Analysis of cervicovaginal fluid metabolome and microbiome in relation to preterm birth

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Dedication

To

My parents

German I. Amabebe and Rose O. Ikiriko
(God rest their souls)

and

My lovely daughter and heiress,

Michelle O. Amabebe

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Amabebe E, Reynolds S, Stern V, Parker J, Stafford G, Paley M, Anumba D. Cervicovaginal fluid acetate: a marker for preterm birth in symptomatic pregnant women. *Reprod Sci* 2016, 23(Suppl 1):81A(O-090). Proceedings of the 63rd annual scientific meeting of the Society for Reproductive Investigation, 2016 (Oral).

Stern V, **Amabebe E**, Reynolds S, Stafford G, Paley M, Anumba D. The association of cervicovaginal fluid metabolites at 20-22 weeks gestation with quantitative fetal fibronectin, ultrasound cervical length, and gestational age at delivery in asymptomatic pregnant women. *Reprod Sci* 2016, 23(Suppl 1):190A-191A(F-014). Proceedings of the 63rd annual scientific meeting of the Society for Reproductive Investigation, 2016 (Poster).

Parker J, Stafford G, Stern V, **Amabebe E**, Reynolds S, Paley M, Anumba D. Prognostic microbiomial and metabolite markers of preterm birth in second trimester cervicovaginal fluid from women at risk. *Reprod Sci* 2016, 23(Suppl 1):128A(T-86). Proceedings of the 63rd annual scientific meeting of the Society for Reproductive Investigation, 2016 (Poster).

Amabebe E, Reynolds S, Stern V, Parker J, Stafford G, Paley M, Anumba D. Elucidating potential metabolite markers of preterm birth in cervicovaginal secretions by Magnetic Resonance Spectroscopy. *BJOG* 2015, 122(Suppl 2):103(PP.8). Proceedings of the 17th annual conference of the British Maternal & Fetal Medicine Society, 2015 (Poster).

Stern V, Parker J, **Amabebe E,** Stafford G, Anumba D. Diversity of the vaginal microbiome and cervicovaginal fetal fibronectin levels: is there an association? *BJOG* 2015, 122(Suppl

2):58(PF.13). Proceedings of the 17th annual conference of the British Maternal & Fetal Medicine Society, 2015 (Poster).

Amabebe E, Stern V, Parker J, Reynolds S, Stafford G, Paley M, Anumba D. Elucidating potential metabolite markers of preterm birth in cervicovaginal secretions by Magnetic Resonance Spectroscopy. *Reprod Sci* 2015, 22(Suppl 1):130A(T-069). Proceedings of the 62nd annual scientific meeting of the Society for Reproductive Investigation, 2015 (Poster).

Amabebe E, Reynolds S, Stern V, Parker J, Stafford G, Paley M, Anumba D. Correlation between cervicovaginal fluid metabolites and gestational age at delivery by MRS. Proceedings of the 21st annual scientific meeting of the British chapter of the International Society for Magnetic Resonance in Medicine, 2015, P031 (Poster and 2 minute poster pitch).

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Amabebe E, Reynolds S, Stern V, Parker J, Stafford G, Paley M, Anumba OCD. Elucidating potential metabolite markers of preterm birth in cervicovaginal secretions by Magnetic Resonance Spectroscopy. Proceedings of the 4th annual symposium of the Academic unit of Reproductive and Developmental Medicine, University of Sheffield, 2014 (Oral-poster presentation).

Amabebe E, Stern V, Parker J, Reynolds S, Stafford G, Paley M, Anumba OCD. Determining changes in vaginal microbiome and ^{1}H NMR metabolite spectrum during pregnancy. Proceedings of the British chapter of the International Society for Magnetic Resonance in Medicine, 20th annual scientific meeting (awarded 11 CPD credits by the Royal College of Radiologists), 2014 (Oral).

Amabebe E, Stern V, Parker J, Reynolds S, Stafford G, Paley M, Anumba OCD. Predicting preterm birth by elucidating changes in the vaginal microbiome and metabolome. Proceedings of the 10th anniversary of the University of Sheffield medical school annual research meeting, 2014 (Poster).

Amabebe E, Stafford G, Paley M, Anumba OCD. Predicting preterm birth by elucidating changes in the vaginal microbiome and metabolome. Proceedings of the 3rd annual symposium of the Academic Unit of Reproductive and Developmental Medicine, University of Sheffield, 2013 (Poster).

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Abbreviations

AHR – Asymptomatic high risk

ALR - Asymptomatic low risk

ASYM – Asymptomatic low and high risk pregnant women combined

AUC – Area under the ROC curve

AUROC - Area under the ROC curve

AV – Aerobic vaginitis

BCAA – Branched chain amino acids

BV – Bacterial vaginosis

BVAB – Bacterial vaginosis-associated bacteria

CI – Confidence interval

CL – Cervical length

CST – Community state type

CVF – Cervicovaginal fluid

DNA – Deoxyribonucleic acid

EMMPRIN – Extracellular matrix metalloproteinase inducer

FFN – Fetal fibronectin

GAAD – Gestational age at delivery

GBS – Group B Streptococcus

GC-MS – Gas chromatography mass spectrometry

G-CSF – Granulocyte Colony Stimulating Factor

IGFBP - Insulin-like growth factor-binding protein 1

IFN-γ – Interferon gamma

IL – Interleukin

IL-1RA – Interleukin 1 receptor antagonist

LC-MS – Liquid chromatography mass spectrometry

LPS – Lipopolysaccharide

LR – Likelihood ratio

MIAC – Microbial invasion of the amniotic cavity

MMP – Matrix metalloproteinase

MRS – Magnetic Resonance Spectroscopy

MS – Mass Spectrometry

N.I. – Normalised integral

NICU – Neonatal intensive care unit

NMR – Nuclear Magnetic Resonance

NPV – Negative predictive value

OTU- Operational Taxonomy Unit

OR – Odds ratio

PCR – Polymerase chain reaction

PGN – Peptidoglycan

pIGFBP – Phosphorylated insulin-like growth factor-binding protein

PPROM – Preterm Premature Rupture of Membranes

PPV – Positive predictive value

PROM – Premature Rupture of Membranes

PTB – Preterm Birth

PTL – Preterm Labour

qPCR – Quantitative polymerase chain reaction

rDNA – Ribosomal Deoxyribonucleic acid

RDS – Respiratory distress syndrome

RNA – Ribonucleic acid

ROC - Receiver Operating Characteristics curve

RR – Relative risk/Risk ratio

SCFA – Short chain fatty acid

sPTB – Spontaneous Preterm Birth

SYM – Symptomatic pregnant women

TLR – Toll-like receptor

TNF – Tumor necrosis factor

UV – Ultraviolet light

Vis – Visible light

VVC – Vulvovaginal candidiasis

Summary

The biochemical activities and resultant metabolic by-products of the vaginal microbial community during gestation can provide useful insight into the pathophysiology of preterm birth (PTB), as well as help in identifying women at risk. These metabolic changes leave specific signature fingerprints that can be investigated by Magnetic resonance spectroscopy (MRS). Therefore, we hypothesised that women who ultimately deliver prematurely will have significantly different vaginal microbiota metabolite signatures compared to their term counterparts even in the absence of clinical infection.

In order to characterise and validate the cervicovaginal fluid (CVF) metabolite profiles and determine their predictive capacities for PTB, high-vaginal swabs were obtained from asymptomatic and symptomatic pregnant women sub-classified depending on a previous history of PTB and/or short cervix (< 25 mm) into: asymptomatic low risk (ALR) women with no prior PTB nor short cervix, 20-22 gestational weeks (w), n = 183; and asymptomatic high risk (AHR) women with prior history of PTB and/or short cervix, 20-22 w, n = 186. A subset of these women were assessed again at 26-28 w (due to their high-risk status), n = 129. The fourth cohort comprised women presenting with symptoms of threatened preterm labour (PTL) 24-36 w, n = 89 (SYM).

CVF dissolved in phosphate buffered saline was analysed with a 9.4T MR spectrometer. Metabolites were identified, integrated for peak area and normalised to the total spectrum integral (excluding water signal). Acetate concentrations (AceConc) were also determined from a randomly selected subset of SYM women (n = 57), by a spectrophotometric technique. Additionally, clinical parameters such as cervical length (CL), fetal fibronectin (FFN), and vaginal pH were recorded and correlated to the metabolites. Furthermore, the 16S rDNA of vaginal bacterial species were PCR-amplified and the vaginal cytology was also determined by Gram, Hematoxylin and eosin, and Papanicolaou staining methods.

We observed that acetate normalised integral (N.I.) (P = 0.002), and acetate/lactate ratio (P = 0.002) were higher in the SYM women who delivered preterm. These were also predictive of PTB < 37 w (AUROC: acetate N.I. = 0.75; acetate/lactate ratio = 0.76), < 32 w (AUROC: acetate N.I. = 0.73; acetate/lactate ratio = 0.79), and within 2 weeks of the index assessment

(AUROC: acetate N.I. = 0.77; acetate/lactate ratio = 0.78), whilst glutamine/glutamate N.I.s was predictive of PTB < 32 w (AUROC = 0.71), and within 2 weeks of the index assessment (AUROC = 0.68) only.

Also, in the AHR20-22w and ALR women, acetate (AUROC = 0.61) and branched chain amino acids N.I.s (AUROC = 0.75) were predictive of PTB < 37 w respectively. Normalised integrals of succinate, formate, lactate, and glucose did not differ in relation to PTB in any of the groups.

Like the acetate N.I.s, AceConc in the SYM women was higher (P = 0.006) in the preterm-delivered women and was predictive of PTB < 37 w (AUROC = 0.74), and within 2 weeks of the index assessment (AUROC = 0.68), with an optimal cut-off of > 0.53 g/l. AceConc also correlated with acetate N.I. (r = 0.69; P < 0.0001).

PCR revealed a higher prevalence of potentially pathogenic anaerobic bacteria species in the preterm-delivered women across the groups except the ALR women.

Apart from correlating with clinical parameters, the prediction of PTB was improved especially in the SYM women when metabolite N.I.s, CL and FFN were combined.

In conclusion, elevated CVF acetate showed clinically useful discriminative propensity for preterm delivery and delivery within 2 weeks of presentation in symptomatic women. A ratio of acetate to lactate showed similar discriminatory capacity in symptomatic women, whilst branched chain amino acids appeared predictive of preterm delivery in asymptomatic women at low risk of PTB. These metabolite differences were supported with the association of higher prevalence of mixed anaerobes in the vaginal melieu and preterm birth.

Chapter 1

1.0. General Introduction

Preterm birth (PTB), defined as birth before 37 weeks of gestation, is the world's commonest cause of perinatal morbidity and mortality.[1] Approximately 15 million PTBs (~12% of all births) occur annually,[2, 3] costing in excess of $26 billion.[4] Preterm babies that survive commonly have greater risk of developing respiratory, gastrointestinal and neurodevelopmental complications.[1, 5] Accordingly, the prediction and possible prevention of PTB has become a major health care priority.[6]

Approximately two-thirds of PTBs occur without an identifiable cause and follow spontaneous onset of preterm labour (PTL), and preterm premature rupture of membranes (PPROM).[5] About 40-50% of spontaneous PTBs are associated with ascending intrauterine infection,[1] which could be related to changes in the vaginal microflora,[7, 8] that often eludes detection by conventional culture-dependent techniques.[9]

The human vaginal microflora is a community of microorganisms (mainly bacteria) that represents a balanced mutualistic relationship which critically influences the reproductive physiology and immunity of the host.[10] The magnitude of the bacterial population of healthy vaginal fluid is 10^{6-8} per gram. Although, opportunistic pathogens are present, this diverse microbial community constitutes a first line of defence against infection by competitive exclusion of foreign pathogenic organisms.[10] The composition of the vaginal microbial community is dependent on host-environmental interactions varying between and within individuals over time.[11]

The human vagina, apart from being a passage for sperm, menstruum, or neonates, is a highly versatile organ with profound capacity to influence the health of women and their newborns. The vaginal ecosystem can influence conception, the timing of delivery, and risk of acquiring sexually transmitted infections (STIs).[12] The cervicovaginal epithelium contributes to maintaining an ecosystem that is supportive of commensal organisms but hostile to invading pathogens. The epithelium is laden with glycogen which allows commensal *Lactobacillus* sp. to thrive, whilst the resulting lactic acid produced by anaerobic glycolysis, renders the vaginal milieu unfavourable to invading pathogens, many of which require a relatively alkaline medium to thrive.[13-15] In addition, the vaginal microbiota provides protection against bacterial vaginosis (BV), candidiasis, STIs (e.g., chlamydia, gonorrhoea, human papillomavirus (HPV), urinary tract infections (UTIs),[16-18] human

immunodeficiency virus (HIV) infection etc. [19, 20] This protective function is mostly due to lactic acid/hydrogen peroxide (H_2O_2)–producing bacteria species, predominantly *Lactobacillus* that inhabit the vagina.[21] The microbiota also plays a crucial role in determining the metabolic and inflammatory profile of the vaginal ecosystem.[12]

1.1. Normal Vaginal Microflora

The normal physiological vaginal microflora was initially described as homogenous, consisting of only Gram-positive bacilli (Doderlein's bacilli),[22] now known to be a part of the genus *Lactobacillus*.[23] Presently, the healthy vaginal microbiota is described as a diverse ecosystem comprising of anaerobic and aerobic microorganisms, with *Lactobacilli* as the predominant species.[21, 24-26] The most often identified species of *Lactobacillus* are *L. crispatus*, *L. jensenii*, *L. gasseri* and *L. iners*.[25, 27-29] The female lower genital tract ordinarily becomes predominantly inhabited by *Lactobacilli* with the onset of puberty. This corresponds with an increase in circulating oestrogen which stimulates the maturation of vaginal epithelium and deposition of glycogen.[30, 31] Glycogen is metabolised to lactic acid by vaginal bacteria[22] and/or epithelial cells,[32, 33] thereby enhancing the proliferation of *Lactobacilli*-dominated vaginal microbiota.[1, 30, 34] Lactic acid is the preponderant acid metabolite in the cervicovaginal fluid (CVF) of healthy asymptomatic women (Table 1.1). The normal pH of the vagina in reproductive-age women ranges between 3.5-4.5,[35-38] driven by oestrogen, glycogen, and *Lactobacilli*.[36] *Lactobacilli* play a critical role in inhibiting the proliferation of opportunistic pathogens such as *Gardnerella vaginalis*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, anaerobes, and HIV, by maintaining the vaginal pH at ≤ 4.5 through lactic acid permeabilisation of bacterial lipid membrane, by production of bacteriostatic and bacteriocidal substances including H_2O_2,[39-41] bacteriocins, bactriocin-like substances[42-44] and biosurfactants, stimulation of local immune responses,[23, 45, 46] and by adherence and competitive exclusion.[28, 40, 47-52] Lactic acid enhances the protective activities of H_2O_2 and bacteriocins.[53, 54] At physiological concentrations (55-111 mM), lactic acid is markedly more lethal to bacterial vaginosis-associated bacteria (BVAB) compared to HCl or acetic acid acidified media (pH 4.5). Glycogen and lactic acid are diminished in BV infected women (Table 1).[38] Accordingly, a *Lactobacillus*-dominated vaginal microflora is indicative of vaginal health.[25, 55] Interestingly, in the absence of *Lactobacillus* dominance, some

women ostensibly maintain healthy vaginal ecosystems. These women have been found to harbour other lactic acid producers such as *Atopobium vaginae*, *Megasphaera*, *Leptotrichia*, *Streptococcus* and *Staphylococcus*.[56, 57]

Racial/ethnic variations in the composition of the "normal" vaginal microflora have been reported. Vaginal bacterial communities devoid of *Lactobacillus* dominance have been observed to be normal in black and Hispanic women.[21, 58, 59] H_2O_2-producing *Lactobacilli* sp. are lower in black women,[55] who show higher vaginal pH compared to white women with/without infection.[60-62] Black and Hispanic women also showed higher vaginal pH compared to white and Asian women, and this correlated with bacterial communities dominated by species other than *Lactobacillus*. For these women, this diverse microbiota appears normal and healthy.[21, 25] Host differences in innate and adaptive immunity, volume and composition of vaginal fluid, expression of epithelia cells surface ligands,[21] as well as other human behaviours and practices (such as smoking, sexual intercourse, douching, contraceptive and antibiotics use etc.) have been implicated.[63]

The composition of the vaginal microbiota could be highly dynamic,[21, 64-66] changing over time in response to changes in sex hormone levels, sexual activity, antibiotic therapy, vaginal douching, oral contraceptives, stage of menstrual cycle, menopause status, pregnancy, lactation, diabetes mellitus and stress.[25, 35, 65, 67-69] For instance, the vaginal microbiota prior to puberty is dominated by anaerobes.[70] At puberty, the rising levels of oestrogen leads to the accumulation of glycogen in the vaginal epithelial cells whose metabolic substrates enhance vaginal colonisation with *Lactobacilli* sp.[70, 71] *Lactobacilli* dominance decreases as oestrogen levels decline following menopause.[72, 73] The vaginal microbiota in normal pregnancy is predominated by *Lactobacilli* sp. and is more stable than that in non-pregnant state.[74, 75] This can be explained by the rising level of oestrogen during pregnancy resulting in increased vaginal glycogen deposition which enhances the proliferation of *Lactobacilli*-dominated vaginal microbiota.[1, 74] Also, replacement of *Lactobacillus* by *G. vaginalis* microflora has been associated with unprotected vaginal intercourse.[76] The number of *G. vaginalis* and *L. iners* is elevated remarkably during menses and decrease thereafter without intervention, whereas *L. crispatus*-dominated flora remains fairly stable during menses.[64] Other studies have shown that menses significantly alters the vaginal microbial diversity with about a 100-fold decrease in *L. crispatus* and significant increase in *L.*

iners, G. vaginalis, Prevotella bivia and *A. vaginae*.[77] Gajer et al. have also reported that in some women, there is a high species turnover and low constancy of vaginal microbial communities, which is widely variable among women including those that exist in the same bacterial community class. Despite these fluctuations, bacterial community function is still preserved in some cases. [65] In summary, there is a crucial inter-individual inconstancy in the vaginal microbiota, and replacement of *L. crispatus* (associated with increased vaginal community stability) with *L. iners* and/or *G. vaginalis* during menstruation.[25] However, lack of substantial change in the vaginal microbiota during menses has been reported.[78]

1.2. Bacterial Vaginosis

Bacterial vaginosis is defined as a dysbiosis or shift from the normal (Döderlein's) vaginal microflora. It is a polymicrobial, superficial vaginal infection characterised by a decreased in *Lactobacillus* sp., a rise in vaginal pH[79, 80] and overgrowth of a variety of anaerobic and facultative bacteria.[76, 80-82] The magnitude of the vaginal bacterial population increases from 10^{6-8} cells/gram in normal conditions to 10^{8-9} cells/gram in BV and 10^{10-11} cells/gram in BV biofilms.[36] BV was originally called nonspecific vaginitis, Gardnerella vaginitis or Haemophilus vaginitis.[83] However, this is only one point of view as it is now described to be a rather poorly characterised disease both symptomatically and microbiologically.[38, 84, 85] BV is the most common vaginal infection affecting reproductive-age women globally,[86] with a prevalence rate of 5-70%.[87] The ratio of anaerobes to aerobes in the normal vaginal ecosystem is 2:1 – 5:1, but this is increased to about 100:1 – 1000:1 in BV.[69, 88] This is due to a decrease in the amount of H_2O_2-producing *Lactobacilli* and increase vaginal pH. H_2O_2 inhibits the growth of catalase-negative anaerobic organisms by production of hydroxyl free radicals and by combining with chloride ions in the vaginal fluid to form chloridinium ions. [89, 90] *Lactobacilli* lose the H_2O_2-producing capacity at pH >4.5.[83, 90] Vaginal microbiota dominated by H_2O_2-producing *Lactobacillus* species is inversely correlated to the presence of BV-related anaerobes.[26] Hence, BV is usually associated with a decrease in *Lactobacilli*, influencing the normal vaginal microbiota signature, as opposed to an increase in other endogenous pathogens.[91] The BVAB are also part of the normal vaginal microflora, but become virulent when they proliferate and increase in quantity above the lactic acid/H_2O_2-producing *Lactobacilli*. These organisms are Gram-negative and/or Gram-variable anaerobic bacteria species such as *Gardnerella vaginalis*, *Mycoplasma hominis*, *Ureaplasma urealyticum*,

Bacteroides fragilis, *Prevotella* sp. *Mobiluncus* sp., *Fusobacterium*, *Atopobium vaginalis*, *Megasphaera* sp., *Eggerthella*, *Leptotrichia*, *Dialister*, *Sneathia, Peptostreptococcus, Porphyromonas* and the newly identified but poorly resolved Bacterial vaginosis-associated bacterium (BVAB-1), BVAB-2, and BVAB-3 in the *Clostridiales* Order.[88, 92-97] These organisms increase vaginal pH by producing short chain fatty acids (SCFAs: acetate, propionate, butyrate, isobutyrate, succinate, formate, valerate etc.) and amines (putrescine, cadaverine, trimethylamine), and sometimes utilise lactic acid as energy source.[38, 98, 99] Overall, in contrast to healthy women, the CVF metabolite profile of BV-infected women is characterised by increased concentration and variety of SCFAs and amines, and a low lactic acid concentration (Table 1.1).[38] SCFAs produced by BVAB act as virulence factors that inhibit the migration of immunocompetent cells capable of initiating an inflammatory response against the invading pathogens, thereby permitting the establishment of dysbiosis and contributing to the pathogenesis of BV.[38, 100] BVAB also act synergistically to promote a woman's risk of BV and PTB. For instance the presence of *G. vaginalis* and *M. hominis* in the vagina are enhanced in the presence of other anaerobic BVAB.[101, 102] Also *Prevotella* sp. via a commensal symbiotic relationship encourages the growth and survival of *G. vaginalis* and *Peptostreptococus anaerobius* through the provision of nutrients such as ammonia and amino acids.[21, 83, 103-105] *G. vaginalis* and other anaerobes such as *Atopobium, Prevotella, Mobiluncus* have been reported to form biofilms that contribute to the pathogenesis of BV.[106-109] Though *G. vaginalis* is often noticed in healthy women especially during menstruation, its proportion in BV infected women is higher.[64] Of the four major *Lactobacillus* species found in the vaginal microflora (i.e. *L. crispatus*, *L. gasseri*, *L. iners,* and *L. jensenii*), *L. iners* is more often associated with BV.[110-112] This has been attributed to its inability to produce H_2O_2[55, 113] and D-lactate.[1, 114] It has also been reported to be a predominant component of the intermediate transitional stage from normal to abnormal microflora.[95, 97, 115-117] Its presence may be correlated with the colonisation of the vagina by BV-associated organisms.[110,115, 118]

BV was previously described as a *sexually enhanced* rather than *sexually transmitted* infection.[119] This was because no evidence of direct heterosexual transmission of BVAB was recorded, but BV is positively associated with frequent sexual intercourse[119] with new and multiple sexual partners.[120-122] Also, treatment of male sexual partners with antibiotics was not beneficial to the women.[123] However, direct transmission may occur in female to female sexual act.[119, 124] Presently, epidemiological evidence supports the hypothesis that BV is sexually transmitted.[36, 106,

[107] This is as a result of the detection of BVAB including *Gardnerella* and *Atopobium* in the male genital microniche,[36, 106, 125, 126] decreased BV infection with consistent use of condom,[63, 127] and recurrence after treatment when the patient remained with the same sex partner.[128] Other factors associated with prevalence of BV include, black race,[86, 88, 121, 122, 129] smoking,[122, 129-131] contraceptive practice,[88, 129, 130]vaginal douching,[121, 129, 132] menstruation,[133, 134] pregnancy,[122, 135] low educational and socioeconomic status.[121, 122, 130] Most women with BV (about 85%) are usually asymptomatic.[122, 136] However, symptoms could appear in the form of a non-itchy but irritating,[35] creamy vaginal discharge with a fishy odour, that may be more prominent after sexual intercourse and during menses.[94] [137] Symptomatic BV is a syndrome characterised by the appearance of clinical features in the absence of identifiable aetiologic factors.[12] The asymptomatic women usually have a lower risk of adverse pregnancy outcomes compared to symptomatic women.[138] There is usually no obvious local inflammation due to the absence of leucocytes in the vaginal fluid, hence the term vaginosis rather than vaginitis.[83, 88, 139] The absence of inflammation may be due to co-evolution determined immune tolerance between the distal gut microbiota (source of vaginal microbiota) and the immune system.[36, 140-143] Also, the immunomodulatory functions of certain SCFA produced by BVAB have been implicated.[100, 144]

Bacterial vaginosis is a common syndrome among reproductive-age women associated with increased risk of acquisition of STI such as *N. gonorrhoea*, *C. trachomatis*, *T. vaginalis*, Herpes simplex virus (HSV), HPV, and HIV,[38, 87, 145-148] pelvic inflammatory disease,[149-151] endometritis,[152] chorioamnionitis,[83, 153] amniotic fluid infection,[154-156] PROM and PTB.[81, 94, 157-161] Increased prevalence of PTB in women diagnosed with BV by Nugent criteria has being reported.[162-164] Six – 35% of asymptomatic pregnant women have BV.[88] About 10-15% of women with BV deliver preterm, and the risk is increased notwithstanding the presence or absence of symptoms.[163] The rate of PTB is increased by 7-fold if BV is diagnosed <16 weeks of gestation and > 4-fold if detected by 20 weeks.[88] However, most pregnant women with BV do not deliver preterm.[163, 165]A gene-environment interaction between BV and PTB has been observed.[165,166] Interaction between genetic susceptibility [e.g. presence of TNF-α allele 2 and single nucleotide polymorphism (SNP) alleles of protein kinase C-α (PRKCA)] and environmental factors (BV) is associated with increased risk of spontaneous PTL and PTB.[166, 167]

Several probable factors may operate independently or contribute to the increased risk of PTB in women with BV. BVAB may ascend from the vagina to the upper genital tract, resulting in chorioamnionitis, preterm rupture of membranes (PROM), and subsequent PTB.[168] Host immune response triggered by BVAB within the vagina and cervix e.g. release of cytokines and chemokines.[165, 168, 169]The differences in the risk of PTB associated with BV can be attributed to immune hypo- or hyperresponse.[165, 170] Individuals vary in their ability to initiate an immune response against BV-associated organisms.[165] Intense immune response may stimulate the inflammatory pathways to PTB, while low response may allow more ascending genital infection.[171] An "appropriate/optimal" immune response (especially during pregnancy) to BV capable of regulating the changes in the vaginal milieu without leading to an adverse pregnancy outcome is required.[88] Also, BV-associated organisms produce hydrolytic and proteolytic enzymes including sialidase, mucinase and proteases, capable of altering the permeability of the vaginal mucosal epithelium leading to ascending intrauterine infection,[168] transudation and discharge.[83]

BV is commonly diagnosed by two methods in clinical research: 1) clinically (Amsel criteria) and 2) microscopically (Gram staining of vaginal fluid and Nugent criteria). The Amsel criteria involves the diagnosis of BV based on the presence of 3 of the 4 following criteria:[172] 1) the presence of a thin, homogenous, milky and adherent vaginal discharge; 2) a vaginal fluid pH > 4.5; 3) presence of "clue cells" (desquamated vaginal epithelial cells studded with BVAB) on saline wet mount; 4) positive "whiff test" i.e. an amine (trimethylamine), fishy odour after application of 10% potassium hydroxide (KOH) solution to the vaginal discharge.

The Nugent criteria (Table 1.2), which is more objective, reliable and reproducible,[88, 171] with considerable inter-observer concordance in the examination of vaginal specimens,[88] is the method widely used in research.[93] The Nugent scoring is based on microscopically identifying the morphotypes of 3 different bacterial species following Gram stain, and evaluated broadly on the prevalence of *Lactobacilli*.[173] A score of 7 – 10 is diagnostic of BV, while 0 – 3 is considered normal. A score of 4 – 6 is referred to as intermediate because it is a transition between normal flora and BV. There is a good correlation between clinical features of BV and Gram stain scores.[88]

Other less commonly used methods in the diagnosis of BV include BVBlue test (detection of sialidase, a microbial enzyme that cleaves the glycosidic linkages of neuraminic acid),[83, 174, 175] detection of amines (putrescine, cadaverine and trimethylamine),[83, 175] proline aminopeptidase assay,[175, 176] detection of volatile (acetate, butyrate, isobutyrate, propionate), and non-volatile (lactate and succinate) short chain organic acids,[35, 100, 144, 177-179] e.g. a vaginal fluid succinate/lactate ratio ≥ 0.4 is suggestive of BV.[83, 100, 139, 179-183] Treatment of BV is by oral or intravaginal metronidazole (500 mg) twice daily for 7 days, metronidazole gel (0.75%) once daily for 5 days or clindamycin cream (2%) for 7 days,[83] and probiotics.[184-188] Although antibiotic therapy is usually efficacious, recurrence is very common.[38, 64] Antibiotic resistance, biofilm formation, recruitment from extravaginal reservoirs and distortion of the microbiota have been implicated in recurrence by studies reviewed by Aldunate et al.[38] However, rifaximin appears to overcome these limitations by reinstating the vaginal microbiota as well as metabolome in non-pregnant women affected by BV.[189]

Although BV is the most common and widely reported, other forms of abnormal vaginal flora related to poor reproductive outcomes have been identified. An example of such is aerobic vaginitis (AV), an equally disruptive infection of the normal vaginal *Lactobacillus*-dominated microflora but is characterised by overt inflammation, leukocyte and parabasal cells infiltration and proliferation of enteric aerobic bacterial organisms including *Escherichia coli*, *Enteroccoci*, *S. aureus*, and group B *Streptococcus*.[190-192] It has been described as the aerobic equivalent of BV.[190] Like BV, there is decreased lactate concentration due to loss or depressed *Lactobacillus* dominance. However, owing to the absence of anaerobes, succinate concentration is low.[190] It has also been associated with STIs such as *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis*.[193] The clinical features of AV include: red vaginal mucosal inflammation, increased IL-1β and IL-6, vaginal pH > 6, pruritus, dyspareunia and yellowish sticky discharge devoid of fishy (trimethylamine) odour.[160, 190, 191] AV is present in 2-25% of women and has been associated with severe adverse gynaecological outcomes including ascending genital infection/inflammation, preterm rupture of membranes (PROM), PTL and PTB.[1, 160, 191, 192, 194] There is no universally agreed optimal treatment regimen for AV currently, but treatment of AV-infected women with antibiotics (e.g. oral moxifloxacin),[195] and probiotics (e.g. oral/vaginal *L. acidophilus*), have shown significant effectiveness.[191, 194]

Another genital infection with significant adverse gynaecological and obstetric implication is vulvovaginal candidiasis (VVC). This is often caused by an overgrowth of *Candida albicans*.[196] It is the second most common vaginal infection after BV in reproductive-aged women. There are usually no changes in the vaginal microflora and pH, though it can co-exist with BV and AV.[195] The frequency of vaginal colonisation by *Candida* is increased in pregnancy as a result of increased circulating oestrogen and deposition of glycogen in vaginal epithelium.[197] *C. albicans* induces the production of extracellular matrix metalloproteinase inducer (EMMPRIN) by vaginal epithelial cells, which is a potent inducer of matrix metalloproteinase-8 (MMP-8). Elevated MMP-8 and leukocytes could trigger a pro-inflammatory immune response, enhance tissue destruction, ascending genital infection[196] and provoke clinical symptoms. Despite its high prevalence in pregnant women and the tendency of treatment to reduce the incidence of PTB,[197] the association between candidiasis, PROM and PTB is still being debated.[198]

Data on the role of viruses in the pathogenesis of PTL and PTB are relatively limited, though viral DNA of adenovirus, cytomegalovirus, and enterovirus have been detected in amniotic fluid of pregnant women irrespective of their risk of preterm delivery. The presence of hepatitis B virus antigens is a risk factor for spontaneous PTB.[199]

It is plausible that BV and other female genital tract infections during gestation may predispose a woman to ascending intrauterine infection or microbial invasion of the amniotic cavity (MIAC) and consequently initiate the onset of PTL and PTB. Isolation and characterisation of BVAB in gestational tissues and amniotic fluid of pregnant women with adverse delivery outcomes has certainly enhanced our understanding of the association between genital infection and PTB, but the individual organisms involved, and the metabolites they produce as well as the aetiology and pathogenesis of this putatively proinflammatory process is still undergoing scrutiny by various investigators.

Table 1.1: CVF metabolite concentrations in healthy and Bacterial vaginosis-infected non-pregnant women

Metabolite	Concentration, mM	
	Healthy women	BV-infected women
Lactate	~120	<20
Acetate	0-4	<120
Propionate	<1	2-4
Butyrate	<1	2-4
Succinate	<1	≥20

CVF, cervicovaginal fluid; *BV*, Bacterial vaginosis.
Adapted from: Aldunate et al., 2015.[38]

Table 1.2: Nugent scoring system for Gram stained vaginal smears

Morphotypes					
Lactobacilli sp.		*Gardnerella vaginalis, Bacteroides* sp.		*Mobiluncus* sp. (curved gram variable rods)	
Count	Score	Count	Score	Count	Score
≥ 30	0	0	0	0	0
5-30	1	<1	1	1	1
1-4	2	1-4	2	1-4	1
<1	3	5-30	3	5-30	2
0	4	≥ 30	4	≥ 30	2

Nugent Score = sum of 3 scores (0-10) i.e. *Lactobailli* + *Gardnerella* and *Bacteroides* + *Mobiluncus*.
Nugent score of ≥ 7 = Bacterial vaginosis; 4 – 6 (intermediate), and 0 – 3 (normal).
Adapted from: Hoffmann et al., 2014.[200]

1.3. Infection and Preterm Birth

PTB may be iatrogenic (e.g. due to fetal growth restriction or preeclampsia) or spontaneous. About 70-80% of premature births are spontaneous (Fig. 1.1).[201] Spontaneous PTL and PTB are associated with heterogeneous conditions with multiple risk factors e.g. previous PTB, cervical dysfunction, idiopathic uterine contractions, antepartum vaginal haemorrhage, intrauterine infection, multiple gestation, malnutrition, maternal BMI, black race, psychological or social stress, depression, smoking, low socioeconomic and educational statuses, and spontaneous preterm premature rupture of membranes (PPROM).[161, 201-203] Studies have suggested four distinct pathways for the pathogenesis of PTL and PTB: a) premature activation of the fetal hypothalamic-pituitary axis, b) infection/inflammation and matrix remodeling, c) uterine over distension, and d) placenta abruption/decidual haemorrhage.[204, 205] Approximately 40-50% of spontaneous PTBs are associated with ascending upper genital tract infection (Fig. 1.1).[1,161, 206] Changes in the vaginal microbiota have been implicated in the pathogenesis of ascending intrauterine infection and microbial invasion of the amniotic cavity (MIAC),[7,8, 207] which may be undetectable using conventional culture-dependent techniques.[9] Vaginal bacterial invasion and infection, and consequently choriodecidual colonisation triggers the release of lipopolysaccharides (LPS), peptidoglycans (PGN), lipoproteins and SCFAs, that elicit deciduitis and chorioamnionitis. Subsequently proinflammatory chemokines (e.g. IL-8, C-C motif ligands 5), and cytokines [e.g. TNF-α and β, IL1-α and β, IL-2, IL-6, and granulocyte colony stimulating factor (G-CSF)], are synthesised and released.[144, 208-212] These act in a positive feed-forward fashion to activate a cascade of inflammatory responses including the synthesis and release of matrix metalloproteinases (MMP) e.g. MMP-8 and 9,[114, 207, 212, 213] and prostaglandins (e.g. PGE_2 and $PGF_{2\alpha}$,).[7, 214] MMPs (collagenases) degrade the extracellular matrix proteins of the cervix, cervical plug and gestational tissues causing cervical remodeling, permitting more ascending bacterial infection,[213] and rupture of the fetal membranes. PGs apart from stimulating myometrial contraction are capable of triggering cervical ripening and release of MMPs, leading to PPROM and PTB (Fig. 1.2).[1, 7, 114, 211]

During pregnancy, microorganisms invade the uterus and fetus most frequently by ascending through the vagina and cervix.[5, 8] Other routes include: haematogenous circulation across the

placenta (e.g. from periodontitis),[215] by descending from the peritoneal cavity via the fallopian tubes,[208] inadvertently during invasive prenatal diagnostic procedures (e.g. amniocentesis or cordocentesis) (Fig. 1.1).[216] The frequency of intrauterine infection is greater with earlier presentations of PTL.[217] Most spontaneous PTBs at mid-second trimester (21-24 weeks gestation) or less than 30 weeks are associated with infection and inflammation of the gestational tissues compared with about 10% at 35-36 weeks.[5, 211, 218] The most commonly identified microorganisms in the amniotic cavity and placenta are genital *Mycoplasma* sp. especially *U. urealyticum*. Others include *G. vaginalis*, *M. hominis*, *Bacteroides* sp, group B *Streptococcus*, *Fusobacterium*, *Peptostreptococci* and *E. coli*.[5, 219, 220] These infections are usually subclinical as the organisms are typically of low virulence.

Chamydia trachomatis is another STI associated with short cervix and BV, both of which are known risk factors of PTB.[221] Mid–second trimester *C. trachomatis* infection predisposes pregnant women to a 2-3 fold risk of spontaneous PTB.[222] Their risk of having PROM, PTB and low birth weight infants is also increased,[223] and are significantly more susceptible to harbouring vaginal Mycoplasmas differing from *U. urealyticum* (noted for its lack of cell wall and present in 70% of sexually active humans).[224] As a result, potential subjects for PTB prediction studies are usually screened for these organisms prior to recruitment as they have been identified as possible risk factors.[225-227] However, the link between *Chlamydia* infection and PPROM, PTB, low birth weight and infant death is not without controversy. Andrew *et al*. [228] studied 2,470 pregnant women recruited in an antibiotic treatment trial for BV and *T. vaginalis* infections and reported that there was no link between mid-trimester *Chlamydia* infection and increased risk of PTB, neither did the frequency of PTB decline after treatment of *Chlamydia* infection. A similar study demonstrated that early treatment may be the reason for lack of association.[229] Also, *Chlamydia* infection was not associated with low birth weight and infant mortality.[230] Therefore, first trimester screening and treatment may alter the possible determination of an association between *Chlamydia* infection and adverse pregnancy outcomes.[231]

U. urealyticum and *Mycoplasma* isolated from the lower genital tract and amniotic fluid at mid-trimester trigger the synthesis of prostaglandins resulting in PPROM, PTL and PTB.[216,187, 188] This may have a significant influence on pregnancy outcomes among unmarried pregnant women.[232] However, it has been argued that *U. parvum*, but not *U. urealyticum*, is linked to late pregnancy

loss or PTB regardless of a previous history of PTB.[224] This contradicts previous observation that *U. urealyticum* poses more danger to pregnancy outcome.[233] Another study demonstrated that vaginal and amniotic fluid contained about 80% of *U. parvum* and *U. urealyticum*.[224] This indicates that these organisms may invade the amniotic cavity at approximately equal rates, albeit, *U. parvum* was detected in significantly higher amounts compared to the other mycoplasmas in women with PPROM and PTB.

The amniotic cavity was previously considered to be 'sterile'.[6, 7] Consequently, isolation of bacteria from the amniotic fluid was regarded as a pathological finding and termed microbial invasion of the amniotic cavity (MIAC).[220] However, recent studies have reported that the amniotic cavity[234] and fetal membranes[235, 236] are not always sterile, as bacteria can cross intact maternal-fetal membrane barriers and may not always cause PTL.[220, 237] Most MIAC result from ascending bacterial infection from the vagina and cervix and are present in 25% of preterm deliveries.[6, 7] MIAC is promoted by inflammatory products such as MMPs that degrade the collagen of the extracellular matrix of the cervix, thereby allowing potential pathogenic organisms into the putatively 'sterile' amniotic cavity with subsequent activation of PTL, PPROM and PTB.[114, 207]

There is a strong causal relationship between infection/inflammation and PTB.[238-241] Inflammation could be a defence mechanism through which the mother (host) expels infected fetal membranes and/or fetus, thereby preventing further fetal and maternal complications. This could also be a survival mechanism with which a fetus is emancipated from the unpleasant intrauterine environment.[7] The inflammatory process is initiated by the ability of the innate immune system to recognise microorganisms and their products. This is accomplished by specific pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) 1-10.[38, 45] The TLRs expressed by the epithelium of the female reproductive tract are capable of recognising microbial products such as lipopolysaccharide (LPS), peptidoglycan (PGN), lipoprotein, dsRNA, and flagellin,[45, 220] and induce the production of cytokines, chemokines, prostaglandins, and proteases leading to parturition.[212] The fetus also has the capacity to recognise and mount an immune response to pathogenic organisms through TLR-initiated pathways, production of antimicrobial peptides, and LPS-binding protein.[240] This has been termed fetal inflammatory

response syndrome (FIRS).[242] Therefore, an inadequate signalling mechanism will reduce infection-induced PTL. Inflammation is vital to reproductive success.[238]

A gene-environment interaction has also been implicated in the pathogenesis of PTB. A specific polymorphism in the promoter region of TNF-α at position -308 referred to as TNF-α allele 2 is associated with an increased production of TNF-α in response to microbial stimulation during genital tract infection.[243, 244, 245] TNF-α promotes the production of collagen-digesting MMP-3, -8, and -9 (from maternal plasma and amniotic fluid),[246-248] which results in an inadequate and exaggerated inflammatory response leading to cervical ripening, MIAC, PPROM and PTB.[243, 246-248] TNF-α allele 2 carriers are about three times more likely to deliver preterm than non-carriers.[166, 249, 250] This association is enhanced by co-occurrence with BV,[166] and a synergistic effect has been suggested between these two risk factors for PTB.[243] A similar synergistic effect exists between BV and SNPs in 3 other genes that regulate the inflammatory pathway i.e. IL-6, fms-like tyrosine kinase 1, and PRKCA.[167] Also, individuals belonging to the black race have higher ratio of amniotic fluid TNF-α and soluble TNF receptor (sTNFR) concentrations indicative of a TNF-α mediated immunological response of PTB.[251] Genetic polymorphisms of MMP-9 (-14CA),[252] MMP-1 (-1607),[253] IL-4 (-590)[254] and Fas gene (TNFRSF6, -670)[255] promoters in relation to PPROM and PTB have also been documented. Conversely, polymorphisms that promote reduced inflammatory response are associated with reduced risk of PTB.[243, 250]

There are indications that the host response rather than mere colonisation or overgrowth of the bacteria themselves, may be the determining factor for an increased risk of PTB.[82, 256] More so, assessment of the degree of the association between infection and PTB is daunting as there are racial/ethnic, gestational ages and geographical variations in genital bacterial colonisation rates during pregnancy.[214, 257, 258] In addition, the mode or pattern of bacterial invasion of the uterine cavity and the possible involvement of viral, protozoan or other bacterial infections in PTB is rather unclear.[214]

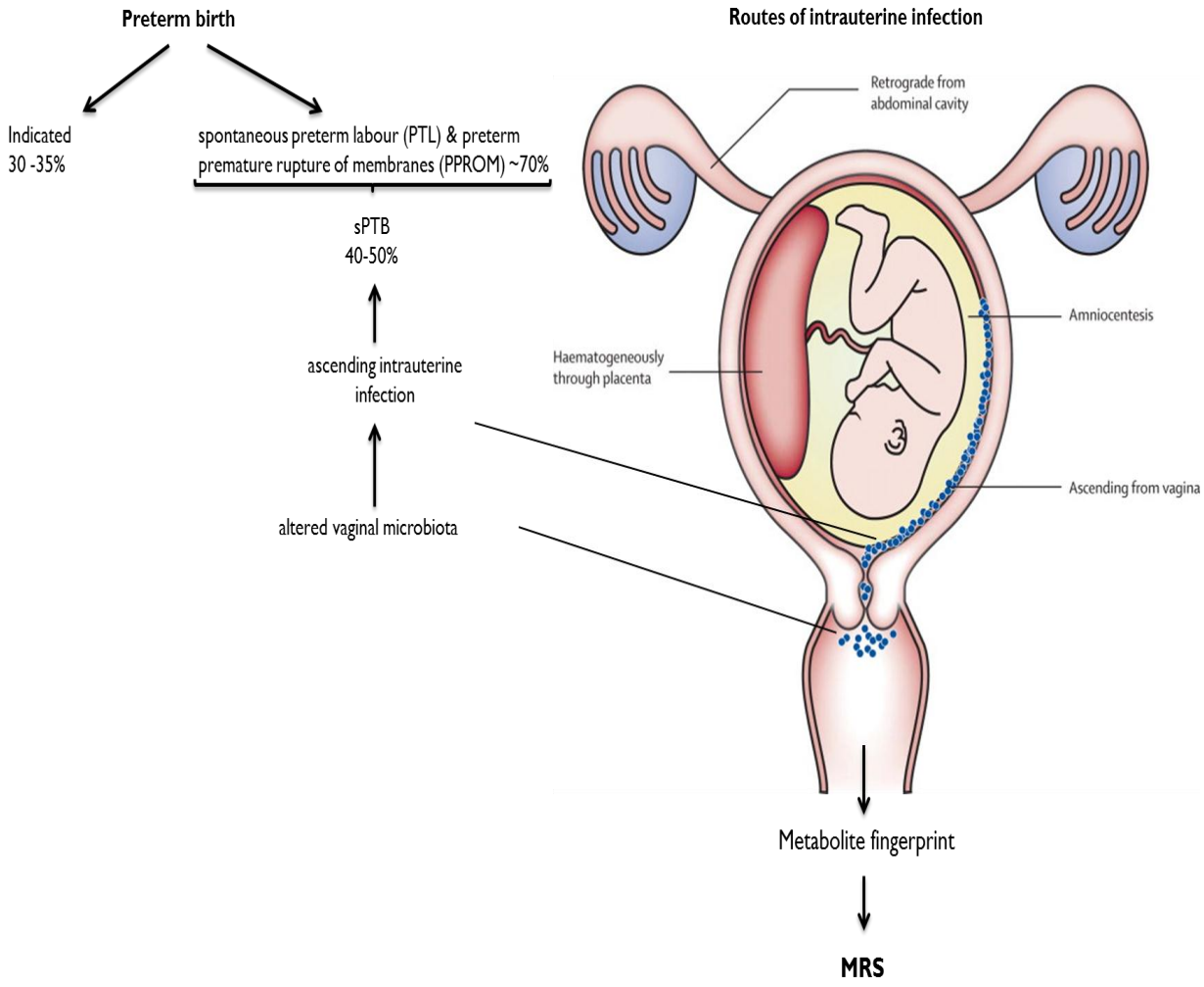


Figure 1.1. Major routes for intrauterine infection leading to spontaneous preterm birth (sPTB) and role of Magnetic Resonance Spectroscopy (MRS). Changes in vaginal microbiota implicated in the pathogenesis of intrauterine infection which accounts for about half of spontaneous PTB, leave metabolic fingerprints detectable by MRS.

Adapted from: Goldenberg et al 2008[5]

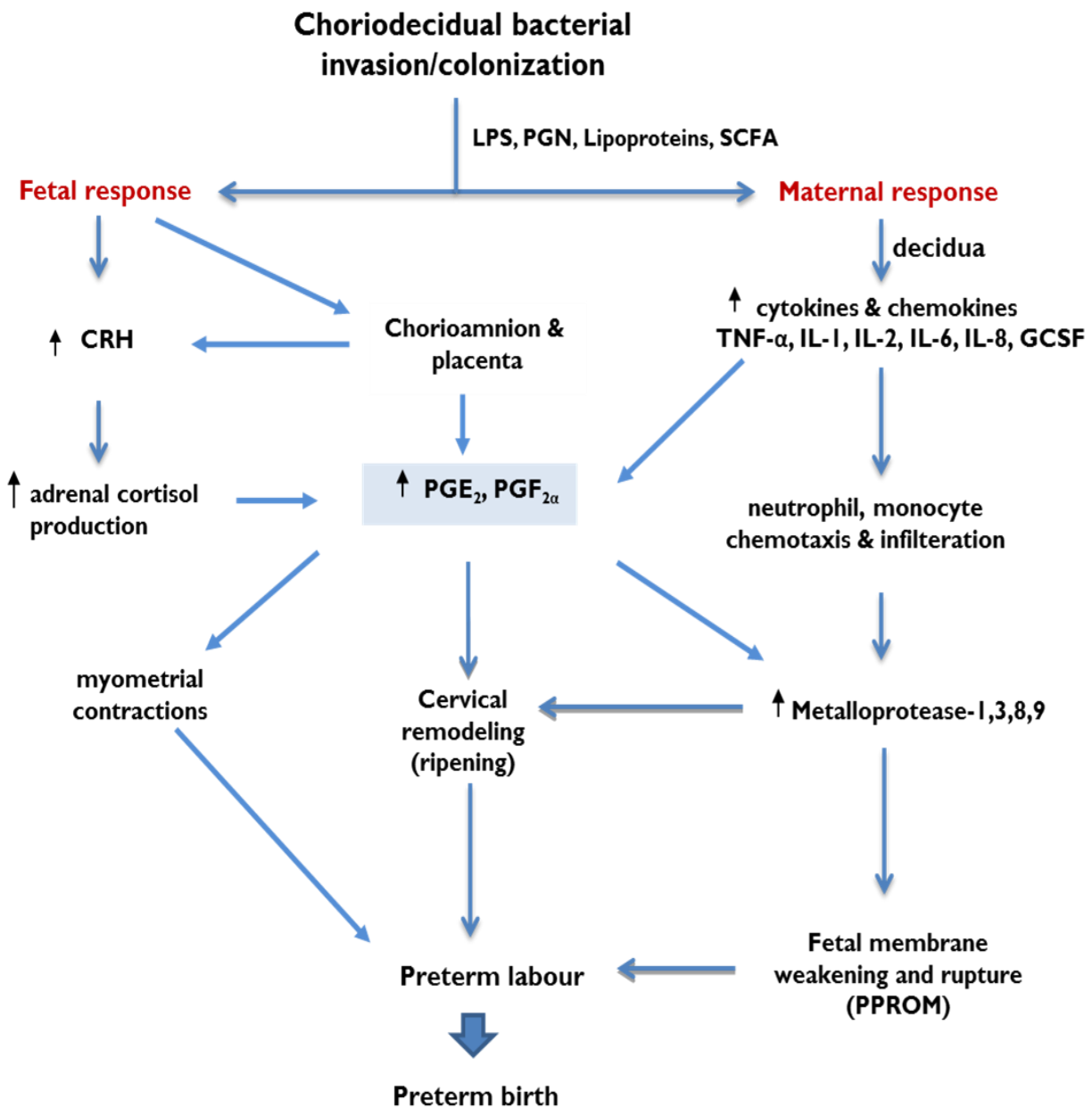


Figure 1.2. Pathogenesis of infection in PPROM and PTB.
BV, bacterial vaginosis; *CRH,* Corticotropin-releasing hormone; *GCSF*, Granulocyte colony stimulating factor; *IL*, Interleukin; *LPS*, Lipopolysaccharide; *PGE_2*, Prostaglandin E_2; *$PGF_{2\alpha}$*, Prostaglandin $F_{2\alpha}$; *PGN*, Peptidoglycan; *PPROM*, Preterm premature rupture of membranes; *PTB*, Preterm birth; *SCFA*, Short chain fatty acids; *TNF*, Tumour necrosis factor.
Adapted from: Goldenberg et al. 2002,[211] and Pararas et al. 2006.[89]

It is highly probable that the epithelium plays a role in preventing cervical inflammation and remodelling during pregnancy, and in causing inflammation and remodelling leading up to parturition at the end of pregnancy. Ascending genital infection may perturb this equilibrium during pregnancy by triggering inflammatory changes within cervical tissue, a process that appears to include changes in cell permeability, tissue hydration, and leukocyte infiltration of cervical stroma, triggered by key cytokines and chemokines. Although these inflammatory changes occur in the cervix and uterus prior to term labour, they characterise PTB more dramatically, even in the absence of positive microbial cultures from vaginal swabs and amniotic fluid.[259]

Increasing vaginal pH, as a consequence of reduced activity of protective commensal *Lactobacillus* species, has been observed in women who deliver prematurely,[21, 260] and the lower the gestational age at birth, the more the likelihood of an infection in the fetal compartment.[261, 262]

1.3.1. *Other risk factors of preterm birth*

Although intrauterine infection accounts for about 50% of spontaneous PTB,[1] several other interacting factors can influence the initiation of PTL and PTB. Hence, PTB has been described as a syndrome. These risk factors can be classified into: maternal background, pregnancy history and pregnancy characteristics.[5]

There is a potential genetic component in the pathways involved in the pathogenesis of PTB. Sisters of women who delivered premature babies have about 80% greater risk of PTB. The history of PTBs in some women has been traced to their grandparents.[5] Mothers born preterm are also at a greater risk of having preterm babies (OR = 1.4).[263] Single nucleotide polymorphisms (SNP) of several genes associated with PTB have been identified through candidate gene-based association studies.[264, 265]

Women of the black, African-American, and Afro-Caribbean decent have been reported to be at higher risk of PTB with an incidence of 16-18% compared with <12% in Caucasians.[5, 266] The likelihood of a very early PTB is about 4 times greater in black women than women from other racial/ethnic population.[5] This disparity could be attributed to differences in the composition of

the normal vaginal microflora, susceptibility to genital infection (e.g. BV), and immune response to genital infection. For instance, the normal vaginal pH of black women is higher than that of their white counterparts and related to higher prevalence of vaginal bacterial community state types (CST) devoid of *Lactobacillus* dominance.[21, 45] This also corresponds to a higher Nugent score and may account for an increased incidence of BV infection and consequently PTB in black women.[59, 88, 266, 267]

The expression and function of inflammatory cytokines in relation to infection and PTB appear to be different across racial/ethnic groups. African-American women exhibit elevated TNF-α and IL-β, while Caucasians show elevated IL-6 and IL-8 in association with PTB. These observations may be due to genetic variation as demonstrated by SNP analyses of genes encoding these inflammatory mediators.[268] Black women have an imbalance of amniotic fluid TNF-α and soluble TNF receptor (sTNFR) concentrations indicative of a heightened TNF-α-mediated immunological response of PTB compared to white women.[251, 269, 270] A gene-environment interaction whereby a SNP of TNF-α at position -308 resulting in TNF-α risk allele 2 common among blacks, and the presence of BV or periodontitis further enhances the risk of spontaneous PTB.[166, 269] In addition, black women with the IL-6 allele and BV infection showed a 2-fold higher risk of PTB. This was not seen in white women.[5] Polymorphisms in genes coding for MMP-1, MMP-8, MMP-9, IL-1RN, IL-1R2, IL-2, TNF-R1, IFN-γ etc. in association with race/ethnicity and PTB have also been reported.[265]

Other maternal demographic factors associated with PTB include maternal age (<18 or >35 years), type of work and level of physical activity (e.g. standing for long hours), low socioeconomic and educational status, single marital status, inter-pregnancy interval less than 6 months, poor nutrition, body mass index (BMI, underweight or obese), alcohol and drug abuse, smoking and stress.[2, 5, 271, 272]

Women with a previous PTB have about 2.5-fold greater risk of recurrent PTB. The gestational age of a previous PTB is inversely related to the risk of subsequent PTB.[5] Many recurrent PTB can be attributed to persistent or recurrent genital tract infection. Diabetes, hypertension, preeclampsia, obesity, thyroid disease, asthma, anaemia, periodontal disease and mental disorders (depression), are common comorbidities that increase a woman's risk of PTB.[2, 5, 271]

Multiple gestations, short cervix at mid-trimester, history of cervical cone biopsy, fetal abnormalities, intrauterine growth restriction, vaginal bleeding, placenta previa or abruption, polyhydramnios or oligohydamnios, maternal abdominal surgery, pregnancy resulting from in vitro fertilisation are all risk factors associated with PTB.[2, 271, 272] The mechanistic pathways of how these factors stimulate PTL and PTB is poorly defined. However, they interact to provoke a transition from uterine quiescence to an active contraction state resulting in PTL or PPROM.[2, 5]

Although, it has been hypothesised that the incidence of PTB in women at risk could be reduced by routine evaluation for, and eventual treatment of common vaginal infections,[273] the microbial link between infection/inflammation and PTB remains unclarified by conventional microbiological assay techniques. Hence the need for more study of the changes in vaginal microflora in PTB using advanced molecular techniques such as 16S rRNA pyrosequencing and targeted species specific PCR. This technique has the advantage of detecting fastidious or culture-insensitive organisms and has provided a great insight into the hitherto "unculturable" or "uncharacterised" pathogenic and non-pathogenic microorganisms present in natural microbial communities such as the vagina and gut.[21, 27] Some of these organisms include *Atopobium vaginae, Megasphaera* sp., *Actinobacteridae, Bifidobacterium biavatii, Dialister* sp, *Finegoldia, Gemella, Parvimonas, Peptoniphilus, Prevotella* sp., *Leptotrichia* sp., *Sneathia sanguinagens, Eggerthella honkongensis, Veillonella* sp. etc.[21, 27] This has enabled researchers to compare the composition of the normal and BV inflicted vaginal microbiota signatures with tremendous success.[274]

1.4. 16S ribosomal RNA Pyrosequencing

The use of this high throughput technique, which involves pyrosequencing of barcoded 16S rRNA genes, has provided a clearer understanding of the vaginal microbiota signature in health and during an infection or disease.[21] It involves the use of specific and highly sensitive universal primers for amplification of the variable and/or hypervariable regions of the 16S rRNA genes,[27, 275] producing lengthy unidirectional amplicon reads required to characterise microbial community diversity and functionality devoid of cloning bias. The primers must include 454 specific sequencing adaptors, amplicon (DNA target) specific primer sequences and a multiplex identifier (MID) to barcode each amplicon and sample. After the PCR, the purified amplicon mixture can then be sequenced by the GS FLX or GS Junior sequencing systems. This will eventually produce a data set of high quality sequences that can be classified using the Ribosomal Database Project (RDP) Naive Bayesian Classifier, SILVA or "BLASTed" against a GenBank. [276, 277] This culture independent molecular technique is currently frequently applied to study human and animal microbial flora (including those of the mouth and gut segments), in health and various pathological conditions. [56, 278-282]

The 16S rRNA gene (1.542 kb long) is a constituent of the 30S small subunit of prokaryotic ribosomes encoded for by the 16S rDNA gene. It has highly conserved primer-binding sites, hence used for phylogenetic studies. Its gene sequences also contain species-specific signature sequences (5 – 10 bases long, in hypervariable regions) necessary for identification of bacteria. It has found great use in the identification and reclassification of novel species and genera of bacteria including other uncultured organisms.[283-287] The data obtained from pyrosequencing of barcoded 16S rRNA genes clearly differentiates strains of organisms and provides accurate microbiota signature. This is useful for determining the diversity and functional characteristics of the vaginal microbial community in healthy and disease conditions.

