gestational window for FFN and cervical length assessment recognised in clinical practice (Abbott et al., 2015; Esplin et al., 2017; Hezelgrave et al., 2016; Hezelgrave et al., 2017; Jwala et al., 2016; Vandermolen et al., 2016), were selected to determine whether early identification of women at greatest risk may improve prevention of sPTB and its complications. Participants were asymptomatic pregnant women at high-risk of PTB on the basis of a previous history of sPTB and/or a short cervix measuring < 25 mm on transvaginal ultrasound (in previous or current pregnancy), attending the specialist antenatal clinics at the Jessop Wing Maternity Hospital, Sheffield, UK, between May 2013 and September 2015. Women presenting with symptoms suggestive of threatened PTL, prelabour ruptured membranes or carrying multiple gestations were excluded from the study, as were those with a recent vaginal examination, or evidence of genital tract infection (e.g. bacterial vaginosis, BV), urinary tract infection or abnormal cervical cytology. PTB outcome was defined as spontaneous delivery before 37 completed weeks of gestation.

At presentation and before any intervention was administered, CVF was obtained from the posterior vaginal fornix of each woman by two high vaginal swabs (HVS, sterile Dacron swabs – Deltalab Eurotubo 300263, Fisher Scientific, UK), after passage of a sterile Cusco's vaginal speculum. One swab was used for the current study and the other for an independent metabolomics examination. For this study, one swab saturated with CVF was immediately processed or stored in −20 °C and then to −80 °C for approximately 3 days pending analysis. The specimens were subsequently processed by washing the CVF off the swab in a clean 1.5 µl microfuge tube containing 400 µl isotonic Phosphate Buffered Saline (PBS). This was done by vortexing the cut end of the swab in PBS solution for 5 min. From the solution, 250 µl was aspirated and transferred into a fresh 1.5 µl microfuge tube for 16S ribosomal DNA extraction to determine the microbial composition of the vaginal environment, while 50 µl was transferred into a separate tube for determination of cytokine concentration. The remnants (i.e. swabs in 100 µl solution) were stored at −80 °C as part of a growing biorepository.

### 2.2. CVF quantitative fetal fibronectin, vaginal pH and cervical length measurements

Study participants also had vaginal swab specimens assessed for qFFN and pH while cervical length was measured by transvaginal ultrasonography. FFN concentrations were quantified using the 10Q Rapid FFN analyser (Hologic, MA, USA), while vaginal pH was determined by a narrow range pH paper (pH-Fix 3.6–6.1, Machery-Nagel, Düren, Germany) (Jespers et al., 2015). The pH indicator paper apart from being a standard (Miller et al., 2016), has the advantage of measuring pH values of unbuffered or weakly buffered solutions/samples. Vaginal pH determination by this method is highly accurate (reading accuracy: ± 0.1 pH), rapid and reliable. ftp://ftp.mn-net.com/english/Flyer_Catalogs/Test_Sticks_Test_Papers/Fl.%20pH-FixTest_StripsEN.pdf.

ftp://ftp.mn-net.com/english/Instruction_leaflets/Testpapers/pHFix/92130en.pdf.

All clinical samples and measurements were obtained by clinical research staff after written informed consent of participants.

### 2.3. CVF cytokine measurement

The concentrations of 10 cytokines (IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, RANTES, TNF-α and IFN-γ) were determined by a multiplexed bead-based immunoassay, using the BD™ Cytometric Bead Array (CBA) from BD Biosciences, CA, USA. Analysis was carried out according to the BD CBA Human Soluble Protein Master Buffer Kit instruction (http://www.bdbiosciences.com/ds/pm/others/23-13480.pdf), and as previously published (Castillo and Maccallum, 2012; Elshal and Mccoy, 2006). Twenty five microlitres of each sample was pipetted into ELKAY 1.1 ml microtubes and placed onto a 96-well plate. On each plate, lyophilised standards corresponding to each cytokine under investigation were added. Standards for each of the cytokines were combined (pooled) to form a Universal (Top Standard). The Top Standard was then diluted by a factor of 2 until 9 dilutions were