The 16S rRNA genes of bacteria broadly consist of alternating hypervariable (V1-V9) and more conserved regions (Fig. 1.3). The hypervariable regions possess appreciable sequence heterogeneity between diverse bacterial species that can be amplified and sequenced to detect and distinguish these bacterial species,[288] while the conserved regions which are parts of the genes that have experienced little degree of changes or mutations in their nucleotide sequence,

are perfect positions for universal primer hybridisation during PCRs.[289-292] The variable regions are more correctly characterised than the conserved regions.[276] Of all the hypervariable regions, V2 (137-242 bp), V3 (433-497 bp) and V6 (986-1043 bp) were shown to have the highest sequence heterogeneity and the highest discriminatory capacity in over 100 bacterial species studied.[293] V6 has the highest density of sequence heterogeneity although it is the shortest. Primer sets designed to amplify the V3-V6 regions produced reliable results in PCR assays.[293] Increased accuracy for and broadened peaks have been observed around the V2 and V4 variable regions. Thus, the semiconserved regions flanking these hypervariable regions may provide good binding sites for PCR as the read length of sequencing techniques increases.[276]

No single hypervariable region has the ability to determine the distinction among all bacterial species. Therefore, sequencing as many hypervariable regions as possible is more beneficial as this further enhances the high sensitivity, specificity and reliability of microbiome assays.[293] Specie identification with 16S rRNA genes is best when almost the whole gene is sequenced. For this reason, the regions with the highest amount of taxonomic information in the shortest nucleotide sequence within the target gene must be identified. The most useful hypervariable regions with less intra-species diversity are V1 – V6.[290, 293]

Pyrosequencing technique has been useful in the assessment of the number of OTUs (richness) and relative abundance (evenness) in microbial ecosystems.[294] Shorter amplicons (e.g. < 400 bp), produce higher richness and primer choice influenced the relative abundance of OTUs. Sequence variability of the actual hypervariable region amplified accounts for the difference in specie richness. The sequence variability of the V1 – V2 region was higher than that of V8. Differences in species evenness occur when mismatches in DNA templates are not resolved by degeneracies in the primer sequence.[294]

Also pyrosequencing has been employed in the identification and determination of genus/specie level composition and diversity. Ravel et al.[21] using barcoded broad spectrum primers in amplifying the V1 – V2 regions of 16S rRNA genes, identified the dominant genera and species in the vaginal microbiome of about 400 non-pregnant asymptomatic women and classified them into 5 different communities designated as Community State Types (CSTs). Four of the 5 communities identified were dominated by *Lactobacillus* sp., while the fifth (CST IV) had higher

proportions of mixed anaerobes such as *Atopobium, Leptotrichia, Prevotella, Megasphaera* etc. CST I was dominated by *L. crispatus*, while CSTs II, III and V had predominantly *L. gasseri*, *L. iners*, and *L. jensenii*. Women who harboured higher amounts of *L. crispatus* and *L. iners* (i.e. CST I and III), had lower vaginal pH values, whilst those with greater proportion of CST IV (mixed anaerobes), indicated the greatest pH values. Both high vaginal pH and Nugent score were directly associated with predominance of non-*Lactobacillus* sp.

Again, amplifying the V1 – V2 regions of 16S rRNA genes of 32 healthy reproductive-age women, Gajer et al. identified similar CSTs except CST V due to low prevalence of CST V in their relatively small study population. However, they reported that temporal changes in the vaginal microbiome e.g. from one CST to another produces detectable changes in the metabolite profile. For instance a change from an *L. iners* (CST III) to *Atopobium* sp. (CST IV)-dominated microbiota produced considerable changes in the metabolome characterised by high amounts of acetate and succinate with a corresponding decrease in lactate. Also, women with *L. crispatus* (CST I) and *L. gasseri* (CST II)-dominated microbiota experienced fewer transitions with low Nugent scores, indicating that these CSTs confers some degree of stability to the vaginal bacterial community.[65]

Similarly, Bai et al. analysing 23 vaginal fluid samples from 8 women, explored the same region of the 16S rRNA genes and reported higher lactate production by *L. crispatus*-dominated CST I compared with other *Lactobacillus* sp.-dominated CSTs. Interestingly, vaginal samples from CST IV dominated by other lactate producing bacteria including *Atopobium* and *Megasphaera* also exhibited varying quantities of lactate although not as high as that of CSTs I, II, III and V dominated by *Lactobacillus* sp.[295]

Furthermore, Drell *et al.*[277] obtained a total of 828, 551 16S gene sequences (average read length = 300 bp) from 494 non-pregnant asymptomatic women. Again 5 groups were identified based on their OTU composition with *Lactobacillus* sp. dominating 4, whilst the last group had predominantly *Gardnerella* sp. Increased vaginal microbial diversity was associated with abnormal discharge and high pH.

More recently, it has been reported that the prevalence of a *Lactobacillus*-deficient vaginal microbial CST IV is inversely associated with gestational age at birth. Women in this group had greater amounts of *Gardnerella* or *Ureaplasma* and were at a significantly greater risk of delivering preterm. The researchers amplified the V3 – V5 variable regions of the 16S gene of bacterial species from 4 body sites (vaginal, distal intestine, saliva, and tooth/gum) of 40 pregnant women.[75]

Collectively, these reports demonstrate that vaginal bacterial communities dominated by commensal *Lactobacillus* sp. (CST I, II, III, and V), are more stable, resistant to infection, associated with low pH, and thus indicative of a 'healthy' vaginal environment and good reproductive outcome. While CST IV predominated by mixed anaerobes with pathogenic propensity is relatively unstable, less resistant to infection, associated with high pH and Nugent score, and indicative of poor vaginal and reproductive health. However, vaginal microbiome dominated by *L. iners* (CST III), has been found to be associated with the presence of BVAB seen in CST IV, predisposing such women to BV infection.[110]

Apart from its application to the characterisation of the vaginal microbiota, 16S rRNA pyrosequencing has proved useful in the study of the bacterial composition of other sites of the human body e.g. the mouth and gut. [56, 278-282] Hutter *et al.* [296] using 27F/519R and 515F/1525R primer sets established a satisfying degree of diversity in the microbiota of periodontal lesions. The 27F/519R primer showed a greater proportion of diversity and this was attributed to the discriminatory properties of the hypervariable regions of the 16S gene. The effectiveness of this primer set can be exploited if the data available in the database are sufficient.

16S rRNA genes have found considerable suitability in the study of bacterial phylogeny and taxonomy because it is ubiquitous among bacteria, has evolutionary conserved activities, and the relatively short (1.5 kb) nucleotide sequence is easily sequenced and sufficient for extracting substantial amount of data.[286]

Overall, a multidisciplinary approach combining fetal and maternal clinical parameters, 16S rRNA pyrosequencing and other molecular techniques to characterise the complex vaginal microbiota and metabolite signatures should be more advantageous in the quest to identify

potential markers of PTB. Biochemical makers which are either intermediates or end products of several metabolic pathways determined originally by the genetic composition of the host and microbial population are a promising source of information capable of resolving the multifaceted PTB syndrome.

Figure 1.3. Bacterial 16S rRNA gene demonstrating the conserved and variable regions. Universal (broad-range) primers bind to the conserved regions to unselectively amplify the unique signature DNA sequences of the variable regions.

1.5. Biochemical markers of preterm birth

Underpinning the clinical process of cervical remodelling prior to labour is a complex series of molecular events that include increasing vascularisation and infiltration of the cervix by inflammatory cells, release of hydrolytic and proteolytic enzymes that degrade cervical matrix, and chemicals that modulate these processes (e.g. prostaglandins and nitric oxide). These molecular events are not fully understood but necessarily involve altered metabolism within cervical and vaginal cells. Whether the aerobic or anaerobic form of glucose metabolism predominates has never been described. It is plausible that the process of cervical remodelling involves a dramatic shift in the energy balance and aerobic glucose breakdown.

The pathophysiology of PTB still remains unclear; hence the need for a search for more accurate predictors. Chorioamnionitis due to female genital tract colonisation by anaerobic bacteria (that produce metabolic by-products in relation to the host), can lead to cervical remodelling, disruption of fetal membranes and leakage of FFN. Recently, biological fluids such as amniotic fluid, CVF, urine, serum, plasma, whole blood, placenta, cord blood and saliva, have been analysed to assess the predictive value of biomarkers for PTB.[297-299] Analyses of biomarkers has enhanced the understanding of the pathogenesis of conditions leading to spontaneous PTB, but only a handful of such markers have attained clinical relevance.[298] Table 3 shows the diagnostic utility of various biochemical markers for spontaneous PTL, PPROM and PTB, obtained at different points in the mid-trimester. The 2 most widely used biochemical predictors of PTB are fetal fibronectin (FFN),[300-302] and phosphorylated insulin-like growth factor binding protein-1 (phIGFBP-1).[201, 303] These are proteins indicative of choriodecidual disruption when present in CVF.

1.5.1. *Fetal Fibronectin*

Fetal fibronectin (FFN) is an extracellular matrix glycoprotein (450 kDa) produced by cells of the amnion and cytotrophoblast localised at the feto-maternal interface between the chorion and decidua, where it serves as an adhesive binding the fetal membranes to the decidua.[201, 304] In normal pregnancies, FFN is present at undetectable amounts in CVF between 16–22 weeks

gestation. However, levels ≥50 ng/ml between 22-34 weeks gestation is associated with an increased risk of spontaneous PTB.[201, 304] In fact, detection of FFN in the CVF is currently the most effective marker of PTB especially in women with threatened PTL, and is one of the best risk assessment markers for PTB in all populations.[303, 305] Detection of FFN at levels >50 ng/ml at mid to late second trimester is suggestive of membrane activation and premature separation of the fetal membranes from the uterine lining and spontaneous PTB.[306-309] In symptomatic women, the initial study to determine the predictive capacity of FFN reported sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of 81.7%, 82.5%, 83.1%, and 81.0% respectively, based on a cut-off value of ≥50 ng/ml. More recently, FFN predicted PTB within 7 days (sensitivity: 50%; specificity: 80.2%; PPV: 9.1%; NPV: 97.6%), at < 34 weeks (sensitivity: 62.5%; specificity: 82.5%; PPV: 22.7%; NPV: 96.4%), and <37 weeks (sensitivity: 50%; specificity: 85.9%; PPV: 45.5%; NPV: 88.0%), in women studied at 24-34 weeks' gestation.[310]

In asymptomatic women, Goldenberg et al. reported a high sensitivity of FFN at 22-24 weeks for predicting >60% of spontaneous PTB (<28 weeks) (sensitivity = 63%, and RR = 59.2). However, the sensitivity and PPV were low in predicting PTB at <34 weeks (i.e. 20–29% and 17–25% respectively); but a high NPV (96-97%) was maintained.[311] A similar specificity (97%) and sensitivity (22%),[312] likelihood ratio, LR of 4.0,[313] and RR = 3.8[314] predictive of delivery within 7 days as well as <34 weeks of gestation were observed in other studies of asymptomatic women. Also, high NPVs and specificities have been recorded for vaginal FFN as a predictor of PTB in similar cohorts within 14 and 21 days of index assessment.[309]

FFN is putatively the most clinically useful test for predicting PTB within 7–14 days of assessment due to its high NPV.[313, 315, 316] However, its predictive accuracy is limited by poor sensitivity and false positive results due to recent unprotected vaginal intercourse, vaginal digital examination, uterine contractions, vaginal bleeding or amniotic fluid contamination from ruptured fetal membranes.[317-319] The more recent approach of combining cervical length and FFN screening in asymptomatic[320, 321] and symptomatic women[302, 322-324] compared to FFN alone, results in a higher sensitivity and PPV to predict PTB risk, while maintaining high NPV. This guides acute management and reduces unnecessary interventions particularly in low risk women.[302]

1.5.2. *Phosphorylated insulin-like growth factor binding protein-1 (phIGFBP-1)*

phIGFBP-1 is necessary for growth and development of gestational tissues, such as the decidua, placenta, chorion and amnion.[325] It is a 25 kDa protein secreted by maternal decidual cells regardless of the isoform of IGFBP-1 in amniotic fluid, fetal plasma and maternal plasma.[303, 325] At the onset of second trimester when the amnion and choriodecidua become fused, the level of IGFBP-1 and extent of phosphorylation in amniotic fluid and decidua rises up to late gestation.[303] phIGFBP-1 concentrations have been used to evaluate cervical maturation.[201] Non-phosphorylated IGFBP-1 (npIGFBP-1) concentration is about 1000-fold higher in amniotic fluid than serum, and elevated levels of npIGFBP-1 in CVF can be diagnostic of PPROM and PTB (LR+ 3.1).[326, 327] Small quantities of phIGFBP-1 can be detected in cervical secretions at the beginning of labour when fetal membranes begin to detach from the parietal decidua. Detection of phIGFBP-1 in CVF, like FFN, at the onset of labour indicates detachment of fetal membranes from the parietal decidua and is a valuable predictor of PTB in asymptomatic and symptomatic women.[325, 328-330]

A positive test has been employed in the diagnosis of PPROM and PTB with a high sensitivity (71-94%), specificity (82-94%) and NPV (70-99%); but unsatisfactory PPV (0-83%).[329, 331-334] In symptomatic women, cervical phIGFBP-1level ≥10 µg/l is associated with a 10-fold greater risk[335] and was predictive of spontaneous PTB with high sensitivity, specificity, NPV, and considerably high PPV (48-50%).[328, 336, 337] Again, positive phIGFBP-1 in women with threatened PTL was a useful predictor of PTB at <32 weeks (LR = 2.2), and <34 weeks (LR =1.8).[325] In asymptomatic women, a pooled sensitivity (33%), specificity (79%), LR+ =1.6 and LR- = 0.8 have been demonstrated.[338]

Like FFN, phIGFBP-1 is a dependable negative predictor of PTB.[201, 303] However, in asymptomatic women, the PPV of phIGFBP-1 is significantly lower than that of FFN, while their NPVs are similar for predicting PTB.[331] But in symptomatic women the NPVs of both tests are comparable in predicting PTB within 7 days,[310, 339] at <34 and <37 weeks.[310] Fortunately, the level of phIGFBP-1 is not altered by the presence of semen or vaginal bleeding,[303] and perhaps a better test than FFN in predicting births before 34 weeks' gestation.[310]

1.5.3. *Other biomarkers*

Due to the relatively low sensitivity and PPV of the FFN and phIGFBP-1 tests in predicting spontaneous PTL, PPROM and PTB especially in asymptomatic pregnant women, alternative biomarkers have been investigated for their predictive accuracy.[303, 338] However, none of these biomarkers have been consistent in predicting spontaneous PTB in clinical settings.[338] They include activin–A, albumin/vitamin D-binding protein (VDBP),[340] alkaline phosphatase (AP), alpha-fetoprotein (AFP), angiogenin,[338] β-human chorionic gonadotrophin (hCG), Cluster of Differentiation 163 antigen,[327] Corticotropin releasing hormone (CRH), C-reactive protein (CRP), creatinine, defensins, elastase, endoglin,[338] ferritin, G-CSF, glucose,[341] IGFBP-4,[342] IL-1, 2, 6, 8, 10, 18,[327, 343, 344] IL-1 receptor antagonist (IL1RN),[345] interferon-gamma (IFN-γ), interstitial cell adhesion molecule-1, lactate, lactoferrin, lipocalin-type prostaglandin D_2 synthase (L-PGDS),[346] MMPs,[338, 347] nitric oxide,[348, 349] placental alpha macroglobulin-1 (PAMG-1), placental protein 13,[338] pregnancy associated plasma protein-A (PAPPP-A),[338, 350] pregnancy-specific β-1-glycoprotein,[338] prolactin, relaxin, salivary estriol[351] and progesterone,[352-354] sex hormone-binding globulin (SHBG),[342] sialidase, thioredoxin,[345] TNF-α, thrombin-antithrombin III complex,[338] urea, vascular endothelial growth factor[347] and proteomic profile: stratifin, desmoplakin isoform-1, and thrombospondin-1 precursor.[202, 297, 298, 326, 327, 338, 344, 349, 355] Table 1.3 summarises the sources of these biomarkers investigated and their predictive performance for prognosticating PTB. The heterogeneity of the pathogenesis of PTL and PTB, experimental design (e.g. sample sizes, inclusion/exclusion criteria, gestation age at sampling etc.), and reproducibility are the common challenges in identifying accurate biomarkers for prediction of PTL and PTB.[303] Hence, improvement in study design and methodology, combining multiple biochemical diagnostic tests that incorporate environmental and behavioural risk factors, cervical length assessment, uterine activity check, fetal DNA and genetic polymorphism, and microbiome test may improve the prediction of spontaneous PTB across diverse populations.[201, 303, 307, 356] However, increasing the range of predictive biomarkers may improve accuracy but may become cost-ineffective.

Analyses of the genome (genomics), transcriptome (transcriptomics) and protein composition (proteomics) have aided the discovery of biomarkers of PTL and PTB.[298, 357] Nonetheless, metabolomics i.e. the study of metabolites <1 kDa and characterisation of the metabolic

network (profile) of cells, is more advantageous as the metabolites are downstream products of gene expression and protein synthesis.[299, 357] This provides a closer insight into the functional activity of the cells.[299, 357] Also, it provides information on the relationship between the host, the microbiome, and the environment to give a comprehensive understanding of physiological and pathological states.[358] Metabolomics appears to be a promising novel tool in obstetrics and gynaecology that may be employed to seek to improve health care during pregnancy.[299] The metabolomics techniques currently employed include liquid chromatography-mass spectrometry (LC-MS), ultra-performance liquid chromatography-mass spectrometry (UPLC-MS), gas chromatography-mass spectrometry (GC-MS) and magnetic resonance spectroscopy (MRS).[65, 98, 295, 299, 357-360]

Evaluation and comparison of biological ecosystems by characterisation of their metabolome and finger printing using high throughput techniques, such as MRS, in pregnant women at risk of PTB compared to their term counterparts may improve the accuracy of predicting PTB.[359]

Table 1.3: Diagnostic utility of biochemical markers obtained at midtrimester for spontaneous PTL, PPROM and PTB (< 37 weeks)

Biomarker	Source of marker	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference
Albumin/VDBP	CVF	≥19000/≥90	77.8	100	100	98	340
Alkaline phosphatase	serum	90th*	14	3			298, 326
Alpha-fetoprotein	serum	>30 µg/l	90-94	95-100	94-100	91-94	202, 297, 327
β-human chorionic gonadotrophin	CVF/serum	>40-65 µIU/ml	68-95	70-95	73-91	78-97	202, 338, 350
CRH	serum	90th*	39	13			298, 326
C-reactive protein	plasma	8 mg/l	26	15			298, 326, 361
Estriol	saliva	≥2.1 ng/ml	44	92	19	98	326, 351
Ferritin	serum	>30 mg/dl	62	45			298, 362
Fetal fibronectin	CVF	≥50 ng/ml	97-98	70-97	36-93	98-100	298, 327, 340
G-CSF**	serum	90th*	49	15			298, 326
Glucose **	amniotic fluid	<25 mg/dl	59.3	91.0	85.4	71.8	341
phIGFBP-1	CVF	>10 µg/l	52.9	89.2	48.7	90.8	310, 338
IGFBP-4/SHBG	serum		75	74			342
IL-6	amniotic fluid	≥99.3 pg/ml	89.6	80.2	40.0	98.1	326, 347, 363
IL-8**	amniotic fluid	≥13.3 ng/ml	67.8	95.5	93	77.1	341, 344
IL1RN**	CVF	0.4 µg/mg	57.1	97.8	72.7	95.7	345
Lactate***	CVF	≥4.5mmol/l	86	92	92	87	364
MMP-8	amniotic fluid	5.14 pg/ml	69.4	68.3			338, 347

Nitric oxide	cervix	87.6 µmol/l	88.2	86.5	53.6	97.6	[349]
PAPP-A	blood	5th†	11	93			[338, 350]
Progesterone	saliva	3950 pg/ml	82	69	66	83	[352]
Prolactin	CVF	>30-50 µIU/ml	83	74			[202, 365]
Relaxin	serum	90th*	38	58			[298, 327, 338]
Thioredoxin**	CVF	0.4 µg/mg	64.3	97.8	75.0	96.4	[345]
TNF-α	amniotic fluid	6.3 pg/ml	81.3	79.2	36.2	96.7	[327, 363]
VEGF	amniotic fluid	25 pg/ml	91.7	75.0			[347]

† Centile
* Percentile
** Endpoint was PTB < 32-35 weeks' gestation.
*** PPROM > 34 weeks' gestation.
CRH, corticotropin-releasing hormone; *CVF*, cervicovaginal fluid; *G-CSF*, granulocyte colony stimulating factor; *IGFBP-4*, insulin-like growth factor binding protein-4; *IL1RN*, interleukin 1 receptor antagonist; *PAPP-A*, pregnancy associated plasma protein-A; *phIGFBP-1*, phosphorylated insulin-like growth factor binding protein-1; *SHBG*, sex hormone-binding globulin; *VEGF*, vascular endothelial growth factor; VDBP, vitamin D-binding protein.

1.6. Cervical length assessment in preterm birth

In many women who deliver preterm, the cervix begins to shorten weeks prior to the occurrence of PTL and PPROM. These changes can be detected by cervical ultrasonography.[366, 367] Transvaginal ultrasonography (TVUS) has been employed since the 1980s and is currently the gold standard for measuring cervical length (CL) and estimation of internal cervical os dilation.[303, 366] TVUS has the advantage of direct and clear visualisation of the cervix without the interference of bowel gas in comparison to transabdominal ultrasound.[366, 368] The average CL (at 24 weeks' gestation) i.e. the distance between the internal cervical os and external cervical os is about 35 mm and cervix <25 mm and funnelling, is considered a short cervix by several authors although there is no consensus regarding the threshold CL below which the risk of PTB increases significantly.[369] There is an association between delivery outcomes and both CL and gestational age at diagnosis of short cervix.[370] Mid-trimester CL is the single most predictive risk factor of PTB.[371-376] CL screening, an estimate of cervical competence, has some predictive clinical utility in low and high-risk women for PTB: a shortened CL <25 mm (at any gestation < 34 weeks) is associated with increased risk of spontaneous PTB.[372, 377-379] About half of the women with a CL ≤15 mm experience early spontaneous PTB.[376] Women with progressively shorter cervices seen on a mid-trimester sonogram experience increased rates of PTB (≤35 weeks)[380-382] and a reasonable proportion of these women have intra-amniotic inflammation, with a 40% risk of PTB within 7 days.[383, 384] In another study, 9% of patients with short cervix measured between 14-24 weeks had MIAC, of which 40% delivered preterm (<32 weeks).[385] In addition, mid-trimester CL is inversely related to vaginal pH, Nugent score and BV, but not after adjustment for relevant confounders.[386] In a study of women with spontaneous PTL, CL and plasma nitric oxide (which activates MMPs) measured between 24-33 weeks' gestations were negatively correlated.[349] Also, obstetric risk factors such as vaginal bleeding[387] and multiple pregnancies could influence the optimal CLs and their predictive value for PTB.[388] However, in high risk women a <25 mm CL at 16-19 weeks' gestation appears highly predictive (75%) of recurrent spontaneous PTB,[372, 378, 382]as neither the number of cases nor the gestational ages of previous PTBs impact its predictive value.[373] Its predictive value improved when CL is assessed sequentially.[382] A combination of the patients' obstetric history and CL provides a more

accurate predictive tool for spontaneous PTB than either factors alone, with improvement in the sensitivity and specificity of the screening as the degree of prematurity increases.[389]

In asymptomatic high risk women, CL <25 mm predicted spontaneous PTB (<35 weeks) with LRs+ of 4.3 (<20 weeks' gestation), 2.8 (20-24 weeks), and 4.0 (>24 weeks).[377] Among asymptomatic women with twin pregnancies, mid second trimester CL ≤20 mm was predictive of PTB (<32 and <34 weeks) with sensitivities, specificities, LR+ and LR- of 39% and 29%, 96% and 97%, 10.1 and 9.0 and 0.64 and 0.74, respectively. A CL ≤25 mm at same gestation predicted PTB (<28 weeks) with LR+ of 9.6.[390]

In women with threatened PTL and intact membranes, CL (≤20.5 mm) had a sensitivity of 88% and specificity of 54% for prediction of delivery at <34 weeks, <32 weeks, and within 48 hours and 7 days of assessment.[325] An earlier study using a cut-off of <25 mm had reported sensitivity of 80%, specificity of 97%, PPV of 80% and NPV of 97%, for prediction of delivery <35 weeks and within 7 days.[391] Using a criteria of <15 mm, Sotiriadis et al. reported that CL measurement can identify symptomatic women at risk of delivering within 7days (sensitivity = 59.9%, specificity = 90.5%, LR+ = 5.7, and LR- = 0.5).[392] The LR+ for PTB within 7days in another study was higher (8.7) at a cut-off of <15 mm compared to 0.4 when the cut-off is ≥15 mm.[393] At a higher cut-off of 30 mm (26-34 weeks), the predictive accuracy of CL for PTB was 83.0 with sensitivity, specificity, PPV and NPV of 68-100%.[354] In cases of CL between 15-30 mm, a combination with FFN test enhances the detection of women at low risk of spontaneous delivery within 7 days.[394] There is inadequate indication to suggest routine CL screening for both symptomatic and asymptomatic women. Further investigation is necessary as a non-significant association between knowledge of CL result and lower prevalence of PTB (<37 weeks) in symptomatic women has been observed.[395]

Ultrasonographic CL assessment is a safe and clinically useful technique to determine increased risk of PTB in selected women.[396] However, there is currently no consensus regarding the threshold CL below which the risk of PTB is considerably increased. Also, as a result of its low PPV and sensitivities, routine CL screening is not recommended in low risk women with about 4.3% incidence of PTB.[397] Consequently, some investigators have argued that universal screening in the general obstetrical population is unwarranted.[396] Its effectiveness has also been challenged

due to the absence of a proven intervention once a short cervix is diagnosed.[398] Hence, there is a need for a more accurate biomarker of PTB with potential universal applicability regardless of risk and/or symptom statuses of the patients.

1.7. Preventive interventions to reduce the incidence and complications of preterm birth

Over the years, evidence based insight into the pathophysiology of PTB has led to the development of several therapeutic interventions to prevent its occurrence and/or reduce the effects of its deleterious complications. The primary objective of these treatments is to defer delivery until the fetal organs are fully developed to sustain the survival of the newborn outside the uterine compartment or to when adequate perinatal care is available. There are also treatments administered to protect the vital organs of the conceptus from the devastating complications that accompany uncontrolled intrauterine infection and inflammation leading to PTL. Lifestyle modifications before and during pregnancy such as smoking cessation, nutritional adjustment, reduced BMI as well calcium, folic acid and vitamins supplementation have been advocated.[399, 400] Generally, these interventions are directed to all women of reproductive age (i.e. lifestyle modification, dietary supplementation, prenatal care and screening of low risk women), targeted at minimising or eradicating identified risk factors (e.g. antibiotic, progesterone, cervical cerclage and tocolytics), or intended to prevent complications and improve survival of preterm infants (e.g. corticosteroids).[399]

Antibiotics

Antibiotics treat BV and other bacterial genital infections by reducing the overgrowth of pathogenic aerobic and anaerobic species and reinstate the stability of the protective Lactobacilliary microflora thereby preventing the development of inflammation-induced PTL and PTB.[188, 195] Routine prescription of antibiotics for women at risk of PTB has been controversial. Antibiotic treatment eradicates BV during pregnancy but does not reduce the risk of PTB significantly.[188, 401] In women with PPROM, antibiotics delay delivery and improve neonatal morbidities in the short term but does not significantly reduce perinatal mortality.[402] Although maternal infection may be reduced, prophylactic antibiotics did not show great improvement in

important neonatal outcomes for women in PTL with intact membranes.[403] Despite these limitations, reduction in the incidence of PTB has been reported with treatment before 20 weeks gestation.[399]

Progesterone

Progesterone (often administered as17-hydroxyprogesterone caproate) reduces the risk of PTB by preventing uterine contraction and cervical remodelling, decreasing the production of prostaglandins and inflammatory mediators (e.g. IL-6).[199, 404] In a recent randomised, placebo-controlled study with singleton pregnancies, prophylactic vaginal progesterone reduced the rate of PTB, the risk of a low birth weight, the rates of respiratory distress syndrome (RDS) and admission to neonatal intensive care unit (NICU) in women at risk of PTB. Also, with a shortened second trimester CL (≤ 25 mm), vaginal progesterone reduced the risk of PTB and neonatal morbidity and mortality without any neurodevelopmental adverse effect. Hence, regardless of the history of spontaneous PTB, vaginal progesterone has been recommended for ultrasound-indicated short cervix at 18-24 weeks gestation.[404, 405] However other reports did not indicate any benefit of progesterone in relation to PTB and poor neonatal outcome.[406]

Cervical cerclage

The length of the cervix at mid-trimester is inversely related to risk of PTB. Reduction in CL and effacement (widening) can arise from biochemical, infection and inflammation-related factors.[399, 407] Cervical cerclage is a surgical procedure that involves sticking a suture (stitch) around the uterine cervix aimed at preventing cervical shortening and effacement, thereby reducing the risk of PTB.[408] It appears to be most adequate in women with structurally (anatomical) defective or incompetent cervix,[399] and history of PTB.[409] It reduces the rate of PTB in women with singleton pregnancies at risk of recurrent PTB but has not shown significant effectiveness against perinatal morbidity and mortality.[410] Its effectiveness in women with multiple gestations has not been established,[408] and poor delivery outcomes have been reported when inflammation is present.[399] However, in combination with prolonged antibiotic therapy (e.g. azithromycin), its prophylactic and therapeutic efficacy for PTB, fetal mortality, low birth weight and rate of abortion is significantly improved.[411]

Corticosteroids

Infants born prematurely may have underdeveloped lungs and other vital organs inevitable for survival ex-utero. Corticosteroids have proven effect on fetal lung development and maturation. They also stimulate the development of other important organs that sustain postnatal adaptation including brain, heart, kidneys, thyroid etc.[412] Corticosteroids improve fetal lung function by inducing the production of surfactant, stimulating the development of alveolar structure, increased vascularisation and alveolar fluid clearance. As a result, antenatal corticosteroids reduce the incidence of neonatal mortality, respiratory distress syndrome (RDS), and other neonatal complications such as intraventricular haemorrhage, necrotising enterocolitis, and patent ductus arteriosus.[412, 413] Oral or intramuscular dexamethasone and bethamethasone are currently the widely used corticosteroids clinically and have been effective in improving short- and long-term sequelae in premature infants.[413]

Tocolytics

Tocolytics are anti-contraction drugs also known as labour represents. They are medications used to delay PTL and PTB, providing enough time (usually 24-48 hours) for intrauterine transfer to a specialist centre, permit overall fetal maturation and antenatal corticosteroids to have clinical effect i.e. production of adequate amount of surfactant in the lungs.[414] There is no evidence that tocolytics reduce the rate of PTB.[399]

A wide variety of tocolytic agents have been employed to inhibit uterine contraction and postpone delivery by affecting the interaction of contractile proteins in the myometrium or inhibiting the synthesis/ blocking the activity of myometrial stimulants.[414] They include: β-adrenergic agonists (Salbutamol, terbutaline, fenoterol), Calcium channel blockers (nifedipine), oxytocin receptor antagonists (atosiban), cyclo-oxygenase inhibitors (indomethacin), and myosin light chain inhibitors (Magnesium sulphate).[414-418] Amongs these agents, the β-adrenergic agonists are the most widely used but with a high frequency of severe maternal side effects.[417] Interestingly, calcium channel blockers and oxytocin receptor antagonists have lesser side effects[417] and are capable of delaying delivery for up to 7 days.[399]

1.8. Magnetic Resonance Spectroscopy

An approach to demonstrating the vaginal host-microbial changes associated with PTB is to determine the metabolite fingerprints resulting from these interactions. These metabolic changes which are direct reflections of vaginal bacterial community function can be detected by Magnetic Resonance Spectroscopy (MRS) also known as Nuclear magnetic resonance (NMR).[65, 295] MRS is a research technique that utilises the magnetic characteristics of certain atomic nuclei to ascertain the physical and chemical properties of atoms or molecules in which they exist. It differs from magnetic resonance imaging (MRI), in that it detects the resonance frequency of chemical compounds other than water allowing for depiction of internal chemistry, whilst MRI which is achieved through the application of linear magnetic field gradient on an existing magnetic field, detects the resonance of hydrogen nuclei in water and fat to produce high-resolution images of the gross internal anatomy of body tissues and organs.[419, 420] Nuclear magnetic resonance occurs when an MR-active nucleus (i.e. ^{1}H, ^{13}C, ^{19}F, ^{23}Na, ^{29}Si, ^{31}P etc.),[419, 421-423] changes its spin state driven by the absorption of electromagnetic radiation at a specific resonance frequency (Larmor precession), that is dependent on the strength of the magnetic field and the magnetic properties of the atomic isotope.[424, 425] MRS signals arise from the interaction between MR-active nuclei and a strong magnetic field.[423, 424] This can provide useful insight into the molecular structure, dynamics, reaction state, and chemical environment of molecules.[424, 426] It has the capacity to determine the relationships between specific nuclei within or between molecules. Though MRS can be applied to any sample containing MR-detectable nuclei (nuclei possessing spin), it is often used to study the molecular properties of organic molecules. One-dimensional (^{1}H or ^{13}C), 2-dimensional (^{1}H-^{1}H or ^{1}H-^{13}C) (for small compounds) and 3, 4, or 5-dimensional experiments (for macromolecules e.g. proteins, nucleic acids and polysaccharides)[422] can be performed. At least one MR-active isotope exists in almost every element.

1.8.1. Nuclear spin, resonance and magnetisation

The charged and spinning nuclei of atoms have a nuclear spin quantum number, I, which is in multiples of ½ e.g. ½, 1, 3/2 etc. Atomic nuclei with $I = 0$ possess no nuclear spin and are 'MR-

inactive' e.g. ^{12}C and all nuclei with even atomic mass and atomic numbers. The spin > 0 nuclei possess angular momentum and are capable of forming magnetic dipoles.[427] These magnetic dipoles, like those of bar magnets (north and south poles), are normally randomly oriented. MR signal is not generated at this state as there is no net magnetic field emanating from them. However, when placed in a strong external and static magnetic field, $\boldsymbol{B_0}$, $2I+1$ spin energy states are created, e.g. for a spin-½ nucleus, 2 spin states are formed; one energetically favourable spin (α state) parallel to the external magnetic field and a higher energy spin (β state) antiparallel to the external magnetic field (Fig. 1.4). At equilibrium a small excess of nuclei exist in the α spin state. The parallel spin which is of lower energy aligns with $\boldsymbol{B_0}$, while the other opposes it resulting in a net magnetic moment, $\boldsymbol{M}$, from the sample.[419] Although MR is a quantum mechanical phenomenon it can be more intuitively understood from a classical interpretation (Fig. 1.4).

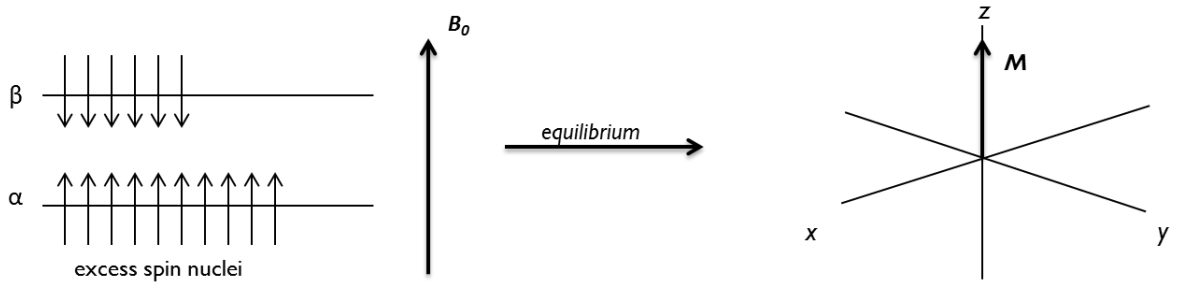


Figure 1.4. At equilibrium, the excess α spin state places the net magnetic moment, *M*, parallel to B_0 on the +z axis.

The application of a second magnetic field, $\boldsymbol{B_1}$, perpendicular to $\boldsymbol{B_0}$, displaces the net magnetic moment, $\boldsymbol{M}$, from alignment with the $\boldsymbol{B_0}$ i.e. away from equilibrium towards the *x-y* plane. Removal of $\boldsymbol{B_1}$ allows the magnetic moment to precess about $\boldsymbol{B_0}$ along the *x-y* plane, thereby generating radio frequency (rf) signals in the detection coil (Fig. 1.5).[419] A pulse is applied when an rf irradiation is turned on and then off after a specific time interval e.g. 90° pulse. In MR experiments, pulse sequences (series of timed rf pulse magnetic field gradients) are used to exploit the magnetic properties of nuclear spins in order to obtain the required data.[427]

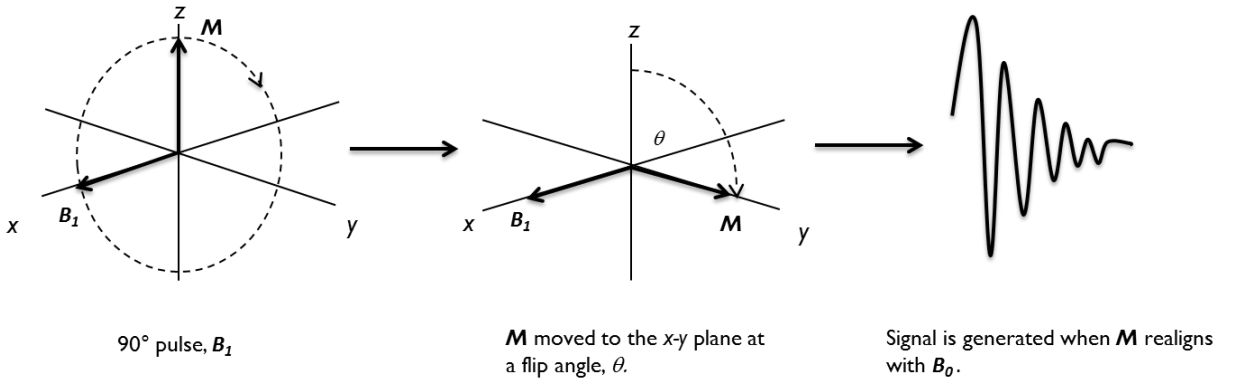


Figure 1.5. Signal is generated when the net magnetic moment is "flipped" out of equilibrium (z axis), towards the *x*-*y* plane by the applied rf pulse.

The circular motion of the magnetic moment around $\boldsymbol{B_0}$ is called Larmor precession. The rate of precession (Larmor frequency) is defined as:

$$\nu = \frac{-\gamma B_0}{2\pi}$$

where γ = magnetogyric ratio (a measure of the magnetic properties of a particular nuclide), ν is expressed in Hertz (Hz), and $\boldsymbol{B_0}$ in Tesla (T). The direction of the motion though constant for any given nuclide, can be either clockwise on anticlockwise depending on the sign of γ.

NMR is achieved when an MR-active nucleus changes its spin state stimulated by the absorption of electromagnetic radiation, which is at a specific resonance frequency (Larmor precession), that depends on the strength of the magnetic field, $\boldsymbol{B_0}$, and the magnetic properties of the isotope of the atom. The energy difference (ΔE) between the 2 spin states is proportional to $\boldsymbol{B_0}$. This is expressed as:

$$\Delta E = \frac{h\gamma B_0}{2\pi}$$

where *h* is the Plank's constant. For the resonance condition to be met, the excitation field must be at the same frequency as the Larmor precession i.e. the resonance frequency of the spin nuclei, hence the name nuclear magnetic resonance.

Through a process called Fourier transformation (FT), nuclear spins can be stimulated simultaneously using a single pulse radio frequency (rf), acquiring time-domain response and converting it to frequency-domain spectrum. This allows averaging of the signal to increase the signal-to-noise ratio and an MR spectrum from which metabolites can be identified is generated.[419, 427]

The signals detected as the resonating nuclei realigns along $\boldsymbol{B_0}$, possess a distinctive frequency which is dependent on the magnetic properties of the isotopes of the atoms (e.g. ^{1}H, ^{13}C, ^{23}Na, ^{31}P), and the strength of the magnetic field. In other words, MR signals are isotope specific with each signal strongly linked to an element or nuclide.[419, 428] The size of the signal is proportional to the number of nuclei present provided the signal is acquired with adequate relaxation decay. All signals are detected simultaneously. These signals decay via the transfer of energy from the excited nuclei into its surroundings (i.e. relaxation). The relaxation varies from one sample to another depending on the molecular environment. Signals relax to equilibrium via 2 energy transfer processes; T_1 and T_2.[419, 427]

T_1 - longitudinal relaxation, is the loss of excess energy into the surrounding molecular lattice (spin-lattice relaxation), as net magnetisation is returned to the *z*-axis (Fig. 1.6). The lattice is the sample in which the nuclei are contained. Usually T_1 ranges between 0.5-5s for medium-sized organic compounds, and a minimum period of $5T_1$ is required for nuclei to relax completely after a 90° pulse. This is because T_1 decays exponentially. At $5T_1$ about 99.3% magnetisation recovery has taken place.[427]

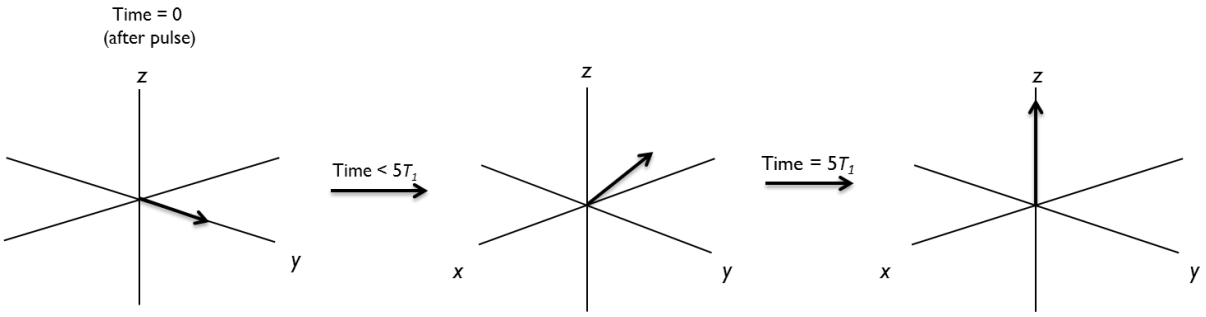


Figure 1.6. Longitudinal relaxation. Recovery of the magnetisation along the z axis establishing equilibrium after pulse excitation.

T_2 - transverse relaxation (Fig. 1.7), is the loss of net magnetisation in the *x*-*y* plane as seen in the free induction decay (FID). It is due to differences in the magnetic field within the sample which arises from 2 sources: heterogeneity of the static magnetic field (reduced by "shimming"), and mainly from the sample intra- and intermolecular interactions-stimulated local magnetic fields. It is also referred to as spin-spin relaxation because it defines the exchange of energy between adjacent nuclei that precess at the same frequencies but different local magnetic fields. In other words, magnetisation decays away due to each spin experiencing different local magnetic fields i.e. loss of phase coherence on the *x*-*y* plane.[427]

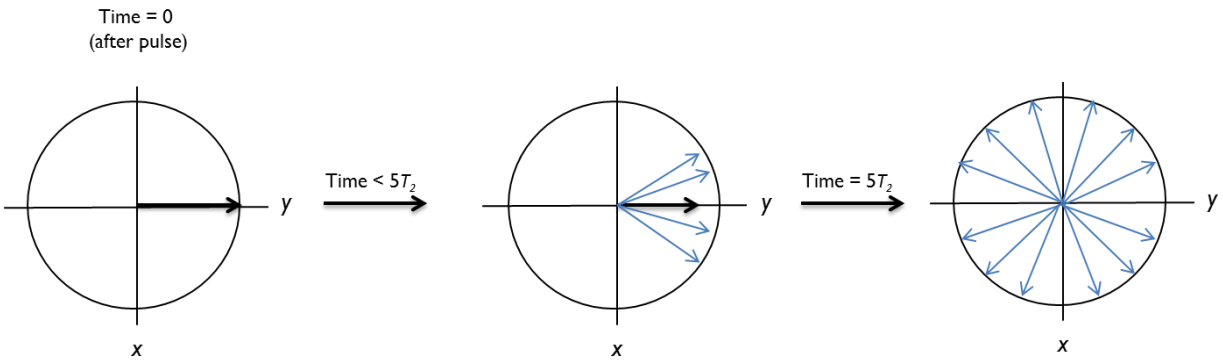


Figure 1.7. Transverse relaxation. No net magnetisation as a result of differences in local magnetic fields within the sample.

Therefore, by placing a sample containing nuclei possessing spin in a magnetic field and exciting it with a second magnetic field (quantum of energy), the structure, dynamics, reaction state and chemical environment of its molecules can be studied noninvasively and non-destructively.[419]

Chemical shift

The practicality of MRS is based on the evidence that atomic nuclei that differ in chemical structure resonate at different frequencies in the same magnetic field. This is due to differences in the electronic environment of the protons. With the aid of their electrons, different chemical compounds shield their nuclei from the applied $\boldsymbol{B_0}$ by different degrees. For resonance to occur, the applied magnetic field must be greater than the local magnetic field generated by the electrons "shielding effect". This is called chemical shift,[419, 423, 428] and described by:

$$v = \frac{\gamma}{2\pi} B_0 (1 - \sigma)$$

where σ is the shielding constant and usually reported in parts per million (ppm). The chemical shift provide information about the structure of compounds as the different chemical groups contained in the sample generate correspondingly varying signals at different frequencies.[419] In ^{1}H-MR, most molecules have a chemical shift range of 0-20 ppm and defined relative to a reference compound, e.g. 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP). An example as shown in Fig. 1.8, is the ^{1}H-MR spectrum of ethanol (CH_3-CH_2-OH), in which 3 signals (CH_3 – 1.17 ppm, CH_2 – 3.65 ppm, and OH – 5.19 ppm), corresponding to the 3 hydrogen groups, with the peak intensities proportional to the number of ^{1}H nuclei in each chemical group (Fig. 1.8).[427] In addition, adjacent MR-active nuclei influence the resonance frequencies in a manner dependent on the chemical bonds within the molecule (J or scalar or spin-spin coupling). J-coupling indicates how nuclei can sense the presence and number of neighbouring nuclei and importantly provides additional structural information via chemical bonds present in the molecule.[421, 429] By analysing the shape, area and signal intensities of MR spectral peaks (integration), the unique molecular structure and concentration of a particular compound (metabolites) in a biological sample can be determined.

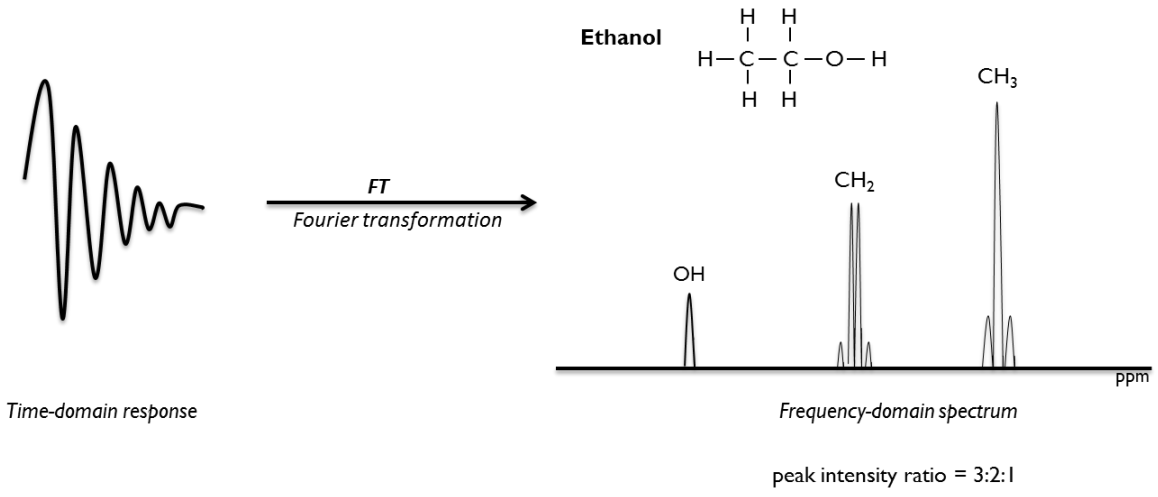


Figure 1.8. ^{1}H-MR chemical shift of ethanol indicating the frequencies and peak intensity ratio. Time-domain signals are converted to frequency domain spectra via Fourier transformation.

MRS can qualitatively and quantitatively elucidate the metabolic profiles of tissues and biofluids, thereby providing biomarkers that indicate physiological and pathological states. It is an efficient non-invasive and non-destructive technique that can be used to distinguish a large variety of metabolites in body fluids including serum, urine, faeces, spinal fluid, vaginal fluid, follicular fluid,[65, 295, 430-433] and provide insight into the metabolic activities of organs such as the heart, breast, cervix, prostate, liver, stomach, pancreas, colon, skeletal and vascular smooth muscle, kidneys and brain.[423, 432, 433] It has also been applied to identify the chemotypes of microoganisms,[434] as well as in food,[435] drug and plant biochemistry.[421, 436]

1.9. Aim of Study

The interaction between genital tract bacterial colonisation and PTB remains unsatisfactorily determined by conventional assay techniques. There may be an association between the vaginal microbial community composition and the metabolite signature of cervicovaginal fluid. An altered vaginal ecosystem have been indicated in the progression of ascending intrauterine infection or MIAC leading to PTL,[8] and could influence the initiation of PTB.[1] The biochemical activities and resultant metabolic by-products of the vaginal microbial population during gestation can provide useful insight into the pathophysiology of PTB as well as help in identifying women at risk.[358] These metabolic changes, which are reflective of vaginal bacterial community function, leave fingerprints that can be investigated by MRS.[65]

Accurate identification of women at risk of PTB remains challenging. Given that two-thirds of spontaneous PTBs are associated with infection/inflammation in reproductive and gestational tissue, investigation of the microbiome and metabolic patterns associated with PTB is topical.

Hypothesis: It was hypothesised that PTB is associated with detectable early linked "signature" microbial and metabolite patterns. In other words, women who go on to deliver prematurely will have significantly different vaginal bacterial specie composition and ^{1}H-MR metabolite signatures compared to their term counterparts even in the absence of clinical infection.

Therefore, in order to characterise the functional identity of the vaginal microbiome during pregnancy and elucidate an integrated picture of microbiome composition and activity in relation to delivery outcome, we performed an MRS metabolite study and PCR assessment on CVF of different cohorts of asymptomatic pregnant women and women presenting with symptoms of threatened preterm labour across the second trimester.

This study focussed on metabolic changes in the vaginal microenvironment early in pregnancy in (2nd trimester), i.e. where an early diagnostic tool aligned with standard pregnancy checkpoints would be advantageous. In parallel, we assessed the prevalence of specific vaginal commensal and potentially pathogenic bacterial species in these patients while assessing other factors indicative of the state of the vaginal environment (e.g. pH).

On the whole, we studied three broad cohorts of pregnant women:

- A group of asymptomatic pregnant women at a lower risk of PTB presenting at 20-22 weeks gestation with a normal cervical length (>25 mm), and/or without prior PTB.
- A group of asymptomatic high-risk pregnant women with a previous history of PTB at 20-22 weeks, and at 26-28 weeks. Primary study end point was delivery before 37 weeks and the secondary endpoints was delivery before 32 weeks.
- A group of symptomatic pregnant women presenting on labour ward with symptoms reminiscent of preterm labour (regular uterine contractions but cervix <3 cm dilated). Predictive endpoint was delivery within two weeks of index assessment while secondary endpoints were delivery before 32 and 37 weeks of gestation.

1.9.1. Specific study objectives

- To determine if the ^{1}H-MR spectrum of CVF can identify women who ultimately deliver prematurely from cohorts of women at high or low risk of PTB, and pregnant women presenting with symptoms of threatened preterm labour.

- To characterise and validate the metabolite profile of CVF of a cohort of pregnant women by ^{1}H-MRS and spectrophotometry, and determine their predictive capacity for PTB.

- To investigate the relationship between CVF fetal fibronectin, ultrasound cervical length, and vaginal microbiota metabolites of pregnant women in relation to gestational age at delivery.

- To identify the cellular composition of the CVF sample and determine the vaginal bacterial specie composition by PCR during pregnancy.

Chapter 2

Determination of cervicovaginal fluid metabolite profile during pregnancy by Magnetic Resonance Spectroscopy

2.1. Recruitment of study participants at different gestations and sample collection

2.1.1. Ethical approval

These studies were reviewed and approved by the Yorkshire & Humber (Sheffield) Committee of the UK National Research Ethics Service (REC Number 13/YH/0167).

2.1.2. Recruitment of study participants

The study participants (N = 458), mainly Caucasians (>80%), comprised of two clinical categories of pregnant women: those that had no symptoms of PTL (asymptomatic group) and those presenting to the delivery suite with symptoms of, but not established, PTL. The asymptomatic pregnant women were further classified into 2 gestationally-matched groups based on a prior history of PTB: a low-risk group (ALR, n = 183), who had no history of PTB (assessed at 20-22 gestational weeks, w), and a high-risk group (AHR, n =186), who had a previous history of PTB and/or short cervix (< 25 mm) on transvaginal ultrasonography (assessed at 20-22w and repeated at 26-28w). The third study group (SYM, n = 89) comprised women presenting with uterine contractions, cervix < 3cm dilated, and intact fetal membranes (24-36w). All participants were recruited via the antenatal clinics and Triage Delivery Suites of the Jessop Wing Hospital, Sheffield, UK. The asymptomatic women were sampled at 20-22w and 26-28w as part of their normal routine visits for antenatal care. Also, these are early critical gestational time points in which an accurate diagnostic marker for PTB can promptly inform therapeutic decisions and interventions before the onset of PTL and adverse pregnancy outcome. On the other hand, the symptomatic women presented at random and were studied between 24-36w in other to exclude pregnant women with miscarriages (i.e. loss of pregnancy before the first 23w). These women were recruited via the Triage Delivery Suites as they presented with symptoms suggestive of PTL.

2.1.3. Sample collection

At presentation, a pair of high vaginal samples was obtained with dry polystyrene Dacron swabs (Deltalab Eurotubo 300263, Fisher Scientific, UK) from each woman by a single clinical research staff following written informed consent. This was to ensure consistency in the sample collection protocol. With a subset of the AHR women assessed at 2 gestational time points i.e. at 20-22w and 26-28w, the total number of CVF samples obtained rose to 587 (i.e. ALR20-22w

= 183; AHR20-22w = 186; AHR26-28w = 129; SYM = 89).The collected samples were immediately processed or stored in a refrigerator at -20°C, for up to 3 days, pending analysis. The clinical course and delivery outcomes of participants were subsequently ascertained. Women with multiple gestation, bacteriologically proven infection, history of abnormal cervical cytology within 3 years, ruptured fetal membranes, and prior vaginal examination at presentation were excluded from the study. Parity was not considered because it is not a known risk factor of PTB independently.[437, 438]

Also at presentation, commonly employed clinical diagnostic methods of PTL and PTB including CVF FFN level, vaginal pH and ultrasound cervical length (CL) were examined. Quantitative CVF FFN levels were analysed using the 10Q Rapid FFN analyser (Hologic, MA), according to manufacturer's instruction, and cervical length (CL) by transvaginal ultrasonography. Vaginal pH was determined by obtaining a sample of vaginal fluid from the lateral vaginal wall with the aid of a dry swab and smeared on a high quality narrow range pH paper (pH-Fix 3.6-6.1, #92130, Macherey-Nagel, Düren, Germany). All measurements were performed by the same clinical research staff at the Jessop Wing Maternity Hospital.

2.1.4. Subject details and Pregnancy outcomes

Table 2.1 summarises the clinical details of the study cohorts. At present (30/10/2015) only 408 out of the 458 (89%) pregnant women recruited in this study have their delivery outcomes ascertained. About 4% (n = 5) of the ALR women delivered preterm and this was the lowest prevalence of PTB observed compared to women in the other groups: AHR 20-22w (33%, n = 35), and AHR 26-28w (28.6%, n = 26). Of women presenting with symptoms suggestive of PTL (SYM), 81.7% (n = 67) went on to deliver at term, while 18.3% (n =15) delivered preterm, with mean gestation between presentation and delivery of 15.7±3.5 and 60.3±3.2 days for preterm- and term-delivered SYM women respectively. All the preterm-delivered women in the SYM group delivered approximately within 2 weeks (15.7±3.5 days) of presentation at the Triage Delivery Suites of the Jessop Wing Hospital, Sheffield, UK.

Table 2.1: Clinical characteristics of the study participants

Characteristics	Asymptomatic Low risk women, 20-22w		Asymptomatic High risk women, 20-22w		Asymptomatic 20-22w (Combined)		Asymptomatic High risk women, 26-28w		Symptomatic women 24-36w	
	Preterm (N=5)	Term (N=124)	Preterm (N=35)	Term (N=71)	Preterm (N=40)	Term (N=195)	Preterm (N=26)	Term (N=65)	Preterm (N=15)	Term (N=67)
Age (years)	29.8±2.6 (22-36) 5	29.4±0.4 (19-39) 123	31.9±0.9 (19-45) 34	30.9±0.6 (19-39) 70	31.6±0.8 (19-45) 39	29.9±0.4 (19-39) 193	31.1±0.9 (24-40) 26	30.9±0.7 (19-40) 65	31.1±2.0* (22-48) 15	26.6±0.7 (16-44) 67
BMI ($kg.m^{-2}$)	23.4±1.2 (21.1-26.3) 5	25.5±0.4 (14.7-42.2) 123	28.0±1.1 (18.2-46.5) 34	27.5±0.7 (18.2-49.7) 70	27.5±1.0 (18.2-46.5) 38	26.2±0.4 (14.7-49.7) 193	27.2±1.0 (18.2-34.5) 26	28.6±0.8 (18.2-49.7) 65	28.9+1.9[a] (20.1-42.5) 11	25.1±0.6[a] (17.4-41.6) 56
Previous history of PTB (n)	NA	NA	23	48	23	48	21	47	5	10
Cigarette Smokers n(%)	2(40)	11(9)	8(23)	13(18)	10(25)	24(12)	5(19)	13(20)	1(7)	10(15)
Cervical length (mm)	36.2±1.6 (32-40) 1	40.7±0.4 (29-66) 124	29.4±1.9 (13-53) 30	36.7±1.0* (18-52) 65	30.4±1.7 (13-53) 35	39.3±0.5* (18-66) 189	24.8±2.3 (7-44) 26	33.9±0.8* (15-50) 37	21.1±4.8[a] (7-45) 8	30.8±1.6*[a] (11-54) 37
Fetal fibronectin conc. (ng/ml)	6.2±1.2 (4-10) 5	16.0±2.6 (1-187) 121	85.7±22.1* (2-453) 29	32.3±8.6 (1-497) 63	74±19.5* (2-453) 34	21.6±3.4 (1-497) 184	91.8±26.1* (1-442) 24	25.8±6.8 (1-248) 59	188±85*[a] (5-501) 6	15.2±3[a] (1-74) 37
Gestational age at presentation (days)	143±2.1 (139-151) 1	141±0.5 (132-159) 123	146±1.5 (115-165) 35	147±1.2 (103-175) 68	146±1.4 (115-165) 40	143±0.6 (103-175) 191	190±1.1 (182-205) 26	188±0.7 (173-203) 65	206±5.4 (177-237) 15	213±3.1 (139-254) 67
Gestational age at delivery (days)	225±13.1 (189-254) 5	281±0.8 (258-299) 124	225±5.2 (146-258) 34	274±1.0 (259-292) 71	225±4.8 (146-258) 39	279±0.7 (258-299) 195	233±4.1 (187-258) 25	274±1.1 (259-295) 65	221±5.7 (187-257) 15	275±1.1 (260-295) 61
Vagina pH	4.2±0.2 (3.6-4.8) 5	4.0±0.03 (3.6-5) 124	4.2±0.1 (3.6-5.3) 30	4.2±0.1 (3.6-5.6) 63	4.2±0.1 (3.6-5.3) 35	4.1±0.03 (3.6-5.6) 187	4.3±0.1 (3.6-6.1) 26	4.2±0.1 (3.6-6) 62	4.4±0.3[a] (3.6-6.1) 7	4.2±0.1[a] (3.6-6.1) 36
Prevalence of PTB,%	3.9		33.0		17.0		28.6		18.3	

Data are presented as mean ± SE (range, n); *w*, gestational weeks; *BMI*, body mass index; *PTB*, preterm birth; *NA*, not applicable. *N*, total number of term- or preterm-delivered women in each cohort; *n*, actual number of women for each clinical parameter obtained.

* Differences between preterm and term-delivered women within the group, $P < 0.05$ (shaded yellow).

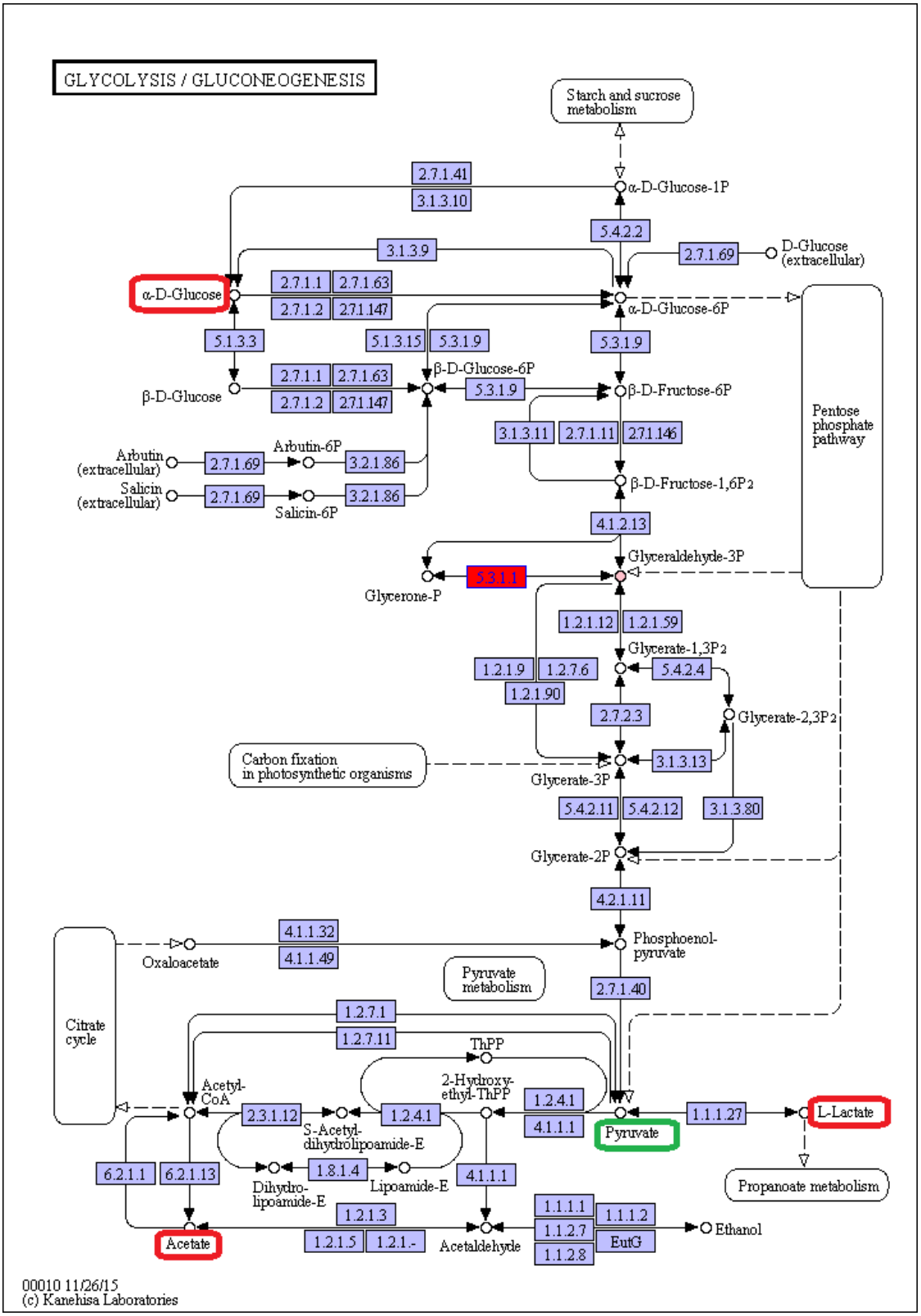
[a] Reduced study population (n) due to absence of participants' consent/data.

2.2. Introduction to ^{1}H-Magnetic Resonance Spectroscopy

With the observation of strong resonance signals from lactate, acetate, succinate, amino acids and glucose in CVF analysed with proton magnetic resonance (^{1}H-MR) spectroscopy,[65, 295, 439] it is plausible that the metabolic profile of these secretions, which is a reflection of the metabolic activities of the host interacting with the vaginal microbiota, can be elucidated both in pregnant and non-pregnant reproductive-age women using this technique. ^{1}H-MR can also provide useful information regarding the functional capacity or chemical consequence of the prevailing vaginal microbial community composition at each instance. ^{1}H-MR metabolite profile provides qualitative and quantitative data on the many different small molecules present in a sample at μM to mM concentration.[440] Additionally, MR-based metabolomics gives direct data on the identity of the metabolites that are implicated in the disease under investigation, because information on the altered metabolite signatures is directly obtained through statistical analysis of the MR metabolite profiles.[441, 442] These unique features of ^{1}H-MR were recently explored to investigate the association between vaginal fluid microbial community composition and metabolic profiles in healthy and BV-infected non-pregnant reproductive-age women.[189, 439] Both human and bacterial metabolites involved in complex metabolic pathways as shown in Fig. 2.1, were identified. As a result, determination of the changes and differences in CVF metabolite profiles which are direct reflections of biochemical events in the vaginal microenvironment could be of immense benefit in the diagnosis and prognostication of vaginal dysbiosis during gestation and PTB.

We therefore sought to characterise the metabolite profile of CVF obtained from cohorts of pregnant women who eventually deliver prematurely compared to those who do not, classifying the participants according to their risk of PTB, and by presentation with symptoms of PTL. We hypothesised that the ^{1}H-MR spectrum of CVF might predict pregnant women who ultimately deliver prematurely, as well as women with symptoms of PTL who deliver shortly after assessment.

A.


GLYCOLYSIS / GLUCONEOGENESIS
Starch and sucrose metabolism
α-D-Glucose-1P
D-Glucose (extracellular)
α-D-Glucose
α-D-Glucose-6P
β-D-Glucose
β-D-Glucose-6P
β-D-Fructose-6P
Pentose phosphate pathway
Arbutin (extracellular)
Arbutin-6P
Salicin (extracellular)
Salicin-6P
β-D-Fructose-1,6P2
Glyceraldehyde-3P
Glycerone-P
Glycerate-1,3P2
Glycerate-2,3P2
Carbon fixation in photosynthetic organisms
Glycerate-3P
Glycerate-2P
Phosphoenol-pyruvate
Oxaloacetate
Pyruvate metabolism
Citrate cycle
ThPP
Acetyl-CoA
2-Hydroxy-ethyl-ThPP
S-Acetyl-dihydrolipoamide-E
Pyruvate
L-Lactate
Propanoate metabolism
Dihydro-lipoamide-E
Lipoamide-E
Acetate
Acetaldehyde
Ethanol
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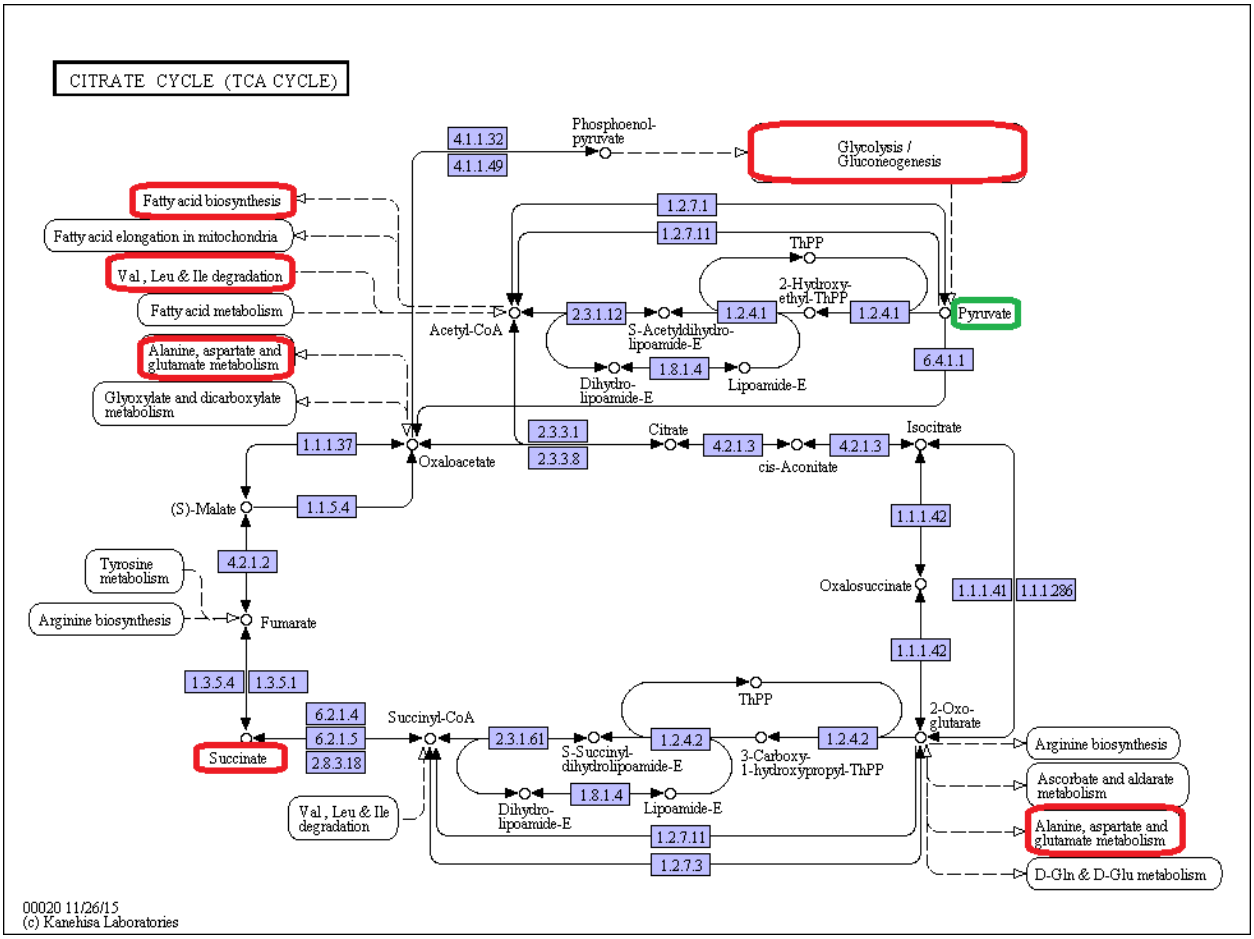
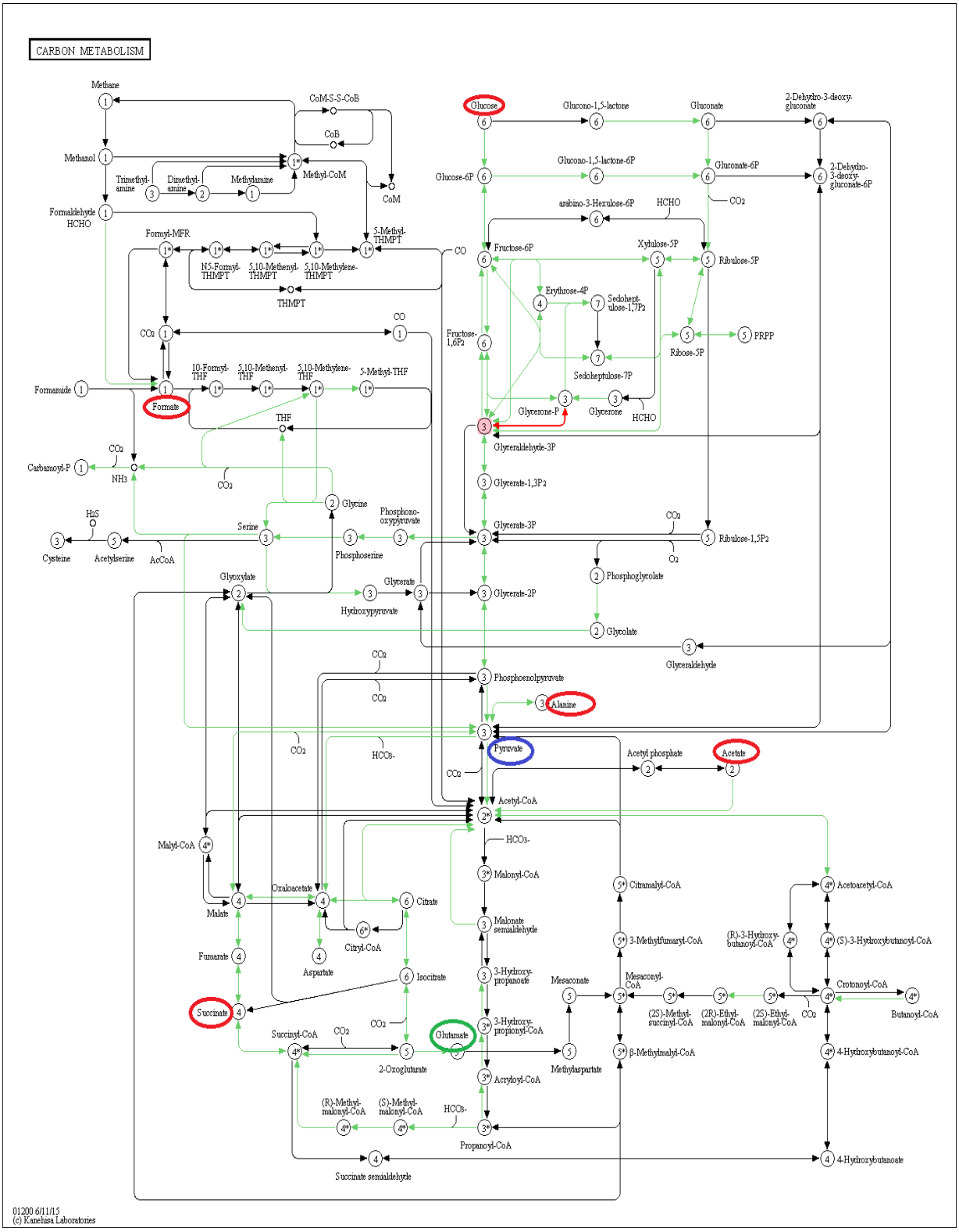
(c) Kanehisa Laboratories

B.


CITRATE CYCLE (TCA CYCLE)
Phosphoenol-pyruvate
4.1.1.32
4.1.1.49
Glycolysis / Gluconeogenesis
Fatty acid biosynthesis
Fatty acid elongation in mitochondria
Val, Leu & Ile degradation
Fatty acid metabolism
Alanine, aspartate and glutamate metabolism
Glyoxylate and dicarboxylate metabolism
1.2.7.1
1.2.7.11
ThPP
2-Hydroxy-ethyl-ThPP
Acetyl-CoA
2.3.1.12
1.2.4.1
1.2.4.1
Pyruvate
S-Acetyldihydro-lipoamide-E
1.8.1.4
6.4.1.1
Dihydro-lipoamide-E
Lipoamide-E
1.1.1.37
2.3.3.1
2.3.3.8
Citrate
4.2.1.3
4.2.1.3
Isocitrate
Oxaloacetate
cis-Aconitate
(S)-Malate
1.1.5.4
1.1.1.42
Tyrosine metabolism
4.2.1.2
Oxalosuccinate
1.1.1.41
1.1.1.286
Arginine biosynthesis
Fumarate
1.1.1.42
1.3.5.4
1.3.5.1
ThPP
6.2.1.4
6.2.1.5
2.8.3.18
Succinyl-CoA
2.3.1.61
1.2.4.2
1.2.4.2
2-Oxo-glutarate
Succinate
S-Succinyl-dihydrolipoamide-E
3-Carboxy-1-hydroxypropyl-ThPP
Arginine biosynthesis
Ascorbate and aldarate metabolism
Alanine, aspartate and glutamate metabolism
D-Gln & D-Glu metabolism
Val, Leu & Ile degradation
1.8.1.4
Dihydro-lipoamide-E
Lipoamide-E
1.2.7.11
1.2.7.3
00020 11/26/15
(c) Kanehisa Laboratories

C.


CARBON METABOLISM
Methane
CoM-S-S-CoB
CoB
Methanol
Trimethyl-amine
Dimethyl-amine
Methylamine
Methyl-CoM
CoM
Formaldehyde
HCHO
Formyl-MFR
5-Methyl-THMPT
CO
N5-Formyl-THMPT
5,10-Methenyl-THMPT
5,10-Methylene-THMPT
THMPT
CO2
10-Formyl-THF
5,10-Methenyl-THF
5,10-Methylene-THF
5-Methyl-THF
Formamide
Formate
THF
Carbamoyl-P
NH3
Glycine
H2S
Cysteine
Acetylserine
AcCoA
Serine
Phosphono-oxypyruvate
Phosphoserine
Glyoxylate
Glycerate
Hydroxypyruvate
Glucose
Glucono-1,5-lactone
Gluconate
2-Dehydro-3-deoxy-gluconate
Glucose-6P
Glucono-1,5-lactone-6P
Gluconate-6P
2-Dehydro-3-deoxy-gluconate-6P
arabino-3-Hexulose-6P
HCHO
Fructose-6P
Xylulose-5P
Ribulose-5P
Erythrose-4P
Sedohept-ulose-1,7P2
Fructose-1,6P2
PRPP
Ribose-5P
Sedoheptulose-7P
Glycerone-P
Glycerone
Glyceraldehyde-3P
Glycerate-1,3P2
Glycerate-3P
Ribulose-1,5P2
O2
Phosphoglycolate
Glycerate-2P
Glycolate
Glyceraldehyde
Phosphoenolpyruvate
Alanine
Pyruvate
HCO3-
Acetyl phosphate
Acetate
Acetyl-CoA
Malyl-CoA
Malonyl-CoA
Oxaloacetate
Citrate
Malate
Malonate semialdehyde
Citryl-CoA
Fumarate
Aspartate
Isocitrate
3-Hydroxy-propanoate
Succinate
Succinyl-CoA
3-Hydroxy-propionyl-CoA
Glutamate
2-Oxoglutarate
Acryloyl-CoA
Methylaspartate
(R)-Methyl-malonyl-CoA
(S)-Methyl-malonyl-CoA
Propanoyl-CoA
Succinate semialdehyde
Citramalyl-CoA
3-Methylfumaryl-CoA
Mesaconate
Mesaconyl-CoA
β-Methylmalyl-CoA
(2S)-Methyl-succinyl-CoA
(2R)-Ethyl-malonyl-CoA
(2S)-Ethyl-malonyl-CoA
Acetoacetyl-CoA
(R)-3-Hydroxy-butanoyl-CoA
(S)-3-Hydroxybutanoyl-CoA
Crotonoyl-CoA
Butanoyl-CoA
4-Hydroxybutanoyl-CoA
4-Hydroxybutanoate
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(c) Kanehisa Laboratories

D.

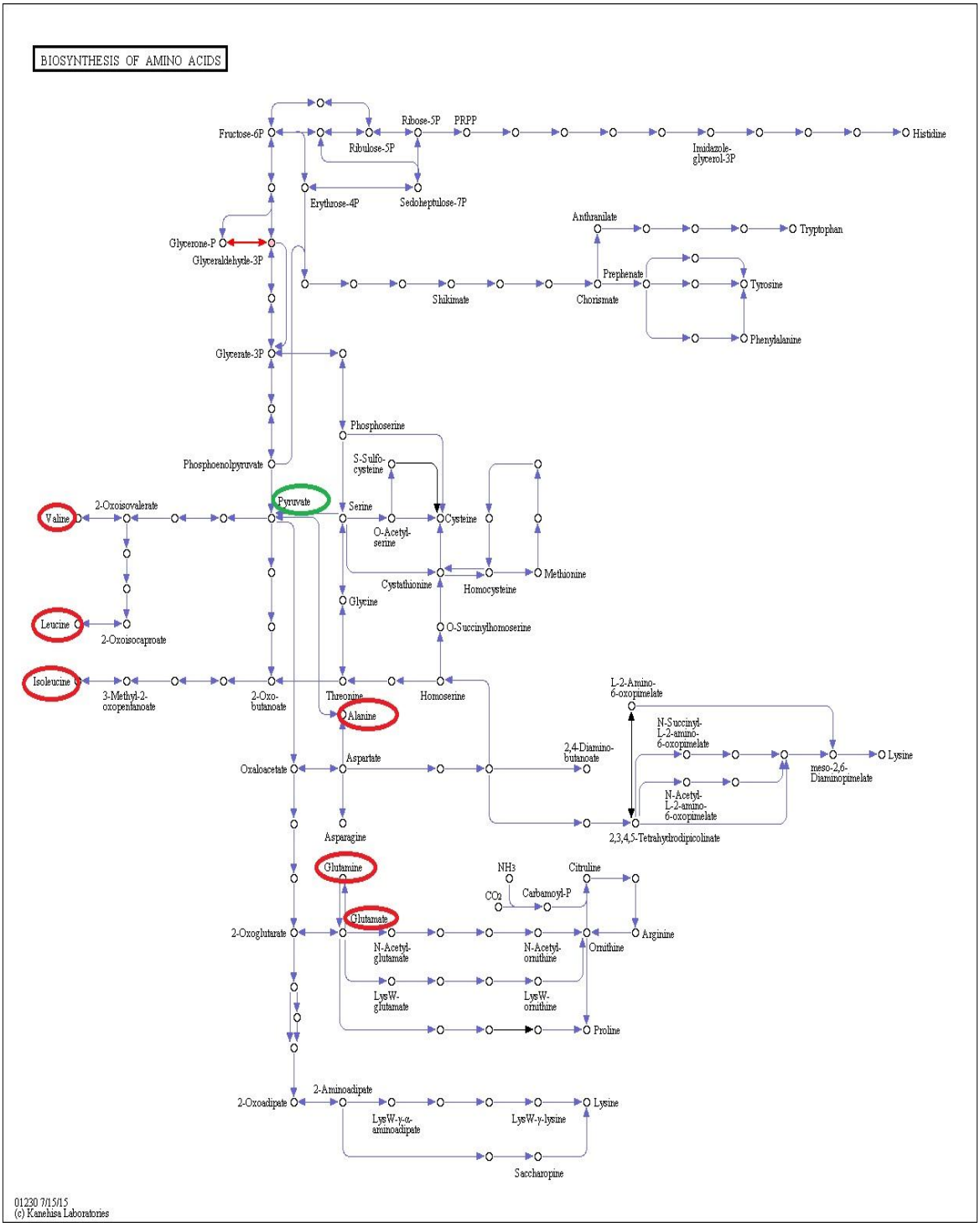


Figure 2.1. Metabolic pathways involving metabolites identified in the vaginal microenvironment. (A) Glycolysis/Gluconeogenesis, (B) Tricarboxylic acid (TCA) cycle, (C) Carbon metabolism, (D) Biosynthesis of amino acids.

Adapted from: KEGG pathway mapping tool (KEGG Mapper v2.5, Kanehisa Laboratories, JP).

2.3. Methods

2.3.1. ^{1}H-MR Sample preparation

The end of the swab soaked with the vaginal fluid sample was cut off and placed in a 1.5 ml microfuge tube. Six hundred microlitres of Phosphate Buffered Saline at pH 7.4 was added to the tube as an extraction solvent. The vaginal fluid was washed off the swab by vortexing for 5 min, after which the swab was removed and safely discarded. The solution was then centrifuged at 13,000 rpm for 3 min to separate swab and bacterial particles from the vaginal fluid solution. The supernatant was carefully aspirated into a separate clean 1.5 ml microfuge tube and stored at -80°C ready for analysis. Prior to ^{1}H-MR analysis, a total of 400 µl of each sample comprising of 380 µl of vaginal fluid in solution and 20 µl of deuterium oxide (D_2O) was immediately transferred into a 5 mm MR sample tube (Norell, Marion, NC) (Fig. 2.2). D_2O was added to the samples to maintain the homogeneity of the static magnetic field and provide field-frequency lock via "shimming". An unused (sterile) polystyrene Dacron swab was also prepared as above and analysed using the same protocol as a background signal control. This was repeated at regular intervals or change of swab batch to control for any swab manufacturing variations.

2.3.2. ^{1}H-MR Spectroscopy

Pulse sequence is a series of timed rf pulse magnetic field gradients used to exploit nuclear spins in order to obtain the required data. For example, in our experiments the Watergate water (excitation sculpting) suppression pulse sequence[427] was used to suppress net magnetisation of water in the samples, thereby eliminating the usually large water signal. This ensures that the metabolite peaks of interest are left unperturbed and easily analysed.[420]

A 9.4T (400 MHz) Bruker Avance III MR spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany), with 5 mm broadband observe probe (Fig. 2.2), was used for the experiments. The 5 mm broadband observe probe is designed for 5 mm MR sample tubes and located in the magnet. It contains rf coil that can be tuned over a wide frequency range (^{15}N-^{31}P, ^{1}H) and as such used to detect most MR-active nuclei.[420] Probes have the greatest impact on sensitivity in any given field strength.[420]

^{1}H-MR spectra were acquired using the Watergate water suppression pulse sequence (number of scans, NS = 256, relaxation time, D1 = 5s, acquisition time, AQ = 1s, sweep width, SW = 20.6 ppm, time domain, TD = 16446), for each of the CVF samples. All ^{1}H-MR experiments were performed at approximately 21°C (294 K). Data was acquired and processed using the Bruker Topspin 2.1.6 software to produce a phase and baseline corrected spectrum.

2.3.2.1. Assigning the ^{1}H-MR spectral peaks

The following 2-D MR spectra were acquired in order to confirm the structure of the metabolites and assign them to the ^{1}H-MR spectral peaks presented in Fig. 2.3:[65, 295] ^{1}H-^{13}C presat-HSQC (Heteronuclear Single Quantum Correlation spectroscopy) – NS = 1024, D1 = 1s, AQ = 0.078 x 0.006s, SW = 10.0 x 150 ppm, TD = 624 x 180; ^{1}H-^{13}C presat-HMBC (Heteronuclear Multiple Bond Correlation spectroscopy) - NS = 1024, D1 = 1s, AQ = 0.128 x 0.005s, SW = 10.0 x 200 ppm, TD = 1024 x 200; ^{1}H-^{1}H watergate-DQFCOSY (Double Quantum Filtered Correlation spectroscopy) - NS = 256, D1 = 0.5s, AQ = 0.832 x 0.022s, SW = 9.0 x 9.0 ppm, TD = 6000 x 160; and ^{1}H-^{1}H presat-cleanTOCSY (Total Correlation spectroscopy) - NS = 16, D1 = 1.5s, AQ = 0.284 x 0.071s, SW = 9.0 x 9.0 ppm, TD = 2048 x 512. A representative 2-D spectrum is shown in Fig. 2.4. All spectral peaks were referenced to the ^{1}H lactate signal at δ = 1.30 ppm. This proved more consistent and reliable than 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP), which was discontinued due to variations in its peak intensities with each sample analysed. The metabolite peak intensities were then assigned by matching their chemical shifts and multiplicity with previous publications,[431, 439, 443] the SDBS Spectral Database for Organic Compounds (AIST, JP) and Chenomx NMR Suite software (Chenomx Inc. CA, version 7.7).

The ^{1}H-^{13}C HSQC correlates chemical shifts of proton-carbon nuclei connected by a single bond in molecules providing carbon and proton chemical shifts to aid molecular assignment. The ^{1}H-^{13}C HMBC is similar to the HSQC but detects correlations over 2 or 3 bonds indicating the location carbon-carbon or carbon-heteroatom bonds. Thereby defining the skeletal framework of the molecule.[427] The ^{1}H-^{1}H watergate-DQFCOSY detects the single bond interactions (J-coupling) between protons, whilst the ^{1}H-^{1}H TOCSY identifies relationships between those protons that share the same coupled network over multiple bonds, but not the

same J-coupling e.g. amino acids and sugars.[427] These 2-D MR techniques were employed for structural and conformational analysis of the metabolites in our samples. With these unique features MR has the ability to characterise the interactions between specific atomic nuclei within and between molecules.[427] This is a significant advantage over other metabolomics techniques e.g. mass spectroscopy (MS), hence our choice of MR in this study. Plausibly the greatest constraint of MRS is its sensitivity. Compared to MS, larger amounts of samples (nanograms) are required for MR analysis.[420] However, the required amount of samples have been significantly reduced with development of stronger magnets e.g. 21 T (900 MHz), and advances in flow cell and probe design.[420]

Identified metabolite signals in the ^{1}H-MR spectra were integrated for peak area (which is proportional to metabolite concentration).[65, 295] To correct for differences in CVF concentration (e.g. variation in the swab sampling), validate and optimise the sample preparation protocol, each metabolite integral was divided by the total spectrum integral (δ = 0.0-10.0 ppm, excluding the residual water signal between δ = 4.7-5.0 ppm) to provide a normalised integral (N.I.).[65, 295] This procedure together with the background control signal obtained from sterile swabs further enhanced the optimization of our sample preparation protocol.

2.3.3. Statistical analysis

All Statistical and Receiver Operating Characteristics (ROC) curve analyses were performed using MATLAB (Mathworks, Natick, MA). The Wilcoxon's rank-sum test was performed to compare differences in metabolite N.I. between and within the groups, while the relationships between clinical data and ^{1}H-MR metabolite N.I. were determined by Pearson's correlation coefficients. *P* values < 0.05 were considered statistically significant. The predictive capacity of the CVF metabolites for PTB was determined by ROC curves for the following comparisons:

- Preterm (< 37 weeks) versus term births in all groups
- < 32 versus > 32 weeks in symptomatic women
- < 2 versus > 2 weeks from presentation to delivery in symptomatic women.

The ROC curve is derived by plotting sensitivity of a test against 1minus its specificity at each possible cut-off point . It is a graphical approach of evaluating the capacity of a diagnostic test to discriminate between the presence or absence of disease. The area under the ROC curve (AUC), provides an estimate of the overall performance of a diagnostic test, with an AUC of 1.0 indicating a perfect test, > 0.9 (high accuracy), 0.7-0.9 (moderate accuracy), 0.5-0.7 (low accuracy), and 0.5 (chance result). The predictive accuracy of metabolite N.I. was also determined by the sensitivity, specificity, negative and positive predictive values, as well as likelihood ratio.[444-447]

Figure 2.2. A 9.4T (400 MHz) Bruker Avance III MR spectrometer and 5mm BBO broadband Observe probe (left), and MR 5 mm glass tube containing cervicovaginal fluid sample (right).

2.4. Results

2.4.1. Integration ^{1}H-MR metabolites

Eight metabolite signals of interest were identified and analysed in the ^{1}H-MR spectra (Fig. 2.3 and 2.4). These were lactate, alanine, acetate, glutamine/glutamate, succinate, formate, glucose and branched chain amino acids (BCAA) (i.e. leucine, isoleucine and valine). Apart from identification of strong their signals in ^{1}H-MR spectra, these metabolites were analysed as a result of their proven association with the vaginal microbiota and impact on vaginal pH from previous reports.[38, 84] To avoid influence from other metabolite peaks in the glucose signal region 3.2-3.9 ppm, the glucose signal at δ = 5.2 ppm was integrated and used in subsequent analysis. There were no differences in the ^{1}H-MR total spectrum absolute integrals, indicating that total CVF metabolite concentration did not vary significantly between and within the study groups (Fig. 2.5). Comparison of ^{1}H-MR metabolite N.I. from the different cohorts in relation to delivery outcomes is shown in Fig. 2.6.

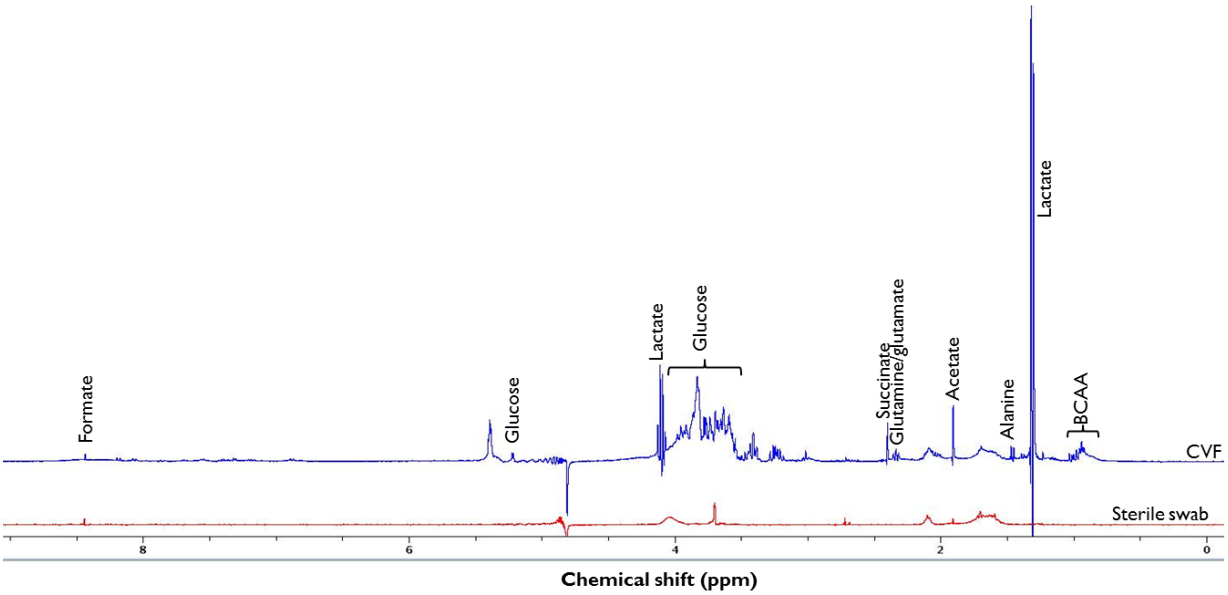


Figure 2.3. 1-D ^{1}H-MR spectrum of identified metabolites in cervicovaginal fluid (CVF) and sterile swab at 400 MHz and 294K. Due to the presence of other additional metabolite peaks at the δ = 3.2 ppm and 3.9 ppm region, the glucose signal at δ = 5.2 ppm was integrated and used in subsequent analysis.

BCAA, Branched chain amino acids (leucine, isoleucine, and valine) (δ = 0.9-1.2 ppm), *ppm*, parts per million.

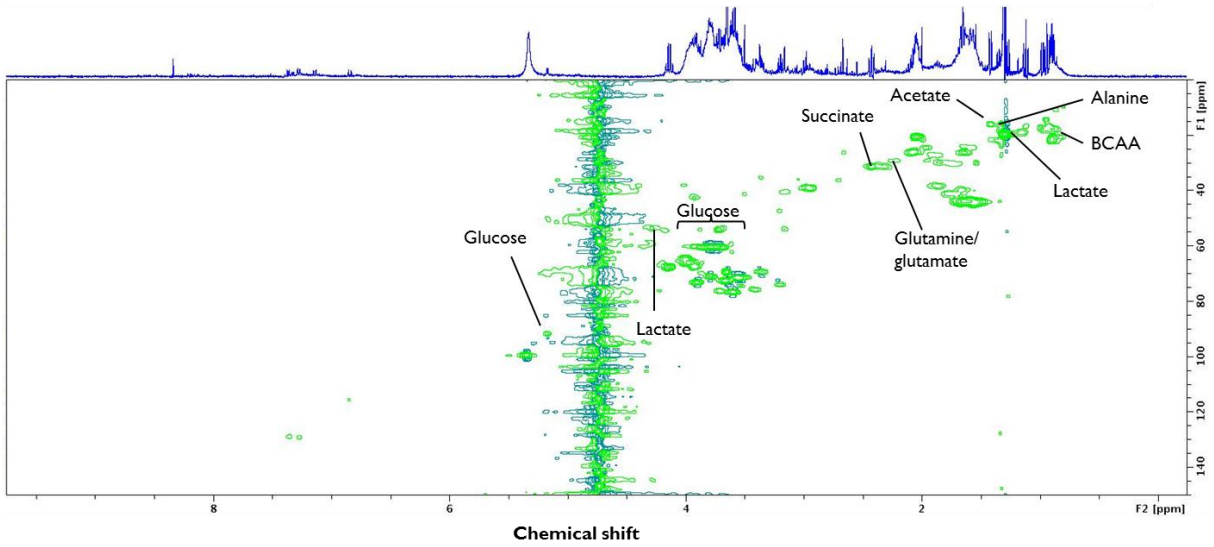


Figure 2.4. 2-D ^{1}H-^{13}C HSQC MR spectrum of cervicovaginal fluid showing confirmed metabolites at 400 MHz and 294K.

BCAA, Branched chain amino acids (leucine, isoleucine, and valine); *HSQC*, heteronuclear single quantum correlation spectroscopy.

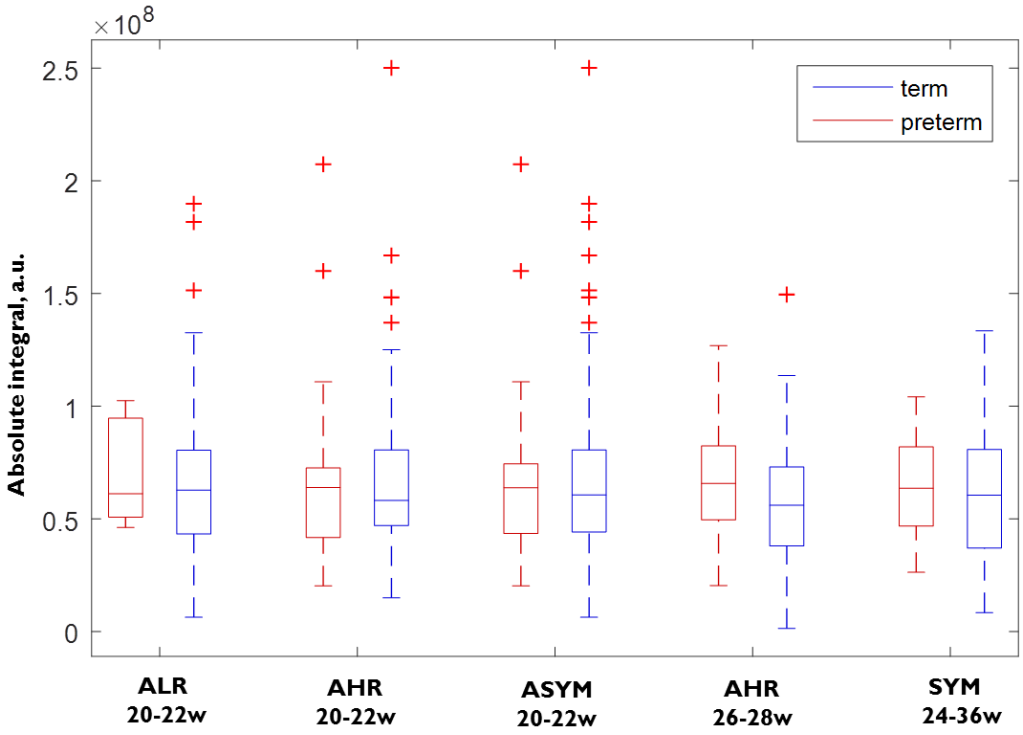


Figure 2.5. ^{1}H-MR total spectrum absolute integrals of cervicovaginal fluid metabolites. Comparison of ^{1}H-MR total spectrum absolute integrals of cervicovaginal fluid in the various cohorts in relation to delivery outcomes indicated no significant difference. Box plots show the median line within the box, with the bottom and top edges of the box representing the 25% and 75% quartiles respectively. Whiskers extend to the furthermost value within 1.5 times the interquartile range from the 25% and 75% quartiles. Outlier values are also indicated (red pluses).

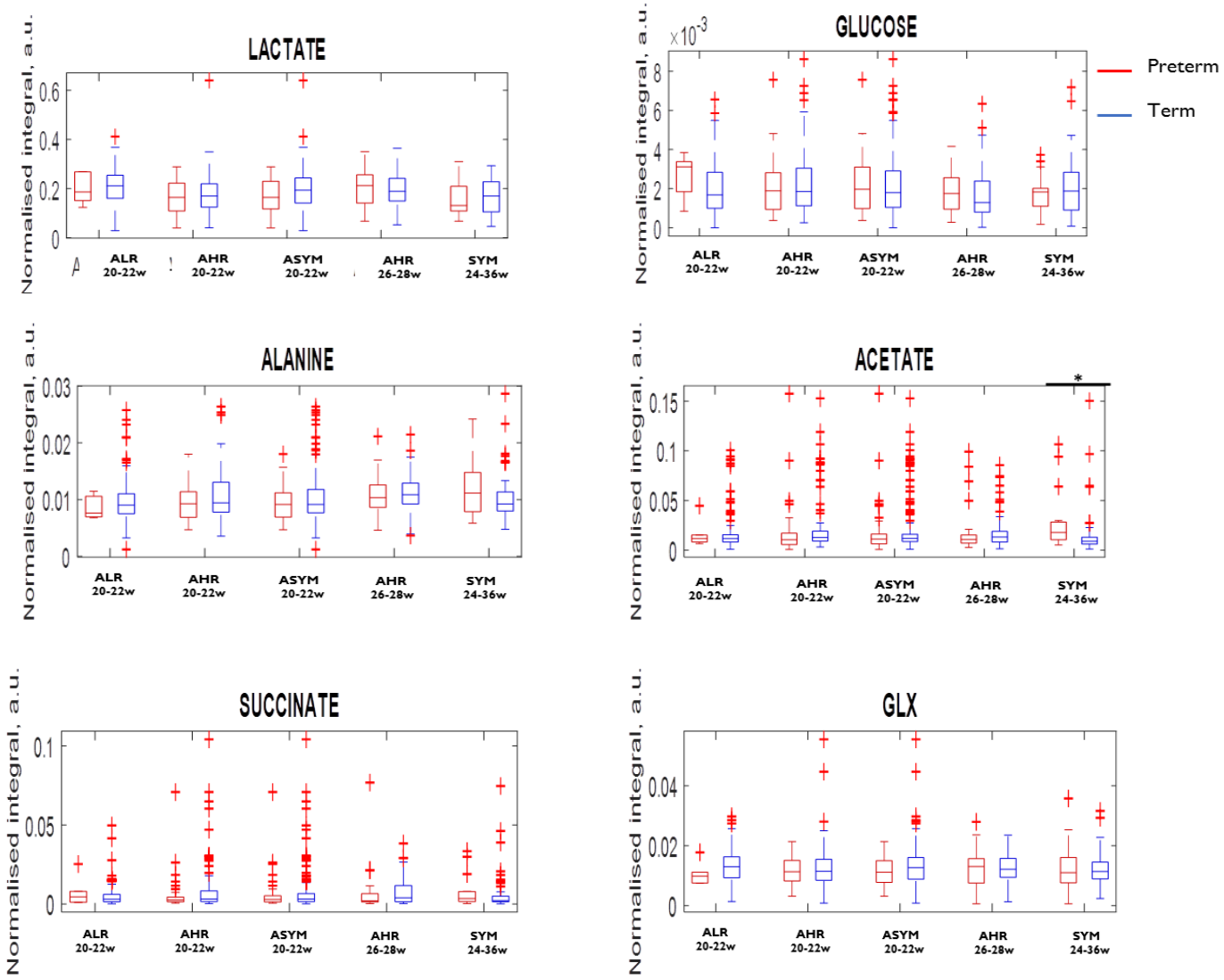
ALR, asymptomatic low risk women; *AHR*, asymptomatic high risk women; *ASYM*, ALR and AHR 20-22w combined; *SYM*, symptomatic women; *w,* gestational weeks.

2.4.2. Differences in CVF ^{1}H-MR metabolites in relation to delivery outcomes

It is plausible that differences in CVF metabolites produced by vaginal microbiota in relation to the host could predispose the women to infection, inflammation and adverse pregnancy outcomes.[38 84, 85] Therefore, comparing the medians of the ^{1}H-MR metabolites identified, we observed that of all the metabolites analysed, only acetate N.I. and acetate/lactate ratio showed significant differences and these were observed in the SYM cohort alone (Fig. 2.6). The acetate N.I. (P = 0.002) and acetate/lactate ratio (P = 0.002) in the SYM women that delivered preterm were significantly higher than those of their term-delivered counterparts. There was over a 2-fold (103%) increase in CVF acetate N.I. of women presenting with symptoms suggestive of PTL who ultimately delivered preterm, whilst the difference in acetate/lactate ratio was only 46%. Though not statistically significant, differences in acetate N.I. between preterm- and term-delivered women were observed in the AHR26-28w (P = 0.17) group.

In addition, the term-delivered women (n = 124), in the ALR group demonstrated non-significant trends to higher lactate (P = 1.0), glutamine/glutamate (P = 0.36) and BCAA N.I.s (P = 0.054), compared to their preterm counterparts (n = 5).

Also, differences in all other metabolite N.I.s between preterm- and term-delivered women in the ALR, AHR20-22w and 26-28w, and SYM were not statistically significant (Fig. 2.6).


LACTATE
GLUCOSE
ALANINE
ACETATE
SUCCINATE
GLX
Normalised integral, a.u.
×10-3
Preterm
Term
*
ALR 20-22w
AHR 20-22w
ASYM 20-22w
AHR 26-28w
SYM 24-36w

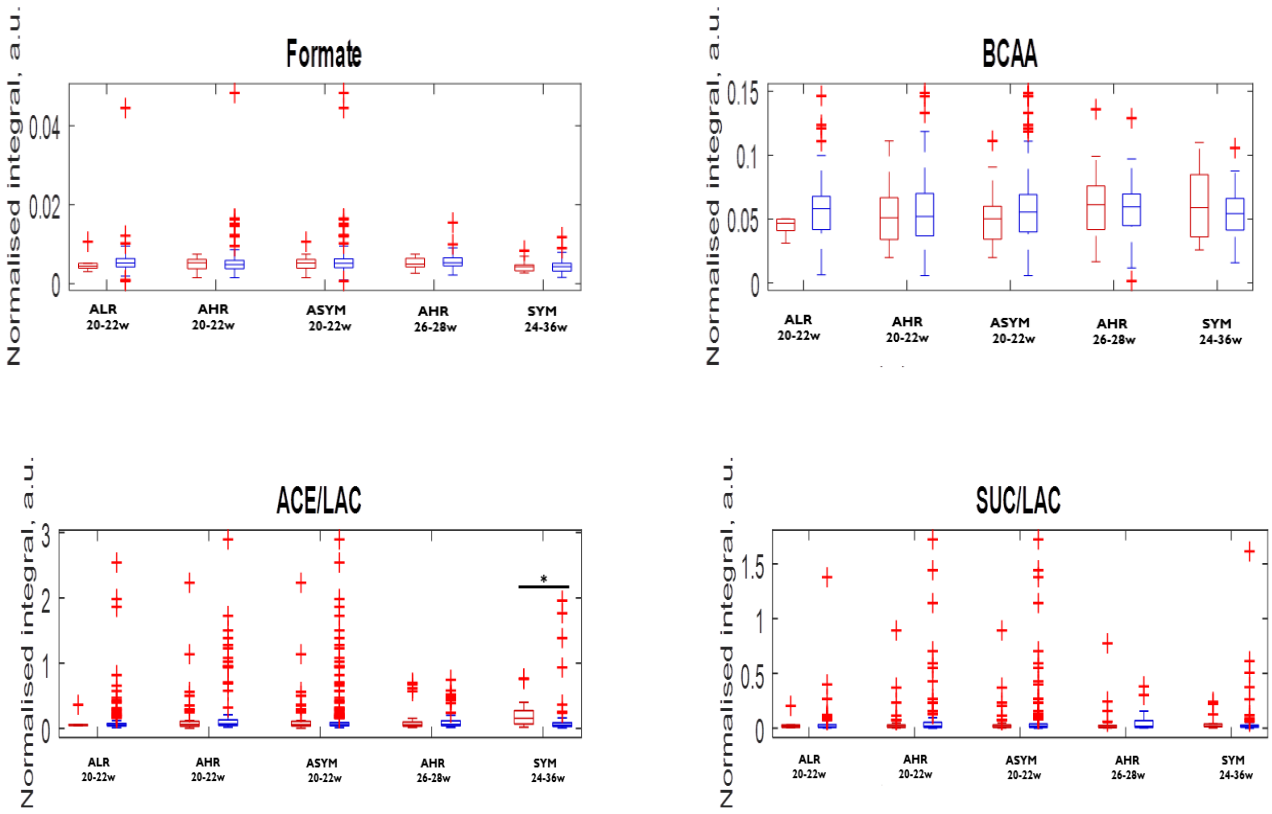


Figure 2.6. Comparison of mean ^{1}H-MR metabolite normalised integrals from different cohorts of pregnant women in relation to delivery outcomes.

Box plots show the median line within the box, with the bottom and top edges of the box representing the 25% and 75% quartiles respectively.

ALR, asymptomatic low risk women; *ACE/LAC*, acetate/lactate ratio; *AHR*, asymptomatic high risk women; *ASYM*, ALR and AHR 20-22w combined; *BCAA*, Branched chain amino acids; *SUC/LAC*, succinate/lactate ratio; *SYM*, symptomatic women; *w*, gestation weeks.

* *P* value < 0.05 considered statistically significant.

2.4.3. Relationship between vaginal pH and CVF ^{1}H-MR metabolites

The interactions between the various metabolites and vaginal pH could be indicative of the prevalent vaginal microbiota signature and the host-microbial interplay in the vaginal microecology.[32, 448, 449] Our data showed vaginal pH correlated with lactate and acetate N.I. in the ALR20-22w, AHR20-22w, AHR26-28w, and SYM women, while it correlated with glucose, glutamine/glutamate, succinate N.I.s, and succinate/lactate ratio in the ASYM 20-22w and AHR26-28w women only (Table 2.2 and Fig. 2.7A-F). Higher CVF lactate N.I. were associated with low vaginal pH, whilst higher acetate N.I. was associated with high vaginal pH in all the study participants irrespective of gestational age at sampling or the presence of symptoms of PTL (Fig. 2.8 and 2.9). Similar to the lactate N.I., higher glutamine/glutamate N.I. was associated with decreased vaginal pH in the ASYM 20-22w and AHR 26-28w women, whilst glucose N.I., succinate N.I. and succinate/lactate ratio were associated with increased pH in all the groups except the SYM women.

Furthermore, across the groups, there were low (r = 0.3) to strong (r = 0.7-1.0) correlations between lactate vs. acetate N.I.s, lactate vs. glucose N.I.s, lactate vs. succinate N.I.s, lactate vs. glutamate/glutamine N.I.s, acetate vs. succinate N.I.s, alanine vs. glutamine/glutamate, alanine vs. BCAA N.I.s etc. (Fig. 2.7A-F).

Table 2.2: Correlation between vaginal pH and metabolites normalised integrals (*r*, *P*)

	Asymptomatic Low risk women, 20-22w	**Asymptomatic High risk women, 20-22w**	**Asymptomatic 20-22w (Combined)**	**Asymptomatic High risk women, 26-28w**	**Symptomatic women, 24-36w**
Lactate	-0.6, < 0.00001	-0.6, < 0.00001		-0.7, < 0.00001	-0.6, < 0.00001
Acetate	0.4, < 0.00001	0.5, < 0.00001		0.5, < 0.00001	0.4, P < 0.01
Glucose			0.2, < 0.0001	0.4, < 0.00001	
Glx			-0.3, 0.04	-0.5, < 0.00001	
Succinate			0.4, < 0.00001	0.2, 0.04	
Suc/Lac			0.4, < 0.00001	0.3, < 0.00001	

GLX, glutamine/glutamate; *Suc/Lac*, succinate/lactate ratio; *r*, Pearson's correlation coefficient; *P*, significance level.

2.4.4. Relationship between CVF ^{1}H-MR metabolites and gestational age at delivery

In order to test for any relationships between ^{1}H-MR metabolites and GAAD, we plotted the identified metabolites against the GAAD in days (Fig. 2.7A-F). Our results showed in the combined asymptomatic low and high risk women studied at 20-22w (ASYM 20-22w), lactate ($r = 0.2$, $P = 0.02$) and glutamine/glutamate N.I.s ($r = 0.2$, $P = 0.04$) correlate modestly with GAAD. However, when these women were subdivided based on their risk of PTB, only the low risk (ALR 20-22w) showed a correlation between glutamine/glutamate N.I. and GAAD ($r = 0.2$, $P = 0.01$). Higher lactate and glutamine/glutamate N.I.s were associated with increased GAAD especially in the ALR women.

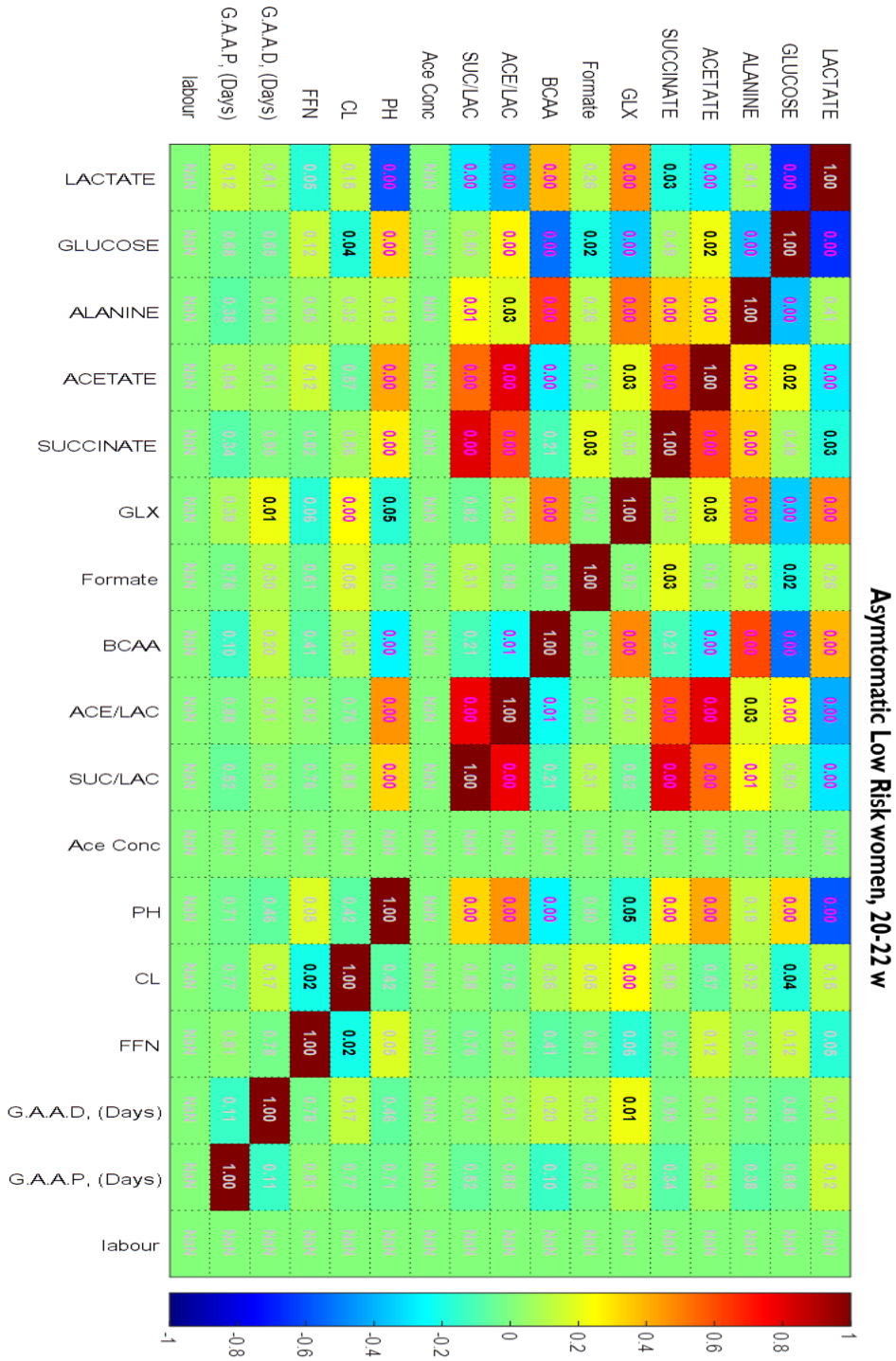


Figure 2.7A. Association of ^{1}H-MR cervicovaginal fluid metabolites and maternal clinical parameters in asymptomatic pregnant women (20-22 weeks gestation), with low risk of preterm birth.

Pearson's correlation coefficients (r, colour) range from -1 (dark blue) to +1 (dark red); and *P*, values (significance: bold black < *0.05*, bold purple < *0.01*). *GLX*, glutamine/glutamate; *BCAA*, branched chain amino acid; *ACE/LAC*, acetate/lactate ratio; *SUC/LAC*, succinate/lactate ratio; *Ace Conc*, acetate concentration, *CL*, cervical length; *BMI*, Body mass Index; *FFN*, fetal fibronectin; *G.A.A.D*, gestational age at delivery; *G.A.A.P*, gestational age at presentation, *Lobour*, number of days between presentation and delivery; *w*, gestation weeks.

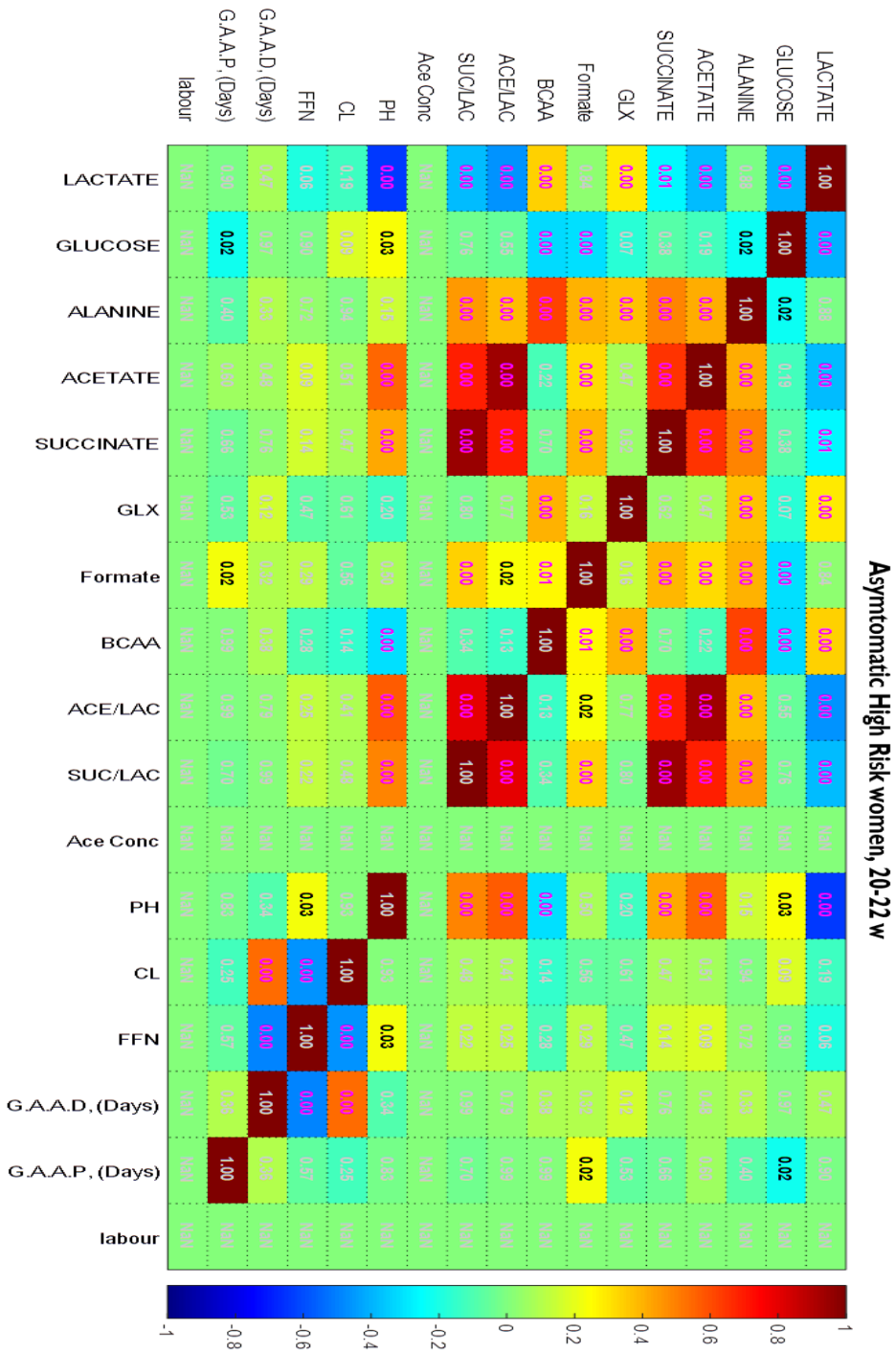


Figure 2.7B. Association of ^{1}H-MR cervicovaginal fluid metabolites and maternal clinical parameters in asymptomatic pregnant women (20-22 weeks gestation), with high risk of preterm birth.

Pearson's correlation coefficients (r, colour) range from -1 (dark blue) to +1 (dark red); and *P*, values (significance: bold black < *0.05*, bold purple < *0.01*). *GLX*, glutamine/glutamate; *BCAA*, branched chain amino acid; *ACE/LAC*, acetate/lactate ratio; *SUC/LAC*, succinate/lactate ratio; *Ace Conc*, acetate concentration, *CL*, cervical length; *BMI*, Body mass Index; *FFN*, fetal fibronectin; *G.A.A.D*, gestational age at delivery; *G.A.A.P*, gestational age at presentation, *Lobour*, number of days between presentation and delivery; *w*, gestation weeks.

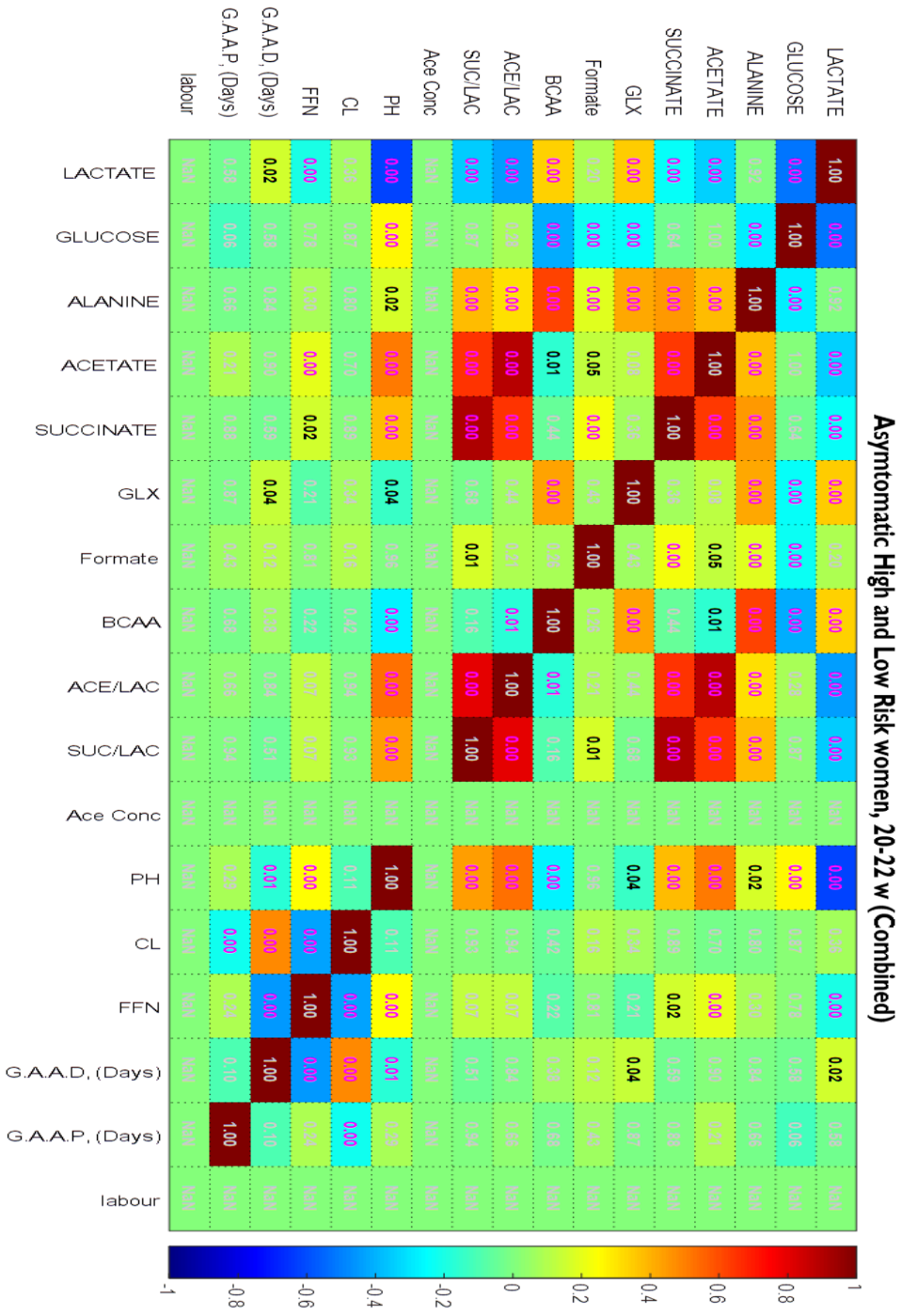


Figure 2.7C. Association of ^{1}H-MR cervicovaginal fluid metabolites and maternal clinical parameters in asymptomatic pregnant women (20-22 weeks gestation), irrespective of risk of preterm birth.
Pearson's correlation coefficients (r, colour) range from -1 (dark blue) to +1 (dark red); and *P*, values (significance: bold black < *0.05*, bold purple < *0.01*). *GLX*, glutamine/glutamate; *BCAA*, branched chain amino acid; *ACE/LAC*, acetate/lactate ratio; *SUC/LAC*, succinate/lactate ratio; *Ace Conc*, acetate concentration, *CL*, cervical length; *BMI*, Body mass Index; *FFN*, fetal fibronectin; *G.A.A.D*, gestational age at delivery; *G.A.A.P*, gestational age at presentation, *Lobour*, number of days between presentation and delivery; *w*, gestation weeks.

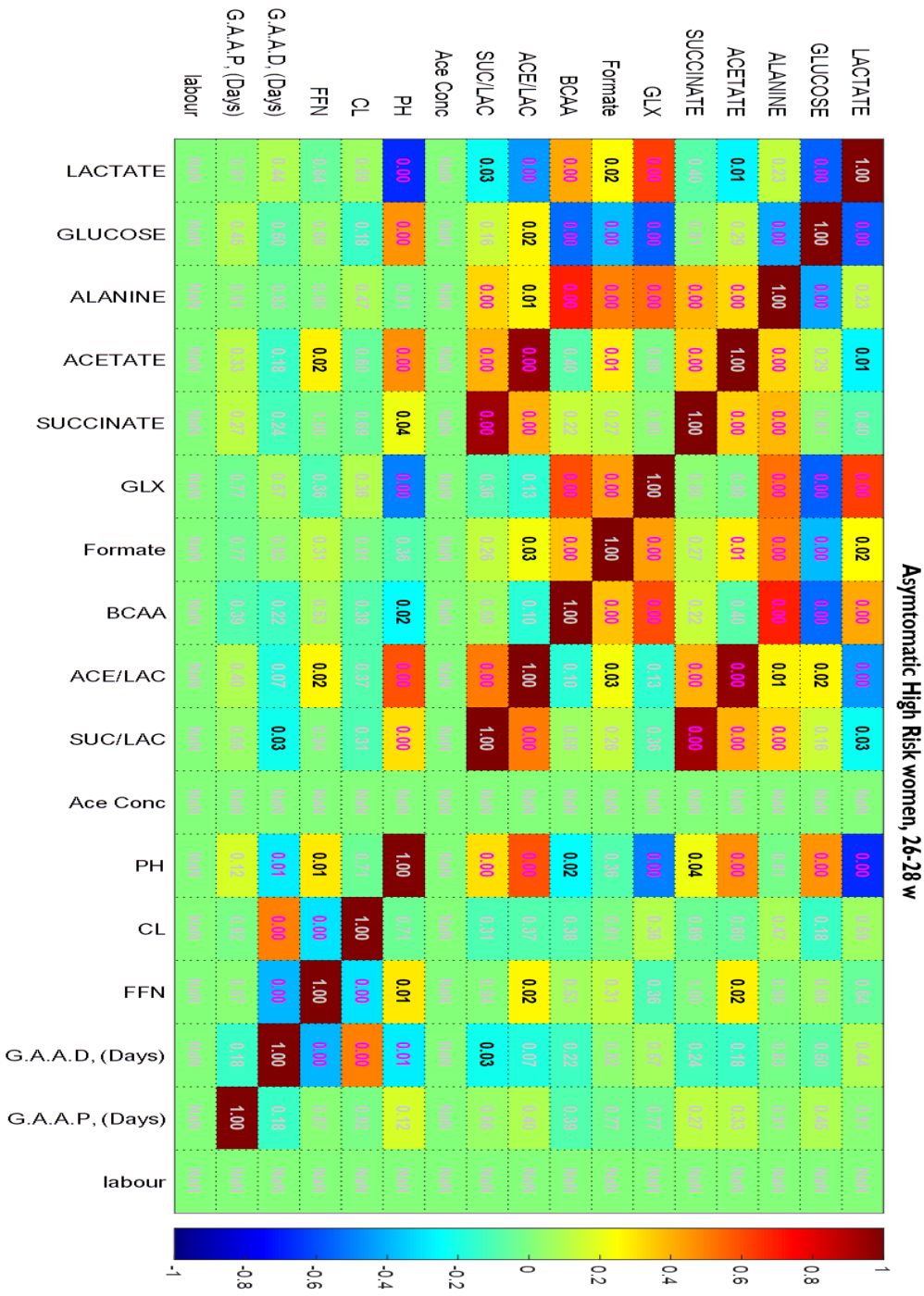


Figure 2.7D. Association of ^{1}H-MR cervicovaginal fluid metabolites and maternal clinical parameters in asymptomatic pregnant women (26-28 weeks gestation), with high risk of preterm birth.

Pearson's correlation coefficients (r, colour) range from -1 (dark blue) to +1 (dark red); and *P*, values (significance: bold black < *0.05*, bold purple < *0.01*). *GLX*, glutamine/glutamate; *BCAA*, branched chain amino acid; *ACE/LAC*, acetate/lactate ratio; *SUC/LAC*, succinate/lactate ratio; *Ace Conc*, acetate concentration, *CL*, cervical length; *BMI*, Body mass Index; *FFN*, fetal fibronectin; *G.A.A.D*, gestational age at delivery; *G.A.A.P*, gestational age at presentation, *Lobour*, number of days between presentation and delivery; *w*, gestation weeks.

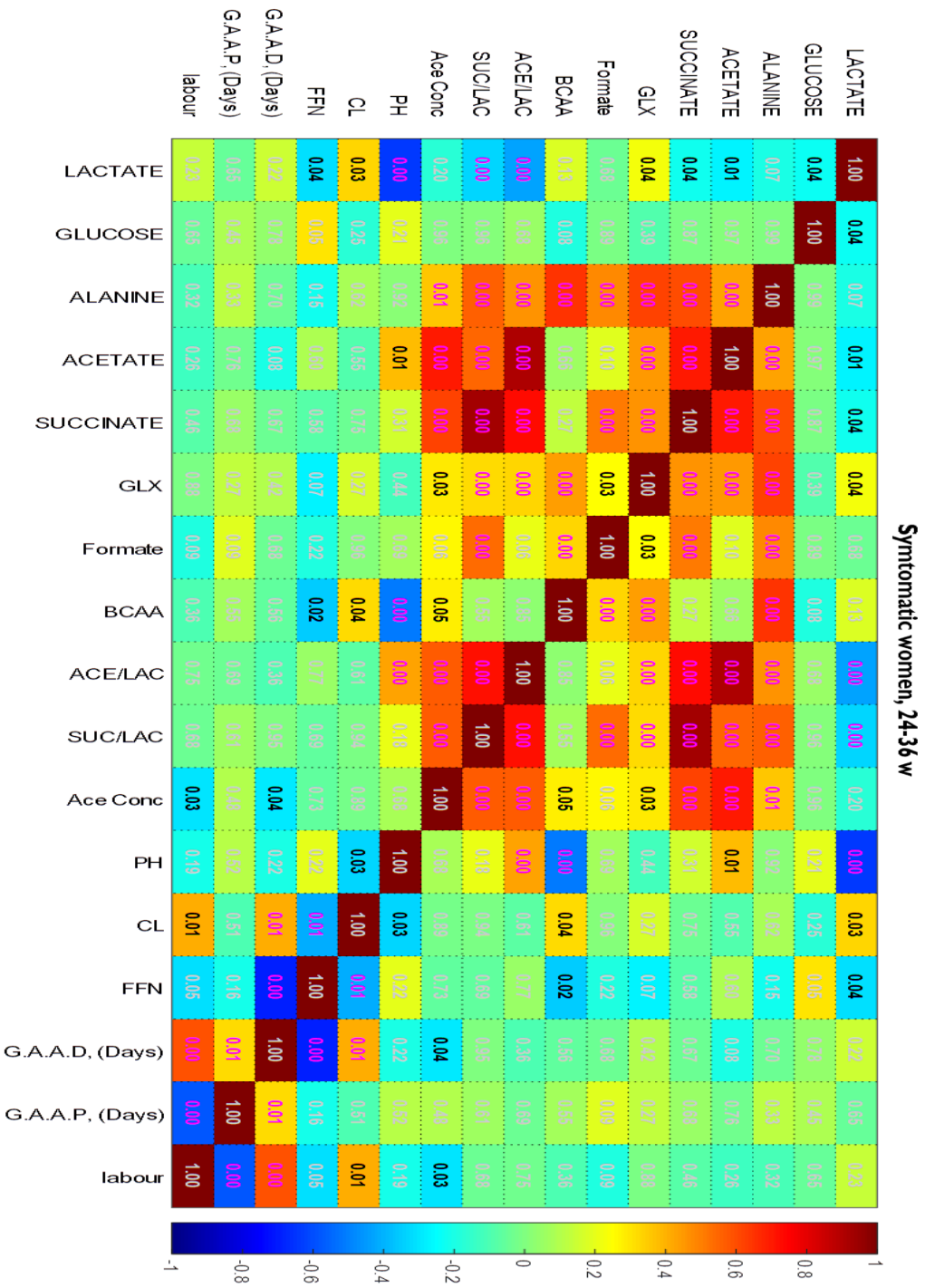


Figure 2.7E. Association of ^{1}H-MR cervicovaginal fluid metabolites and maternal clinical parameters in symptomatic pregnant women (24-36 weeks gestation).

Pearson's correlation coefficients (r, colour) range from -1 (dark blue) to +1 (dark red); and *P*, values (significance: bold black < *0.05*, bold purple < *0.01*). *GLX*, glutamine/glutamate; *BCAA*, branched chain amino acid; *ACE/LAC*, acetate/lactate ratio; *SUC/LAC*, succinate/lactate ratio; *Ace Conc*, acetate concentration, *CL*, cervical length; *BMI*, Body mass Index; *FFN*, fetal fibronectin; *G.A.A.D*, gestational age at delivery; *G.A.A.P*, gestational age at presentation, *Lobour*, number of days between presentation and delivery; *w*, gestation weeks.

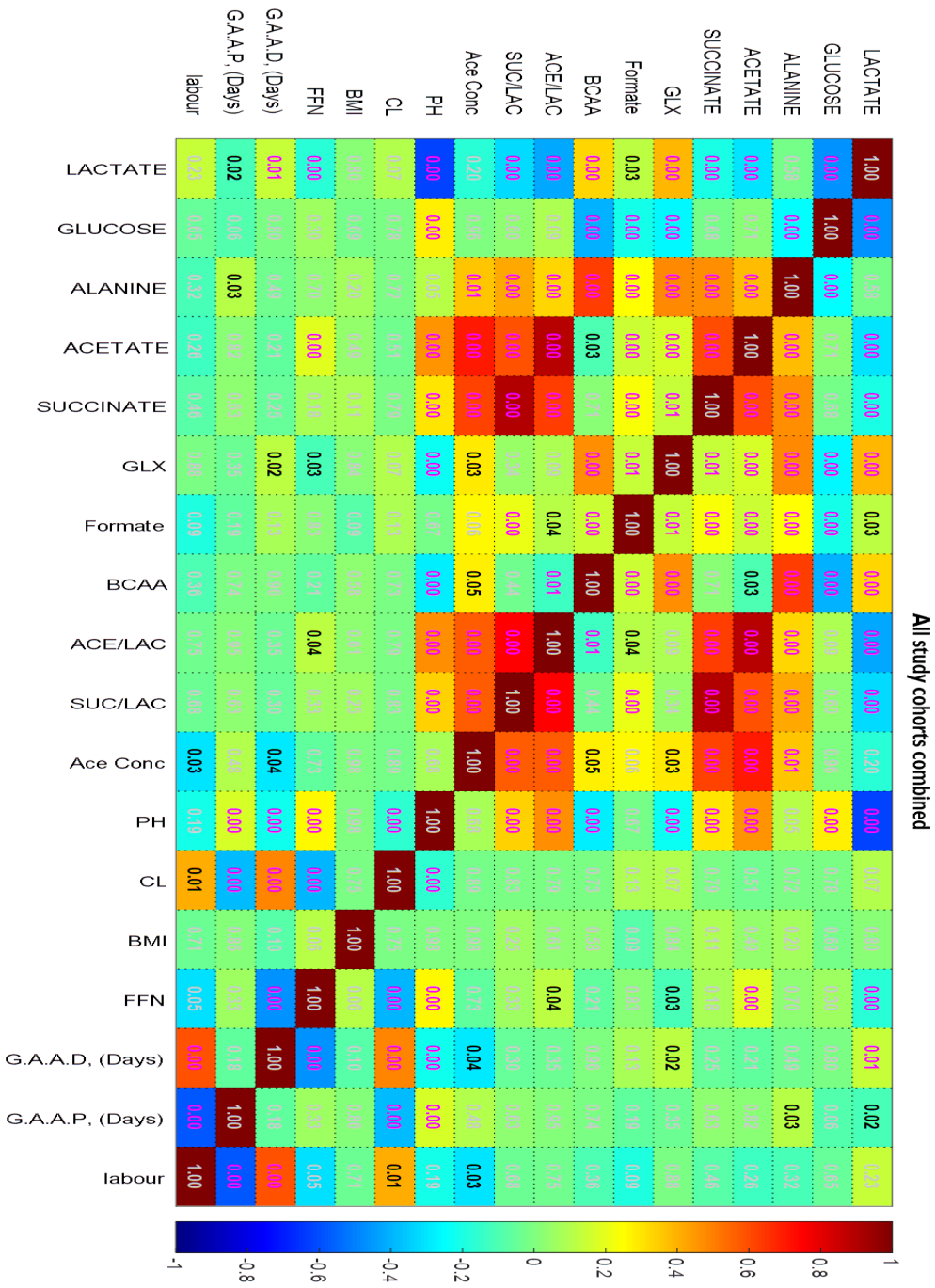


Figure 2.7F. Association of ^{1}H-MR cervicovaginal fluid metabolites and maternal clinical parameters in pregnant women (20-36 weeks gestation), irrespective of risk and symptoms of preterm birth.
Pearson's correlation coefficients (r, colour) range from -1 (dark blue) to +1 (dark red); and *P*, values (significance: bold black < *0.05*, bold purple < *0.01*). *GLX*, glutamine/glutamate; *BCAA*, branched chain amino acid; *ACE/LAC*, acetate/lactate ratio; *SUC/LAC*, succinate/lactate ratio; *Ace Conc*, acetate concentration, *CL*, cervical length; *BMI*, Body mass Index; *FFN*, fetal fibronectin; *G.A.A.D*, gestational age at delivery; *G.A.A.P*, gestational age at presentation, *Lobour*, number of days between presentation and delivery; *w*, gestation weeks.

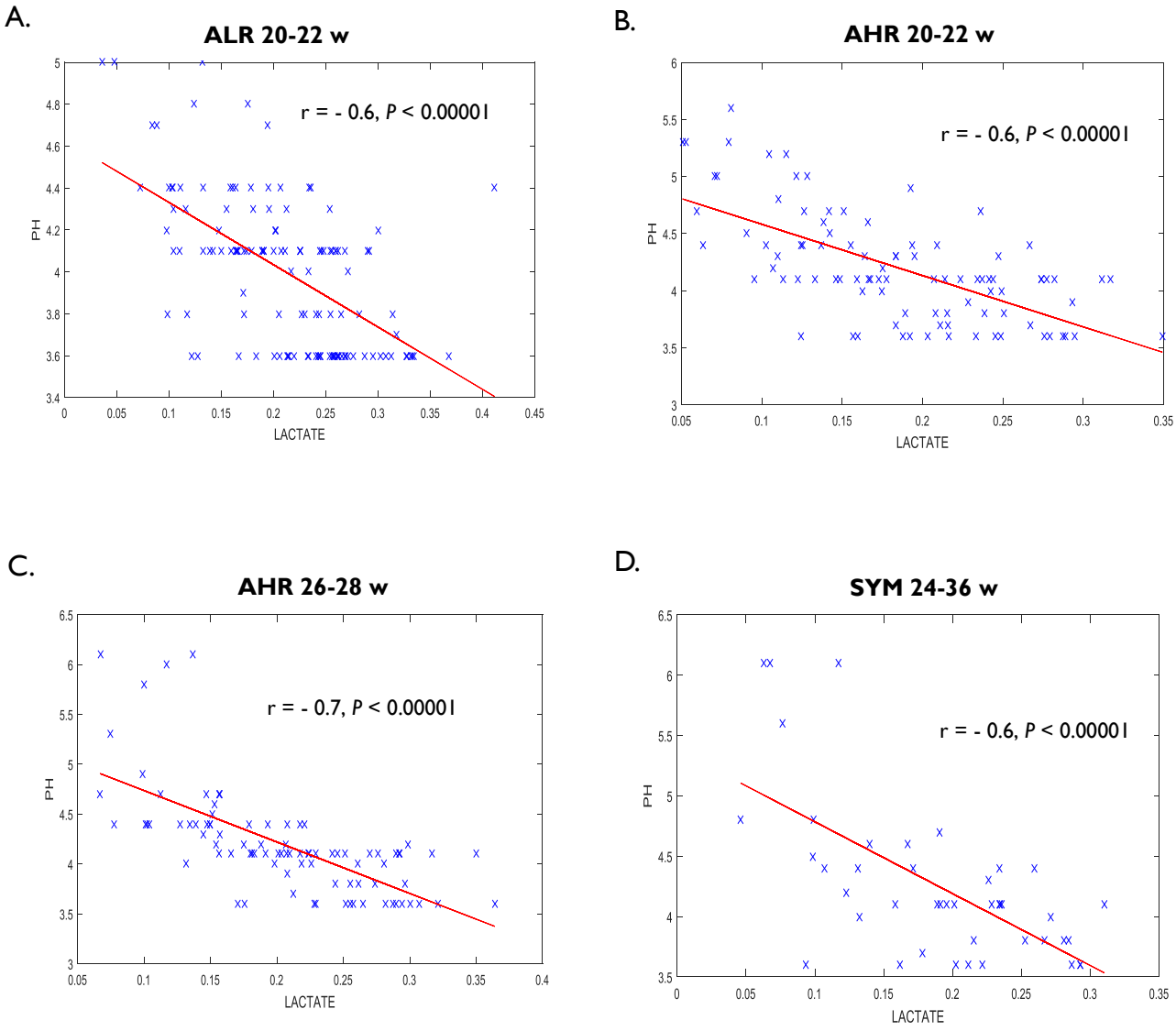


Figure 2.8. Association between vaginal pH and CVF lactate normalised integrals in pregnant women (A) Asymptomatic low risk women 20-22w, (B) Asymptomatic high risk women 20-22w, (C) Asymptomatic high risk women 26-28w, (D) Symptomatic women 24-36w. w, gestation weeks.

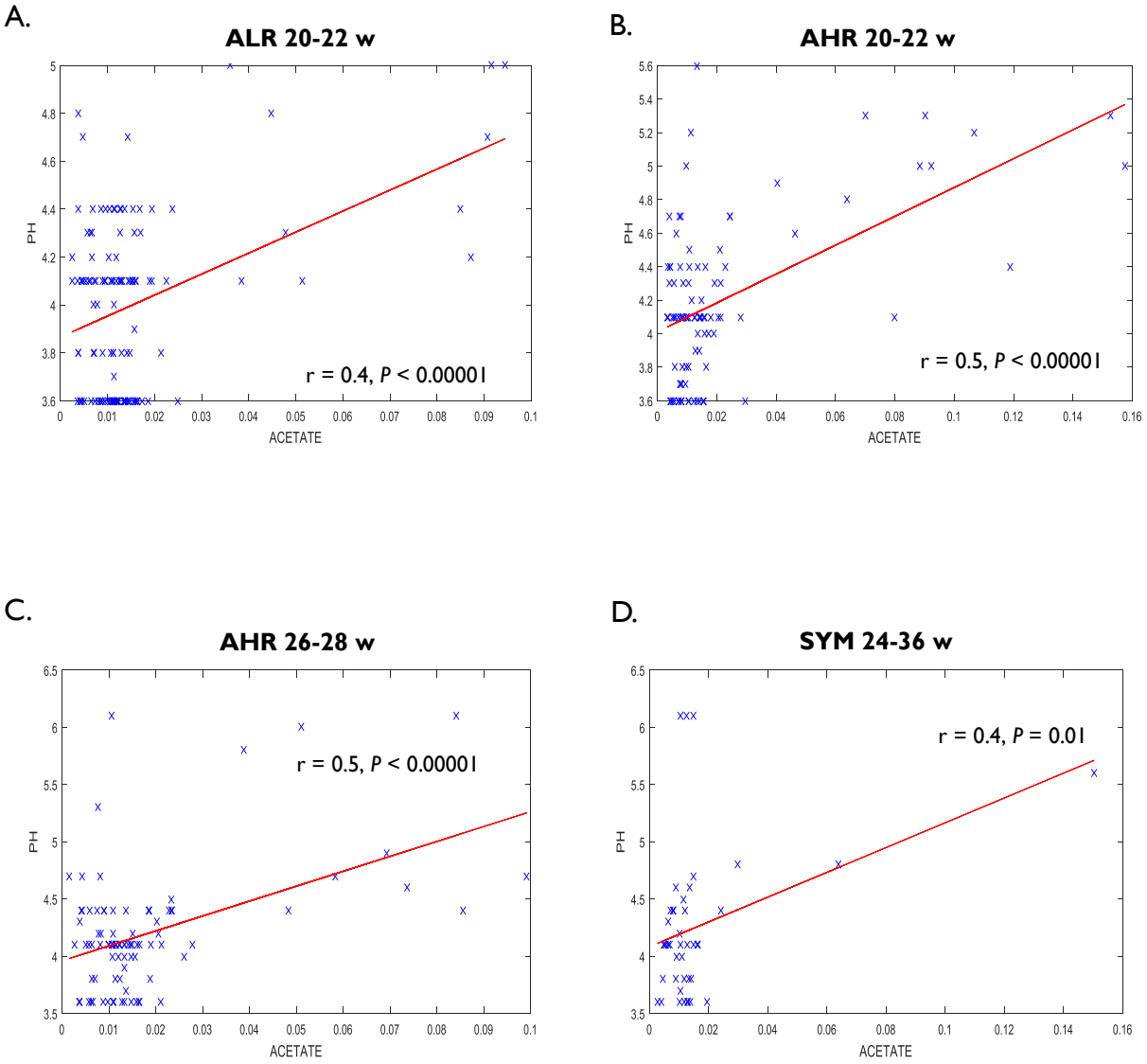


Figure 2.9. Association between vaginal pH and CVF acetate normalised integral in pregnant women (A) Asymptomatic low risk women 20-22w, (B) Asymptomatic high risk women 20-22w, (C) Asymptomatic high risk women 26-28w, (D) Symptomatic women 24-36w. w, gestation weeks.

2.4.5. Predictive capacity of CVF ^{1}H-MR metabolites for preterm birth

Analysis of the area under the ROC curves (calculated by plotting the sensitivity vs. 1 – specificity), for the ^{1}H-MR metabolites showed the acetate N.I. and acetate/lactate ratio were highly predictive of women presenting with symptoms of PTL who delivered prematurely (< 37w and < 32w), and within two weeks of the index assessment (Tables 2.2-2.3 and 2.11, Figs. 2.10-2.11). Also, in the same women, glutamine/glutamate N.I. was predictive of premature delivery (< 32w), and within two weeks of the index assessment (Table 2.4 and Fig. 2.12). In addition, acetate and BCAA N.I. showed useful predictive capacities for PTB in the AHR 20-22w and ALR women respectively (Tables 2.5-2.8 and Fig. 2.13-2.14). The other metabolites did not show significant predictive capacity for PTB in any of the study cohorts (Tables 2.7-2.11).

Table 2.3: Predictive performance of CVF acetate normalised integrals for preterm birth in symptomatic pregnant women

Variables	< 37 weeks gestation	< 32 weeks gestation	≤ 2 weeks of index assessment
Area under the ROC curve (AUC)	0.75	0.73	0.77
Standard Error, SE	0.08	0.11	0.10
95% Confidence Interval	0.60-0.91	0.53-0.94	0.58-0.96
Significance level, *P*	0.0005	0.01	0.003
Youden index, J	0.85	0.59	0.49
Sensitivity, %	60	88	100
Specificity, %	85	59	49
Positive predictive value (PPV), %	47	21	21
Negative predictive value (NPV), %	91	97	100
Positive likelihood ratio, LR+	4.0	2.1	2
Negative likelihood ratio, LR-	0.2	0.5	0.5

Table 2.4: Predictive performance of CVF acetate/lactate ratio for preterm birth in symptomatic pregnant women

Variables	< 37 weeks gestation	< 32 weeks gestation	≤ 2 weeks of index assessment
Area under the ROC curve (AUC)	0.76	0.79	0.78
Standard Error, SE	0.08	0.10	0.09
95% Confidence Interval	0.61-0.91	0.60-0.98	0.60-0.97
Significance level, *P*	0.0004	0.002	0.002
Youden index, J	0.61	0.58	0.64
Sensitivity, %	87	100	89
Specificity, %	61	58	64
Positive predictive value (PPV), %	33	23	25
Negative predictive value (NPV), %	95	100	98
Positive likelihood ratio, LR+	2.2	2.4	2.4
Negative likelihood ratio, LR-	0.5	0.4	0.4

Table 2.5: Predictive performance of CVF glutamine/glutamate normalised integrals for preterm birth in symptomatic pregnant women

Variables	< 32 weeks gestation	≤ 2 weeks of index assessment
Area under the ROC curve (AUC)	0.71	0.68
Standard Error, SE	0.11	0.10
95% Confidence Interval	0.50-0.92	0.47-0.88
Significance level, *P*	0.03	0.045
Youden index, J	0.81	0.73
Sensitivity, %	63	67
Specificity, %	81	73
Positive predictive value (PPV), %	29	25
Negative predictive value (NPV), %	95	94
Positive likelihood ratio, LR+	3.3	2.4
Negative likelihood ratio, LR-	0.3	0.4

Table 2.6: Predictive performance of CVF acetate normalised integrals for preterm birth in asymptomatic high risk (20-22w) pregnant women

Variables	< 37 weeks gestation
Area under the ROC curve (AUC)	0.61
Standard Error, SE	0.06
95% Confidence Interval	0.50-0.73
Significance level, *P*	0.03
Youden index, J	0.78
Sensitivity, %	49
Specificity, %	78
Positive predictive value (PPV), %	52
Negative predictive value (NPV), %	75
Positive likelihood ratio, LR+	2.2
Negative likelihood ratio, LR-	0.5

Table 2.7: Predictive performance of CVF branched chain amino acids normalised integrals for preterm birth in asymptomatic low risk (20-22w) pregnant women

Variables	< 37 weeks gestation
Area under the ROC curve (AUC)	0.75
Standard Error, SE	0.13
95% Confidence Interval	0.50-1.00
Significance level, *P*	0.03
Youden index, J	0.65
Sensitivity, %	100
Specificity, %	65
Positive predictive value (PPV), %	10
Negative predictive value (NPV), %	100
Positive likelihood ratio, LR+	2.9
Negative likelihood ratio, LR-	0.4

Table 2.8: Predictive performance of CVF metabolites normalised integrals for preterm birth (< 37w), in asymptomatic low risk pregnant women (20-22w)

Metabolite	AUC	SE	95% CI	YI	Sen (%)	Spec (%)	PPV (%)	NPV (%)	LR+	LR-	*P* value
Lactate	0.49	0.13	0.24-0.75	0.80	40	80	7	97	2.0	0.5	0.5
Acetate	0.56	0.14	0.30-0.83	0.47	80	47	6	98	1.5	0.7	0.3
Succinate	0.64	0.14	0.37-0.91	0.61	80	61	7	99	2.0	0.5	0.1
Formate	0.57	0.14	0.30-0.84	0.51	80	51	6	98	1.6	0.6	0.3
Glucose	0.62	0.14	0.35-0.89	0.77	60	77	10	98	2.6	0.38	0.2
Alanine	0.50	0.13	0.25-0.78	0.68	60	68	7	98	1.8	0.5	0.5
Glx	0.62	0.14	0.36-0.89	0.63	80	63	8	99	2.2	0.5	0.2
BCAA	0.75	0.13	0.50-1.0	0.65	100	65	10	100	2.9	0.4	0.03*
Suc/Lac	0.63	0.14	0.36-0.90	0.63	80	63	8	99	2.2	0.5	0.2
Ace/Lac	0.44	0.13	0.19-0.69	0.43	80	43	5	98	1.4	0.7	0.7

Ace/Lac, acetate-lactate ratio; *BCAA*, branched chain amino acids; *Glx*, glutamine/glutamate; *Suc/Lac*, succinate-lactate ratio; *AUC*, area under the ROC curve; *SE*, standard error; *CI*, 95% confidence interval; *YI*, Youden index (J); *Sen*, sensitivity; *Spec*, specificity; *PPV*, positive predictive value; *NPV*, negative predictive value; *LR+*, positive likelihood ratio; *LR-*, negative likelihood ratio; *P*, significance level.
*Statistically significant

Table 2.9: Predictive performance of CVF metabolites normalised integrals for preterm birth (< 37w), in asymptomatic high risk pregnant women (20-22w)

Metabolite	AUC	SE	95% CI	YI	Sen (%)	Spec (%)	PPV (%)	NPV (%)	LR+	LR-	*P* value
Lactate	0.52	0.06	0.40-0.64	0.85	29	85	48	71	1.8	0.5	0.4
Acetate	0.61	0.06	0.50-0.73	0.78	49	78	52	75	2.2	0.5	0.03*
Succinate	0.56	0.06	0.45-0.68	0.44	74	44	39	78	1.3	0.8	0.1
Formate	0.42	0.06	0.30-0.53	0.13	100	13	36	100	1.1	0.9	0.9
Glucose	0.51	0.06	0.39-0.63	0.23	88	23	36	79	1.1	0.9	0.43
Alanine	0.55	0.06	0.43-0.67	0.32	86	32	38	82	1.3	0.8	0.2
Glx	0.54	0.06	0.42-0.66	0.32	80	32	37	77	1.2	0.8	0.3
BCAA	0.55	0.06	0.43-0.67	0.51	69	51	41	77	1.4	0.7	0.2
Suc/Lac	0.55	0.06	0.43-0.67	0.38	80	38	39	79	1.3	0.8	0.2
Ace/Lac	0.59	0.06	0.47-0.71	0.51	69	51	41	77	1.4	0.7	0.06

Ace/Lac, acetate-lactate ratio; *BCAA*, branched chain amino acids; *Glx*, glutamine/glutamate; *Suc/Lac*, succinate-lactate ratio; *AUC*, area under the ROC curve; *SE*, standard error; *CI*, 95% confidence interval; *YI*, Youden index (J); *Sen*, sensitivity; *Spec*, specificity; *PPV*, positive predictive value; *NPV*, negative predictive value; *LR+*, positive likelihood ratio; *LR-*, negative likelihood ratio; *P*, significance level.

*Statistically significant

Table 2.10: Predictive performance of CVF metabolites normalised integrals for preterm birth (< 37w), in asymptomatic pregnant women (20-22w), irrespective of risk of preterm birth

Metabolite	AUC	SE	95% CI	YI	Sen (%)	Spec (%)	PPV (%)	NPV (%)	LR+	LR-	*P* value
Lactate	0.57	0.05	0.47-0.67	0.58	60	58	23	88	1.4	0.7	0.07
Acetate	0.57	0.05	0.47-0.67	0.75	45	75	27	87	1.8	0.5	0.08
Succinate	0.46	0.05	0.37-0.56	0.11	95	11	18	92	1.1	0.9	0.8
Formate	0.49	0.05	0.39-0.59	0.20	92	20	19	93	1.2	0.9	0.6
Glucose	0.48	0.05	0.38-0.58	0.17	89	17	18	89	1.1	0.9	0.6
Alanine	0.49	0.05	0.40-0.59	0.21	88	21	18	89	1.1	0.9	0.6
Glx	0.55	0.05	0.45-0.65	0.59	55	59	21	86	1.3	0.8	0.2
BCAA	0.57	0.05	0.47-0.67	0.64	58	64	25	88	1.6	0.6	0.1
Suc/Lac	0.49	0.05	0.40-0.59	0.15	95	15	19	94	1.1	0.9	0.5
Ace/Lac	0.45	0.05	0.35-0.55	0.92	15	92	27	84	1.8	0.6	0.8

Ace/Lac, acetate-lactate ratio; *BCAA*, branched chain amino acids; *Glx*, glutamine/glutamate; *Suc/Lac*, succinate-lactate ratio; *AUC*, area under the ROC curve; *SE*, standard error; *CI*, 95% confidence interval; *YI*, Youden index (J); *Sen*, sensitivity; *Spec*, specificity; *PPV*, positive predictive value; *NPV*, negative predictive value; *LR+*, positive likelihood ratio; *LR-*, negative likelihood ratio; *P*, significance level.

Table 2.11: Predictive performance of CVF metabolites normalised integrals for preterm birth (< 37w), in asymptomatic high risk pregnant women (26-28w)

Metabolite	AUC	SE	95% CI	YI	Sen (%)	Spec (%)	PPV (%)	NPV (%)	LR+	LR-	*P* value
Lactate	0.49	0.07	0.36-0.62	0.59	54	59	34	76	1.3	0.8	0.6
Acetate	0.39	0.06	0.27-0.52	0.97	12	97	60	73	3.8	0.3	1.0
Succinate	0.59	0.07	0.46-0.72	0.65	58	65	39	79	1.6	0.6	0.09
Formate	0.50	0.07	0.37-0.64	0.12	100	12	31	100	1.1	0.9	0.5
Glucose	0.53	0.07	0.40-0.67	0.30	88	30	34	87	1.3	0.8	0.3
Alanine	0.50	0.07	0.37-0.63	0.86	27	86	44	75	1.9	0.5	0.5
Glx	0.50	0.07	0.37-0.63	0.48	65	48	33	76	1.3	0.8	0.5
BCAA	0.50	0.07	0.37-0.64	0.82	35	82	43	76	1.9	0.5	0.5
Suc/Lac	0.38	0.06	0.26-0.51	0.99	8	99	67	73	5.0	0.2	1.0
Ace/Lac	0.41	0.06	0.29-0.54	1.0	12	100	100	74	-	-	0.9

Ace/Lac, acetate-lactate ratio; *BCAA*, branched chain amino acids; *Glx*, glutamine/glutamate; *Suc/Lac*, succinate-lactate ratio; *AUC*, area under the ROC curve; *SE*, standard error; *CI*, 95% confidence interval; *YI*, Youden index (J); *Sen*, sensitivity; *Spec*, specificity; *PPV*, positive predictive value; *NPV*, negative predictive value; *LR+*, positive likelihood ratio; *LR-*, negative likelihood ratio; *P*, significance level.

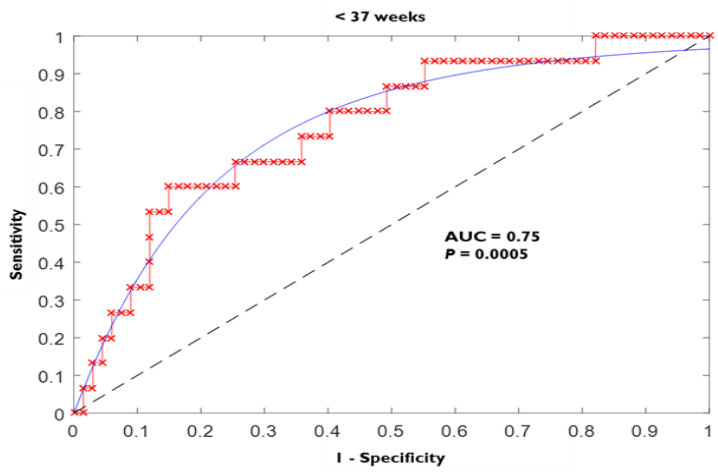
Table 2.12: Predictive performance of CVF metabolites normalised integrals for preterm birth (< 37w), in symptomatic pregnant women (24-36w)

Metabolite	AUC	SE	95% CI	YI	Sen (%)	Spec (%)	NPV (%)	PPV (%)	LR+	LR-	*P* value
Lactate	0.56	0.08	0.39-0.72	0.65	67	65	30	90	1.9	0.5	0.2
Acetate	0.75	0.08	0.60-0.91	0.85	60	85	47	90	4.0	0.2	0.0005*
Succinate	0.55	0.08	0.39-0.72	0.64	53	64	25	86	1.5	0.7	0.3
Formate	0.50	0.08	0.35-0.68	0.38	80	38	23	90	1.3	0.8	0.4
Glucose	0.52	0.08	0.35-0.68	0.46	80	46	25	91	1.5	0.7	0.4
Alanine	0.59	0.09	0.42-0.75	0.83	47	83	38	87	2.8	0.4	0.2
Glx	0.46	0.08	0.30-0.62	0.83	33	83	31	85	2.0	0.5	0.7
BCAA	0.56	0.08	0.39-0.72	0.99	27	99	80	86	17.3	0.1	0.3
Suc/Lac	0.40	0.08	0.25-0.56	0.08	100	8	20	100	1.1	0.9	0.9
Ace/Lac	0.76	0.08	0.61-0.91	0.61	87	61	33	95	2.2	0.5	0.0004*

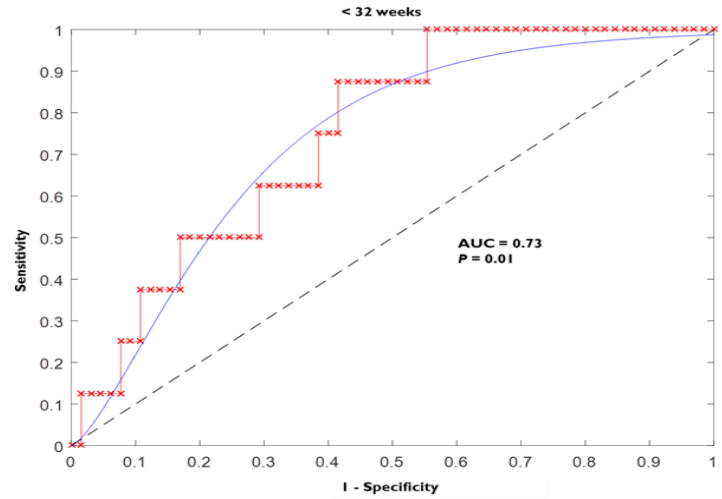
Ace/Lac, acetate-lactate ratio; *BCAA*, branched chain amino acids; *Glx*, glutamine/glutamate; *Suc/Lac*, succinate-lactate ratio; *AUC*, area under the ROC curve; *SE*, standard error; *CI*, 95% confidence interval; *YI*, Youden index (J); *Sen*, sensitivity; *Spec*, specificity; *PPV*, positive predictive value; *NPV*, negative predictive value; *LR+*, positive likelihood ratio; *LR-*, negative likelihood ratio; *P*, significance level.

*Statistically significant

A.



B.



C.

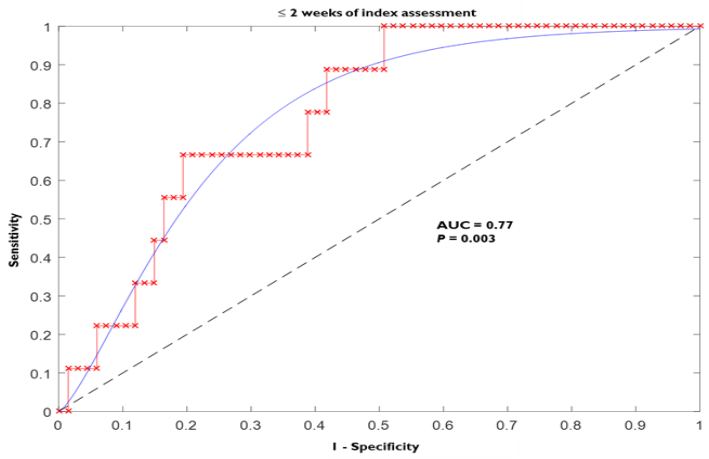


Figure 2.10. Performance of CVF acetate normalised integrals in predicting preterm delivery in symptomatic pregnant women (A) < 37 weeks, (B) < 32 weeks, (C) within 2 weeks of index assessment.

AUC, area under the ROC curve.

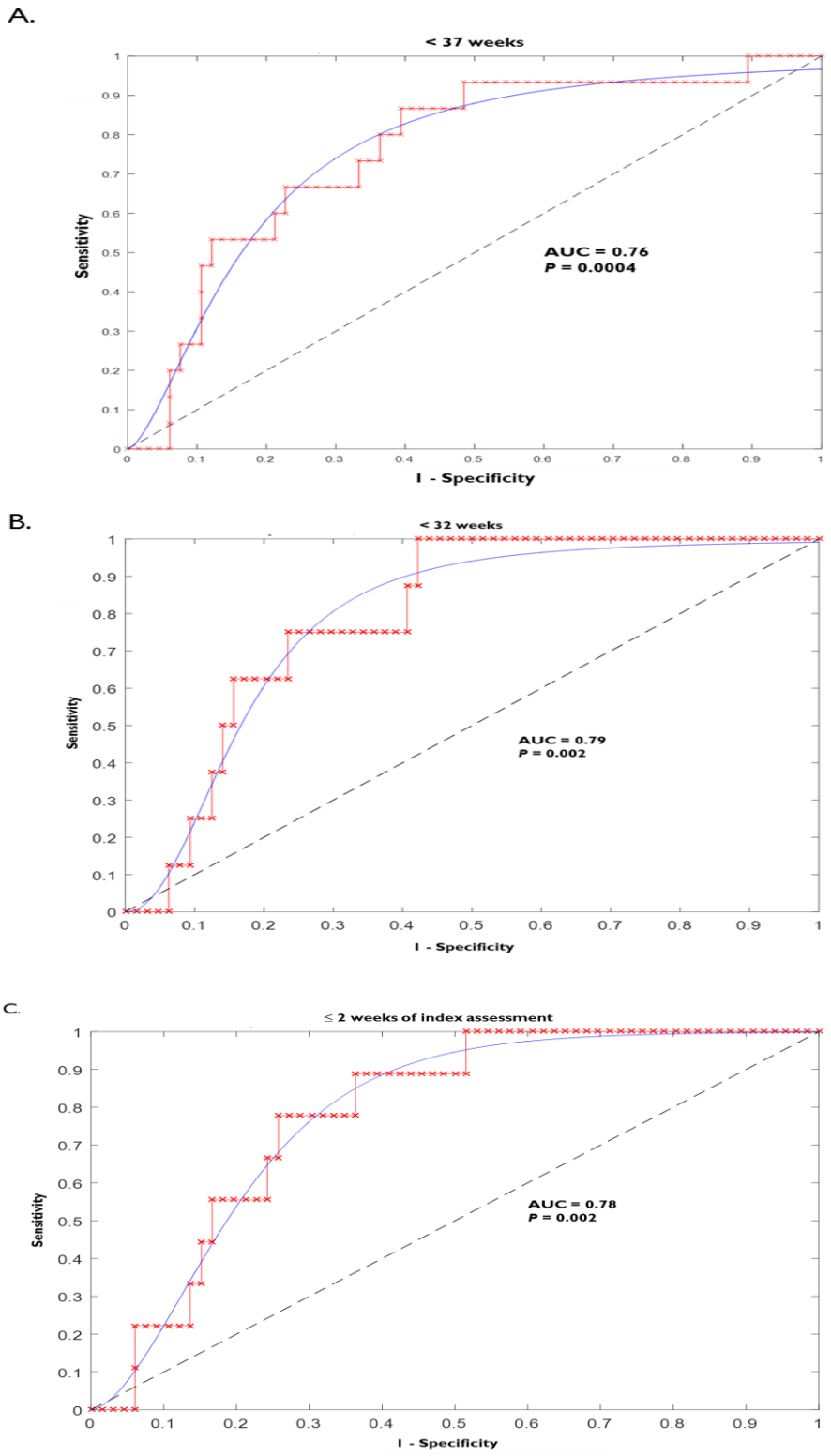
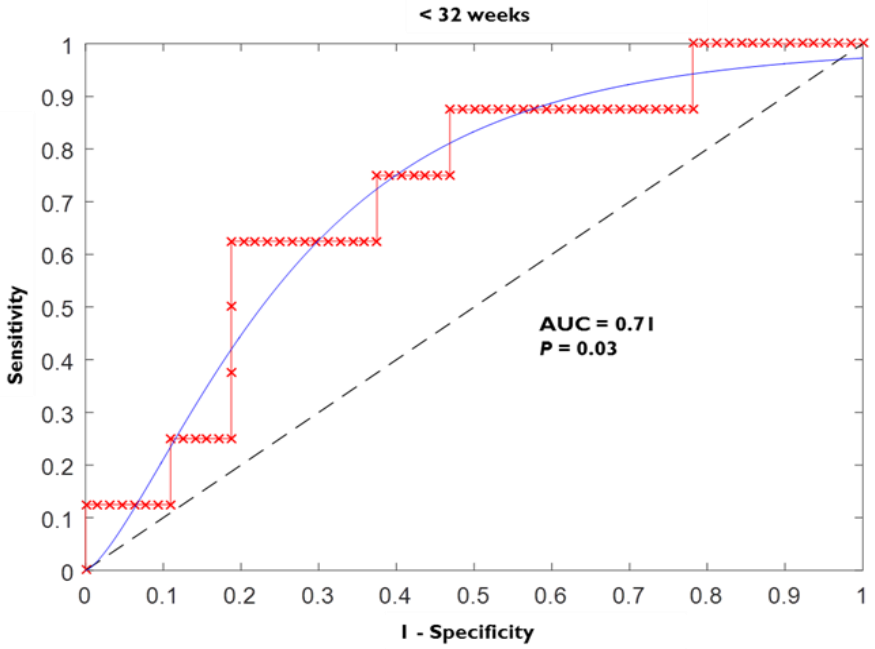


Figure 2.11. Performance of CVF acetate/lactate ratio in predicting preterm delivery in symptomatic pregnant women (A) < 37 weeks, (B) < 32 weeks, (C) within 2 weeks of index assessment.

AUC, area under the ROC curve.

A.



B.

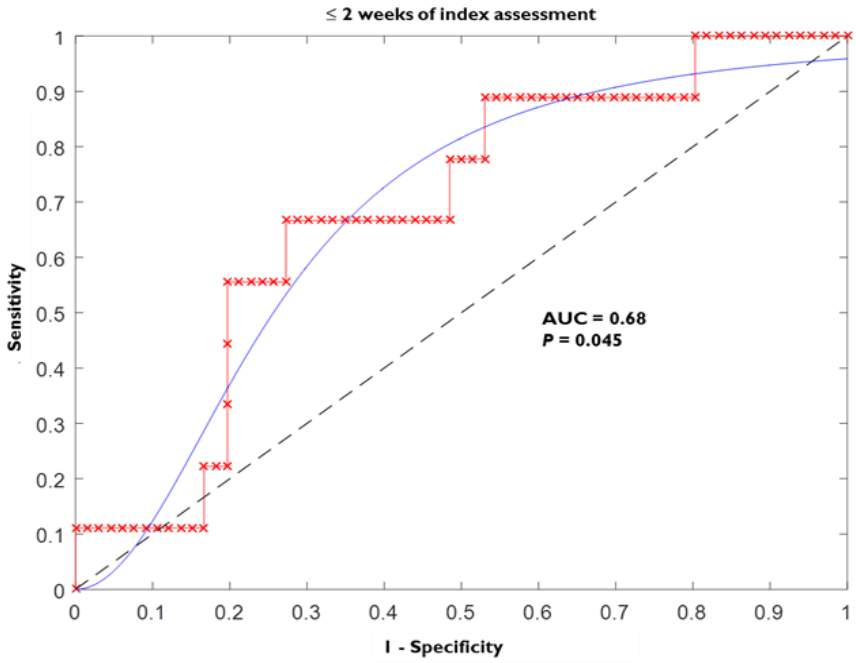


Figure 2.12. Performance of CVF glutamine/glutamate normalised integrals in predicting preterm delivery in symptomatic pregnant women (A) < 32weeks, (B) within 2 weeks of index assessment.

AUC, area under the ROC curve

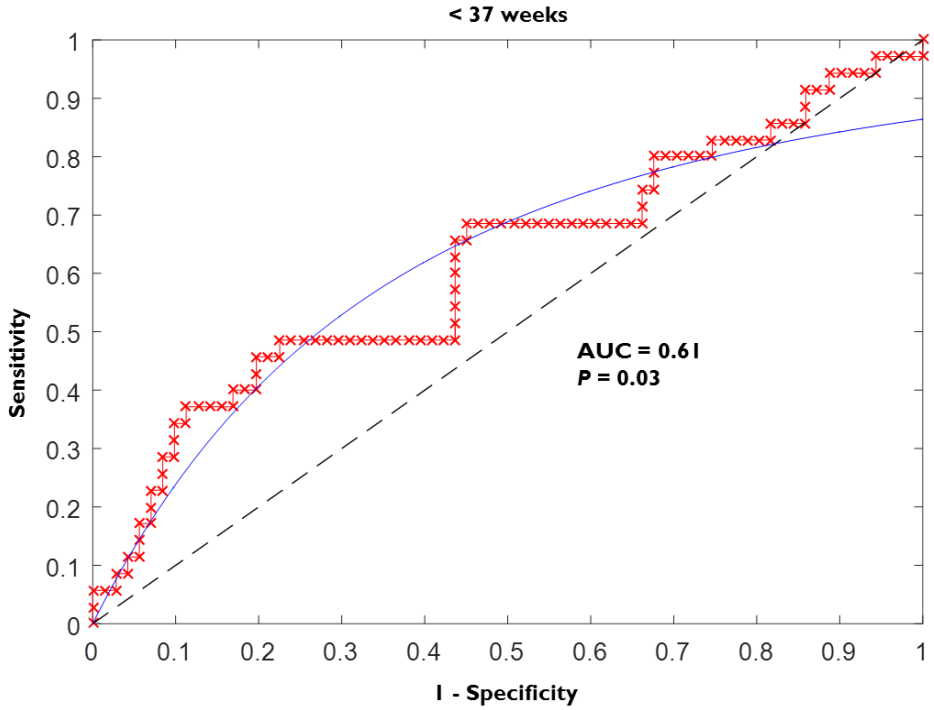


Figure 2.13. Performance of CVF acetate normalised integrals in predicting preterm delivery (<37 weeks) in asymptomatic high risk pregnant women studied at mid second trimester (20-22w).

AUC, area under the ROC curve

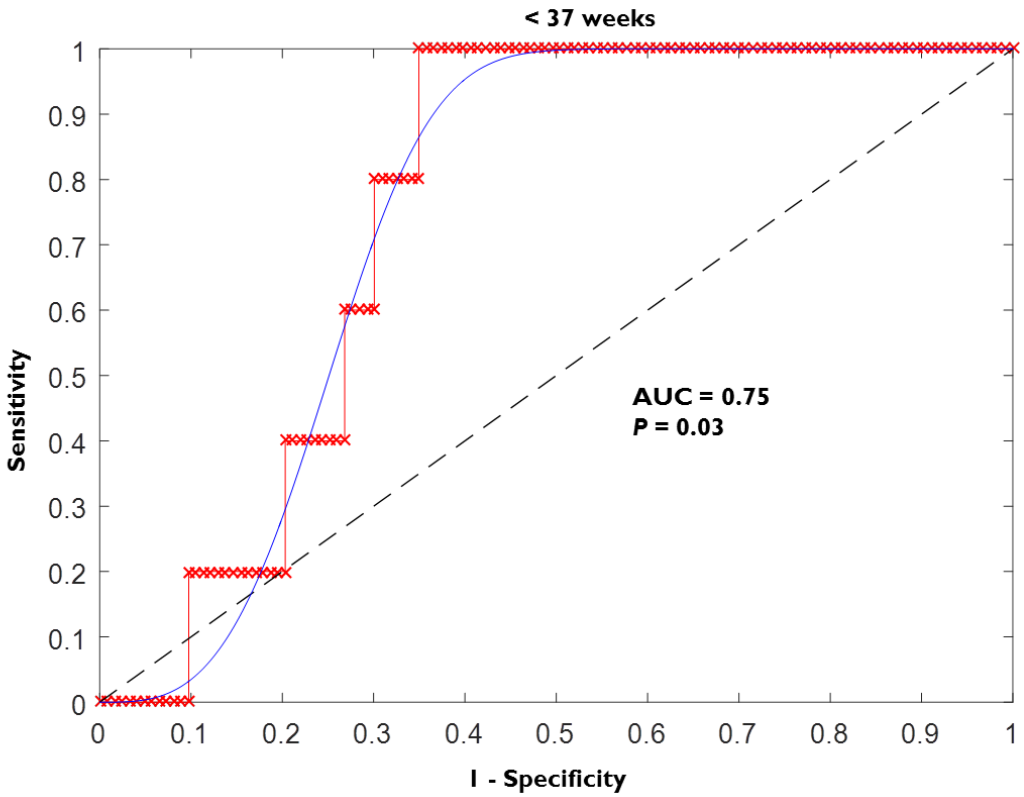


Figure 2.14. Performance of CVF branched chain amino acids normalised integrals in predicting preterm delivery (<37 weeks) in asymptomatic low risk pregnant women studied at mid second trimester (20-22w).

AUC, area under the ROC curve

2.5. Discussion

In this chapter, we sought to characterise the CVF metabolite profile of a unique study cohort of pregnant women who delivered prematurely compared to those who do not, based on their risk of PTB, and by presentation with symptoms of PTL. We speculated that the CVF ^{1}H-MR spectrum may reliably predict pregnant women who deliver prematurely, as well as women with symptoms of PTL who deliver shortly after assessment. With our study cohort adequately designed and stratified, and the commonly used assessment markers of PTB applied (Chapter 5), CVFs obtained by high-vaginal swabs from both asymptomatic and symptomatic pregnant women (n = 408) across the mid trimester, with intact membranes and no evidence of genital infection, were analysed with a 9.4T MR spectrometer. Metabolites (acetate, succinate, formate, lactate, glucose, glutamine/glutamate, alanine and branched chain amino acids) were identified in the ^{1}H-MR spectrum, integrated for peak area and normalised to the total spectrum integral (excluding residual water peak). This was in order to validate and optimise the sample preparation procedure and fairly compare the CVF samples from our study cohorts and prevent errors due to variations in the total sample concentration which can eclipse specific changes in individual metabolites. Normalisation counteracts the influence of variations in total sample concentration.[450] The large residual water signal was omitted as it has intensity in approximately 2 orders of magnitude greater than the other metabolites present and this can immensely obscure the intensities of other metabolite signals if not excluded. Other metabolite peaks associated with infection and PTB that were not identified in the ^{1}H-MR spectra, but still undergoing investigation include propionate, butyrate, isobutyrate, cadaverine, putrescine, trimethylamine etc.

In comparison to other metabolomics techniques, ^{1}H-MR is non-invasive and non-destructive, and has a relatively higher resolution and sensitivity. Samples can be recovered after the experiment and stored for future analyses. Furthermore, it is a highly flexible technique as MR spectroscopy and imaging can be employed in a single experiment, thus making it possible to obtain metabolic, physiological, and anatomical data. Another advantage of MRS over other techniques is the absence of ionizing radiation.[419] However, due to its ability to detect large water signals at significantly greater intensity than other metabolites, some metabolites

especially those around the vicinity of the water signal may not be identified on the spectrum. This greatly affects the sensitivity of the technique compared to other analytical methods including gas and liquid chromatography mass spectroscopy (GC/LC-MS). Fortunately, it can be used to analyse the structural properties of molecules including non-volatile compounds, as opposed to GC/LC-MS which is usually challenging with non-volatile compounds. Recent investigations employing mass spectroscopy have been unable to identify vaginal fluid acetate (a volatile metabolic acid),[65, 84, 358, 451] which is readily detected by ^{1}H-MR.[65, 189, 295, 439] Also, working in a high magnetic-field environment such as that of an MR lab could be dangerous if adequate safety precautions are not observed. More so, MRS systems are usually not readily available and accessible owing to their very huge cost.[419]

Normalisation of the identified metabolite peaks to the total spectrum integral was to correct for any concentration differences in the obtained CVF due to sampling.[65, 295] This was verified by the absence of any statistically significant differences in the ^{1}H-MR total spectrum absolute integrals, indicating that CVF concentration did not vary significantly between and within the study groups (Fig. 2.5). An alternative approach is the probabilistic quotient normalisation method.[189, 439] This method involves determination of the most probable quotient or normalisation factor by dividing the signals of interest in a test spectrum by corresponding signals of a reference spectrum. The median of the resultant quotients has been recommended as the best approach for normalising all variables (metabolite signals) of the test spectrum.[450] This has been argued to be a more robust and accurate method of normalisation in quantitative metabolite analysis compared to the integral normalisation method. This is because integral normalisation is susceptible to alterations due to extreme concentrations of particular metabolites in a given sample. However, the probability quotient still requires normalisation to total spectrum integral as its initial step.[450]

The CVF metabolite fingerprint is reflective of changes in the vaginal microecology that may impact the initiation of PTB.[1] Our metabolite data showed that pregnant women presenting with symptoms of threatened preterm labour who eventually delivered preterm had significantly higher acetate N.I. (> 2 fold, 103%) and acetate/lactate ratio (46%) (P = 0.002), than their term counterparts (Fig. 2.6). In all study groups i.e. both symptomatic and asymptomatic pregnant women, acetate and lactate N.I.s correlated with vaginal pH, whilst glucose N.I.,

glutamine/glutamate N.I., succinate N.I. and succinate/lactate ratio only correlated with vaginal pH in the asymptomatic low and high risk women studied at 20-22w (ASYM 20-22w), and asymptomatic high risk women studied at 26-28w. Consistent with their relationship with the vaginal pH, the metabolites also correlated among themselves across the groups (Fig. 2.7A-F). For instance, lactate N.I. was inversely related to acetate, succinate and glucose N.I.s, but was directly related to glutamine/glutamate N.I. Acetate and succinate N.I.s, alanine, glutamine/glutamate and BCAA N.I.s were also directly related.

All these metabolites are part of a complex metabolic interaction that occurs between the host vaginal epithelial cells and microbial population as illustrated in Fig. 2.1. The direction of these metabolic pathways in the vaginal microenvironment is also influenced by hormonal factors such as the sex hormones and the menstrual cycle. For instance, high oestrogen levels as seen in premenopausal and pregnant women enhance the maturation and deposition of large amounts of glycogen in the vaginal epithelium. Glycogen is further metabolised via the glycolytic pathway to glucose and then lactate predominantly by *Lactobacillus* sp. Consistent with our data, elevated lactate is related to low pH and decreased risk of PTB, whilst elevated acetate, a SCFA produced by anaerobes associated with BV, is related to higher pH and increased risk of PTB.[37, 38]

The vaginal pH in women whose vaginal microflora is dominated by *Lactobacillus* is strongly related with elevated CVF lactic acid levels,[37] and is protective against infection [38, 160, 452, 453] and PTB.[1, 207, 454] On the other hand, acetate and other SCFAs are related to abnormal vaginal flora, BV and PTB.[100, 207, 210, 452, 455] Also, there were correlations between lactate and succinate N.I.s in all the groups, as well as between elevated vaginal succinate/lactate ratio, a marker of dysbiosis (e.g. BV)[38,84] and high pH in the asymptomatic women. Though these were modest relationships, they indicate the importance of succinate/lactate ratio as a predictor of abnormal vaginal flora and consequently adverse delivery outcome. However, with the recent report that vaginal succinate is not associated with high vaginal microbial diversity and clinical BV,[85] and whether such metabolite marker can be applied irrespective of symptoms or identified risk,[451] the data indicate that the predictive utility of these markers should be elucidated by further investigation with larger and well stratified study population.

The positive association between acetate and succinate N.I.s across the groups, and their negative correlation with lactate N.I. observed was not unexpected. Acetate and succinate are produced by anaerobic BVAB and have been reported to exhibit immune modulatory activities inhibiting optimal host immune response against pathogens, contributing to a dysbiotic vaginal microflora and subsequently leading to increased risk of poor sexual and reproductive outcomes.[38, 100, 144, 210, 455, 456] Like acetate, elevated succinate N.I. was associated with high vaginal pH in all the groups except the symptomatic women. Combined together, elevated CVF acetate and succinate N.I.s are associated with low lactate N.I., high vaginal pH and hence, altered vaginal microbial environment and poor delivery outcome.

The relationship between vaginal pH and glutamate levels in relation to abnormal vaginal flora has been highlighted in recent studies.[84, 439] It was reported that elevated vaginal glutamate level (like lactic acid) is associated with low vaginal pH but inversely related to an *L. iners* and BVAB-dominated microbiota.[84] In line with the above observation, our data showed an inverse association between glutamine/glutamate N.I. and vaginal pH in the asymptomatic women studied at 20-22w and 26-28w (Fig. 2.7C-D); and a direct correlation between glutamine/glutamate and lactate N.I.s in both symptomatic and asymptomatic women (Fig. 2.7A-F). This is exciting as glutamine/glutamate could be another metabolite that influences vaginal pH and by extension vaginal microenvironment with conceivable prognostic value. In depth analysis of the vaginal microbiota community compositions of these study groups using broad-range PCR and pyrosequencing in relation to glutamine/glutamate and other identified metabolites may improve our understanding of the diagnostic implications of these associations.

Although the amino acids identified in this study did not differ significantly between term- and preterm-delivered women, there were modest associations and trends between them. Alanine correlated positively with both glutamine/glutamate and BCAA N.I.s. The implications of these associations are unclear. Nonetheless, it is known that BVAB utilise amino acids as carbon and nitrogen sources as opposed to *Lactobacillus* species which preferentially metabolise carbohydrates, such as glycogen.[84, 457] Accordingly, a high level of intact amino acids in the vaginal ecosystem is associated with *Lactobacillus* sp. dominance and/or low amount of anaerobic BVAB. This is supportive of our data as we observed firstly, positive associations between lactate and the amino acids especially glutamine/glutamate and BCAA; and secondly, positive

associations between the amino acids themselves (Fig. 2.7A-F). Furthermore, Srinivasan et al.[84] demonstrated a positive association between the BCAAs (valine, leucine, and isoleucine) and glutamate with *Lactobacillus* sp. particularly *L. crispatus* and *L. jensenii*; and a negative association between these amino acids and BVAB (e.g. *G. vaginalis*, *A. vaginae*, *Eggerthella*, *Megasphaera* etc.). This was recently validated by Vitali et al.[439] who by employing the same technique as ours (i.e. ^{1}H-MR), demonstrated decreased levels of glutamate, leucine and isoleucine in BV-infected women compared to their healthy counterparts. This is another identifiable source of future experiments as the potential diagnostic utility of these specific amino acids remains unresolved.

We also examined the relationship between the identified ^{1}H-MR metabolite N.I.s and the gestational age at delivery. There were no significant associations except in the asymptomatic women studied at 20-22w combined regardless of their risk statuses. In this combined group, only lactate and glutamine/glutamate N.I.s correlated with gestational age at delivery (Fig. 2.7C). When they were reclassified into low and high risk group, only the low risk women showed a positive association between glutamine/glutamate N.I. and gestational age at delivery (Fig. 2.7A). These observations buttress the negative relationship between lactate, glutamine/glutamate N.I.s and vaginal pH on one hand, and the negative relationship between vaginal pH and gestational age at delivery on the other hand (Fig. 2.7C). Taken together, elevated CVF lactate and glutamine/glutamate N.I.s in asymptomatic women sampled at mid second trimester regardless of a prior PTB and/or short cervix, are associated with lower vaginal pH and normal gestational age at delivery.

Another metabolite identified in the CVF ^{1}H-MR spectra of our study participants was formate, a SCFA also produced by BVAB. Though there were no significant differences in this metabolite in relation to delivery outcomes, we observed certain associations with other SCFAs, organic acid and amino acid metabolites across the groups (Fig. 2.7A-F). The implications of these associations are unclear but like acetate and succinate, high levels of formate are associated with BV and poor reproductive outcomes.[38, 439]

With the identification of metabolite differences and associations with other maternal clinical parameters currently used as assessment markers for PTB, we investigated the predictive potential of the CVF metabolites identified by MRS for risk of PTB in our unique study

population. Acetate N.I. and acetate/lactate ratio were predictive of < 37 weeks and < 32 weeks premature deliveries, as well as delivery within 2 weeks of presentation and measurement in women presenting with symptoms of threatened preterm labour (Tables 2.2-2.3 and Figs. 2.10-2.11). Similar predictive potential for delivery before 32 weeks gestation and within 2 weeks of presentation was observed for glutamine/glutamate N.I. in the same women (Table 2.4 and Fig. 2.12). These metabolites showed high sensitivities, specificities and NPVs but low PPVs for PTB comparable to those of FFN[309, 310, 458] and ultrasound CL.[354, 459-461] The high NPVs achieved in our series also indicate that low acetate N.I., just like low FFN and high CL,[303, 459] may demonstrate a good "exclusion" test for imminent premature birth, referring women to outpatient care, providing maternal reassurance and thus minimizing waste of scarce resources. Combining measurements of FFN, CL and acetate could enhance the diagnostic performance of these markers in both asymptomatic and symptomatic women. This is discussed later in this report (Chapter 5). We also observed that women with increased acetate N.I. and acetate/lactate ratio had 4-fold and 2-fold chances of delivering preterm (< 37 weeks) respectively. A 2-fold increase in CVF acetate N.I. produced a 4-fold likelihood of delivery before 37 weeks gestation, and a 2-fold likelihood of delivery before 32 weeks gestation and within 2 weeks of presentation. This is intriguing as all the PTBs recorded in the symptomatic women in this study occurred approximately within 2 weeks of presentation. Also, in women presenting with symptoms of threatened PTL, the prediction of the likelihood of spontaneous preterm delivery occurring within 2 weeks of testing is of utmost interest as this determines the level of management.[313] In other words it ensures adequate "diagnostic evidence in therapeutic decision making".[313] Glutamine/glutamate N.I. also showed 3-fold and 2-fold probability for delivery before 32 weeks gestation and within 2 weeks of presentation respectively (Table 2.2-2.4). These observations suggest probable clinical utility of these metabolites for predicting PTB. Whether this is achievable independently or in combination with FFN and CL remains undetermined. A multivariate analysis of these markers could enhance predictive performance compared to CL or FFN alone. This was attempted by our group and will be seen later in this report (Chapter 5).

In addition to its predictive value in symptomatic pregnant women, acetate N.I. also showed considerable discriminative capacity for premature delivery (< 37 weeks) in the asymptomatic

high risk women studied at mid second trimester (20-22w), with high sensitivity, specificity, PPV, NPV, and a LR+ of 2.2 (Table 2.5 and Fig. 2.13). Again, this can be compared to the predictive capacities of FFN and ultrasound CL in this specific cohort of pregnant women.[311, 312, 320, 321]

Also, in the asymptomatic low risk cohort, apart from showing a trend to higher branched chain amino acids (BCAA), in the women who delivered at term compared to those that delivered preterm, the BCAAs were predictive of preterm birth (< 37w), with high sensitivity, specificity, NPV, but low PPV, and LR+ of 2.9 (Table 2.6 and Fig. 2.14). This is not only comparable to the data obtained from FFN and ultrasound CL in other studies with asymptomatic women,[303] but was even a better predictor of spontaneous preterm birth than FFN (AUC: 0.75 vs. 0.65[313]), with a significantly higher sensitivity (BCAA:100% vs. FFN: 20-29% vs. CL: 33-54%[303]). Women with high BCAA had about 3-fold likelihood of delivering at term. This is corroborated by the observation of decreased vaginal protein/amino acid catabolism in normal healthy women,[84] such as the term-delivered low risk women in our study. High amounts of BCAA (as seen in the term-delivered women) are associated with *L. crispatus* and *L. jensenii* dominance characteristic of a healthy vaginal ecosystem with good reproductive prognosis.[84, 439] CVF amino acids, including the BCAA (leucine, isoleucine and valine) are catabolised and used up as energy sources by BVAB. Therefore, high amounts of intact BCAA, could be suggestive of absence of BVAB and/or dominance of *Lactobacillus* sp.[84] To our knowledge this is the first of its kind in the quest for identification of CVF metabolite markers with prognostic utility for spontaneous preterm delivery in a low risk population. More so, as will be seen subsequently in this report, FFN and ultrasound CL were not predictive of preterm birth in this group of women in our study (Chapter 5).

The associations between CVF FFN and metabolites especially acetate in the asymptomatic women (20-22w) (Fig. 2.7C), and the comparable predictive capacity of acetate in the high risk women of this group, suggests that combining these analytes may offer additional predictive potential for preterm delivery. A more robust approach using MR spectrometers with stronger magnetic field (e.g. 600 MHz (14.1T), 700 MHz (16.4T) and 900 MHz (21.1T)), that allow more sensitive analysis of metabolites; and a larger study population with more stringent inclusion and exclusion criteria is necessary.

One limitation to the current study which is a critical subject for future investigation is the identification of the chiral forms of lactate in CVF of our cohorts in relation to reproductive health. Lactate has 2 optical isomers (D- and L-lactate). D-lactate is almost exclusively produced by bacterial cells, while L-lactate can be synthesised by both human and bacterial cells.[1, 114] The vaginal epithelial cells, like other human cells produce L-lactate only because they lack the enzyme D-lactate dehydrogenase.[1, 114] As could be the case in our study, the vaginal epithelium secretes L-lactate into the vaginal environment irrespective of the composition of the vaginal bacterial community. L-lactate is less potent in protecting the host against microbial invasion of the upper genital tract compared to D-lactate which is produced by *Lactobacillus* sp. including *L. crispatus*, *L. gasseri* and *L. jensenii* but not *L. iners*. *L. iners*, like human cells, lacks D-lactate dehydrogenase and so does not synthesise D-lactate.[1, 114, 196] Because of its significant protective capacity against ascending intrauterine infection in relation to L-lactate, via the inhibition of extracellular matrix metalloproteinase inducer (EMMPRIN) and MMPs, D-lactate has been recommended as a prebiotic in the treatment of BV and prevention of preterm birth.[1, 114, 196] Determination of CVF L-/D-lactate ratio in pregnant women at risk of preterm birth will provide insight into the source(s) of lactate identified, the composition of the vaginal microbial population as well as the predominant *Lactobacillus* sp. at any given gestational time point. Fortunately, CVF D- and L-lactate optical isomers can be measured colourimetrically by commercially available assay kits.[1, 114,196] This warrants further study.

Lastly, in these experiments we were unable to identify significant differences or predictive utility of any of the metabolites in the asymptomatic women who were studied again at 26-28 weeks. For example, the AUC for acetate was 0.39, significantly lower than those of the other groups. These observations could be attributed to the administration of several therapeutic interventions including antibiotics to women in this group due to their high risk status (Chapter 5). Antibiotics were administered in cases of suspected subclinical genital bacterial infection acquired in later gestation (after sampling at 20-22 w). This may have altered the vaginal microbiome towards normal[403, 462] and by extension metabolite profile, hence, an alteration in certain metabolite levels. Such interventions could not be prevented as it will be unethical to abstain from treating such cases when identified. Nevertheless, some of these women ultimately delivered preterm. An in-depth investigation of the influence of antibiotic treatment and other

interventions such as cervical cerclage and progesterone on the vaginal microenvironment at mid-trimester is required.

Chapter 3

Quantification of cervicovaginal fluid acetate in pregnant women by biochemical assay

3.1. Introduction

Magnetic resonance spectroscopy is a powerful tool to establish the metabolite profiles of tissues and biofluids, and in identifying potentially useful biomarkers in relation to health and disease conditions. It is highly analytical, sensitive and capable of detecting volatile compounds such as acetate.[65, 295, 430, 432, 439] However, one setback of this approach is that additional steps are required to determine absolute concentrations of metabolites, e.g. concentration standards added to the sample, and this poses a challenge when cut-off values of a metabolite marker are required for diagnosis in the clinical setting. Also constraints due to its relatively low sensitivity compared to other techniques (e.g. MS), and cost effectiveness are prevalent. With these limitations, application of MRS in the clinical setting where rapid and accurate tests are needed to inform diagnosis and treatment may not be feasible. Hence there is the need to provide more rapid, cost-effective and easily assessable techniques of quantifying these CVF metabolites in clinical settings.

A relatively cost-effective and readily assessable technique capable of measuring the absolute concentrations of metabolites in biofluids is spectrophotometry. Vaginal fluid acetate and lactate have been measured spectrophotometrically with great success.[37] Spectrophotometry is a quantitative analytical technique that measures the concentrations of analytes in samples by their ability to absorb or transmit light in form of electromagnetic radiation, over certain range of wavelengths. With the use of a spectrophotometer, the intensity of light (amount of photons) absorbed by a sample solution can be measured, and the concentration of the analytes can be determined from the intensity of light detected. There are 2 types of spectrophotometers based on the range of wavelength of the light source.

The first is the Ultraviolet-Visible (UV/Visible) spectrophotometer, which utilises electromagnetic radiations between wavelengths of 190-400 nm (ultraviolet), and 400-900 nm (visible).[420] The other is the Infrared (IR) spectrophotometer, which utilises electromagnetic radiations between wavelengths of 700-1500 nm (infrared).

The mechanism of spectrophotometry as depicted in Fig. 3.1 involves 2 components: a spectrometer that provides the desired light in a range of colours (wavelengths), and a

photometer that detects the intensity of light that is absorbed which can be read on digital monitor or electric meter.

The spectrometer comprise of a lens (collimator) that transmits photons (beam of light). The beam of light is refracted (dispersed) as it passes through a prism (monochromator), into a spectrum of a range of wavelengths. Only light rays with the required wavelengths are transmitted with the aid of a wavelength selector (aperture). When the required wavelength of light traverses the sample contained in the cuvette, the intensity of light beam absorbed is detected by the photometer, with the aid of a light detector (photoelectric transducer). The signals are then amplified and recorded by an absorbance meter.

The concentration of a metabolite in a sample can be determined from the absorbance of the sample using the Beer-Lambert Law.[463] The Beer-Lambert Law states that absorbance of a given sample is directly proportional to its concentration i.e.

$$A = \mathcal{E}\, l\, c = -Log\ T = -Log \left[\frac{I_1}{I_0} \right]$$

where A is the measured absorbance with no unit, $\mathcal{E}$ is a constant called molar absorbtivity coefficient ($M^{-1}cm^{-1}$). It is unique for each molecule and is wavelength-dependent. l is the path length i.e. the length of sample solution the light passes through and it is dependent on the width of the cuvette (usually 1 cm), c is the concentration of the analyte (M or $molL^{-1}$), I_0 is the initial (incident) light intensity, I_1 is the transmitted light intensity (i.e. after it passes through the sample), while T is the Transmittance (i.e. the fraction of light that is not absorbed).

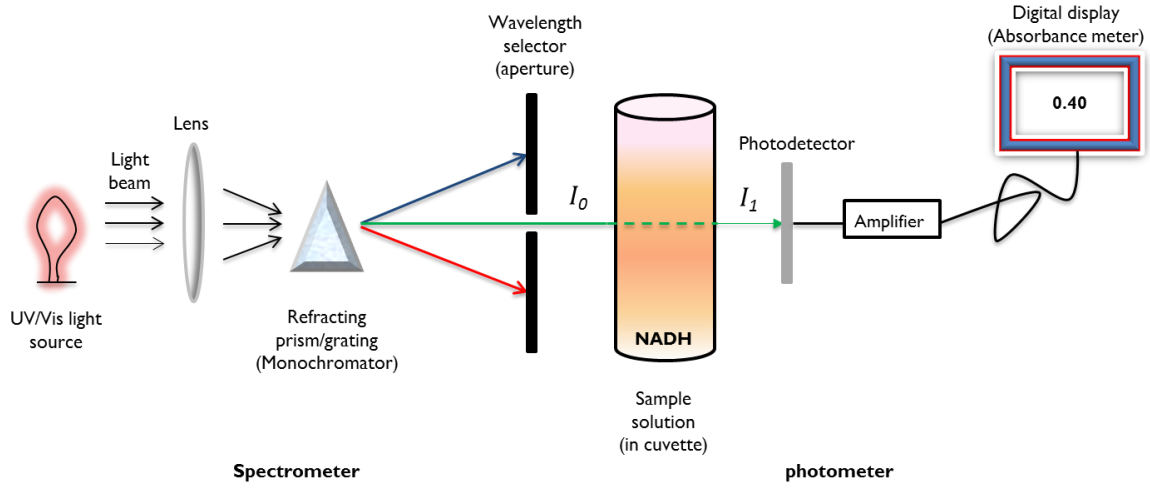


Figure 3.1. Schematic representation of the components and mechanism of a spectrophotometer

Analysis of CVF from pregnant women at risk of PTB across mid gestation using ^{1}H-MR, revealed significantly different acetate N.I. between term- and preterm-delivered SYM women. Also, acetate N.I. showed significant prognostic utility for PTB (< 37w, < 32w), and delivery within 2 weeks of presentation with symptoms suggestive of PTL (Chapter 2). With these fascinating findings, we sought to further validate the data obtained by ^{1}H-MR and attempt to provide a biochemical assay to assess the concentrations of acetate and other metabolites in CVF of pregnant and even non-pregnant women in relation to reproductive health. As a result we measured the concentrations of acetate in the same CVF samples of the symptomatic women (as in the ^{1}H-MR experiments) by enzyme-based spectrophotometric technique.

3.2. Methods

Acetate concentrations were determined from a randomly selected subset of CVF samples from pregnant women presenting with symptoms of threatened PTL (n = 57), by spectrophotometric absorption of NADH using an acetic acid assay kit (ADP-Glucokinase format, K-ACETGK 08/14, Megazyme, IE). The assay principle is based on the determination of acetate concentration by the generation of NADH from NAD^+, which is then quantified by the increase in absorbance at 340 nm.[464] The enzymes involved in the 4-step reaction and supplied with the assay kit include (1) acetate kinase (AK), (2) phosphotransacetylase (PTA), (3) ADP-glucokinase (ADP-GK), and (4) glucose-6-phosphate dehydrogenase (G6P-DH). Acetate is converted through the following reaction which is positively related to the eventual formation of NADH:

1. Acetic acid + ATP $\xrightarrow{\textit{Acetate kinase}}$ acetyl-phosphate + ADP

2. Acetyl-phosphate + CoA $\xrightarrow{\textit{Phosphotransacetylase}}$ acetyl-CoA + P_i

3. ADP + D-glucose $\xrightarrow{\textit{ADP-glucokinase}}$ glucose-6-phosphate + AMP

4. Glucose-6-phosphate + NAD^+ $\xrightarrow{\textit{G6P-dehydrogenase}}$ 6-phosphogluconate + NADH + H^+

The reaction is a positive reaction indicated by the increase in absorbance as demonstrated by the calibration curve (R^2 = 0.975, detection limit: 1.8 g/l) (Fig. 3.2). The acetic acid GK assay kit is an endpoint type assay specific for acetate with its lowest sensitivity limit ~0.1 g/l. All reagents were prepared, mixed, and stored according to manufacturer's instruction. A single reaction mixture containing 3 µl CVF sample in PBS, 200 µl of reagent 1 (distilled water, buffer and AK/PTA/ADP-GK/G6P-DH), and 20 µl of reagent 2 (NAD^+/ATP/D-glucose/CoA/PVP), was assayed for each sample. The reaction time was ~ 5 mins at 37°C after which the absorbance of the end product NADH, was read at 340 nm. A calibration curve indicating the linearity of K-ACETGK 08/14 was produced by plotting absorbance at 340 nm against concentrations of the

acetic acid standards (g/l) (Fig. 3.2). Different concentrations (calibrators) of the acetic acid standard (1.8 g/l), were made be serial dilution (i.e. 1.8, 1.2, 0.9, 0.6, 0.45, 0.36, 0.3, 0.225, 0.2, and 0.15 g/l). From the calibration curve concentrations of acetate in the samples were ascertained after measurement of their absorbance at 340 nm. All assays were performed using optical grade 96 well plates on a computer controlled Infinite M200 microplate reader (TECAN, CH), capable of detecting UV/Visible light, fluorescence, and luminescence.

3.2.1. Statistical analysis

All Statistical analyses were performed using MATLAB (Mathworks, Natick, MA). Values are presented as median ± quartile range and the Wilcoxon's rank-sum test was performed to compare differences in acetate concentration within the groups. The relationship between acetate concentration and ^{1}H-MR acetate N.I. was determined by Pearson's correlation coefficient and *P* values < 0.05 were considered statistically significant. The predictive capacity of the CVF acetate concentrations for PTB was determined by ROC curves for the following comparisons:

- Preterm (< 37 weeks) versus term births,
- < 32 versus > 32 weeks and
- < 2 versus > 2 weeks from presentation to delivery in symptomatic women.

A cut-off value of acetate concentration for predicting PTB < 37 weeks was also calculated from the ROC curve.

3.3. Results

By exploring the spectrophotometric absorption of NADH produced by a 4-step phosphorylation reaction involving acetic acid and D-glucose, we were able to measure the concentration of acetate in the CVF obtained from 57 women presenting with symptoms suggestive of threatened PTL in whom we hitherto observed significant difference in ^{1}H-MR acetate N.I. in relation to term and PTB (Chapter 2). Acetate concentration was determined by the generation of NADH from NAD^{+} and quantified by the increase in absorbance at 340 nm.[464]

Similar to our observation with acetate N.I., acetate concentrations measured by spectrophotometric technique was significantly higher in the preterm-delivered vs. term-delivered women in the symptomatic cohort (P = 0.006), and correlated strongly with the ^{1}H-MR acetate N.I. (r = 0.69, P < 0.00001) (Fig. 3.3A-B). Though there were significant differences in both acetate concentration and ^{1}H-MR acetate N.I. between preterm- and term-delivered women in the symptomatic cohorts, the difference observed in relation to ^{1}H-MR acetate N.I. was significantly greater (> 2-fold, 103%), compared to ~17% difference in acetate concentration (Fig. 2.6 and Fig. 3.3A). Compared to the ^{1}H-MR acetate N.I., the difference in acetate concentration was marginal. However, acetate concentrations in concordance with the acetate N.I. were predictive of PTB (< 37 w) and delivery within 2 weeks of index assessment with an optimal cut-off value for delivery < 37 w of > 0.53 g/l (0.009 mol/L). Its predictive ability for PTB < 32w did not attain statistical significance (AUC = 0.63, P = 0.13) (Table 3.1 and Fig. 3.4).

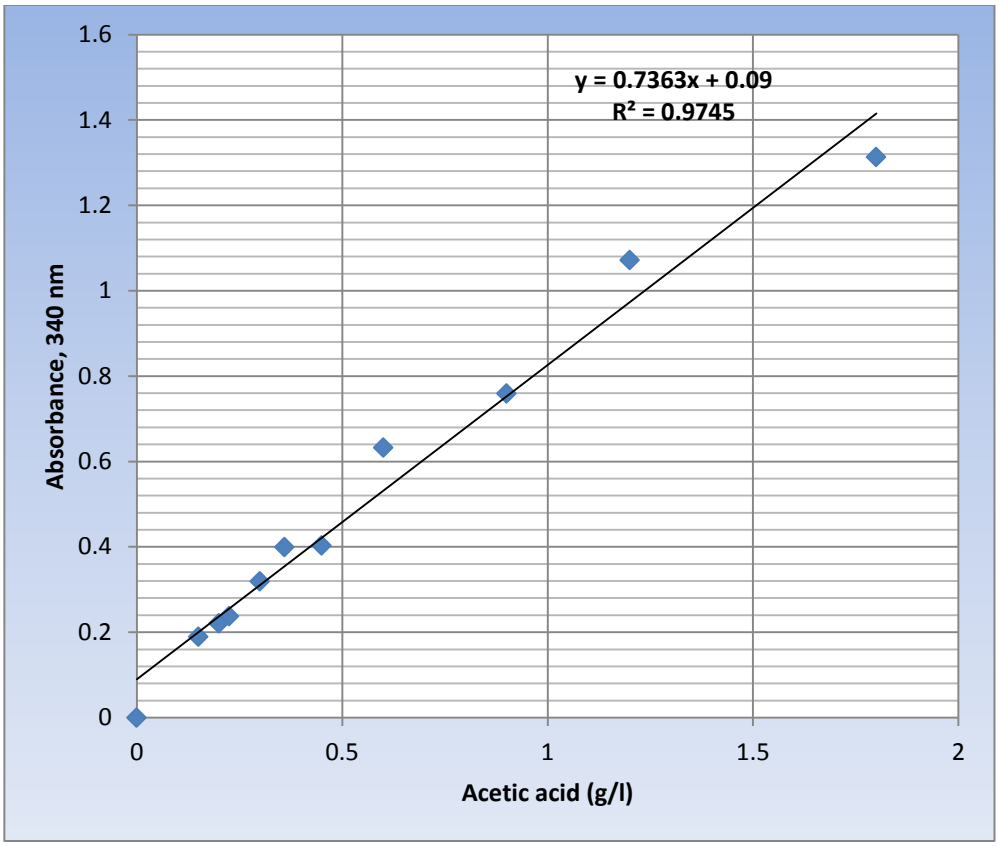
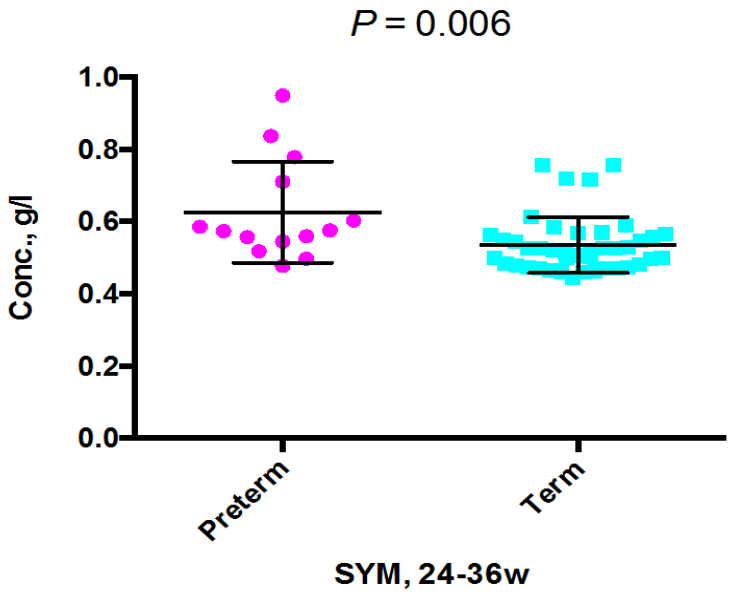


Figure 3.2. Calibration curve indicating the linearity of K-ACETGK. The reactions from which this calibration curve was generated were performed at 37°C for 5 mins using a TECAN Infinite M200 microplate reader (detection limit = 1.8 g/l or 0.03 mol/l).

A.



B.

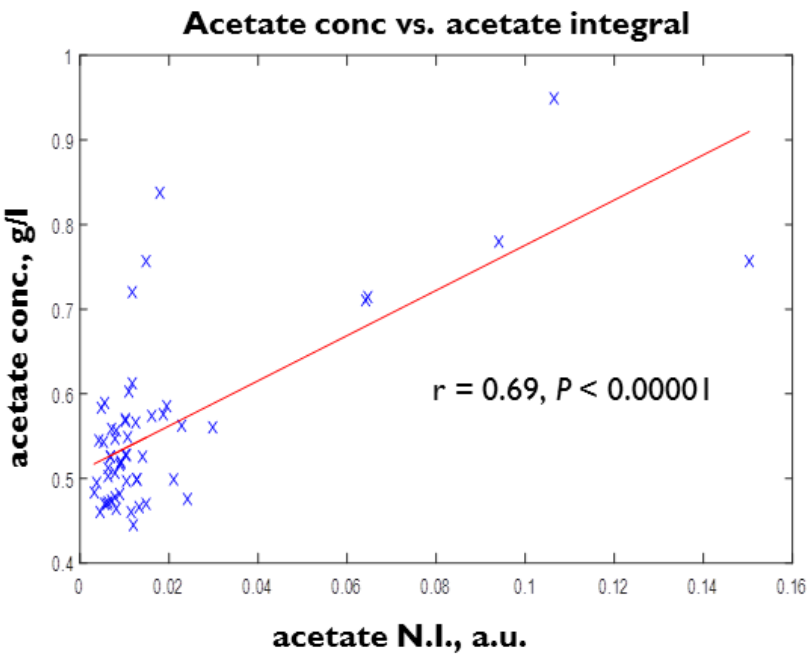


Figure 3.3. Validation of the differences in CVF acetate levels in pregnant women presenting with symptoms of preterm labour between 24 and 36 weeks gestation (A) Comparison of acetate concentration between preterm- and term-delivered women, (B) Association between acetate normalised integrals (N.I.) measured by ^{1}H-Magnetic Resonance Spectroscopy and acetate concentrations measured by spectrophotometry.

Table 3.1: Predictive performance of CVF acetate concentration for preterm birth in symptomatic pregnant women

Variables	< 37 weeks gestation	< 32 weeks gestation	≤ 2 weeks of index assessment
Area under the ROC curve (AUC)	0.74	0.63	0.68
Standard Error, SE	0.08	0.12	0.11
95% Confidence Interval	0.57-0.90	0.40-0.87	0.47-0.89
Significance level, *P*	0.002	0.13	0.045
Youden index, J	0.71	0.63	0.79
Sensitivity, %	71	71	67
Specificity, %	71	63	79
Positive predictive value (PPV), %	36	19	29
Negative predictive value (NPV), %	92	95	95
Positive likelihood ratio, LR+	2.5	2.0	3.1
Negative likelihood ratio, LR-	0.4	0.5	0.3
Optimal cut-off, g/l	0.53		

Acetate concentration was not predictive of preterm birth < 32 weeks gestation (*P* = 0.13).

Table 3.2: Predictive accuracy of CVF acetate ^{1}H-MR normalised integral and concentration for preterm birth

	AUC	95% CI	Sens (%)	Spec (%)	PPV (%)	NPV (%)	LR+	LR-	*P* value
Delivery < 37 weeks gestation									
Acetate N.I.	0.75	0.60-0.91	60	85	47	91	4.0	0.2	0.001
AceConc	0.74	0.57-0.90	71	71	36	92	2.5	0.4	0.002
Delivery < 32 weeks gestation									
Acetate N.I.	0.73	0.53-0.94	88	59	21	97	2.1	0.5	0.01
AceConc	0.63	0.40-0.87	72	63	19	95	2.0	0.5	0.13
Delivery within 2 weeks of assay									
Acetate N.I.	0.77	0.58-0.96	100	49	21	100	2.0	0.5	0.003
AceConc	0.68	0.47-0.89	67	79	29	95	3.1	0.3	0.045

N.I., ^{1}H-MR normalised integral; *AceConc*, acetate concentration; *AUC*, area under the ROC curve; CI, 95% confidence interval; *Sens*, sensitivity; *Spec*, specificity; *PPV*, positive predictive value; *NPV*, negative predictive value; *LR+*, positive likelihood ratio; *LR-*, negative likelihood ratio; *P*, significance level.

3.4. Discussion

In this chapter we validated our data obtained by MRS and attempted to identify a possible clinical assay to assess concentrations of CVF acetate in pregnant women in relation to premature delivery. A subset of randomly selected CVF samples from the women presenting with symptoms of threatened preterm labour were analysed for acetate by spectrophotometric absorption using cheaper, but sensitive commercially available assay kit. To our knowledge, this comparative analysis is the first of its kind.

It can be recalled that of all the metabolites identified in our ^{1}H-MR experiments (Chapter 2), only acetate N.I. was significantly different between term- and preterm-delivered women and this difference was only observed to attain significance in the symptomatic cohort. Similar to this observation, acetate concentration measured by a spectrophotometric technique also differed significantly between term- and preterm-delivered women in the symptomatic group. In other words, the symptomatic women that delivered preterm had higher vaginal acetate concentrations compared to their term counterparts. Furthermore, acetate concentrations measured correlated with acetate N.I. measured by ^{1}H-MR (Fig. 3.3A-B), and was likewise predictive of preterm birth (< 37 weeks) and delivery within 2 weeks of presentation with comparable sensitivities, specificities, PPVs, NPVs and likelihood ratios. An optimal cut-off value of > 0.53 g/l for predicting deliveries before 37 weeks was obtained from the ROC curve (Table 3.1 and Fig. 3.4). In other words, at a concentration greater than 0.53 g/l, acetate is considerably capable of discriminating symptomatic pregnant women at a greater risk of PTL and delivery before 37 weeks of gestation.

Though acetate concentration measured by spectrophotometric technique produced similar results as and correlated with acetate N.I. measured by ^{1}H-MR in this study, it was not as sensitive and powerful as acetate N.I. measured by ^{1}H-MR. This is clearly depicted by the marginal difference (17%) in acetate concentration between term- and preterm-delivered women as opposed to the over 2-fold (103%) difference observed with acetate N.I. Additionally, the acetate N.I. was a better predictor of delivery within 2 weeks of presentation (AUC: 0.77 vs. 0.68), and < 32 weeks (AUC: 0.73 vs. nil), compared to acetate concentration. These disparities could be due to the higher sensitivity of ^{1}H-MR over spectrophotometry.

However, the lower sample population analysed by spectrophotometric technique (n = 57) vs. ^{1}H-MR (n = 82) may partly account for the differences noted. Fewer samples were analysed when the cut-off for preterm birth was restricted to less than 32 weeks of gestation (n = 8). This may account for the poorer performance of acetate concentration in predicting preterm birth less than 32 weeks. Also, the use of microplate reader instead of an auto-analyser as indicated by the manufacturer may have impacted the current data.

Some investigators, using the same assay kit as that employed in our study, analysed arterial blood acetate levels in patients after cardiopulmonary bypass with great success.[464] Another group, applying the same principle, but with a different commercially available assay kit validated the measurement of CVF lactate and acetate concentrations by spectrophotometric technique which was comparable to High Performance Liquid Chromatography (HPLC) analysis.[37] Despite its relatively weaker sensitivity and difficulty in discovering unknown metabolites, spectrophotometric measurement of acetate concentrations in CVF of pregnant women with symptoms suggestive of threatened PTL as shown by our data could provide a cheaper, rapid and more accessible tool for predicting PTL and premature delivery in the clinical setting. This is in contrast to ^{1}H-MR which is a great technique for discovery of metabolites, but with less affordability and accessibility requiring a great deal of expertise, sophistication and safety precautions as a high magnetic field environment is required. Furthermore, apart from ^{1}H-MR, other techniques with relatively higher sensitivity and affordability particularly GC/LC-MS have been unable to detect reasonable amounts of acetate in CVF probably due to its high volatility and as such were unable to make useful inferences in relation to this metabolite and vaginal health.[84, 85, 358, 451]

Going forward, a more robust analysis using an auto-analyser (e.g. ChemWell® 2910 Auto-Analyser), in place of a microplate reader as used in this study, will enhance the accuracy and output of these assays. The ChemWell® 2910 is an automated (robotic) bench top open system auto-analyser supplied with optimised programming protocols (for Megazyme assay kits), with the capacity to perform biochemical assays. It is equipped with a 96 well plate layout where the assays are performed, 27 reagents and 96 sample racks, as well as a flush system that washes the 96 well plate ready for re-use at the end of the experiment. It employs continuous flow analysis to perform repetitive stages of sample analysis. It can simply be described as an

"automatic spectrophotometer" capable of mixing precise quantities of reagents and samples incubated at the right temperature and duration, reading the absorbance at the appropriate wavelength (e.g. UV-Vis light range), and washing up the micro-wells at the end of the assay. Though the sensitivities of the microplate reader and auto-analyser are similar, increasing the volume of sample used during the reaction can enhance the sensitivities of both methods. However, for improved precision, repeatability and simultaneous analysis of multiple metabolites in CVF sample from different study populations, the auto-analyser is preferred. Depending on the duration of the reaction, the auto-analyser can perform up to 200 endpoint assays per hour (https://secure.megazyme.com/ChemWell-2910-Chemistry-Analyser). Meanwhile, not only does this spectrophotometric assay validate our data obtained by ^{1}H-MR, it also kindles the possibility of a specific, rapid and affordable clinical assay for the assessment of risk of preterm birth at least in symptomatic women. Also, we are currently unaware of any impediments to its utility by health care systems involved in maternal and child care, and the UK's National Health Service (NHS) could benefit tremendously from the application of such technique. There is also the possibility that by using spectrophotometry, interesting data could emerge from other metabolites in which we were unable to demonstrate any statistically significant difference by ^{1}H-MR in relation to delivery outcomes in this study.

Chapter 4

Investigation of the vaginal microbiome in the second trimester of pregnancy in relation to delivery outcomes

4.1. Introduction

The vaginal bacterial community comprises a mixed population of diverse species, and the relative abundance of these organisms impact the woman's urogenital health and disease.[465] A normal and healthy vaginal microbiome is characterised by the predominance of *Lactobacillus* sp., while the dominance by genera other than *Lactobacillus* is termed abnormal and unhealthy.[24, 25] Loss of vaginal *Lactobacillus* dominance and the subsequent proliferation of facultative and strictly anaerobic bacteria (e.g. *Gardnerella*, *Prevotella Atopobium, Fusobacterium, Mycoplasmas, Mobiluncus* etc.), describes a common syndrome among reproductive-age women known as BV.[21, 64, 66, 466] BV is an established risk factor of PTB and other adverse pregnancy outcomes.[94, 160, 196] A considerable amount of these anaerobes are unculturable and of low abundance,[465, 466] hence the need for high throughput cultivation-independent characterisation of the vaginal microecology in health and disease conditions.

Characterisation of vaginal microbial composition has been enhanced recently by cultivation-independent broad-range and species-specific PCR techniques.[64, 465-467] The initial crucial procedure employed for vaginal microbiome study is the extraction of 16S rDNA from the crude samples (See section 4.2.1. of this chapter for a general description). Contaminants such as molecules and artefacts capable of hindering the efficiency of the PCR and sequencing processes in producing adequate quantities of useable reads, need to be eliminated. This is then followed by PCR amplification of the isolated DNA with primers specific for the hypervariable regions of the 16S rRNA genes, and purification of amplicons generated. These processes will produce pure amplicon libraries that can be sequenced using the 454 - Next Generation sequencing platforms. Sequencing produces Operational Taxonomy Units (OTUs) which can be classified by BLAST, SILVA, Greengenes, EzTaxon-e and RDP classifiers.[21, 66]

In this chapter, PCR assays to determine the prevalence and relative abundance of 10 vaginal bacterial species (*Lactobacillus* sp., *L. crispatus*, *L. jensenii*, *Bacteroides-Prevotella*, *Gardnerella*, Group B *Streptococcus*, *Fusobacterium* sp., *Mycoplasma, Mobiluncus curtisii,* and *Mobiluncus mulieris*) were performed. These bacterial species were amplified because they are commensal endogenous vaginal microorganisms detected both in healthy and deranged vaginal microbiota. Some of

them have been implicated in infection and pathogenesis of adverse delivery outcome.[12, 21, 74, 84, 85, 168]

Furthermore, the presence of these bacterial species has been linked to various metabolites we have already identified in the previous chapters of this report (Chapter 2 and 3). For instance, *Lactobacillus* sp. dominance is associated with elevated lactate and low levels of acetate, succinate, formate etc., while decreased amounts of *Lactobacillus* sp. is associated with proliferation of BVAB, elevated acetate, succinate and other SCFAs.[38] In other words alterations in the vaginal microbial population are also reflected in the metabolite profile. Whether either of these markers could be used as a proxy for the other necessitated our analyses of the bacterial species by PCR. Collectively, investigation of the composition of the vaginal bacterial population and their metabolic by-products could provide a holistic approach to the predictive value of these potential markers for PTB.

These experiments were applied on vaginal fluid samples from both asymptomatic and symptomatic pregnant women presenting at the antenatal clinics and Triage Delivery Suites of the Jessop Wing Hospital, Sheffield, UK, with intact membranes and no clinically defined genital infection. These women were studied at 20-22 and 26-28w (asymptomatic women), and 24-36w (symptomatic women). The symptomatic women were those presenting with regular uterine contractions but < 3 cm dilated cervical os. Knowledge of the prevalence and relative abundance of vaginal bacteria is required to ascertain their impact on pregnancy outcomes and identify women at risk of PTB in relation to the metabolites identified. PCR is capable of identifying and quantifying both culturable and unculturable bacteria at the species level and has gained immense usefulness in the determination of the vaginal bacterial community composition in relation to infection and adverse pregnancy outcome.[465, 468-470]

4.2. Methods

4.2.1. DNA Extraction

The sample DNA was extracted using a standard operating procedure for DNA extraction from vaginal swabs with QIAamp DNA mini kit (Qiagen, 51304, UK) performed according to amended manufacturer's instructions as described below.

The cut off swab tips were placed in 1.5 ml microfuge tubes with 400 µl Phosphate Buffered Saline (PBS) (Sigma-Aldrich, D8537, UK) and vortexed thoroughly for 5 minutes. To each tube of 250 µl sample, 75 µl lysozyme (10 mg/ml; Sigma-Aldrich, L1667-1G, UK), and 10.5 µl Tris-EDTA buffer at pH 8.0 (Fisher, BP2473-100, CA) were added and incubated at 37°C for 1 hour to degrade the bacterial cell wall and preserve the genomic DNA from degradation. Proteins and RNA were digested by adding 20 µl proteinase K (Qiagen, 19131, UK) and 4 µl RNase A (100 mg/ml; Qiagen, 19101, UK) and 250 µl of buffer AL (lysis buffer i.e. 1:1 ratio) to each tube, mixed by inverting tube 2–3 times and incubated for 10 minutes at 56°C. 400 µl of absolute ethanol was then added to each tube and vortexed for 15s to precipitate the DNA out of solution. DNA was further precipitated by adding each sample to a spin column in 2 ml collection tube and centrifuged at 6,000 x g for 1 minute. The filtrate was discarded and the column was transferred into a clean collection tube and recentrifuged briefly. We then added 500 µl buffer AW2 (wash buffer) to each column and centrifuged at 20,000 x g for 3 minutes. This was followed by another centrifugation for 1 minute at full speed. Each column was transferred to a clean 1.5 ml microfuge tube, 100 µl of nuclease free water added and incubated at room temperature. After a minute, the samples were centrifuged at 6,000 x g for 1 minute and the DNA sample was obtained. The quantity and purity of the isolated DNA was assessed by spectrophotometry using the Thermo Scientific NanoDrop (ND1000) spectrophotometer (Labtech International LTD, UK). The extracted DNA samples were then preserved at -20°C until required for further analysis.

4.2.2. Targeted PCR amplification of vaginal bacterial species

Bacterial genus-specific primers (Sigma-Aldrich, UK), targeted at the bacterial 16S rDNA were used to examine for a range of commensal and potentially pathogenic bacterial species which included *Lactobacillus*, *Bacteroides* (*Prevotella*), *Gardnerella*, Group B *Streptococcus*, *Fusobacterium*, *Mycoplasma* and *Mobiluncus*. The names of the primers, their sequences and targets, annealing temperatures, amplicon product size and sources are represented in Table 4.1. A pair of primers specific for *Lactobacillus* sp. was designed by first identifying the *Lactobacillus* 16S rRNA gene and then identifying base sequences complementary to the gene of interest. The primer sequences were confirmed with the National Centre for Biotechnology Information Basic Local Alignment Search Tool (BLAST). PCR amplification experiments were performed using 12.5 µl AmpliTaq Gold DNA polymerase (Applied Biosystems, UK), 5 ng genomic DNA template, 1 µl each of forward and reverse primers (10 µM), in a total reaction volume of 25 µl. Reactions were run in an Applied Biosystems 2720 Thermal cycler (Life Technologies, UK) with the following cycling criteria: 95°C (5 mins) – denaturation, followed by 35 cycles of 95°C (1 min) - denaturing, 50 - 62°C depending on the primer sets (1 min) – annealing, 72°C (1 min) – elongation, with a final extension at 72°C (7 mins). The results were visualised on a UV-transilluminator by 1% agarose gel electrophoresis and ethidium bromide staining. Positive results were assigned according to the presence of bands of the appropriate size. The chi-square test alongside a benchmark of ≥10% was then applied to determine any differences in prevalence of vaginal bacterial species based on delivery outcome.

4.2.3. Quantitative (Real-time) PCR analysis of vaginal bacterial species

Real-time PCR (qPCR) amplification and detection was performed using 16S rDNA specific primers for *Gardnerella* and *Mobiluncus curtisii* only. This was not only because of the availability of a suitable calibrant DNA for these bacterial species used to produce accurate standard calibration curves (absolute quantification), but due to the high prevalence of *Gardnerella* in all women among the groups and its association along with *M. curtisii* to BV and PTB. A relatively lower number of women across the groups had positive results for *Bacteroides* compared with *Gardnerella* and *Mobiluncus curtisii*, thus it was not a priority due to finite resources. The PCR reactions were performed using Power SYBR green master mix (Applied biosystems, 1503489,

UK), on a 7900HT Fast Real-Time PCR system (Applied Biosystems, UK) using optical grade 96 well plates. The total reaction mixture (20 µl) contained 10 µl SYBR green master mix, 1 µl genomic DNA template (1 ng), 1 µl each of forward and reverse primers (10 µM), and 7 µl of DNAse free water. The temperature cycling conditions for all experiments was 50°C for 2 mins, 95°C for 10 mins, and 40 cycles of 95°C for 15 s, and 65°C (*Gardnerella*) or 57°C (*M. curtisii*) for 1 min. For each experiment vaginal samples and calibrant bacterial DNA were run in triplicates. Fluorescence was measured at the final stage of each cycle and the threshold cycle (C_t) obtained. Data obtained were analysed by Sequence Detection Software (version 1.2.2) supplied by Applied Biosystems. Final DNA concentration in the samples was extrapolated from the equation of the fit line of the standard curve (Fig. 4.2), and compared in relation to delivery outcomes. The equation is as follows:

$$\mathbf{y = M \log X + B}$$

Where:

y = C_t value
M = Slope
X = Concentration of bacterial genomic DNA
B = Y-intercept

4.2.4. Bacterial strains and culture conditions

Bacterial strains were grown in different selective media for use as PCR standards/calibrant DNA (controls) confirming the quality of bacterial 16S rDNA primers used for amplification. The bacteria and their respective culture media are shown in Table 4.2. The organisms were cultured in an anaerobic chamber containing a gas mix of 10% CO_2, 80% N_2 and 10% H_2, at 37°C for 24-72 h (*Gardnerella* and *Mobiluncus*) and 16 hours (*Bacteroides* and *Fusobacterium*), followed by gram staining to confirm their growth. With confirmation of growth, bacterial DNA was isolated using QIAamp DNA mini kit (Qiagen, 51304, UK) and the same procedure as applied to the vaginal bacterial DNA.

Table 4.1: Bacterial genus-specific primers, targets, and annealing temperatures

Primer[a]	Sequence (5'-3')	Target	Annealing temp (°C)	Amplicon size (bp)	Reference
LABF LABR	AGAGTTTGATYMTGGCTCAG CACCGCTACACATGGAG	*Lactobacillus*	62	667	[467]
LJ2F LJ2R	GCACGTAGTTAGCCGTGACT GAAACAGATGCTAATACCGG	*Lactobacillus*	61	~350	This study
LBF LBR	ATGGAAGAACACCAGTGGCG CAGCACTGAGAGGCGGAAAC	*L. jensenii* *L. crispatus*	50	~150	[471]
FBF FBR	ACTCCTACGGGAGGCAGCAGT CGAATTTCACCTCTACACTTGT	*Fusobacterium*	60	341	[472]
GV1F GV3R	GGAAACGGGTGGTAATGCTGG CGAAGCCTAGGTGGGCCATT	*G. vaginalis*	65	125	[465]
BAC32F BAC708R	AACGCTAGCTACAGGCTT CAATCGGAGTTCTTCGTG	*Bacteroides-Prevotella*	53	676	[473]
Sag59 Sag190	TTTCACCAGCTGTATTAGAAGTA GTTCCCTGAACATTATCTTTGAT	*GBS*	55	153	[474]
M. curt-440F M. curt-1026R	TTCTCGCGAAAAAGGCACAG CTGGCCCATCTCTGGAACCA	*M. curtisii*	57	586	[466]
Mobil-577F M.mulie-1026R	GCTCGTAGGTGGTTCGTCGC CCACACCATCTCTGGCATG	*M. mulieris*	62	449	[466]
Mh1-F Mh2-R	CAATGGCTAATGCCGGATACGC GGTACCGTCAGTCTGCAAT	*M. hominis*	62	334	[471]

GBS, group B *Streptococcus*.

[a] Primers designed to detect the species-specific regions of the 16S rRNA gene.

Table 4.2: Bacterial strains and media

Bacteria species	Culture medium	DNA conc. (ng/µl)
Bacteroides fragilis (NCTC 9343)	FAA	112
Fusobacterium nucleatum (ATCC 25586)	FAA	5.3
Fusobacterium polymorphum (ATCC 10953)	FAA	-
Gardnerella vaginalis (NCTC 11292)	CA and CBA	6.7
Mobiluncus curtisii (NCTC 11656)	CA, CBA and BHI	3.2
Streptococcus agalactiaea (clinical isolate)	CBA	3.8

BHI, Brain heart infusion broth (Oxoid, CM1135, UK) containing 5% horse serum (Oxoid, SR0035C, UK); *CA*, Casman agar (Becton, Dickson and Company, 211106, UK) containing 5% defibrinated sheep blood (Oxoid, SR0051B, UK); *CBA*, Columbia blood agar (Oxoid, CM0331, UK) containing 5% defibrinated sheep blood (Oxoid, SR0051B, UK); *FAA*, Fastidious anaerobe agar (Lab M, LAB090, UK) supplemented with 5% oxalated horse blood (Oxoid, SR0049C, UK).

4.2.5. Vaginal cytology

To detect the cells present in the vaginal eluate obtained, various staining procedures were also performed. This was necessary to provide an insight into the predominant bacterial sp., morphology of vaginal epithelial cells and consequently the possible source(s) of the metabolites identified in the cervicovaginal fluid. The staining techniques performed include Hematoxylin and Eosin (H & E), Papanicolaou (Pap) stains, which stain human cells e.g. vaginal and cervical epithelial cells; and Gram stain which stains bacterial cells.

Gram stain is the gold standard in the diagnosis of BV. It is used to identify vaginal bacteria based on morphology.[475] The vaginal flora can then be categorised as normal, intermediate, or BV.[173] It has a sensitivity of 62-100% and a specificity of 79-100%.[475]

Pap stain is commonly used in screening women for cervical dysplasia.[83] It can also be used for detecting clue cells and BV microflora with significantly high sensitivity and specificity.[476, 477] [175]However, Pap test is not a routine test for the diagnosis of BV.[83]

4.2.5.1. Smear preparation

For Hematoxylin and Eosin (H & E) and Papanicolaou (Pap) stains, a smear of vaginal swab was prepared on a clean glass slide and allowed to air dry for 0.5–1 min. The slide was then placed in a coplin jar containing 90% alcohol for 1 hr. This was done in order to dehydrate the cells, denature the proteins, and dissolve and remove lipids (Fixation).

4.2.5.2. Hematoxylin and Eosin (H & E) stain

Hematoxylin is a dark blue basic (positive) stain, that binds to basophilic substances (such DNA/RNA - which are acidic and negatively charged), hence staining the nuclei blue/violet. Eosin is a red or pink acidic (negative) stain that binds to acidophilic substances (such as proteins - which are basic and positively charged) staining them pink.

The H & E stain was performed on an automatic Leica ST4040 linear staining system (Leica biosystems, UK).

4.2.5.3. Papanicolaou stain (Pap stain)

The solutions used for this method include: Harris haematoxylin (stains the nuclei blue), Orange G-6 (stains keratin yellow or orange red), Eosin Azure (EA50) (stains the cytoplasm of non-keratinized cells blue-green), 0.1% hydrochloric acid in 70% alcohol and Scott's tap water substitute.

The first part of the procedure was done on the automatic Leica ST4040 linear staining machine, from 70% industrial methylated spirit (IMS) to the running tap water bath before the eosin. The slide was then removed from the staining machine and rinsed with 95% IMS. This was followed by staining with Orange G-6 for 2 mins on the rails across the special stain sink. The slides were then rinsed in 95% IMS and stained with EA 50 for 2 mins. The slides were then returned to the first dehydrating 99% IMS bath on the staining machine after being rinsed in 95% IMS and allowed to proceed to xylene. Finally the slides were mounted and viewed under light microscope.

4.2.5.4. Gram stain

This staining technique differentiates bacteria by detecting the peptidoglycan (PGN) layer of the cell wall. Gram-positive bacteria retain the primary dye (crystal violet) and stain purple (bluish) due to their thick PGN layer. While Gram-negative bacteria with thinner PGN layer take up the counter stain (carbol fuschin) and appear pinkish.

10 µl of vaginal fluid sample dissolved in PBS was placed on a glass slide and allowed to air dry for 10 mins and then heat fixed. This was to stick the bacterial cells to the glass slide to prevent them from being washed away during the following staining process. The staining process involved application of a primary stain (crystal violet) for 2 mins after which the slide was rinsed with running water. Then the slide was fixed with a mordant (Gram's iodine) for 2 mins and rinsed again with running water. This binds to the primary stain and increases the affinity of the bacterial cell wall for the stain. Iodine-acetone (decolourizer) was then added to the slide for 30 s after which the slide was rinsed again. Finally, a counter stain (Carbol fuschin) was applied for 2 mins and then rinsed off. The slides were ready for viewing after allowed to dry.

4.2.6. Statistical analysis

All Statistical analyses were performed using GraphPad Prism 6.0c (GraphPad Software, Inc. CA). The Wilcoxon's rank-sum test was performed to compare differences in bacterial DNA concentration within the groups. For the qualitative PCR, The chi-square test alongside a benchmark of ≥10% was then applied to determine any differences in prevalence of vaginal bacterial species based on delivery outcome. *P* values < 0.05 were considered statistically significant.

4.3. Results

4.3.1. PCR confirmation of vaginal bacterial species

Two hundred and eighteen vaginal fluid samples from 181 pregnant women across the second and third trimesters classified into 4 groups were analysed: SYM (n = 80, preterm = 14, term = 66); AHR(20-22w) (n = 50, preterm = 19, term = 31); AHR(26-28w) (n = 37, preterm = 17, term = 20); and ALR (n = 51, preterm = 5, term = 46). PCR amplification of vaginal bacterial 16S rRNA genes from these participants revealed the presence of both commensal (e.g. *Lactobacillus* sp.) and potentially pathogenic anaerobic bacterial organisms (Table 4.3). These bacterial organisms were chosen for analysis because they are normal endogenous vaginal organisms only becoming virulent when there is a distortion in the vaginal ecosystem. A representative picture of bacterial DNA bands observed on agarose gel is shown in Fig. 4.1. As presented in Table 4.4., qualitative assessment of vaginal microbial composition in the SYM group indicated higher prevalence of *Fusobacterium* sp., *M. mulieri*s and *M hominis* in the preterm-delivered women, except for *M. curtisii* ($P < 0.0001$).

Similar trends were observed in the AHR (26-28w) group, where the preterm-delivered women showed a higher prevalence of *Fusobacterium* sp., *Bacteroides* sp., *M. curtisii*, *M. mulieri*s and *M. hominis* ($P < 0.0001$).

There were no significant differences observed in the AHR (20-22w) group except for *M. mulieri*s and *M. hominis* ($P = 0.006$). However, in the ALR group, the term-delivered women showed higher prevalence of *Fusobacterium* sp., *Gardnerella*, *M. curtisii*, *M. mulieri*s and *M hominis* compared to the preterm-delivered women, except for *Bacteroides* sp. ($P < 0.001$). Only 1.5%(1/66) and 5%(1/20) of the term-delivered women in the SYM and AHR(26-28w) groups respectively, had positive results of group B *Streptococcus*. Group B *Streptococcus* was not observed in any preterm-delivered woman across the groups. At least one specie of *Lactobacillus* which is commensal to the host was identified in all vaginal samples analysed.

Comparing the prevalence of the different bacteria species in the vaginal microbiome of asymptomatic women studied at 20-22w, i.e. ALR vs. AHR (20-22w), we observed that there were significant differences between the preterm-delivered women in these groups. The

preterm-delivered women in the AHR(20-22w) group had higher prevalence ($P < 0.0001$) of *Gardnerella* (100%(19/19) vs. 60%(3/5)), *Fusobacterium* sp. (16%(3/19) vs. 0%(0/5)); and *M hominis* (11%(2/19) vs. 0%(0/5)), but lower prevalence of *Bacteroides* sp. (16%(3/19) vs. 40%(2/5); compared to the preterm-delivered women in the ALR group. The term-delivered women did not show any significant differences (Table 4.3 and 4.4). In the women that delivered preterm, the prevalence of *Gardnerella*, *Fusobacterium* sp., and *M hominis* was higher in the AHR (20-22w), than the ALR group.

A similar comparison was made for the asymptomatic high risk women studied at two gestations i.e. AHR (20-22w) vs. AHR (26-28w). Again, the term-delivered women in these groups did not show any significant differences. However, the preterm-delivered women in the AHR (26-28w) group had higher prevalence ($P = 0.007$) of *Fusobacterium* sp. (41%(7/17) vs. 16%(3/19); *Bacteroides* sp. (71%(12/17) vs. 16%(3/19)); and *M. mulieris* (29%(5/17) vs. 0%(0/19). The prevalence of *Fusobacterium* sp., *Bacteroides* sp., and *M. mulieris* increased with gestation in the preterm-delivered asymptomatic high risk women (Table 4.3).

Also, we observed that compared to preterm-delivered women in other groups, the preterm-delivered women in the SYM cohort had the greatest prevalence of *M. hominis* (SYM: 29% vs. AHR (26-28w): 6% vs. AHR (20-22w): 11% vs. ALR: 0%).

When the groups were analysed as a whole (preterm vs. term), we observed a higher prevalence of *Fusobacterium* sp. (25%(14/55) vs. 11%(18/163)); *Bacteroides* sp. (45%(25/55) vs. 34%(56/163)); *M. mulieris* (15%(8/55) vs. 6%(9/163)); and *M hominis* (13%(7/55) vs. 5%(8/163)) ($P < 0.01$), although the differences in the prevalence of *M. mulieris* and *M. hominis* were less than 10% but ≥8%. The prevalence of *Lactobacillus* sp. and *Gardnerella* were similar in both groups, while only 1%(2/163) of the term women had group B *Streptococcus* positive vaginal fluid samples.

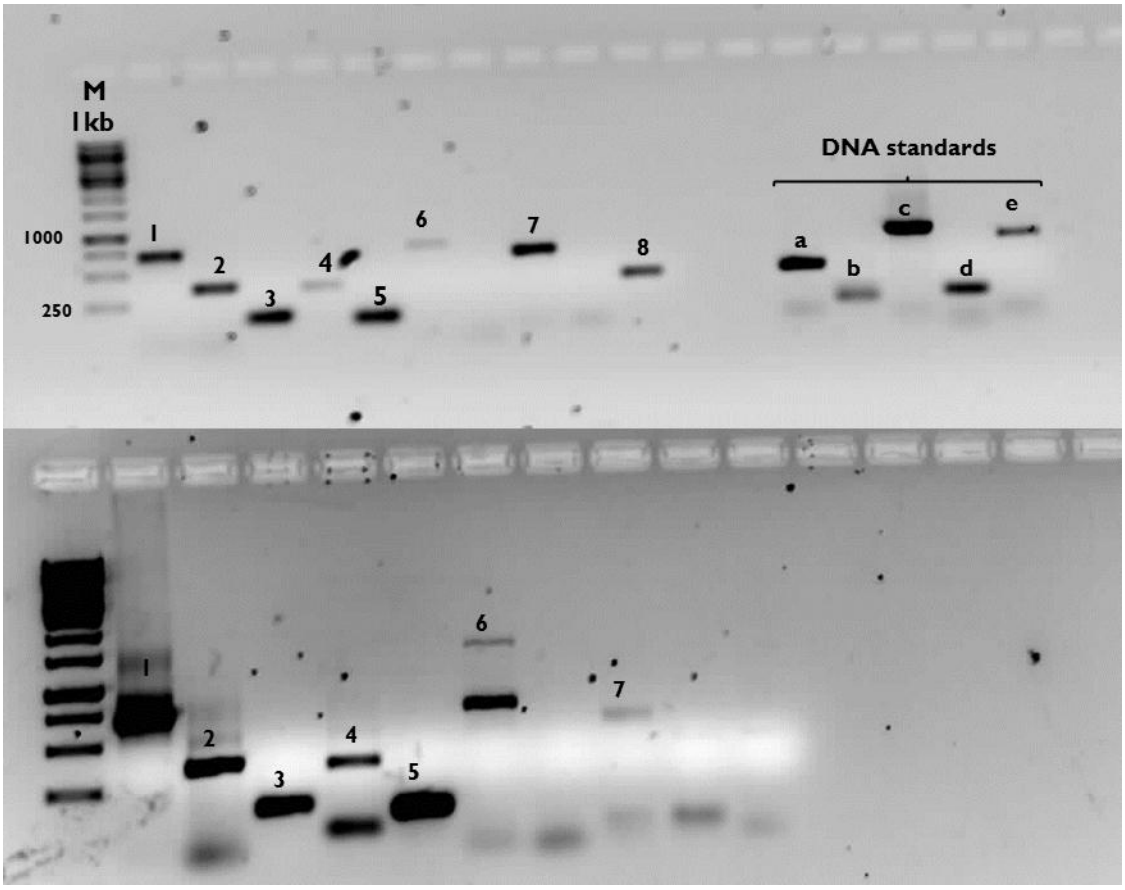


Figure 4.1. Presence of bacterial species in vaginal fluid of pregnant women. Bacterial genomic DNA bands visualized on a UV-transilluminator by agarose gel electrophoresis : 1) *Lactobacillus* sp., 2) *L. jensenii*, 3) *L. jensenii* and/or *L. crispatus*, 4) *Fusobacterium* sp., 5) *G. vaginalis*, 6) *Bacteroides-Prevotella* sp., 7) *Mobiluncus cutisii*, 8) *Mycoplasma hominis*. Bacterial DNA standards used as positive controls: a) *Fusobacterium nucleatum* (ATCC 25586), b) *Gardnerella vaginalis* (NCTC 11292), c) *Bacteroides fragilis* (NCTC 9343), d) *Streptococcus agalactiaea* (clinical isolate), e) *Mobiluncus curtisii* (NCTC 11656).

Table 4.3: Prevalence of endogenous vaginal bacterial species identified by PCR

Bacterial sp., %	Asymptomatic Low risk women, 20-22w		Asymptomatic High risk women, 20-22w		Asymptomatic High risk women, 26-28w		Symptomatic women 24-36w	
	Preterm N=5	Term N=46	Preterm N=19	Term N=31	Preterm N=17	Term N=20	Preterm N=14	Term N=66
Lactobacillus sp.[a]	100	100	100	100	100	100	100	100
L. crispatus[a] *L. jensenii*	100	100	95	97	100*	90	100	99
Fusobacterium sp.[b]	0	13*	16	16	41*	10	29*	8
Gardnerella vaginalis[b]	60	96*	100	94	77	82	86	82
Bacteroides-Prevotella[b]	40*	26	16	23	71*	25	57	49
GBS[b]	0	0	0	0	0	5	0	2
Mobiluncus curtisii[b]	0	30*	26	29	29*	20	21	33*
Mobiluncus mulieris[b]	0	9	0	3	29*	10	21*	3
Mycoplasma hominis[b]	0	2	11	3	6	0	29*	9

[a] Commensal bacterial sp.

[b] Potentially pathogenic bacterial sp. associated with abnormal vaginal microflora, infection and PTB.

* Differences in the prevalence of bacterial sp. between term and preterm-delivered women ≥10%.

GBS, group B *Streptococcus*.

Table 4.4: Differences in prevalence of vaginal bacterial species

Symptomatic women 24-36w					
	Fusobacterium sp., %(n)	*Mobiluncus curtisii*, %(n)	*Mobiluncus mulieris*, %(n)	*Mycoplasma hominis*, %(n)	*P* value
Preterm, N=14	29(4)	21(3)	29(4)	21(3)	<0.0001
Term, N=66	8(5)	8(2)	9(6)	33(22)	

Asymptomatic High risk women, 26-28w						
	Fusobacterium sp., %(n)	*Bacteroides-Prevotella*, %(n)	*Mobiluncus curtisii*, %(n)	*Mobiluncus mulieris*, %(n)	*Mycoplasma hominis*, %(n)	*P* value
Preterm, N=17	41(7)	71(12)	29(5)	29(5)	6(1)	<0.0001
Term, N=20	10(2)	25(5)	20(4)	10(2)	0(20)	

Asymptomatic High risk women, 20-22w			
	Mobiluncus mulieris, %(n)	*Mycoplasma hominis*, %(n)	*P* value
Preterm, N=19	0(0)	11(2)	<0.006
Term, N=31	3(1)	3(1)	

Asymptomatic Low risk women, 20-22w							
	Fusobacterium sp. %(n)	*Gardnerella vaginalis* %(n)	*Mobiluncus curtisii* %(n)	*Mobiluncus mulieris* %(n)	*Mycoplasma hominis* %(n)	*Bacteroides-Prevotella* %(n)	*P* value
Preterm, N=5	0(5)	60(3)	0(5)	0(5)	0(5)	40(2)	<0.001
Term, N=46	13(6)	96(44)	30(14)	9(4)	26(12)	26(12)	

N, total number of term- or preterm-delivered women in each cohort; *n*, subset of women habouring the bacterial sp. in either the term- or preterm-delivered women. *P*, significance level.

4.3.2. Relationship between bacterial DNA quantity and delivery outcome

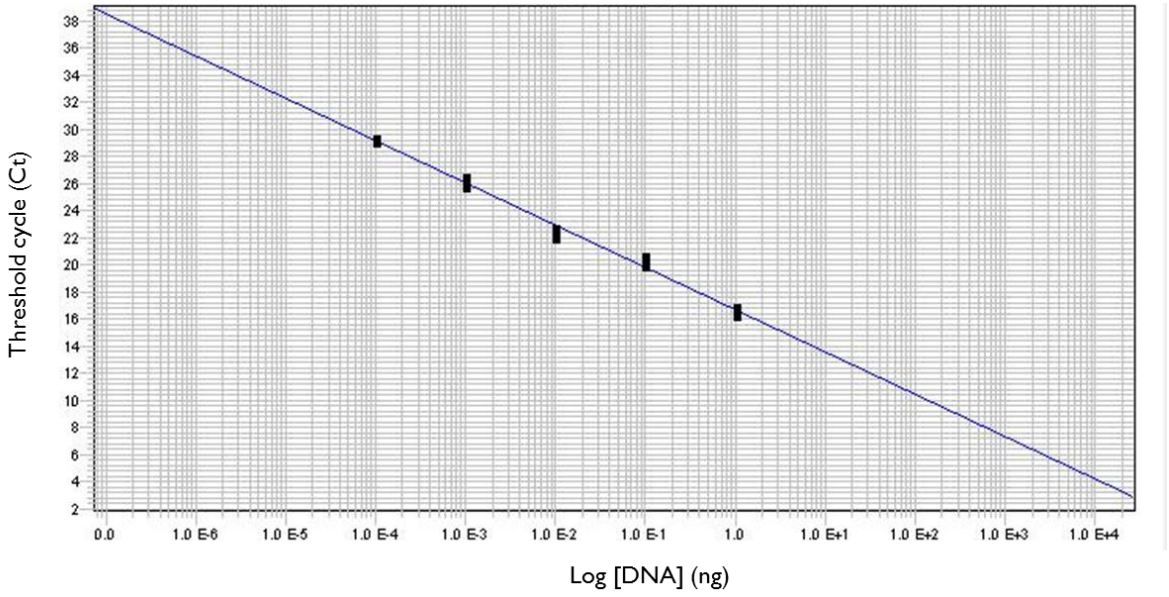
In addition to determining the prevalence of identified commensal and potentially pathogenic vaginal bacteria in the vaginal fluid samples in each cohort, there was the need to ascertain any differences in the relative abundance of these bacterial species in relation to delivery outcome. This is more advantageous than the standard PCR as it does not only detect the presence of bacterial species but also provides an idea of the predominant specie(s) in the vagina microflora in relation to others at a particular time which can be used to ascertain the health status of the vaginal milieu and by extension the host. It indicates the relative quantity and not just the presence of the organisms. It can also be used to calculate the number of bacterial cells in a given sample.[82] This can also influence the type of treatment or intervention (if any) required. For example, the particular species of BVAB can be identified and its susceptibility to the antibiotic agent of choice is ascertained before administration.[82] However resolution to the species level is challenging as more than one specie in a genus can share similar genetic sequence in the amplified region.[82] To this end, a subset of randomly selected CVF samples across the groups: SYM (term = 11, preterm = 11); AHR(20-22w) (term = 12, preterm = 12); AHR(26-28w) (term = 12, preterm = 12), were analysed for the DNA quantity of 2 known BV- and PTB-associated bacterial species (*Gardnerella* and *M. curtisii*). This was due to the relatively low numbers of women with preterm deliveries across the groups and the finite resources available. After a quality control process i.e. removal of sample replicates with differences in Ct values > 1, the sample size reduced to as follows: SYM (*G. vaginalis*: term = 11, preterm = 9; *M. curtisii*: term = 7, preterm = 8); AHR(20-22w) (*G. vaginalis*: term = 7, preterm = 6; *M. curtisii*: term = 10, preterm = 10); AHR(26-28w) (*G. vaginalis*: term = 9, preterm = 6; *M. curtisii*: term = 9, preterm = 7). Samples from participants in the ALR were not included in these experiments because of the very few numbers of preterm deliveries (n = 5), recorded in this group which occurred after the analysis were concluded.

Comparison of DNA quantity between term- and preterm-delivered women within the various cohorts did not indicate any statistically significant difference in relation to either *Gardnerella* or *M. curtisii* except in the AHR(20-22w) group. In this group, the term-delivered women had

significantly higher (4-fold) amounts of *M. curtisii* DNA compared to their preterm counterparts (P = 0.0001) (Fig. 4.3).

Also, a comparison of the abundance of *Gardnerella* and *M. curtisii* DNA in the vaginal samples of term- and preterm-delivered women in all groups considered together indicated a significant difference in the amount of *M. curtisii* DNA, but not for *G. vaginalis* (Fig. 4.3). This is somewhat consistent with data obtained using the standard PCR. From our data, it is noteworthy that *G. vaginalis* DNA constituted about a quarter of total quantity of DNA in the samples and was about 100 – 1500 times more than *M. curtisii* DNA. *Gardnerella* also showed a non-significant trend to higher amounts in the term-delivered women across the groups (Fig 4.3).

A.



B.

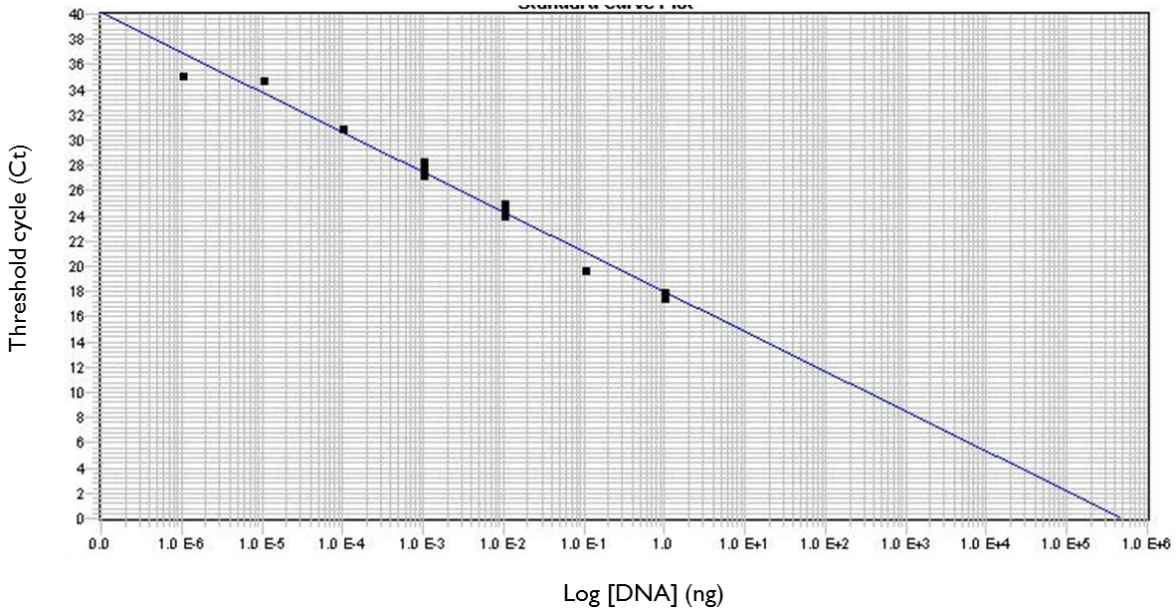
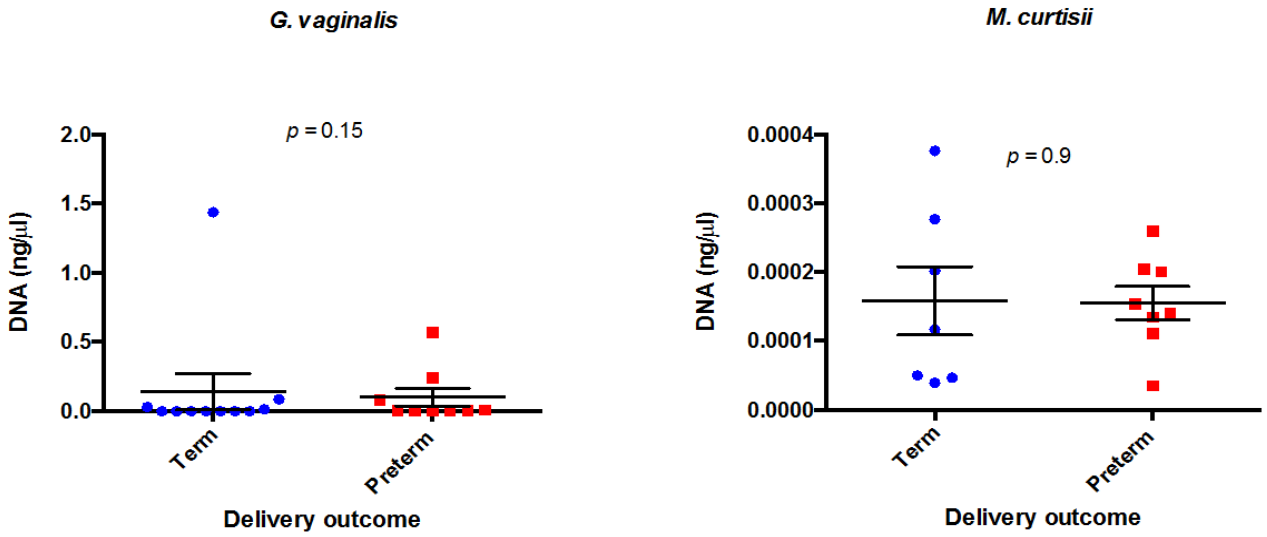
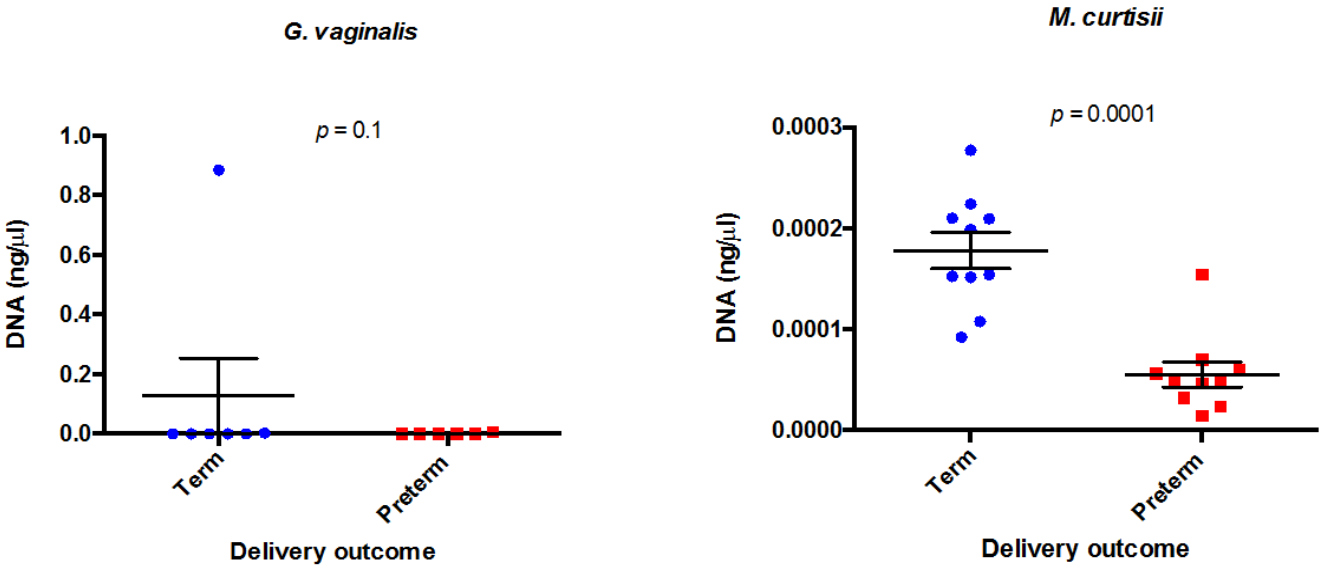


Figure 4.2. Real-time PCR standard calibration curve for (A) *G. vaginalis* and (B) *M. curtisii*. The Log [DNA] (quantity of bacterial 16S rDNA) is plotted against the number of cycles at which the fluorescence (positive signal) exceeds the threshold (threshold cycle or Ct). Both vaginal samples and calibrant bacterial 16S rDNA were run in triplicates. Similar results were obtained for each cohort and a total of 12 independent thermocycler runs were performed.



SYM 24-36w



AHR 20-22w

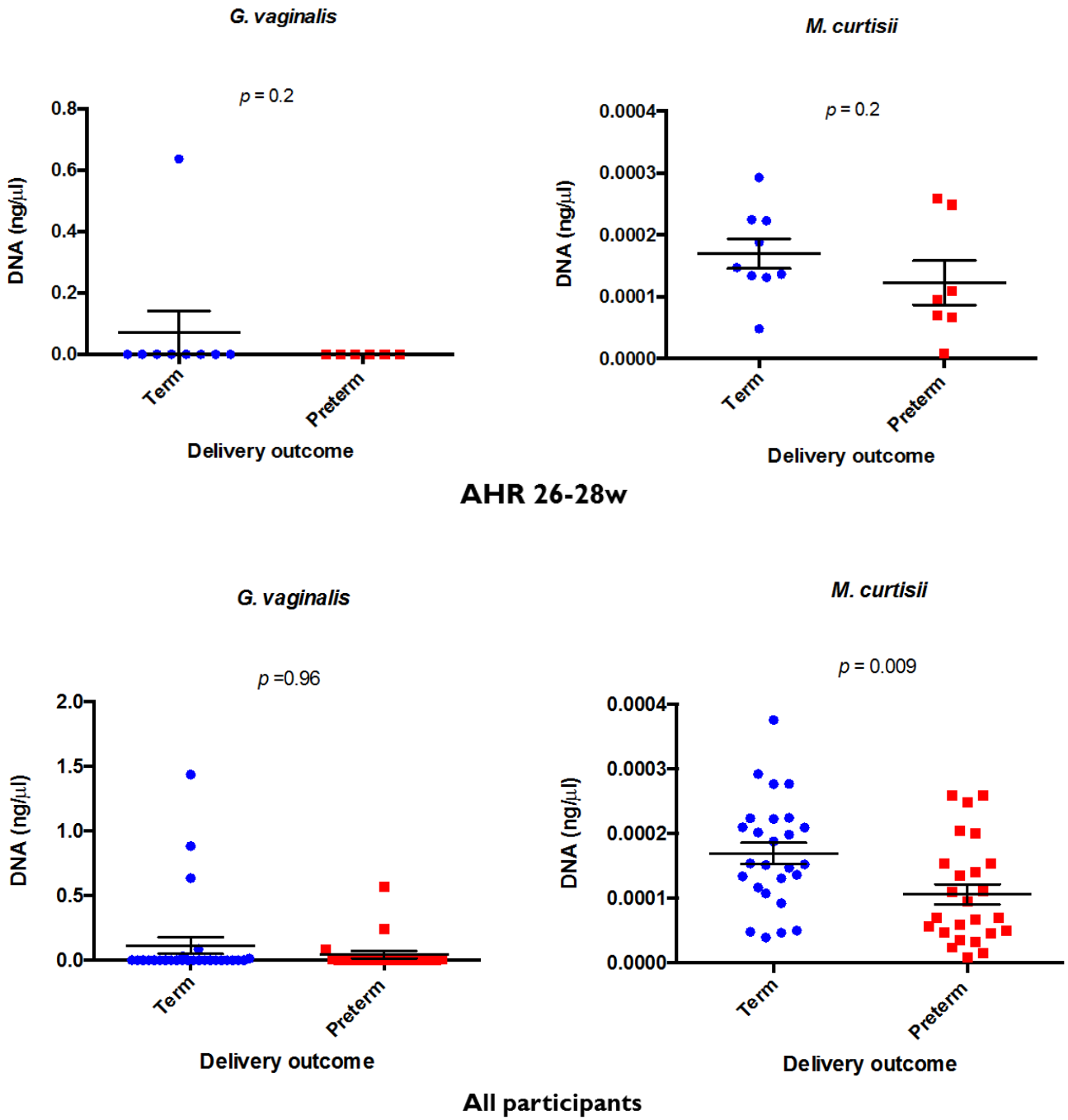


Figure 4.3. Comparison of the relative abundance of 16S rDNA of *G. vaginalis* and *M. curtisii* in vaginal fluid samples of different cohorts of pregnant women sampled at various gestational time points. Data are presented as median ± quartile range.
AHR, Asymptomatic high risk pregnant women
SYM, Symptomatic pregnant women

4.3.3. Cellular composition of vaginal fluid samples

In order to identify the cellular composition of the vaginal milieu of the study cohorts various staining procedures were also performed. This was necessary to provide an insight to the predominant bacterial sp., morphology of cervicovaginal epithelial cells and consequently the potential source(s) of the metabolites identified in the CVF. The metabolite profile of the CVF is a function of both host and microbial activities.[38] Representative vaginal smear micrographs obtained from randomly selected samples (n = 14) are shown in Fig. 4.4-4.6. Both host vaginal epithelial and bacterial cells were identified in the vaginal fluid samples across the various gestational time points studied. No abnormal cells (e.g. clue cells, cancerous cells etc.) were identified giving credence to the strict exclusion criteria applied during participants' recruitment. This provides insight to the source(s) of the metabolites observed. For instance gram-positive rods indicative of *Lactobacillus* morphotypes, as well as Gram-negative and Gram-variable organisms representative of *Gardnerella*, *Bacteroides* and *Mobiluncus* morphotypes were identified (Fig. 4.6). Furthermore, H&E and Pap staining revealed the presence of normal vaginal squamous epithelial cells and some bacterial cells particularly of *Lactobacillus* morphology (Fig. 4.4-4.5).

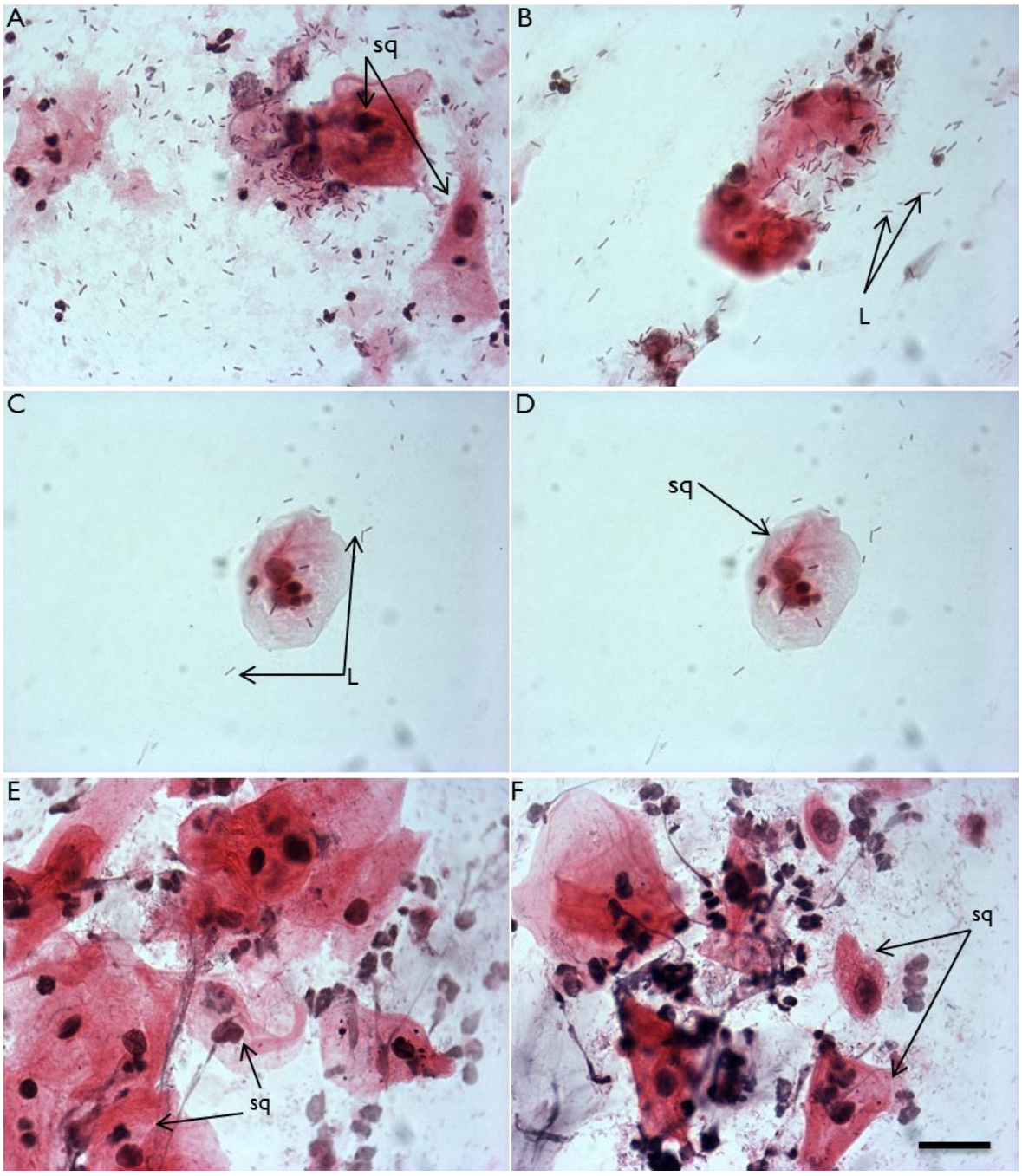


Figure 4.4. Vaginal fluid smears (x100) of pregnant women stained by Hematoxylin and Eosin (H & E) at 20-22 weeks (A-D), 26-28 weeks (E-F). *L*, *Lactobacillus* morphotype; *sq*, vaginal squamous epithelial cells. Scale bar = 20 µm for all pictures.

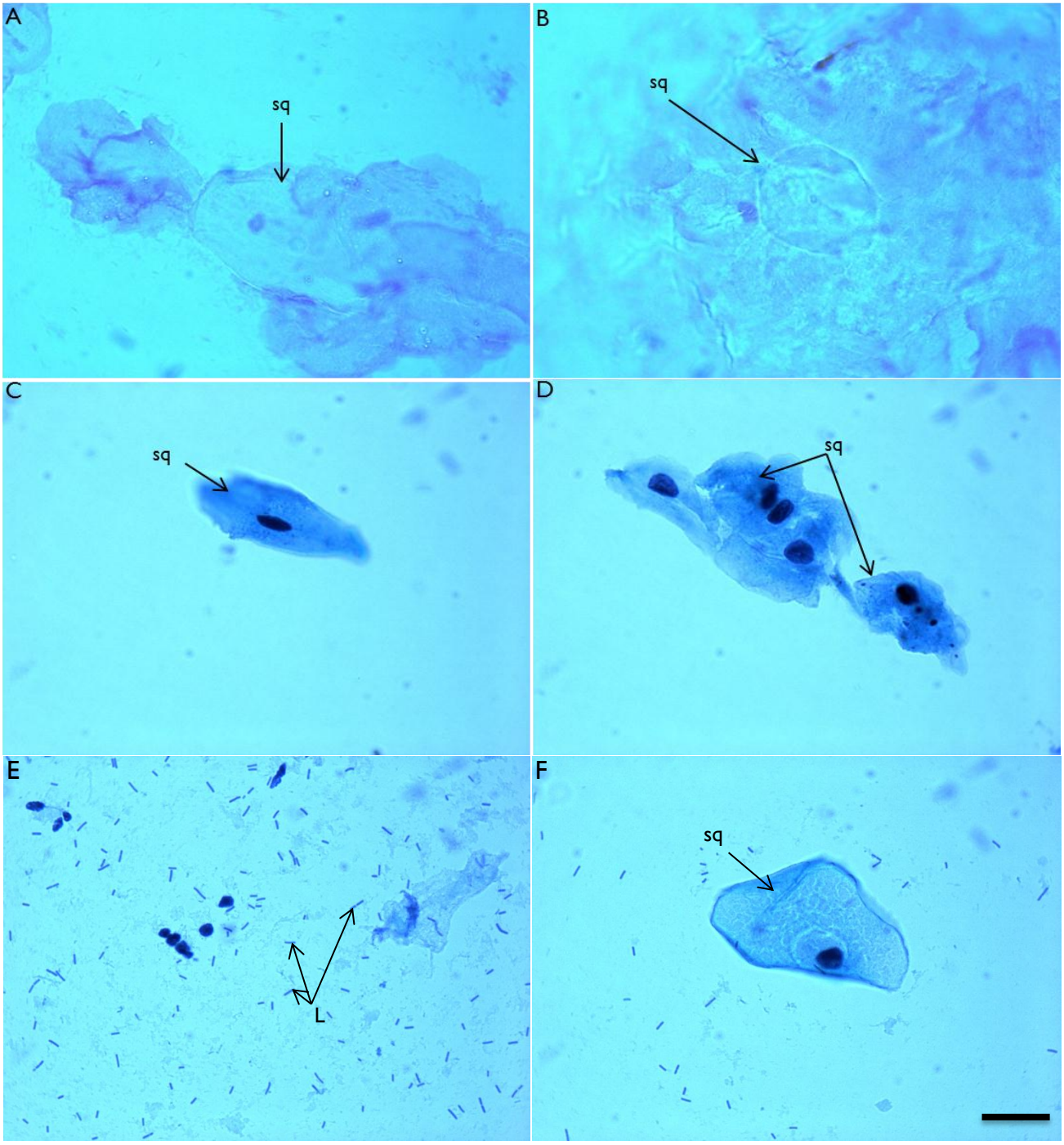


Figure 4.5. Vaginal fluid smears (x100) of pregnant women stained by Papanicolaou stain (Pap stain) at 20-22 weeks (A-B), and 33 weeks (C-F). *L*, *Lactobacillus* morphotype; *sq*, vaginal squamous epithelial cells. Scale bar = 20 μm for all pictures.

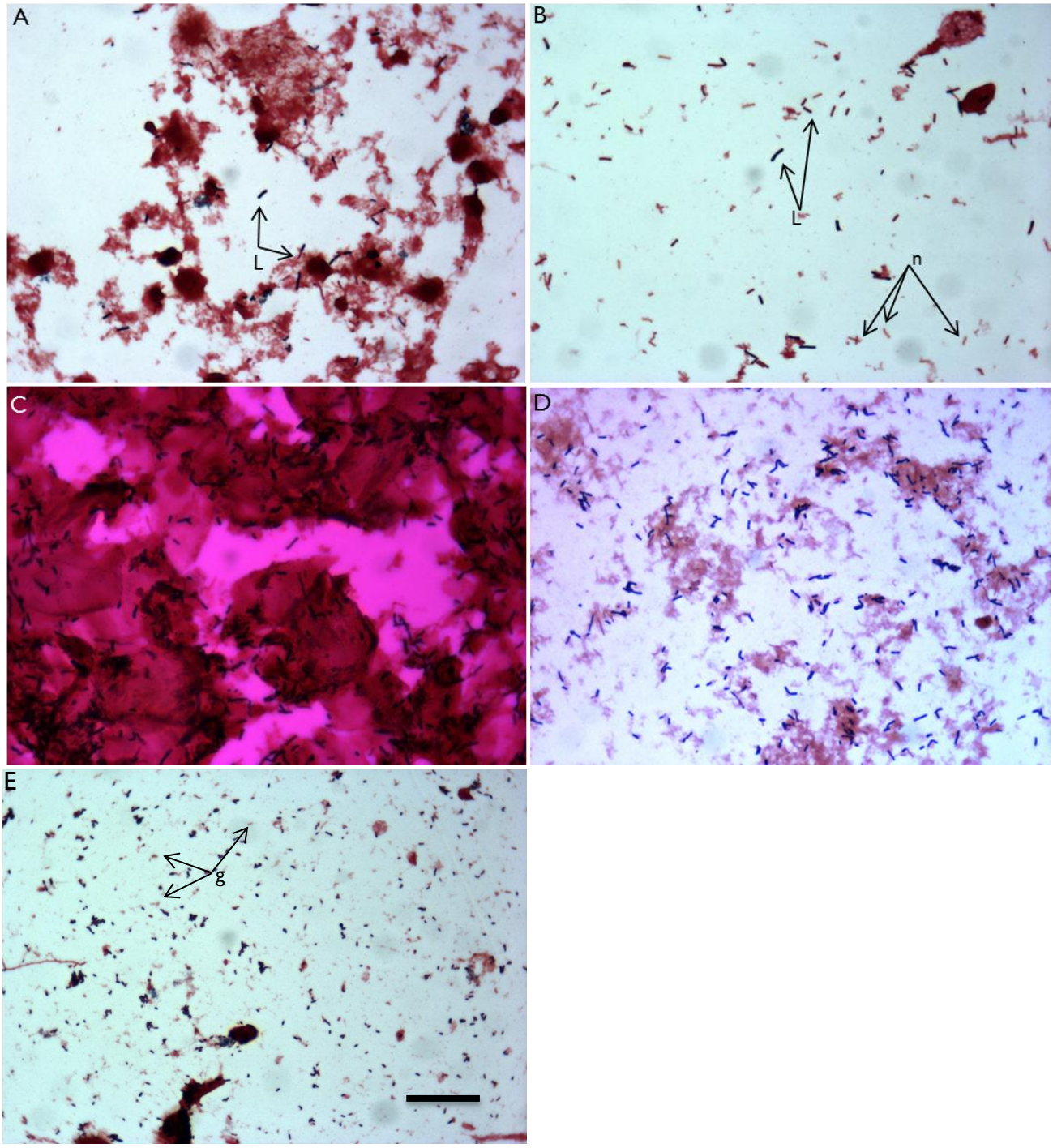


Figure 4.6. Vaginal fluid smears (x100) of pregnant women stained by Gram stain at 20-22 weeks (A-C), and 26-28 weeks (D-E). *L*, *Lactobacillus* morphotype (large Gram-positive rods); *g*, *Gardnerella* morphotype (small Gram-variable cocci); *n*, Gram-negative rods (*Bacteroides* morphotype). Scale bar = 20 µm for all pictures.

4.4. Discussion

In this chapter we present PCR and vaginal cytology data of different cohorts of pregnant women studied at different gestational time points and classified into 2 broad categories i.e. asymptomatic women and women presenting with symptoms of preterm labour. The former who were studied at 20-22w, were further subdivided into low and high risk group based on a previous history of preterm birth. A subset of the high risk women were studied again at 26-28w. In all, the vaginal fluid samples of 4 groups of pregnant women were analysed. All women presented with intact membranes and no clinical signs of genital infection. We then analysed the prevalence and relative abundance of specific vaginal endogenous bacterial species in these participants using standard and real-time PCR techniques and also detected various cell types present in the vaginal specimens obtained. This was in order to identify the potential source(s) of the metabolites observed in the vaginal fluid of these women (Chapters 2 and 3).

The composition of vaginal microbiota in both pregnant and non-pregnant women at any given time is indicative of the health status of the host and is critical to the prevention of several urogenital diseases and adverse delivery outcome.[6, 21, 65, 74, 118, 212, 295] The vaginal microbiota has been classified into 5 major CSTs based on the dominant bacterial species. Four of the CSTs are dominated by *Lactobacillus* sp. while the fifth is comprised of higher proportions of mixed anaerobes and lower amounts of *Lactobacillus* sp. and other lactate producers.[21] The vaginal microbiota in health is said to be predominated by commensal *Lactobacillus* sp. and more stable in pregnant compared with non-pregnant women.[74] While some researchers have identified a strong association between vaginal bacterial community dominated by mixed anaerobes (CST IV) and preterm birth,[75] others argue that the vaginal microbiota of women who deliver preterm does not differ from those that deliver at term,[6] and that the presence of little or no *Lactobacillus* sp. is not associated with preterm birth.[266] This is perhaps because preterm birth is regarded as a syndrome with many causative factors.[212] In addition to 16S rRNA gene quantification and sequencing as employed in most of these investigations, evaluation of the risk of preterm birth may require assessment of the host-microbial interactions as well as metabolic or biochemical activities of both the host cells and microbial population.[6, 478] In line with these assertions, we have employed a more comprehensive approach involving metabolomics, PCR,

and cytology to elucidate probable differences in vaginal microbiota composition between term- and preterm-delivered pregnant women. In addition, other members of our group have characterised the vaginal microbiome by bacterial 16S gene sequencing. Although the data are not presented in this report, 5 Community state types (CSTs), similar to those of previous studies[21, 65, 75, 277, 295] were identified and related to delivery outcomes.

In relation to PCR analysis, several studies have detected the presence of both commensal and potentially pathogenic bacterial organisms in vaginal specimens.[118, 465, 479] The presence in high amounts of anaerobic bacterial species has been associated with unhealthy vaginal microflora, infection and adverse pregnancy outcomes.[465, 469, 480-482] This in conjunction with clinical diagnosis is highly reproducible[480] and has been advocated to be more sensitive and advantageous than Gram staining.[471, 483] Both cultivated and culture-insensitive bacteria have been detected using this method ultimately determining their relationship with preterm birth.[469] It is also a useful technique in elucidating the structure and composition of the vaginal microecosystem in health and disease.[465, 471, 484] The above reasons necessitated our application of targeted PCR amplification of specific bacterial species as well as quantitative PCR to elucidate the microbial structure and composition of the vagina in pregnant women to identify those at higher risk of preterm delivery. The targeted PCR showed a higher prevalence of mixed anaerobes in preterm-delivered women compared to their term counterparts, whilst quantification of the DNA revealed a higher amount of *M. curtisii* in the term-delivered women, but no significant differences in *G. vaginalis* in relation to delivery outcome. The association between the prevalence of mixed anaerobes in the vagina during gestation and eventual preterm delivery supportive of our data has been reported.[5, 7, 111, 160] Some have even implicated uncultured bacteria species.[266] However, there have been contrasting views arguing that the vaginal microbiome between term- and preterm-delivered women does not differ.[6]

In the asymptomatic low risk pregnant women, we observed that *Fusobacterium*, *Gardnerella*, *M. curtisii* and to a lesser degree *M. mulieris* were more prevalent in the women who delivered at term compared to the women with preterm deliveries. In fact none of the 5 preterm-delivered women in this group had positive results of *Fusobacterium*, *M. curtisii*, *M. mulieris* and *M. hominis*. These findings in asymptomatic women at low risk of preterm birth differ from the established link between the prevalence of vaginal mixed anaerobic bacteria and preterm delivery.[82, 469, 485]

The relatively few preterm deliveries recorded in this cohort could account for this disparity. However, in spite of the convincing link between BV and preterm birth, a considerable number of women with high amounts of BV-associated organisms still deliver at term.[469] Some studies were even unable to establish any difference in vaginal microbiome between women who deliver preterm and term.[6] Interestingly, consistent with the association of BV-related organisms and preterm birth, *Bacteroides* sp. another obligate anaerobe was detected in higher proportions in the preterm-delivered women compared to their term counterparts. Unfortunately, due to finite resources, duration of this research and the relatively lower number of women with positive results across the groups, it could not be further quantified and the more prevalent *G. vaginalis* and *M. curtisii* were chosen instead. With the contrasting data obtained from this cohort, it is plausible that the link between the prevalence of mixed anaerobes in the vagina during pregnancy and preterm delivery in women at low or undefined risk of preterm birth is contestable. This warrants further investigation

Consistent with our data in the asymptomatic high risk participants studied at 20-22w, the presence of *M. hominis* (a facultative anaerobe) is associated with increased risk of preterm birth.[82] However, racial/ethnic modifications suggest that the association between *Mycoplasma* and preterm birth may not be causal, but an indication of the presence of cofactors that can cause preterm birth.[160, 193] Though our study population involved predominantly Caucasians, our observations in the subset of these women studied at later gestation (26-28w) were consistent with the established association of higher prevalence of facultative and obligate anaerobic bacterial species with preterm birth. The preterm-delivered women in this group showed significantly higher prevalence of *Fusobacterium*, *Bacteroides*, *Mobiluncus* and *Mycoplasma* species compared to their term counterparts. Whether the prevalence of these organisms change with gestation warrants further investigation. Pro tem, this is thought-provoking as these differences are seen despite the influence of the treatment interventions administered to a considerable number of these women (Chapter 5). This challenge the therapeutic accuracy of current treatments (especially antibiotics) routinely employed in the prevention of preterm births.

Similar to the data observed in the asymptomatic high risk women studied at 26-28w, the women presenting at the labour ward with symptoms of preterm labour who eventually delivered preterm showed significantly higher prevalence of *Fusobacterium*, *M. mulieris* and *M.*

hominis, compared to their term counterparts. In contrast, *M. curtisii* was more prevalent in the term-delivered women, similar to the observations in the asymptomatic low risk women sampled at 20-22w. A similar trend was seen in the asymptomatic high risk women (20-22w), though not statistically significant. When compared to women in the other groups who also delivered preterm, the women who presented with symptoms of threatened PTL and eventually delivered preterm had a higher prevalence of *M. hominis*. Significant prevalence of *M. hominis* and subsequent reduction in preterm birth rate after treatment with antibiotics has been recorded in symptomatic pregnant women.[486] Its impact alongside *U. urealyticum* has been associated with adverse delivery outcomes.[153, 486] Independently, these organisms may not be able to cause sufficient pathology. However, acting synergistically with other BVAB or incompetent cervix, they can initiate significant inflammatory process leading to preterm labour and delivery.[153] This may be responsible for the high incidence of preterm birth observed in the symptomatic women in our study.

In the asymptomatic women, we observed an increase in the prevalence of anaerobic bacteria species in relation to risk of preterm birth and gestational age at sampling in those women who eventually delivered preterm. The prevalence of *Gardnerella*, *Fusobacterium* and *M. homins* was low in the low-risk women compared to the high-risk women. In the high-risk preterm-delivered women, the prevalence of *Fusobacterium*, *Bacteroides* and *M. mulieris* increased between 20-22w (mid second trimester), and 26-28w (late second trimester). The low prevalence of mixed anaerobes in the low risk women is not surprising and could be a reason for the relatively low incidence of preterm birth (10%) recorded in this group. Though the normal vaginal microbiome is increasingly stable as pregnancy progresses with *Lactobacillus* sp. being predominant,[74] the increased prevalence of some anaerobes associated with adverse pregnancy outcome between the mid and late second trimester, and the associated increase in the preterm birth rate (AHR 20-22w: 38% vs. AHR 26-28w: 46%), observed in our study is interesting. Even with the vaginal microbiome stability observed during pregnancy, transitions between community state types dominated by different species of *Lactobacillus* have been recorded.[74] More so, *L. iners* have been reported to confer lesser protection/stability compared to *L. crispatus* and *L. jensenii*, hence, its association with the presence of BVAB.[111] In the event of a transition from an *L. crispatus* or *L. jensenii*-dominated CST to that dominated by *L. iners*, an

environment suitable for the growth and proliferation of mixed anaerobes may be established.[111] This could be the case in the asymptomatic high risk women in our study. Determination of the relative abundance of these species in these women by amplifying and sequencing the 16S rRNA gene could further delineate any differences in relation to delivery outcome.

With the observed inconsistencies in our data, we further analysed our study population as a whole classifying them into 2 groups in relation to delivery outcome (i.e. preterm vs. term). The results indicated that only *Fusobacterium* sp., *Bacteroides* sp., *M. mulieris* and *M. hominis* were significantly associated with preterm birth, while *M. curtisii* was seen in more term-delivered women, although this did not reach statistical significance. High prevalence of *Lactobacillus* sp. and *Gardnerella* were observed and were similar irrespective of delivery outcome, while only 1%(2/163) of the participants had group B *Streptococcus*. The insignificant levels of group B *Streptococcus* in our cohorts was not unexpected as only 25% of women in the general population harbour this bacteria vaginally.[487]

With the plethora of evidence that it is not just the presence or absence of, but the relative quantities (abundance) of bacteria that is related to the risk of preterm birth,[168] we conducted further analysis of the relative abundance of *Gardnerella* and *M. curtisii* using real-time PCR. There was no statistically significant difference in the abundance of *G. vaginalis* in the CVF specimens of both term- and preterm-delivered women in all the cohorts when analysed individually and as a whole, despite the non-significant trend to greater abundance in the term-delivered women. This is in accordance with our previous observation and could be attributed to the very low incidence of BV in our study cohort (due to exclusion) and the narrow racial mix - mainly Caucasian, but it is a shift from the widely reported association of *G. vaginalis* with BV and adverse pregnancy outcome.[481, 482, 488-490] Nevertheless, using the same method as applied in our study, Vitali et al. reported that the amount of *G. vaginalis* does not vary significantly in healthy and BV-infected women.[439] More so, others have opined that *G. vaginalis* does not differ in relation to delivery outcome as it is commonly isolated from women with normal vaginal microflora.[81, 475 274, 491] *G. vaginalis* has also been detected in more healthy samples compared to any other non-*Lactobacillus* sp.[465] It is often cultured from women regardless of infection (e.g. BV) status.[492] In fact, it is found in almost 100% of healthy women.[465] In addition, Fredricks et

al.[466] demonstrated that *G. vaginalis* amidst other organisms, was one of the most frequently identified bacteria in subjects without BV. Besides, no single bacterium has been implicated as the sole cause of BV,[467] hence it has been regarded as a microbiological and immunological conundrum.[36, 38, 493, 494]Whether *Gardnerella* alone in the absence of other BVAB can initiate PTL and PTB is still a subject of controversy. The heterogeneity of this infection, synergistic effects of diverse Gram-negative and Gram-variable anaerobes and host immune response in relation to infection and consequent sequelae is still of great value.[12, 36, 97, 103, 104, 495]

Using primers with high sensitivity (56%) and specificity (93%),[466] we were able to quantify *M. curtisii* genomic DNA from CVF samples of both asymptomatic and symptomatic pregnant women. Though *M. curtisii* has been exclusively recovered from women who deliver preterm,[496] our data revealed otherwise especially in the asymptomatic high risk pregnant women studied at 20-22w. The presence and persistence of this organism has also been shown in recurrent BV,[479] and attributed to its resistance to metronidazole treatment.[497] In contrast, its presence in the vagina was earlier described to be highly specific but not sensitive for the diagnosis of BV.[498, 499] Again, although it is usually isolated from women with BV, it is more common in healthy women than previously suspected.[500] This may explain the higher abundance of *M. curtisii* in the asymptomatic high risk women who delivered at term in particular and the term-delivered women on the whole compared to the preterm-delivered women in this study.

Another possible reason for this variation could be the low incidence of BV and other genital infection in our study cohorts as women with proven genital infection were excluded from the study. Besides, this supports the inability of some investigators to identify a link between the presence of noxious vaginal bacterial species and premature birth.[266] Also, the effect of the antibiotics treatment administered in those women who were diagnosed with BV or other infection in later gestation (after inclusion), alongside other routine preterm birth prevention therapies is suspected. Additionally, variations in host immune response to female genital tract colonisation by bacterial organisms in relation to adverse delivery outcome, and not just the presence of these organisms have been documented.[82, 256] Risk of preterm birth is modified by maternal genetic composition, epigenetics and immune response to altered vaginal microflora during gestation.[166, 167, 243, 245, 501, 502] Also, the relatively small sample size analysed, differences in the concentration of extracted DNA (range = 1-617 ng/µl), could be co-factors capable of

influencing our observations. Though the amount of genomic DNA per PCR mix (5 ng/µl), was normalised to correct for any inter-individual variations, our unique observations may be due to the disparity in quantity of extracted DNA from each participants. Molecular characterisation using high fidelity specie-specific primers and 16S gene sequencing is required to holistically describe and quantify the vaginal bacterial species associated with health and disease. A multiplex PCR approach which involves the amplification of multiple different DNA simultaneously can be applied in the assessment of the vaginal microbiome in relation to disease burden.[468] It uses multiple optimised primers and a temperature-mediated DNA polymerase to amplify several DNA of interest in a single experiment and has being employed in the diagnosis of common genital infections with great success.[468] Applying this technique, we could simultaneously amplify more bacterial DNA associated with dysbiosis and adverse pregnancy outcome including *Bacteroides*, which we were unable to analyse further despite its higher prevalence in the preterm-delivered women in this study. Also, because specie level differentiation of microorganisms is usually difficult using qPCR, 16S gene sequencing and classification of vaginal bacterial species in to CSTs could provide a more lucid picture of the microbiota with greater diagnostic utility.

Our study of the vaginal fluid of pregnant women also permitted us to examine the vaginal cytology of the study participants. This was done because Gram staining alone or in combination with Nugent criteria identifies women with BV infection.[38] In all the samples analysed, we observed the presence of squamous epithelial cells characteristic of the normal vaginal epithelium. Gram-staining demonstrated the presence of numerous long Gram-positive rods characteristic of *Lactobacilli* morphotypes as wells as Gram-negative and Gram-variable short rods and cocci characteristic of *Gardnerella*, *Bacteroides* and *Mobiluncus* morphotypes as amplified earlier using PCR techniques (Fig. 4.6). No abnormal human cells (e.g. clue cells, cancerous cells etc.), or differences in cytology between and within the groups were identified giving credence to the strict exclusion criteria applied during participants' recruitment i.e. women with clinically proven genital infection (e.g. BV) and/or abnormal cervical cytology were excluded from the study. This observation provides an insight to the potential source(s) of the metabolites observed. The presence of host epithelial cells in the samples is suggestive that some of the metabolites identified (Chapters 2 and 3), could originate from these cells. This is

because; the metabolite profile of the CVF is a representation of both host and microbial factors.[38] Also, it is not just the presence of these cells but their relative abundance and level of metabolic by-products that are capable of influencing delivery outcome.[1] The metabolic profile of the vaginal environment at any given instance could be a manifestation of the metabolic activities of both the host epithelium and microbial cells. For instance, D-lactate is almost exclusively of bacterial origin, while the vaginal epithelial cells like other human cells, *L. iners* and other lactic acid-producing bacteria produce L-lactate.[449] D-lactate produced by *L. crispatus*, *L. jensenii* and *L. gasseri* enhances protection against microbial invasion of the upper genital tract, reduce MMP-8 levels via its inducer (EMMPRIN), and prevents the degradation of the cervix and fetal membrane rupture.[114, 196] In this study we were unable to differentiate D/L optical isomers of lactic acid. An in-depth study of the prevailing vaginal host-microbial cellular composition in relation to L/D-lactate ratios and other metabolites in pregnant women sampled at various gestational time points could further improve our understanding of the pathogenesis of preterm birth. In addition, the microscopic examination of vaginal specimen by the Gram staining method is based on the relative abundance of bacterial morphotypes, but it does not distinguish the different bacterial species. However, the proportion of different bacterial species in a vaginal specimen can be predictive of normal or abnormal microflora. For instance, *L. crispatus* is observed mainly in females with healthy vaginal microflora, while *L. iners* is found both in healthy and abnormal vaginal microflora.[97, 465, 470] Using qPCR, these organisms can be quantified and compared in relation to the amount of their metabolic by-products. Furthermore, because certain fastidious or unculturable bacterial cells may have been omitted by the methods applied in this study, analysis with high throughput cytogenetic techniques such as Fluorescence in situ hybridization (FISH) may better resolve the structural composition of the vaginal ecology in health and disease e.g. FISH can determine the localisation or distribution of specific bacterial species in the vaginal microbial ecology.

Chapter 5

Association of Cervicovaginal fluid metabolites with fetal fibronectin, cervical length, and gestational age at delivery

5.1. Introduction

Putatively the most widely used clinical test in prognosticating PTB within 7–14 days especially in symptomatic women is FFN. This is due largely to its high NPV.[313, 315, 316] Its predictive utility has also been investigated in asymptomatic women, although not as effective as in the symptomatic women.[313] FFN is a glycoprotein produced by cells of the fetal membrane located in the feto-maternal interface, binding the fetal membranes to the decidua.[201, 304] In normal pregnancies, FFN is present in insignificant amounts in CVF in early mid trimester (16–22 weeks). Between 22-34 weeks gestation, detection of FFN at levels ≥50 ng/ml is suggestive of membrane activation and premature separation of the fetal membranes from the uterine lining and associated with an increased risk of spontaneous PTB.[201, 304,309]

Although, the detection of FFN in the CVF is currently the most effective marker of PTB especially in women with threatened PTL, and one of the best risk assessment markers for PTB in all populations,[303, 305] it has low sensitivity and PPV especially in asymptomatic women. Its applicability is also affected by common factors such as unprotected vaginal intercourse before testing, recent vaginal digital examination, vaginal bleeding and contamination with amniotic fluid from ruptured membranes leading to a false positive result.[303]

Another widely used clinical assessment tool for the prediction of PTB is ultrasonographic measurement of cervical length (CL).[303, 367] It is a simple and reliable method of objectively evaluating the integrity of the cervix and dilation of the internal cervical os with minimal patient discomfort.[407] The length of the cervix during pregnancy is inversely correlated with the risk of spontaneous PTB, though there is no consensus as to the threshold CL at which such risk becomes significant.[303, 367] In asymptomatic women, a CL <25 mm at <24 weeks is associated with PTB (< 35 weeks), with LR+ of 2.8-6.3. Various CL threshold values (25-35 mm) predicted PTB with 33-55% sensitivity and 73-100% specificity.[303] However, in asymptomatic women at low risk for PTB, its low sensitivity has posed some constraints to its utility as a routine/universal screening tool.[303, 398]

In symptomatic women, CL <25 mm (24-36 weeks) had PPV, NPV and specificity of 24%, 97% and 71% respectively.[329] Also, in high risk symptomatic women, a CL threshold value of 30 mm

(26-34 weeks) produced 72% sensitivity, 100% specificity, 100% PPV and 70% NPV.[354] Despite its predictive potential in symptomatic women, evaluation of CL did not significantly reduce the incidence of PTB (< 37 weeks), and as a result routine screening was not recommended.[395]

An association between a short cervix and intrauterine infection i.e. microbial invasion of the amniotic cavity, and intra-amniotic inflammation have been identified. This is due to chorioamnionitis-stimulated cervical shortening or an already shortened cervix permitting ascending genital tract infection,[407] ultimately leading to PTL and spontaneous PTB. Intrauterine infection and the subsequent inflammatory processes that ensue can also disrupt the fetal membranes with a concomitant leakage of FFN into the cervicovaginal space.[199]

Ultrasound CL, like FFN, has enhanced the prediction of PTB, nonetheless, there is a considerable high rate of false positive results as most women with short cervix (< 25 mm), still go on to deliver at term.[503] More recently, combining CL and FFN screening in asymptomatic[320, 321] and symptomatic women[302, 322-324] compared to either of these tests alone, results in an improved discriminative and predictive ability for risk of PTB. This aids the decision making process during management and reduces unnecessary interventions particularly in low risk or unknown risk population.[302]

Considering the obvious deficiencies of FFN and ultrasound CL, and the awareness that these 2 biomarkers of PTB, as well as changes in vaginal pH are associated with ascending intrauterine infection which can be detected by CVF metabolite profiling, in this chapter we sought to: 1) examine the relationship between FFN, CL, vaginal pH and ^{1}H-MR CVF metabolites, and 2) Combine the predictive abilities of these biomarkers to determine whether the prediction of PTB could be improved.

5.2. Methods

5.2.1. Study participants

As enumerated in chapter 2 of this report, the study participants comprised of the same clinical cohorts of pregnant women (N = 458), recruited via the antenatal clinics and Triage Delivery Suites of the Jessop Wing Hospital, Sheffield, UK: those that had no symptoms of PTL (asymptomatic group) and those presenting to the delivery suite with symptoms of, but not established, PTL. The asymptomatic pregnant women were further classified into 2 gestationally-matched groups based on a prior PTB: a low-risk group (ALR, n = 183), who had no prior PTB (assessed at 20-22 gestational weeks, w), and a high-risk group (AHR, n =186), who had at least 1 prior PTB and/or short cervix (< 25 mm) on transvaginal ultrasonography (assessed at 20-22w and repeated at 26-28w). The third study group (SYM, n = 89) comprised women presenting with uterine contractions, cervix < 3cm dilated, and intact fetal membranes (24-36w). The clinical characteristics of the study participants are represented in Table 2.1 (Chapter 2).

5.2.2. Measurement of CVF fetal fibronectin and cervical length

Also at presentation, commonly employed clinical assessment methods of PTL and PTB including CVF FFN level and ultrasound cervical length were examined. Quantitative CVF FFN levels were analysed using the 10Q Rapid FFN analyser (Hologic, MA), according to manufacturer's instruction, and cervical length (CL) by transvaginal ultrasonography. Both measurements were performed by the same clinical research staff at the Jessop Wing Maternity Hospital. The qualitative FFN cut-off value (50 ng/ml) has the capacity to determine the risk of spontaneous PTB in women with symptoms suggestive of threatened PTL with a considerably high negative predictive value. In addition, we measured quantitative FFN because it is reliable and provides thresholds (e.g. 10 and 200 ng/ml), thereby improving the predictive utility of the test.[301]

5.2.3. Vaginal fluid pH measurement

During vaginal fluid sample collection, the vaginal pH was also determined by obtaining a sample of vaginal discharge from the lateral vaginal wall with the aid of a dry swab and smeared on a

hiqh quality narrow range pH paper (pH-Fix 3.6-6.1, #92130, Macherey-Nagel, Düren, Germany).[504, 505] This pH indicator paper has the advantage of measuring pH values of unbuffered or weakly buffered solutions or samples and the dye does not bleed even in strongly basic solutions. Vaginal pH-determination by this method is highly accurate (reading accuracy: ± 0.1 pH), rapid and reliable as the reference colour chart to which the test strips are compared matches both colour and position of the 4 indicator pads on the strips. The product is certified according to ISO 13485 and CE-marked for in-vitro diagnostic applications (98/79/EG) 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

5.2.4. Treatment Interventions to reduce preterm birth

In order to prevent the occurrence and reduce the complications associated with PTB, as well as improve delivery outcomes, a subset of the study cohort received a range of therapeutic interventions between the 1st study at 22 weeks and the second at 26-28 weeks depending on the particular needs of the patient. The interventions include the administration of antibiotics for asymptomatic bacteriuria or subclinical genital bacterial infection,[188, 402, 403, 462] progesterone for women with previous PTB and short cervix,[506, 507] history and/or ultrasound-indicated cervical cerclage for incompetent cervix,[408, 410, 506] anticontraction medication (tocolytics),[414, 416] and corticosteroids (e.g. betamathasone) effective against respiratory distress syndrome, intraventricular haemorrhage and fetal mortality.[412, 413, 508] The treatment options and the number of participants that were administered such treatments in relation to the eventual delivery outcomes are presented in Table 5.1.

5.2.5. Statistical analysis

All Statistical analyses were performed using MATLAB (Mathworks, Natick, MA). The Wilcoxon's rank-sum with Bonferroni PostHoc tests was performed to compare differences in clinical data between and within the groups. The relationships between clinical data were

determined by Pearson's correlation coefficients and *P* values < 0.05 were considered statistically significant.

Also, the predictive capacities of CVF FFN, CL and vaginal pH for PTB (< 37 weeks), were determined by ROC curves and combined with CVF metabolite N.I. by binary logistic regression.

5.3. Results

5.3.1. Fetal fibronectin concentration and cervical length

In all study cohorts except the ALR group, the preterm-delivered women had significantly higher CVF FFN levels compared to the term delivered women (Table 2.1 and Fig. 5.1). This difference was more pronounced in the SYM cohort (> 12 times). Similarly, the CL of the preterm-delivered women was significantly shorter than that of the term-delivered women in all study cohorts except the ALR group (Table 2.1 and Fig. 5.1). Both FFN and CL correlated with gestational age at delivery (GAAD) (an indication of term or preterm birth), in all groups except the ALR group (Fig. 5.2 and Fig. 5.3).

Analysing all asymptomatic low and high risk women studied at 20-22w irrespective of their risk of delivering preterm (ASYM 20-22w), we observed that the preterm-delivered women had greater than 3-fold higher (> 200%) CVF FFN levels and 29% shorter CL compared to the term-delivered women ($P < 0.00001$) (Table 2.1 and Fig. 5.1). Also, the ALR women generally had a 3-fold (> 200%) lower CVF FFN concentration ($P = 0.0001$) and about 18% longer CL ($P < 0.0001$) compared to their high risk counterparts (i.e. AHR 20-22w).

In addition, modest correlations were observed between CVF FFN and CL in all groups: ALR ($r = -0.2$, $P = 0.02$); AHR 20-22w ($r = -0.5$, $P < 0.00001$); ASYM 20-22w ($r = -0.4$, $P < 0.00001$); AHR 26-28w ($r = -0.4$, $P < 0.005$); and SYM ($r = -0.5$, $P = 0.007$) (Fig. 5.4).

5.3.2. Vaginal pH

Sampling the vaginal pH values of participants whose consents were obtained in this study, we observed a pH range of 3.6-6.1 and there were no significant differences between term- and preterm-delivered women within the groups (Table 2.1). However, the ALR women in general had lower vaginal pH (4.0±0.03 vs. 4.2±0.05, $P = 0.0004$), and correspondingly higher lactate N.I. (0.21±0.006 vs. 0.18±0.006 a.u., $P = 0.0005$), than their high risk counterparts studied at the same gestation i.e. AHR20-22w. Vaginal pH was modestly associated with GAAD in the combined asymptomatic low and high risk women studied at 20-22w (ASYM 20-22w) ($r = -0.2$, $P = 0.009$), and AHR (26-28w) ($r = -0.3$, $P = 0.008$) women only (Fig. 2.7C-D). There was no

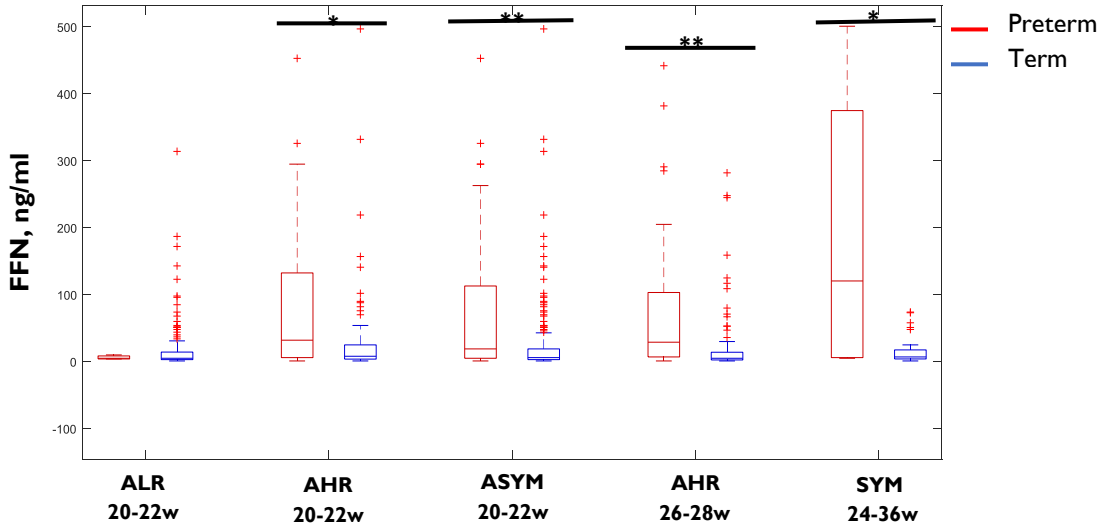
correlation between vaginal pH and GAAD when the participants studied at 20-22w were subdivided into high (AHR) and low risk (ALR) groups. Additionally, there were modest correlations between vaginal pH and CVF FFN in the AHR 20-22w ($r = 0.3$, $P = 0.03$), ASYM 20-22w ($r = 0.2$, $P < 0.00001$), and AHR 26-28w ($r = 0.2$, $P = 0.01$) (Fig. 2.7B-D); and CL in the SYM women only ($r = -0.4$, $P = 0.03$) (Fig. 2.7E).

Table 5.1: Therapeutic interventions administered to prevent preterm birth

Treatment Option	Asymptomatic Low risk women 20-22w		Asymptomatic High risk women 20-22w		Asymptomatic 20-22w (Combined)		Asymptomatic High risk women 26-28w		Symptomatic women 24-36w	
	Preterm (N=5)	Term (N=124)	Preterm (N=35)	Term (N=71)	Preterm (N=40)	Term (N=195)	Preterm (N=26)	Term (N=65)	Preterm (N=15)	Term (N=67)
Antibiotics										
Yes	1	30	15	31	16	61	18	41	5	25
No	4	91	15	34	19	125	5	18	1	12
Progesterone										
Yes	0	0	5	7	5	7	10	10	2	2
No	5	121	25	58	30	179	13	49	4	35
Cervical cerclage										
Yes	0	0	7	6	7	6	7	6	0	0
No	5	121	23	59	28	180	16	53	6	37
Tocolytics										
Yes	0	0	1	0	1	0	1	1	2	7
No	5	120	29	65	34	185	22	58	4	30
Steroids										
Yes	0	0	1	0	1	0	6	3	5	27
No	5	121	29	65	34	186	17	56	1	10

N, total number of term- or preterm-delivered women.

A.



B.

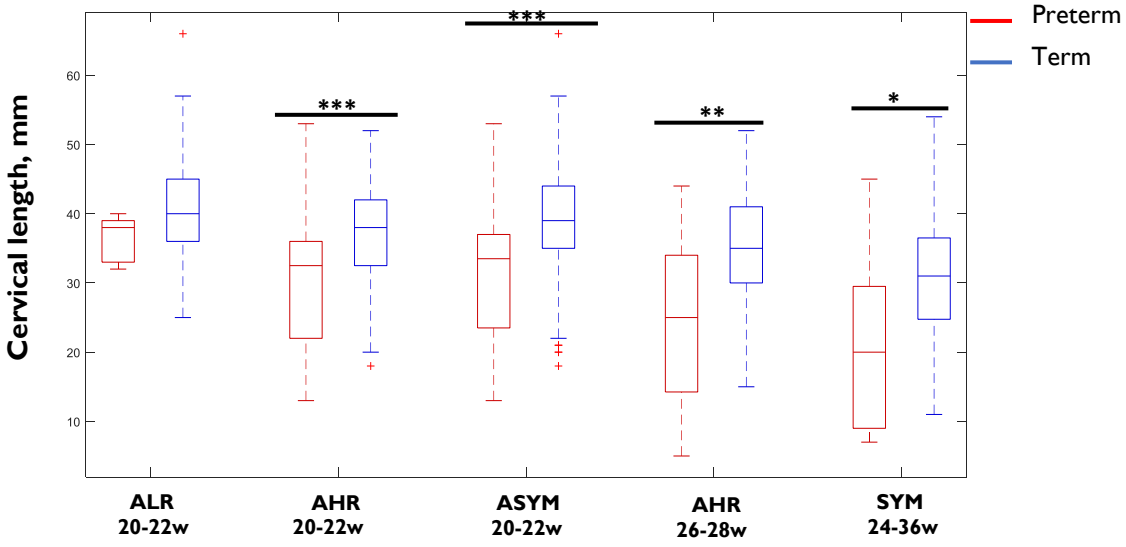


Figure 5.1. Clinical assessment methods for preterm birth (A) quantitative cervicovaginal fluid fetal fibronectin level and (B) cervical length in different cohorts of pregnant women in relation to delivery outcomes. Box plots show the median line within the box, with the bottom and top edges of the box representing the 25% and 75% quartiles respectively.

ALR, asymptomatic low risk women; *AHR*, asymptomatic high risk women; *ASYM*, ALR and AHR 20-22w combined; *SYM*, symptomatic women; *w*, gestation weeks.

* *P* value < 0.05, ** *P* value < 0.01, *** *P* value < 0.001.

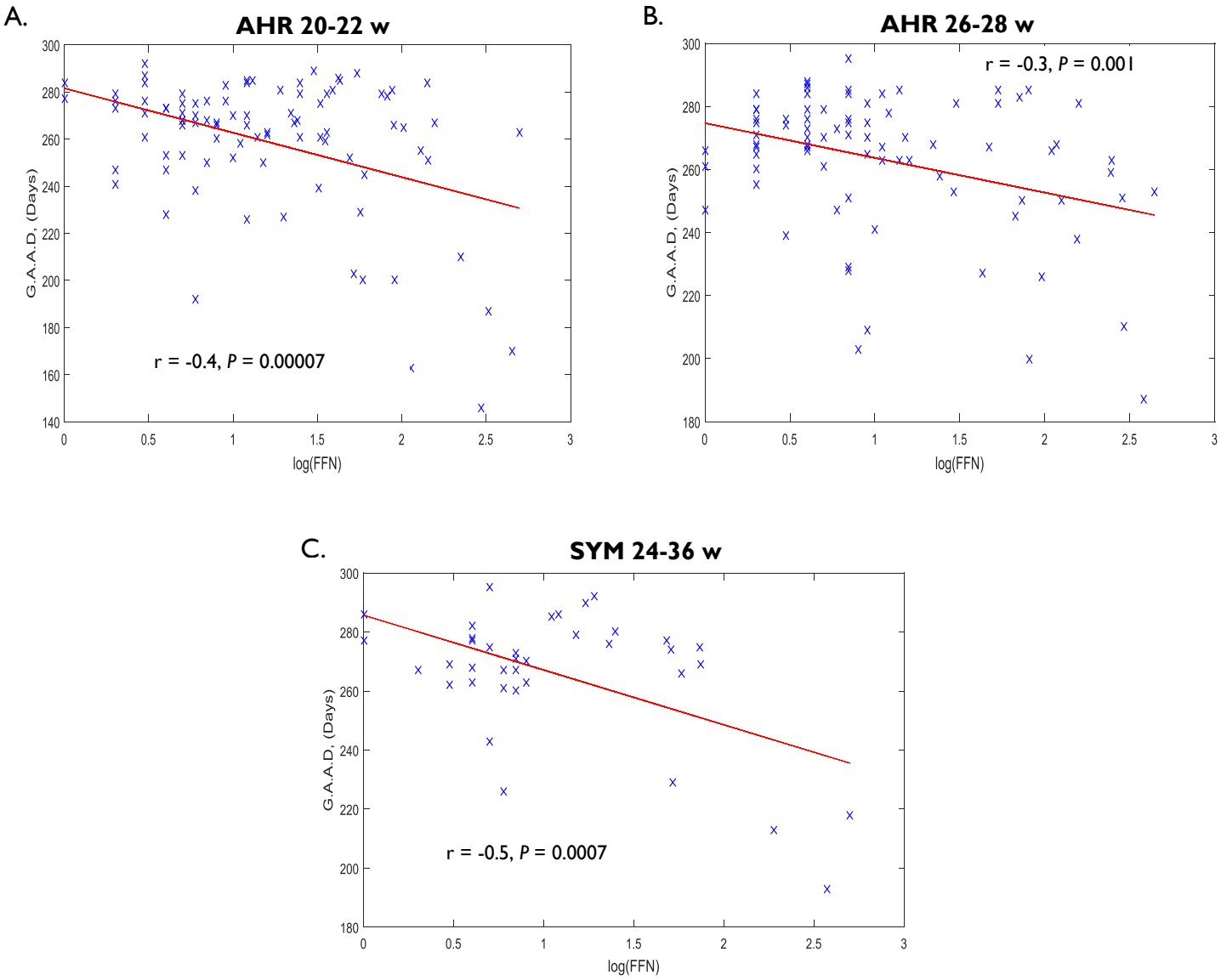


Figure 5.2. Association between cervicovaginal fluid fetal fibronectin (FFN) level and gestational age at delivery (G.A.A.D) (A) Asymptomatic high risk women 20-22w, (B) Asymptomatic high risk women 26-28w, (C) Symptomatic women 24-36w. *w*, gestation weeks.

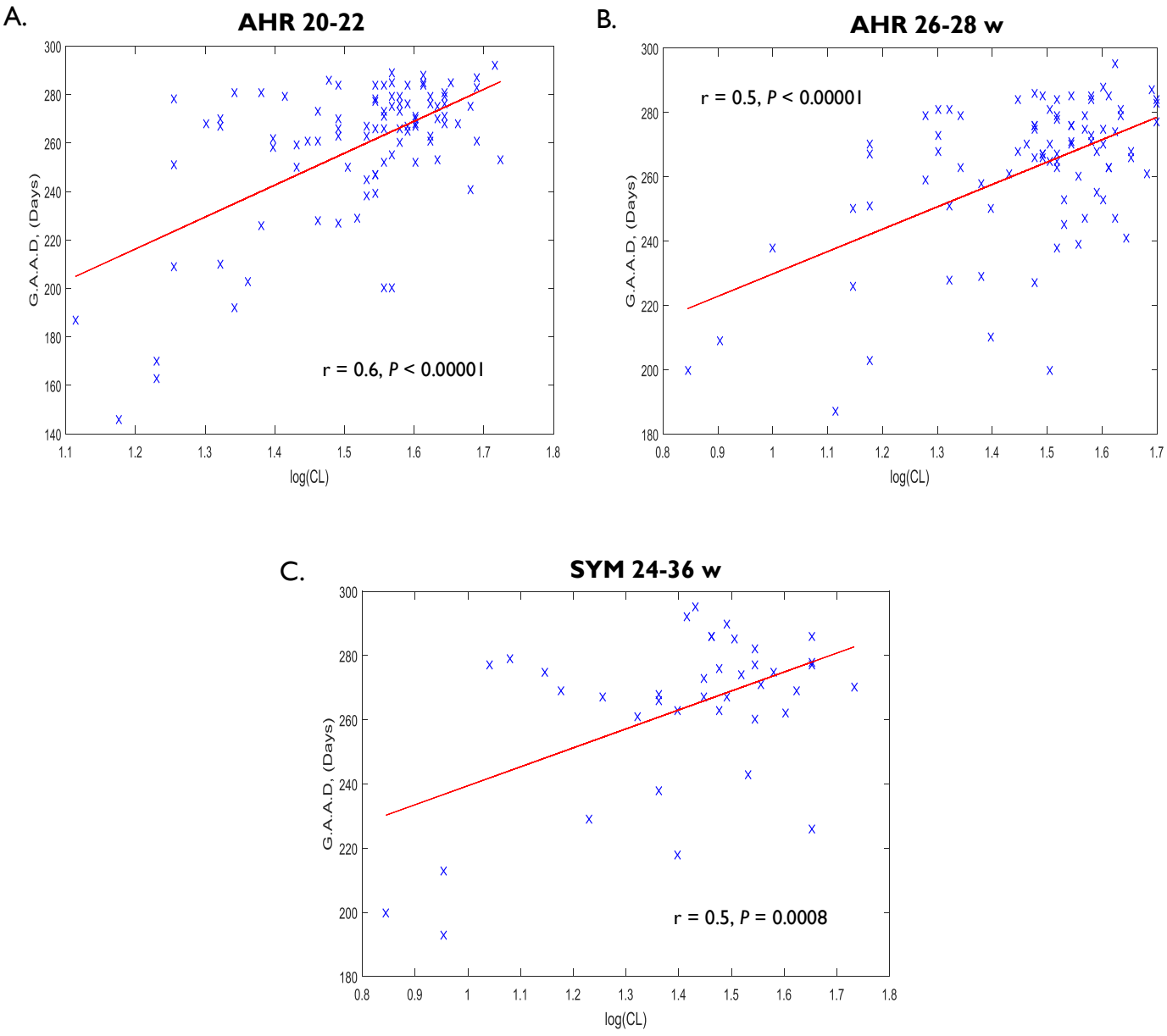


Figure 5.3. Association between ultrasound cervical length (CL) and gestational age at delivery (G.A.A.D) (A) Asymptomatic high risk women 20-22w, (B) Asymptomatic high risk women 26-28w, (C) Symptomatic women 24-36w. *w*, gestation weeks.

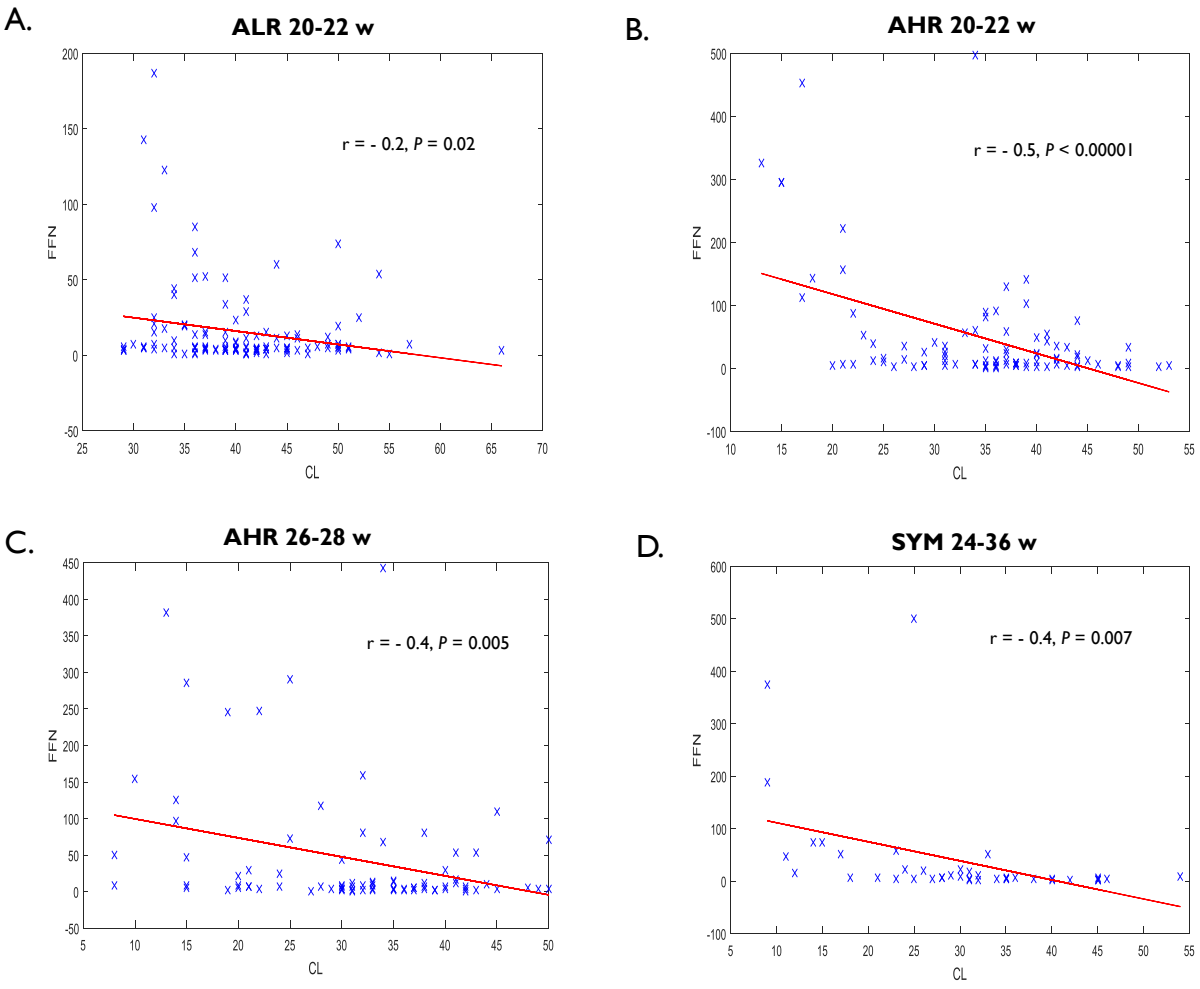


Figure 5.4. Association between CVF Fetal fibronectin (FFN) and ultrasound cervical length (CL) (A) Asymptomatic low risk women 20-22w, (B) Asymptomatic high risk women 20-22w, (C) Asymptomatic high risk women 26-28w, (D) Symptomatic women 24-36w. *w*, gestation weeks.

Correlations between clinical parameters and metabolites are also depicted in Chapter 2 (Fig. 2.7A-F).

5.3.3. Predictive capacity of CVF fetal fibronectin, cervical length and vaginal pH for preterm birth

Analysis of the area under the ROC curves for the measured CVF FFN, CL and vaginal pH showed significant discriminative abilities of CVF FFN and CL in all groups except the ALR women. Vaginal pH was not predictive of PTB in any of the study groups (Table 5.2).

In the asymptomatic women studied at 20-22w irrespective of their risk of PTB, CL had the best predictive utility (AUC = 0.79, $P < 0.0001$), while the combination of CL, FFN, vaginal pH, and ^{1}H-MR N.I.s of acetate, lactate and succinate, produced a slightly better predictive capacity than either of these markers alone (AUC = 0.81, $P < 0.001$) (Fig. 5.5).

Also, in the AHR (26-28w) women, combination of CVF ^{1}H-MR N.I.s of acetate and succinate, and succinate/lactate ratio, FFN and CL, only modestly improved the predictive values of FFN, and CL (Fig. 5.6).

However, in the SYM group, a combination of acetate N.I., FFN and CL improved the predictive value for PTB, compared to either of FFN and CL alone (AUC = 0.85, $P = 0.02$). Addition of ^{1}H-MR N.I.s of lactate and succinate did not change this predictive ability (AUC = 0.85, $P = 0.003$) (Fig. 5.7).

Table 5.2: Predictive performance of Fetal fibronectin and ultrasound cervical length for preterm birth (< 37w), in pregnant women

Metabolite	AUC	SE	95% CI	YI	Sen (%)	Spec (%)	NPV (%)	PPV (%)	LR+	LR-	*P* value
Asymptomatic Low risk women (20-22w)											
FFN	0.50	0.13	0.24-0.76	0.32	100	32	7	100	1.5	0.7	0.5
CL	0.70	0.13	0.44-0.97	0.49	100	49	7	100	2.0	0.5	0.06
Vaginal pH	0.60	0.14	0.33-0.87	0.44	80	44	5	98	1.4	0.7	0.2
Asymptomatic High risk women (20-22w)											
FFN	0.61	0.07	0.48-0.73	0.86	48	86	62	77	3.4	0.3	0.0499*
CL	0.72	0.06	0.60-0.84	0.52	87	52	47	89	1.8	0.6	0.0001*
Vaginal pH	0.49	0.06	0.36-0.62	0.86	23	86	45	69	1.6	0.6	0.6
Asymptomatic High and Low risk women (combined) (20-22w)											
FFN	0.61	0.06	0.50-0.72	0.89	41	89	43	88	3.6	0.3	0.03*
CL	0.79	0.05	0.68-0.87	0.60	83	60	30	94	2.1	0.5	<0.00001*
Vaginal pH	0.57	0.05	0.47-0.68	0.73	46	73	26	87	1.7	0.6	0.09
Asymptomatic High risk women (26-28w)											
FFN	0.70	0.07	0.57-0.83	0.81	58	81	56	83	3.1	0.3	0.001*
CL	0.72	0.06	0.59-0.84	0.84	58	84	59	83	3.6	0.3	0.0004*
Vaginal pH	0.49	0.07	0.35-0.62	0.97	15	97	66	74	4.8	0.2	0.6
Symptomatic women (24-36w)											
FFN	0.76	0.1	0.53-1.0	0.92	67	92	65	92	8.2	0.1	0.01*
CL	0.73	0.1	0.52-0.94	0.73	75	73	38	93	2.8	0.4	0.02*
Vaginal pH	0.50	0.1	0.26-0.74	0.89	29	89	37	85	2.6	0.4	0.5

CL, ultrasound cervical length; *FFN*, fetal fibronectin; *AUC*, area under the ROC curve; *SE*, standard error; *CI*, 95% confidence interval; *YI*, Youden index (J); *Sen*, sensitivity; *Spec*, specificity; *PPV*, positive predictive value; *NPV*, negative predictive value; *LR+*, positive likelihood ratio; *LR-*, negative likelihood ratio; *P*, significance level.
*Statistically significant

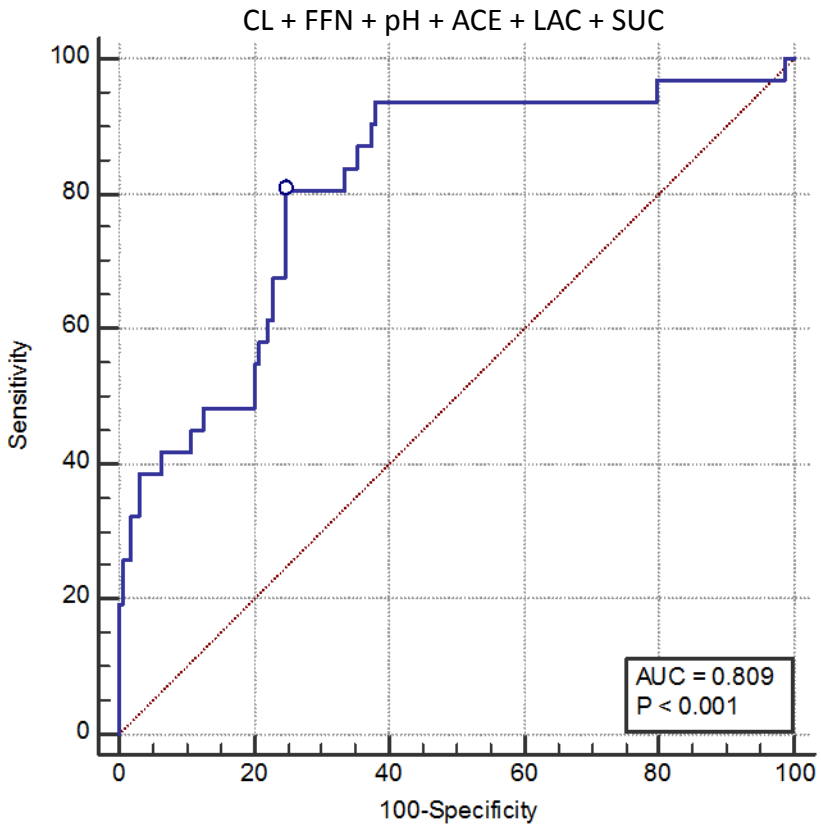
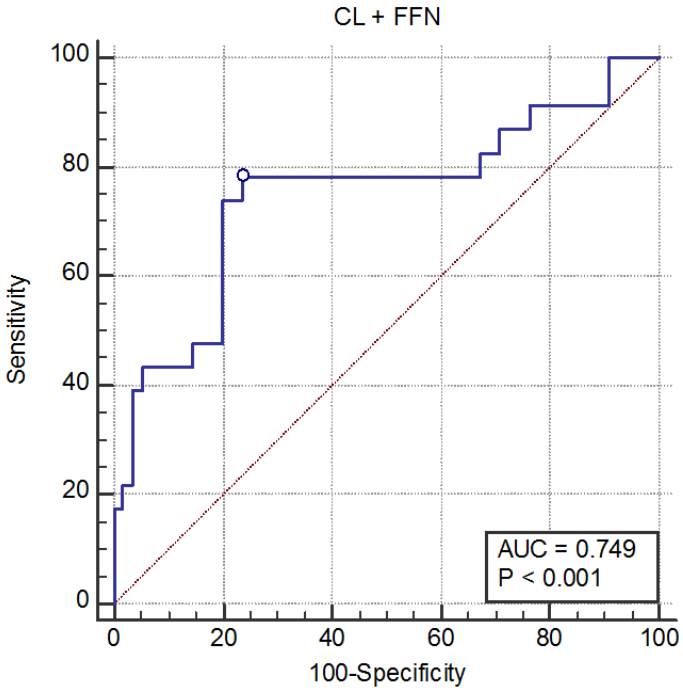
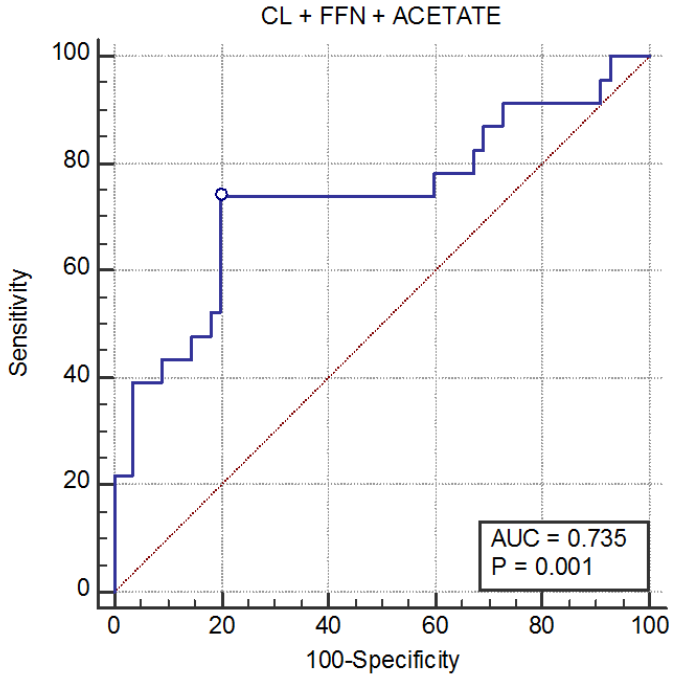


Figure 5.5. Combined ROC curves of cervical length, fetal fibronectin, and ^{1}H-MR normalised integrals of acetate, lactate and succinate for the prediction of preterm birth (< 37 weeks), in asymptomatic high and low risk women (20-22w).

A.


CL + FFN
Sensitivity
100-Specificity
AUC = 0.749
P < 0.001
0
20
40
60
80
100

B.


CL + FFN + ACETATE
Sensitivity
100-Specificity
AUC = 0.735
P = 0.001
0
20
40
60
80
100

C.

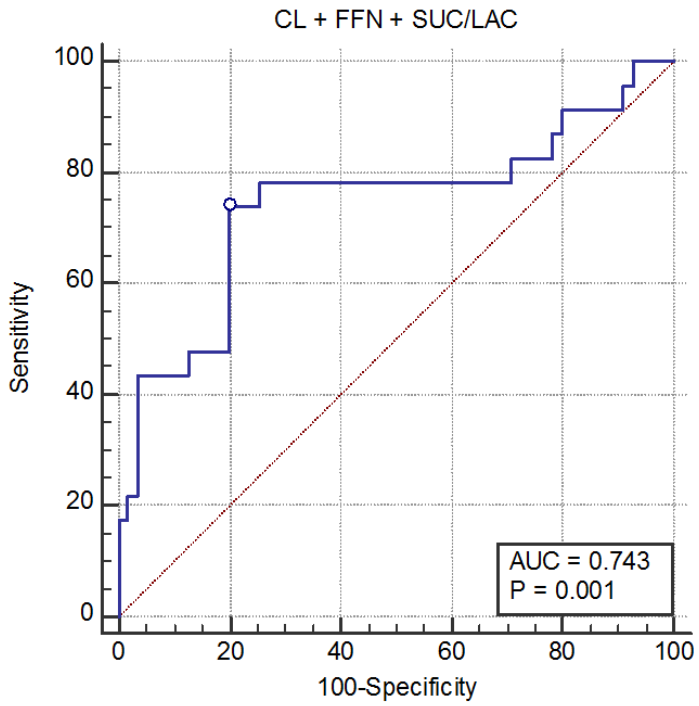
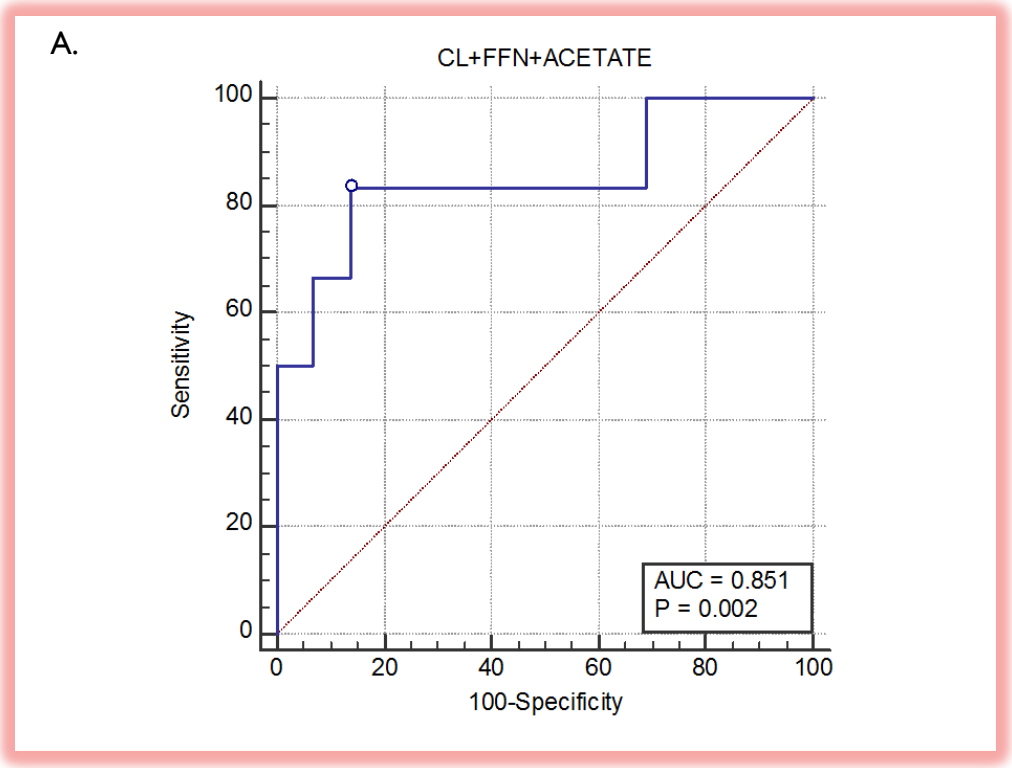


Figure 5.6. Combined ROC curves for the prediction of preterm birth (< 37 weeks), in asymptomatic high risk women (26-28w), (A) cervical length and fetal fibronectin, (B) cervical length, fetal fibronectin, and ^{1}H-MR normalised integrals of acetate, (C) cervical length, fetal fibronectin, and succinate/lactate ratio.



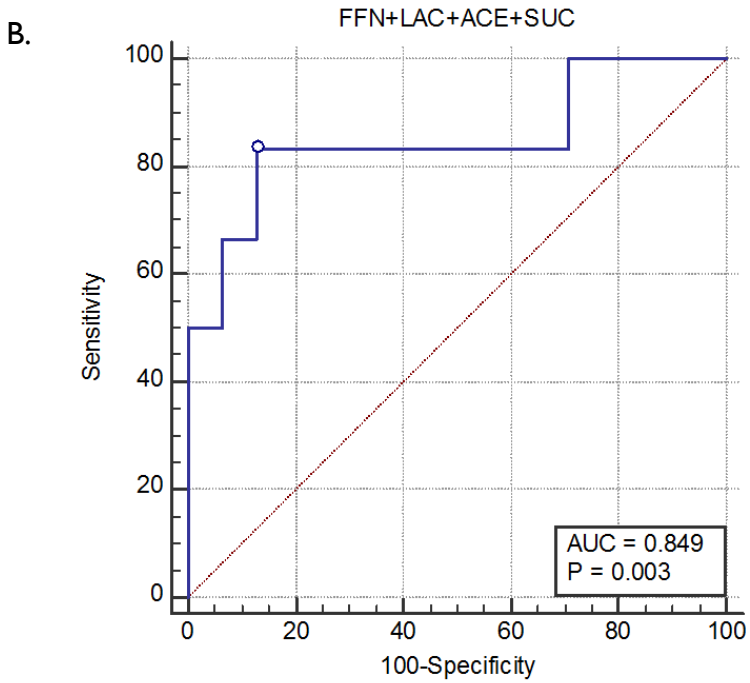


Figure 5.7. Combined ROC curves for the prediction of preterm birth (< 37 weeks) in symptomatic women (24-36w), (A) cervical length, fetal fibronectin, and ^{1}H-MR integrals of acetate, (B) fetal fibronectin, and ^{1}H-MR normalised integrals of lactate, acetate and succinate.

5.4. Discussion

In this study we present a unique cohort of both asymptomatic and symptomatic pregnant women studied across the mid trimester. The asymptomatic women were further subdivided into low and high risk groups based on a previous history of PTB and/or mid trimester ultrasound cervical length < 25 mm. None of the asymptomatic low risk women had a previous history of PTB or short cervix at second trimester, and only 5(3.9%) of these women delivered preterm compared to 35(33%) of their high-risk counterparts studied at similar gestation (20-22w) (Table 2.1). Also, their mean CL was about 18% higher than that of their high-risk counterparts. The prevalence of preterm birth in the low risk asymptomatic women in this study was significantly lower than the average recorded in the general population (3.9% vs. 12%), while that of the high-risk women was above 2.5 times greater than the general population average (33% vs. 12%).[2, 3, 266] This justifies the classification of the asymptomatic pregnant women into low and high-risk groups based on previous PTB and length of cervix at mid second trimester (20-22w). Due to their "high risk" status, a subset of the asymptomatic high risk women who attended for routine antenatal care at 26-28 weeks gestation were assessed again after their first study visit at mid second trimester (20-22w). The strict exclusion criteria employed were in order to prevent contamination of the CVF with microbes and/or fluid from the external environment or any other body compartment particularly the amniotic cavity.

With our study population well defined and stratified, we determined the risk of PTL and PTB using currently available clinical assessment methods i.e. quantitative CVF FFN level, ultrasound CL and vaginal pH. As anticipated, the lowest prevalence of PTB (~4%) was observed in the asymptomatic low risk group (Table 2.1). But more interestingly, all preterm-delivered women in the symptomatic group delivered approximately within 2 weeks of presentation with symptoms suggestive of PTL. This could be a critical window period in which a biomarker with accurate predictive capacity will be clinically useful. Elevated CVF FFN and short cervix were associated with preterm delivery, whilst low CVF FFN and normal/longer cervix were associated with term delivery. Both CVF FFN and second trimester ultrasound CL correlated strongly with gestational age at delivery. These observations were recorded in all the groups

except the asymptomatic low risk women (Table 2.1 and Figs. 5.1-5.3). Our data corroborates the already established relationship between FFN, ultrasound CL and PTB.[332, 459, 509-515] FFN is an extracellular matrix glycoprotein binding the gestational tissues at the feto-maternal interface. It is released after an abruption or cytokine-mediated damage to the membranes or placenta before delivery.[300] FFN is predictive of delivery within 2 weeks with a high negative predictive value and is a useful biomarker of PTB in women with symptoms of PTL.[301, 313] This was obvious in our data especially in the symptomatic group where the FFN concentration in the preterm-delivered women was more than 12 times higher compared to the term-delivered women. FFN was also predictive of preterm delivery in these women as well as the asymptomatic high risk women, but not in the low risk group.

Compared to FFN, the differences in CL observed in the cohorts in relation to delivery outcome was significantly less. However CL had a better discriminative utility for preterm delivery compared to FFN in the asymptomatic high risk women, whilst the reverse was observed in the symptomatic women. Hence, FFN level could be a better assessment tool for risk of preterm birth in women presenting with symptoms suggestive of PTL.[303] However, just like we have observed in our study, other researchers have reported high sensitivities, specificities, PPVs and NPVs for CL in the prediction of preterm birth in asymptomatic high risk and symptomatic pregnant women.[354, 459] A combined measurement of FFN and CL improved the prognostication of preterm birth.[459] Again, both FFN and CL were not predictive of preterm birth in the asymptomatic low risk women. Perhaps, these differences, correlations and predictive utility were not seen in the asymptomatic low risk women in this study because of the low number of preterm delivery in this group. The trend can be seen and at least other studies suggest that FFN is equally useful in this group.[516] It is just not cost-effective as a reasonable number of women are needed to identify one at risk of preterm birth. Also, FFN and CL could be useful in distinguishing between high and low risk women, and then applied to those women at significantly higher risk of PTB. This was obvious in our study population as the asymptomatic women sampled at mid second trimester (20-22w), were clearly differentiated in terms of their level of CVF FFN and ultrasound CL. The asymptomatic low risk women had over 200% lower FFN and about 18% increase in CL compared to their high risk counterparts. Though, in the low risk women FFN and ultrasound CL were not predictive of preterm birth in

this study, the prognostic potential of these two markers were observed in high risk women and those presenting with symptoms of threatened preterm labour. This is in accordance with the views that women with prior term deliveries tend to have lower incidence of short cervix,[398] and FFN levels and ultrasound CL measurements have proven capacity to identify women that are at significant risk for PTB.[304, 458, 459, 506, 517, 518] Although from our data it is obvious that in asymptomatic pregnant women at mid second trimester, ultrasound CL could be a more powerful biomarker in identifying women with higher risk of PTB compared with quantitative FFN. This is strengthened by the absence of significant improvement in the predictive ability of CL when FFN and metabolite markers were added.

Again, for the asymptomatic women studied at 26-28w, combination of the metabolites markers and FFN/CL did not seem to enhance the prediction of preterm birth significantly. FFN and CL remained the most powerful predictors of preterm birth in this group of women.

Because FFN and ultrasound CL are the most widely employed predictors of PTBs clinically, it was not surprising that there were modest inverse relationships between these 2 clinical assessment methods for PTB in all our study groups. Chorioamnionitis due to a perturbed vaginal microbiota signature and the associated ascending intrauterine infection can lead to cervical weakening (remodeling), disruption of fetal membranes and leakage of FFN.[199, 211] Therefore, an elevated CVF FFN level could be indicative of concomitant shortened cervix. Besides, fibronectin has been reported to stimulate the release of MMPs (e.g. MMP-2 and MMP-9) at least in human cancer and inflammatory cells via several signaling pathways.[519-523] MMPs degrade the collagen matrix of the cervix leading to cervical remodeling/shortening and PTB.[524-526]

The normal healthy vaginal pH in reproductive-age women ranges from 3.8-4.5, above which an imbalance in the vaginal microbial environment is suspected.[36, 449] Our data showed a pH range of 3.6-6.1 and no differences in relation to delivery outcome was observed within the groups (Table 2.1). Also, there was a modest relationship between vaginal pH and gestational age at delivery in all asymptomatic women studied at 20-22w irrespective of risk status, and the asymptomatic high-risk women studied at 26-28w. This correlation was not seen when the asymptomatic low and high-risk women were separated according to their risk statuses (Fig.

2.7A-D). Again, although this to an extent confirms the well documented relationship between vaginal pH and PTB,[509, 527-531] whether it can be conveniently applied singly in a low or undefined risk population warrants further investigation. Consistent with other reports,[509, 527] a modest inverse correlation between vaginal pH and ultrasound CL was observed but in the symptomatic women only. This relationship is noteworthy as an increase in vaginal pH could be suggestive of an altered vaginal microbiota and/or an ascending genital infection capable of triggering the release of MMPs via proinflammatory cytokine-mediated neutrophil and macrophage degranulation. MMPs catalyse the degradation of the cervical extracellular matrix and mucus plug leading to shortening, softening and dilation of the cervix and thus, promoting the establishment of an ascending genital infection and consequently PTL and PTB.[1, 114, 196 213]

In addition to the above observations, as gestation progressed and depending on the indication, a subset of our study participants in each group received a range of treatments aimed at reducing or preventing the risk of PTL and PTB as well as the attendant complications (Table 5.1). Nevertheless, some of these women still went on to deliver preterm. Though unethical and beyond the scope of this investigation, an ideal experimental situation would be allowing normal pregnancy progression without any therapeutic intervention. The effects of these treatment measures independently or as a whole on the vaginal microbiota and metabolite signatures at various gestational time points in asymptomatic and symptomatic high and low risk pregnant women is a subject for future investigation. In the meantime, we acknowledge that these measures may have influenced the eventual delivery outcome, although our study cohorts were recruited and sampled at presentation prior to the administration of any treatment. Despite the possible impact of these treatments on the ultimate delivery outcomes, the incidence of PTB in our study cohorts is comparable to that of the general population which ranges from 5-18% of all live births annually.[2, 3, 5, 304, 305, 532, 533] Albeit, evidence supporting routine transvaginal ultrasound screening of all pregnant women regardless of symptoms or a prior term delivery was meager,[395, 398] recent reports have recommended universal screening for both asymptomatic and symptomatic women with or without previous preterm birth, but was not indicated for those with multiple gestation.[506, 534, 535]

Chapter 6

General Discussion

It is conceivable that vaginal microorganisms and their biochemical activities are traceable from the metabolic by-products produced. There is an established association between the vaginal microbial composition and preterm birth.[160, 193, 536] By extension the vaginal microbiota metabolites, which are an indication of not just the presence but the functional activities of the bacterial species, should be associated with preterm birth. Also, adequate analyses of these metabolites can elucidate the microbiology of the female genital tract in pregnant and non-pregnant states.[34, 210] These observations, as well as the requirement of an accurate marker for the prognostication of preterm birth, formed the rationale for investigating CVF metabolites in different cohorts of pregnant women in relation to preterm birth. Specifically, this study was designed; firstly, to determine if the ^{1}H-MR spectrum of CVF can diagnose women who ultimately deliver prematurely from cohorts of women at high or low risk of preterm birth, and in pregnant women presenting with symptoms suggestive of threatened preterm labour. Secondly, this study aimed to characterise and verify the CVF metabolite signature of a cohort of pregnant women by ^{1}H-MRS and spectrophotometry, and determine their predictive utility for preterm birth. Thirdly, we examined the relationship between current clinical assessment methods for preterm birth such as CVF fetal fibronectin and ultrasound cervical length; and vaginal microbiota metabolites of pregnant women in relation to gestational age at delivery. Lastly, to identify the cellular composition of the CVF sample and determine the vaginal bacterial specie composition by PCR during pregnancy. Overall, these were performed in order to provide a comprehensive analysis of the cervicovaginal environment during gestation for the purpose of predicting the likelihood of preterm delivery.

A unique cohort of pregnant women comprising of asymptomatic pregnant women and women presenting with symptoms of threatened preterm labour were studied. The asymptomatic women were sub-classified into low and high risk depending on a prior preterm delivery as well as the presence of a short cervix. These women were studied at mid second trimester (20-22 weeks gestation), but the high risk women had a second study visit at late second trimester (26-28 weeks gestation), due to their high-risk status. The primary endpoint for these women was delivery before 37 weeks gestation (preterm), while the secondary endpoint was delivery before 32 weeks. The women who presented with symptoms reminiscent of threatened preterm labour (symptomatic women), were studied between 24 and 36 weeks gestation. The

predictive endpoint for these women was delivery within 2 weeks of index assessment while secondary endpoints were delivery before 32 and 37 weeks of gestation.

Our choice of this unique cohort was to take into consideration the controversy regarding the usefulness of the predictive potential of CVF metabolite markers obtained from a high risk population when applied in a population of low or undefined risk.[451] Also, it was hoped that potential CVF metabolite markers from this study independently or in conjunction with current clinical assessment makers (i.e. FFN and CL), could be applied effectively and accordingly depending on the risk and symptom statuses of the patients. To our mind, this is somewhat a holistic approach to the investigation of CVF metabolite markers of preterm birth.

Notably, our results revealed that in the asymptomatic women studied at 20-22 weeks gestation, acetate and BCAA normalised integrals measured by ^{1}H-MRS were predictive of preterm birth before 37 weeks gestation in the high and low risk groups respectively. In the symptomatic women, acetate normalised integrals and acetate/lactate ratio were predictive of preterm birth before 32 and 37 weeks gestation as well as within 2 weeks of index assessment. Also, the glutamine/glutamate normalised integral in these women was predictive of preterm birth before 32 weeks gestation and within 2 weeks of index assessment. Furthermore, just like acetate normalised integrals, CVF acetate concentration measured by spectrophotometric assay technique in the symptomatic women was predictive of preterm birth before 37 weeks gestation and within 2 weeks of index assessment, but not at 32 weeks gestation (possibly due to smaller sample population). The predictive abilities of the above metabolites in the different cohorts were comparable to those of FFN and CL, but not in the asymptomatic low risk women where both FFN and CL did not show significant predictive utility for preterm birth. Similar to both FFN and CL, the metabolites showed high sensitivities (49-100%), specificities (61-85%), NPVs (75-100%), but low PPVs (10-52%). No metabolite was predictive of preterm birth in the asymptomatic high risk women studied at 26-28 weeks gestation.

We also observed that in the symptomatic women, apart from being correlated, both acetate normalised integral and acetate concentration were higher in the women who delivered preterm than those that delivered at term. In addition, acetate normalised integral enhanced the predictive abilities of both FFN and CL for preterm birth before 37 weeks gestation.

Although vaginal pH did not differ between term- and preterm-delivered women, and was not predictive of preterm birth in any of the groups in this study, it was associated with acetate and lactate normalised integrals across the groups similar to other reports.[38] Increase in CVF acetate and decrease in lactate as seen during anaerobic bacterial colonisation due to diminished *Lactobacillus* dominance is associated with a corresponding increase in vaginal pH. This provides a favourable environment for mixed anaerobes to thrive leading to infection/inflammation and adverse reproductive outcomes.[38]

In an attempt to determine the cells responsible for the metabolites identified, we observed that the CVF samples comprised of both vaginal squamous epithelial cells and Gram positive and Gram negative bacterial cells. Generally, there was a higher prevalence of mixed anaerobes in preterm-delivered women compared to their term counterparts, whilst quantification of the bacterial 16S rDNA using qPCR revealed a higher amount of *M. curtisii* in the term-delivered women particularly in the asymptomatic high risk women studied at 20-22 weeks gestation, but no significant differences in *G. vaginalis* was observed in relation to delivery outcome. In contrast to this trend, the asymptomatic low risk women showed an unexpected higher prevalence of mixed anaerobes in the term-delivered women compared to their preterm counterparts. This is somewhat consistent with the qPCR-identified higher abundance of *M. curtisii* in the term-delivered asymptomatic high risk women. This warrants further investigation as our observations in the asymptomatic group, and especially the low risk women are inconsistent with the established relationship between the prevalence of mixed anaerobes including *M. curtisii* with preterm delivery.[75, 111, 118, 160, 469] However, there are evidences that *M. curtisii* is not sensitive for the diagnosis of vaginal dysbiosis such as BV, and is even more frequently isolated from healthy women.[500]

Taken together, the CVF metabolite profile, microbiota signature, vaginal pH, FFN, and CL can be linked in a common pathological pathway. Bacterial colonisation of the female genital tract during gestation and the subsequent ascending genital infection, which could be subclinical in some cases, triggers both maternal and fetal immune responses. This induces cytokine-mediated synthesis and release of prostaglandins and MMPs. The prostaglandins stimulate uterine smooth muscle contraction and cervical remodeling (softened, shortened and dilated cervix), and release of MMPs, whilst the MMPs triggers membrane rupture in addition to cervical ripening.

Ruptured membranes, compromised cervix and degraded cervical plug allow leakage of FFN into the vaginal compartment on one hand and ascension of more potentially virulent bacterial organisms into the upper genital tract on the other hand. An infection-triggered inflammatory positive feed-forward cycle is then established and if allowed uncontrolled could lead to preterm labour and ultimately preterm birth.[1, 7, 114, 214] Therefore, during pregnancy, the detection of CVF microbiota composition, microbiota metabolic by-products and/or the products of the inflammatory processes initiated by these microorganisms could be useful singly or in combination with other clinical diagnostic techniques for the prognostication of adverse reproductive outcomes.

To our knowledge this is the first report of CVF metabolic profiling in such a unique classification of pregnant women. It is also the first report of the prognostic utility of branched chain amino acids i.e. leucine, isoleucine and valine, in asymptomatic pregnant women at low risk of preterm birth against even the widely employed clinical assessment methods. In addition, an intriguing diagnostic capacity of CVF acetate for preterm birth less than 32 and 37 weeks of gestation and within 2 week of presentation in women presenting with symptoms suggestive of preterm labour, and less than 37 weeks only in asymptomatic women at high risk of preterm birth was observed. This further enhanced the predictive abilities of FFN and CL particularly in the symptomatic women. However, it should be noted that these observations were obtained from relatively smaller samples and require confirmation in larger study populations. These metabolites offer potentially cheaper, more accurate and less invasive clinical assays for the prediction of preterm deliveries that can be applied even in primary health care facilities in which other clinical assessment methods may be inadequate.

Future investigations will benefit from acknowledging the interrelations of vaginal microbiome, CVF metabolites profiles, pH, fetal fibronectin, ultrasound cervical length as well as pro- and anti-inflammatory cytokines in the prediction of preterm delivery.

6.1. Conclusion

In conclusion, we have reported that apart from FFN and CL, there are differences in CVF metabolite signatures in asymptomatic and symptomatic pregnant women that can be exploited

for the identification of women at risk and the prognostication of preterm delivery. Elevated CVF acetate showed clinically useful discriminative propensity for preterm delivery and delivery within 2 weeks of presentation in symptomatic women, and for only preterm delivery in asymptomatic high risk women. A ratio of acetate to lactate showed similar discriminatory capacity in symptomatic women, whilst branched chain amino acids appeared predictive of preterm delivery in asymptomatic women at low risk of preterm birth. These metabolite differences were supported with the identification of an association of higher prevalence of mixed anaerobes in the vaginal melieu and preterm birth.

6.2. Future work

Though the data presented in this report is promising, we have identified the limitations and prospective improvements:

Participants:

- Continuous recruitment of pregnant women especially women presenting with symptoms suggestive of preterm labour.
- Reclassification and analysis of CVF metabolites and microbiome data of currently available study cohorts.
- Recruitment of non-pregnant women at the proliferative and secretory phases of menstrual cycle attending the gynaecological clinics.
- Recruitment of pregnant women from other ethnic/racial inclination e.g. African and Hispanic women and determination of racial differences in CVF metabolite and microbiota signatures in relation to preterm birth.

Metabolite study:

- Identification of more metabolites from the ^{1}H-MR spectra and the possible use of MR spectrometer with stronger magnetic field strength e.g. 900 MHz (21T).
- Normalisation of identified ^{1}H-MR signals using the probabilistic quotient normalisation method in comparison to the integral normalisation approach.
- Analyses of CVF metabolite concentrations of both asymptomatic and symptomatic pregnant women using spectrophotometric technique e.g. the ChemWell® 2910 auto-analyser and determination of their predictive utility for preterm birth.
- Evaluation of the relationship between CVF total lactic acid, D-lactic acid, L-lactic acid, L/D-lactic acid ratio and preterm birth in our cohort.
- Investigation of the predictive capacity of branched chain amino acids in asymptomatic low risk women for preterm birth and in comparison to their high risk counterparts.
- Comparison and combination of the predictive abilities of metabolite concentrations and those of FFN and CL as well as pro- and anti-inflammatory cytokines associated with preterm birth.

Microbiome study:

- Molecular characterisation of CVF bacterial gene using high fidelity specie-specific primers and 16S gene sequencing.
- Application of multiplex PCR technique which involves the amplification of multiple different DNA simultaneously in the assessment of the vaginal microbiome in relation to delivery outcomes.
- Detection of fastidious and unculturable vaginal bacterial species by Fluorescence in situ hybridization (FISH). FISH can determine the localisation or distribution of specific bacterial species in the vaginal microbial ecology.
- Evaluation of the relationship between CVF total lactic acid, D-lactic acid, L-lactic acid, L/D-lactic acid ratio and *Lactobacillus sp.* dominance in relation to preterm birth.
- Determination of the relationship between branched chain amino acids and vaginal microbiome in asymptomatic low risk women.

7.0. References

1. Witkin S. The vaginal microbiome, vaginal anti-microbial defence mechanisms and the clinical challenge of reducing infection-related preterm birth. BJOG: an International Journal of Obstetrics and Gynaecology 2015;122(2):213-8.

2. Blencowe H, Cousens S, Chou D, Oestergaard M, Say L, Moller A-B, et al. Born too soon: the global epidemiology of 15 million preterm births. Reproductive Health 2013;10(Suppl 1):S2.

3. Mysorekar IU, Cao B. Microbiome in parturition and preterm birth. Seminars in reproductive medicine 2014;32(1):50-5.

4. MacDorman MF, Munson ML, Kirmeyer S. Fetal and perinatal mortality, United States, 2005. National vital statistics reports 2007;56(3).

5. Goldenberg RL, Culhane JF, Iams JD, Romero R. Preterm birth 1 - Epidemiology and causes of preterm birth. Lancet 2008;371(9606):75-84.

6. Romero R, Hassan SS, Gajer P, Tarca AL, Fadrosh DW, Bieda J, et al. The vaginal microbiota of pregnant women who subsequently have spontaneous preterm labor and delivery and those with a normal delivery at term. Microbiome 2014;2:18.

7. Romero R, Espinoza J, Kusanovic JP, Gotsch F, Hassan S, Erez O, et al. The preterm parturition syndrome. BJOG : an International Journal of Obstetrics and Gynaecology 2006;113 Suppl 3:17-42.

8. Racicot K, Cardenas I, Wünsche V, Aldo P, Guller S, Means RE, et al. Viral infection of the pregnant cervix predisposes to ascending bacterial infection. Journal of Immunology 2013;191(2):934-41.

9. DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, et al. Microbial Prevalence, Diversity and Abundance in Amniotic Fluid During Preterm Labor: A Molecular and Culture-Based Investigation. PloS ONE 2008;3(8).

10. Ma B, Forney LJ, Ravel J. The vaginal microbiome: rethinking health and diseases. Annual review of microbiology 2012;66:371.

11. Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R. Bacterial Community Variation in Human Body Habitats Across Space and Time. Science 2009;326(5960):1694-7.

12. Srinivasan S, Fredricks DN. The human vaginal bacterial biota and bacterial vaginosis. Interdisciplinary perspectives on infectious diseases 2009;2008.

13. Boris S, Barbes C. Role played by lactobacilli in controlling the population of vaginal pathogens. Microbes and Infection 2000;2(5):543-6.

14. Charlier C, Cretenet M, Even S, Le Loir Y. Interactions between Staphylococcus aureus and lactic acid bacteria: an old story with new perspectives. International Journal of Food Microbiology 2009;131(1):30-9.

15. Tlaskalova-Hogenova H, Stepankova R, Hudcovic T, Tuckova L, Cukrowska B, Lodinova-Zadnikova R, et al. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. Immunology Letters 2004;93(2-3):97-108.

16. Donders GG. Pathogenesis of abnormal vaginal bacterial flora. American Journal of Obstetrics and Gynecology 2000;182:872-8.

17. Gupta K. Inverse association of H2O2-producing lactobacilli and vaginal Escherichia coli colonization in women with recurrent urinary tract infections. Journal of Infectious Diseases 1998;178:446-50.

18. Sobel JD. Is there a protective role for vaginal flora? Current Infectious Disease Reports 1999;1:379-83.

19. Martin HL. Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. Journal of Infectious Diseases 1999;180:1863-8.

20. Lai SK. Human immunodeficiency virus type 1 is trapped by acidic but not by neutralized human cervicovaginal mucus. Journal of Virology 2009;83:11196-200.

21. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK, McCulle SL, et al. Vaginal microbiome of reproductive-age women. Proceedings of the National Academy of Sciences of the United States of America 2011;108:4680-7.

22. Weinstein L, Bogin M, Howard J, Finkelstone B. A survey of the vaginal flora at various ages with special reference to the Doderlein bacillus. American Journal Obstetrics and Gynecology 1936;32:211-8.

23. Cook RL, Sobel JD. Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. Review of Infectious Diseases 1990;12(5):856-72.

24. Smith BC, McAndrew T, Chen Z, Harari A, Barris DM, Viswanathan S, et al. The cervical microbiome over 7 years and a comparison of methodologies for its characterization. PloS ONE 2012;7(7):e40425.

25. Petrova MI, Lievens E, Malik S, Imholz N, Lebeer S. Lactobacillus species as biomarkers and agents that can promote various aspects of vaginal health. Frontiers in physiology 2015;6:81.

26. Hillier SL, Krohn MA, Rabe LK, Klebanoff SJ, Eschenbach DA. The Normal Vaginal Flora, H2o2-Producing Lactobacilli, and Bacterial Vaginosis in Pregnant-Women. Clinical Infectious Diseases 1993;16:S273-S81.

27. Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Delanghe J, Van Simaey L, et al. Cloning of 16S rRNA genes amplified from normal and disturbed vaginal microflora suggests a strong association between Atopobiurn vaginae, Gardnerella vaginalis and bacterial vaginosis. BMC microbiology 2004;4:16.

28. Wilks M, Wiggins R, Whiley A, Hennessy E, Warwick S, Porter H, et al. Identification and H_2O_2 production of vaginal lactobacilli from pregnant women at high risk of preterm birth and relation with outcome. Journal of clinical microbiology 2004;42(2):713-7.

29. Lamont RF, Sobel JD, Akins RA, Hassan SS, Chaiworapongsa T, Kusanovic JP, et al. The vaginal microbiome: new information about genital tract flora using molecular based techniques. BJOG: an International Journal of Obstetrics and Gynaecology 2011;118(5):533-49.

30. Paavonen J. Physiology and ecology of the vagina. Scandinavian Journal of Infectious Diseases Supplementum 1982;40:31-5.

31. Cruickshank R, Sharman A. The biology of the vagina in the human subject. BJOG: an International Journal of Obstetrics & Gynaecology 1934;41(2):208-26.

32. Boskey E, Cone R, Whaley K, Moench T. Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source. Human Reproduction 2001;16(9):1809-13.

33. Boskey ER, Telsch KM, Whaley KJ, Moench TR, Cone RA. Acid production by vaginal flora in vitro is consistent with the rate and extent of vaginal acidification. Infection and Immunity 1999;67(10):5170-5.

34. Mirmonsef P, Hotton AL, Gilbert D, Burgad D, Landay A, Weber KM, et al. Free glycogen in vaginal fluids is associated with Lactobacillus colonization and low vaginal pH. PLoS ONE 2014; 9(7): e102467.

35. Donders GGG. Definition and classification of abnormal vaginal flora. Best Practice & Research Clinical Obstetrics and Gynaecology 2007;21(3):355-73.

36. Danielsson D, Teigen PK, Moi H. The genital econiche: focus on microbiota and bacterial vaginosis. Annals of the New York Academy of Sciences 2011;1230(1):48-58.

37. O'Hanlon DE, Moench TR, Cone RA. Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota. PLoS ONE 2013;8(11):e80074.

38. Aldunate M, Srbinovski D, Hearps AC, Latham CF, Ramsland PA, Gugasyan R, et al. Antimicrobial and immune modulatory effects of lactic acid and short chain fatty acids produced by vaginal microbiota associated with eubiosis and bacterial vaginosis. Frontiers in physiology 2015;6:164.

39. Klebanoff SJ, Coombs RW. Viricidal effect of Lactobacillus acidophilus on human immunodeficiency virus type 1: possible role in heterosexual transmission. Journal of experimental medicine 1991;174(1):289-92.

40. Klebanoff SJ, Hillier SL, Eschenbach DA, Waltersdorph AM. Control of the microbial flora of the vagina by H_2O_2-generating Lactobacilli. Journal of Infectious Diseases 1991;164(1):94-100.

41. Zheng H-Y, Alcorn TM, Cohen MS. Effects Of H_2O_2-producing Lactobacilli on Neisseria gonorrhoeae Growth and Activity. Journal of Infectious Diseases 1994;170(5):1209-15.

42. Pascual LM, Daniele MB, Ruiz F, Giordano W, Pajaro C, Barberis L. Lactobacillus rhamnosus L60, a potential probiotic isolated from the human vagina. Journal of general and applied microbiology 2008;54(3):141-8.

43. Kaur B, Balgir PP, Mittu B, Kumar B, Garg N. Biomedical applications of fermenticin HV6b isolated from Lactobacillus fermentum HV6b MTCC10770. BioMed research international 2013;2013:168438.

44. Donia MS, Cimermancic P, Schulze CJ, Wieland Brown LC, Martin J, Mitreva M, et al. A systematic analysis of biosynthetic gene clusters in the human microbiome reveals a common family of antibiotics. Cell 2014;158(6):1402-14.

45. Witkin SS, Linhares IM, Giraldo P. Bacterial flora of the female genital tract: function and immune regulation. Best Practice & Research in Clinical Obstetrics and Gynaecology 2007;21(3):347-54.

46. Mossop H, Linhares IM, Bongiovanni AM, Ledger WJ, Witkin SS. Influence of lactic acid on endogenous and viral RNA-induced immune mediator production by vaginal epithelial cells. Obstetrics and Gynecology 2011;118(4):840-6.

47. Boris S, Barbes C. Role played by lactobacilli in controlling the population of vaginal pathogens. Microbes and Infection 2000;2(5):543-6.

48. Al-Mushrif S, Jones BM. A study of the prevalence of hydrogen peroxide generating Lactobacilli in bacterial vaginosis: the determination of H_2O_2 concentrations generated, in

vitro , by isolated strains and the levels found in vaginal secretions of women with and without infection. Journal of obstetrics and gynaecology : the journal of the Institute of Obstetrics and Gynaecology 1998;18(1):63-7.

49. Onderdonk AB, Lee ML, Lieberman E, Delaney ML, Tuomala RE. Quantitative microbiologic models for preterm delivery. Journal of clinical microbiology 2003;41(3):1073-9.

50. Boris S, Suárez JE, Vázquez F, Barbés C. Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infection Immunity 1998;66(5):1985-9.

51. Ojala T, Kankainen M, Castro J, Cerca N, Edelman S, Westerlund-Wikstrom B, et al. Comparative genomics of Lactobacillus crispatus suggests novel mechanisms for the competitive exclusion of Gardnerella vaginalis. BMC genomics 2014;15:1070.

52. Lebeer S, Vanderleyden J, De Keersmaecker SC. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nature reviews Microbiology 2010;8(3):171-84.

53. Thoma ME, Gray RH, Kiwanuka N, Aluma S, Wang M-C, Sewankambo N, et al. Longitudinal changes in vaginal microbiota composition assessed by Gram stain among never sexually active pre-and postmenarcheal adolescents in Rakai, Uganda. Journal of pediatric and adolescent gynecology 2011;24(1):42-7.

54. Cadieux P, Burton J, Devillard E, Reid G. Lactobacillus by-products inhibit the growth and virulence of uropathogenic Escherichia coli. Journal of Physioliogy and Pharmacology 2009;60(Suppl 6):13-8.

55. Antonio MA, Hawes SE, Hillier SL. The identification of vaginal Lactobacillus species and the demographic and microbiologic characteristics of women colonized by these species. Journal of infectious diseases 1999;180(6):1950-6.

56. Zhou X, Bent SJ, Schneider MG, Davis CC, Islam MR, Forney LJ. Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. Microbiology-Sgm 2004;150:2565-73.

57. Rodriguez Jovita M, Collins MD, Sjoden B, Falsen E. Characterization of a novel Atopobium isolate from the human vagina: description of Atopobium vaginae sp. nov. International Journal of Systematic Bacteriology 1999;49 Pt 4:1573-6.

58. Zhou X, Brown CJ, Abdo Z, Davis CC, Hansmann MA, Joyce P, et al. Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. ISME journal 2007;1(2):121-33.

59. Zhou X, Hansmann MA, Davis CC, Suzuki H, Brown CJ, Schütte U, et al. The vaginal bacterial communities of Japanese women resemble those of women in other racial groups. FEMS Immunology and Medical Microbiology 2010;58(2):169-81.

60. Stevens-Simon C, Jamison J, McGregor JA, Douglas JM. Racial variation in vaginal pH among healthy sexually active adolescents. Sexually Transmitted Diseases 1994;21(3):168-72.

61. Royce RA, Jackson TP, Thorp Jr JM, Hillier SL, Rabe LK, Pastore LM, et al. Race/ethnicity, vaginal flora patterns, and pH during pregnancy. Sexually Transmitted Diseases 1999;26(2):96-102.

62. Fiscella K, Klebanoff MA. Are racial differences in vaginal pH explained by vaginal flora? American Journal of Obstetrics and Gynecology 2004;191(3):747-50.

63. Schwebke JR. New concepts in the etiology of bacterial vaginosis. Current infectious disease reports 2009;11(2):143-7.

64. Srinivasan S, Liu C, Mitchell CM, Fiedler TL, Thomas KK, Agnew KJ, et al. Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. PloS ONE 2010;5(4):e10197-e.

65. Gajer P, Brotman RM, Bai GY, Sakamoto J, Schuette UME, Zhong X, et al. Temporal Dynamics of the Human Vaginal Microbiota. Science Translational Medicine 2012;4(132).

66. Ravel J, Brotman RM, Gajer P, Ma B, Nandy M, Fadrosh DW, et al. Daily temporal dynamics of vaginal microbiota before, during and after episodes of bacterial vaginosis. Microbiome 2013;1(1):29.

67. Brotman RM, Shardell MD, Gajer P, Fadrosh D, Chang K, Silver MI, et al. Association between the vaginal microbiota, menopause status, and signs of vulvovaginal atrophy. Menopause (New York, NY) 2014;21(5):450-8.

68. Brotman RM, Ravel J, Cone RA, Zenilman JM. Rapid fluctuation of the vaginal microbiota measured by Gram stain analysis. Sexually Transmitted Infections 2010;86(4):297-302.

69. Donders G. Diagnosis and management of bacterial vaginosis and other types of abnormal vaginal bacterial flora: a review. Obstetrical & gynecological survey 2010;65(7):462-73.

70. Farage M, Maibach H. Lifetime changes in the vulva and vagina. Archives of gynecology and obstetrics 2006;273(4):195-202.

71. Spear GT, French AL, Gilbert D, Zariffard MR, Mirmonsef P, Sullivan TH, et al. Human alpha-amylase present in lower-genital-tract mucosal fluid processes glycogen to support vaginal colonization by Lactobacillus. Journal of infectious diseases 2014;210(7):1019-28.

72. Gupta S, Kumar N, Singhal N, Kaur R, Manektala U. Vaginal microflora in postmenopausal women on hormone replacement therapy. Indian journal of pathology & microbiology 2006;49(3):457-61.

73. Cauci S, Driussi S, De Santo D, Penacchioni P, Iannicelli T, Lanzafame P, et al. Prevalence of bacterial vaginosis and vaginal flora changes in peri- and postmenopausal women. Journal of clinical microbiology 2002;40(6):2147-52.

74. Romero R, Hassan SS, Gajer P, Tarca AL, Fadrosh DW, Nikita L, et al. The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. Microbiome 2014;2(1):4.

75. DiGiulio DB, Callahan BJ, McMurdie PJ, Costello EK, Lyell DJ, Robaczewska A, et al. Temporal and spatial variation of the human microbiota during pregnancy. Proceedings of the National Academy of Sciences 2015:201502875.

76. Leppälluoto PA. Bacterial vaginosis: what is physiological in vaginal bacteriology? An update and opinion. Acta obstetricia et gynecologica Scandinavica 2011;90(12):1302-6.

77. dos Santos Santiago GL, Tency I, Verstraelen H, Verhelst R, Trog M, Temmerman M, et al. Longitudinal qPCR study of the dynamics of L. crispatus, L. iners, A. vaginae,(sialidase positive) G. vaginalis, and P. bivia in the vagina. PLoS ONE 2012;7(9): e45281.

78. Chaban B, Links MG, Jayaprakash TP, Wagner EC, Bourque DK, Lohn Z, et al. Characterization of the vaginal microbiota of healthy Canadian women through the menstrual cycle. Microbiome 2014;2(1):23.

79. Wilson J. Managing recurrent bacterial vaginosis. Sexually Transmitted Infections 2004;80(1):8-11.

80. Cauci S, McGregor J, Thorsen P, Grove J, Guaschino S. Combination of vaginal pH with vaginal sialidase and prolidase activities for prediction of low birth weight and preterm birth. American Journal of Obstetrics and Gynecology 2005;192(2):489-96.

81. Hillier SL, Nugent RP, Eschenbach DA, Krohn MA, Gibbs RS, Martin DH, et al. Association between bacterial vaginosis and preterm delivery of a low-birth-weight infant. The Vaginal Infections and Prematurity Study Group. New England journal of medicine 1995;333(26):1737-42.

82. Foxman B, Wen A, Srinivasan U, Goldberg D, Marrs CF, Owen J, et al. Mycoplasma, bacterial vaginosis–associated bacteria BVAB3, race, and risk of preterm birth in a high-risk cohort. American Journal of Obstetrics and Gynecology 2014;210(3):226. e1-. e7.

83. Deb K, Chaturvedi MM, Jaiswal YK. Comprehending the role of LPS in Gram-negative bacterial vaginosis: ogling into the causes of unfulfilled child-wish. Archives of gynecology and obstetrics 2004;270(3):133-46.

84. Srinivasan S, Morgan MT, Fiedler TL, Djukovic D, Hoffman NG, Raftery D, et al. Metabolic Signatures of Bacterial Vaginosis. mBio 2015;6(2):e00204-15.

85. McMillan A, Rulisa S, Sumarah M, Macklaim JM, Renaud J, Bisanz J, et al. A multi-platform metabolomics approach identifies novel biomarkers associated with bacterial diversity in the human vagina. arXiv preprint arXiv:150402816 2015.

86. Tolosa JE, Chaithongwongwatthana S, Daly S, Maw WW, Gaitan H, Lumbiganon P, et al. The International Infections in Pregnancy (IIP) study: variations in the prevalence of bacterial vaginosis and distribution of morphotypes in vaginal smears among pregnant women. American Journal of Obstetrics and Gynecology 2006;195(5):1198-204.

87. Kenyon C, Colebunders R, Crucitti T. The global epidemiology of bacterial vaginosis: a systematic review. American Journal of Obstetrics and Gynecology 2013;209(6):505-23.

88. Guaschino S, De Seta F, Piccoli M, Maso G, Alberico S. Aetiology of preterm labour: bacterial vaginosis. BJOG: An International Journal of Obstetrics and Gynaecology 2006;113(s3):46-51.

89. Pararas MV, Skevaki CL, Kafetzis DA. Preterm birth due to maternal infection: causative pathogens and modes of prevention. European Journal of Clinical Microbiology 2006;25(9):562-9.

90. Lamont RF. Infection in the prediction and antibiotics in the prevention of spontaneous preterm labour and preterm birth. BJOG: an International Journal of Obstetrics and Gynaecology 2003;110(s20):71-5.

91. Klebanoff S, Hillier S, Eschenbach D, Waltersdorph A. Control of the microbial flora of the vagina by H202-generating lactobacilli. Journal of Infectious Diseases 1991;164(1):94-100.

92. Hay P. Recurrent bacterial vaginosis. Current opinion in infectious diseases 2009;22(1):82-6.

93. Donders GG. Definition and classification of abnormal vaginal flora. Best Practice and Research Clinical Obstetrics and Gynaecology 2007;21(3):355-73.

94. Donati L, Di Vico A, Nucci M, Quagliozzi L, Spagnuolo T, Labianca A, et al. Vaginal microbial flora and outcome of pregnancy. Archives of Gynecology and Obstetrics 2010;281(4):589-600.

95. Shipitsyna E, Roos A, Datcu R, Hallen A, Fredlund H, Jensen JS, et al. Composition of the vaginal microbiota in women of reproductive age--sensitive and specific molecular diagnosis of bacterial vaginosis is possible? PloS ONE 2013;8(4):e60670.

96. Manhart LE, Khosropour CM, Liu C, Gillespie CW, Depner K, Fiedler T, et al. Bacterial Vaginosis–Associated Bacteria in Men: Association of Leptotrichia/Sneathia spp. With Nongonococcal Urethritis. Sexually Transmitted Diseases 2013;40(12):944.

97. Srinivasan S, Hoffman NG, Morgan MT, Matsen FA, Fiedler TL, Hall RW, et al. Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. PloS ONE 2012;7(6):e37818.

98. Yeoman CJ, Thomas SM, Miller M, Ulanov AV, Torralba M, Lucas S, et al. A multi-omic systems-based approach reveals metabolic markers of bacterial vaginosis and insight into the disease. PloS ONE 2013;8(2):e56111.

99. Macklaim JM, Fernandes AD, Di Bella JM, Hammond J-A, Reid G, Gloor GB. Comparative meta-RNA-seq of the vaginal microbiota and differential expression by Lactobacillus iners in health and dysbiosis. Microbiome 2013;1(1):12.

100. Al-Mushrif S, Eley A, Jones B. Inhibition of chemotaxis by organic acids from anaerobes may prevent a purulent response in bacterial vaginosis. Journal of Medical Microbiology 2000;49(11):1023-30.

101. Hill GB, Eschenbach DA, Holmes KK. Bacteriology of the Vagina. Scandinavian Journal of Urology and Nephrology. Supplementum 1985:23-39.

102. Holst E, Wathne B, Hovelius B, Mardh PA. Bacterial Vaginosis - Microbiological and Clinical Findings. European Journal of Clinical Microbiology 1987;6(5):536-41.

103. Pybus V, Onderdonk AB. A commensal symbiosis between Prevotella bivia and Peptostreptococcus anaerobius involves amino acids: potential significance to the pathogenesis of bacterial vaginosis. FEMS immunology and medical microbiology 1998;22(4):317-27.

104. Pybus V, Onderdonk AB. Microbial interactions in the vaginal ecosystem, with emphasis on the pathogenesis of bacterial vaginosis. Microbes and infection / Institut Pasteur 1999;1(4):285-92.

105. Datcu R, Gesink D, Mulvad G, Montgomery-Andersen R, Rink E, Koch A, et al. Vaginal microbiome in women from Greenland assessed by microscopy and quantitative PCR. BMC infectious diseases 2013;13(1):480.

106. Schwebke JR, Muzny CA, Josey WE. Role of Gadnerella vaginalis in the Pathogenesis of Bacterial Vaginosis–A Conceptual Model. Journal of Infectious Diseases 2014:jiu089.

107. Muzny C, Schwebke J. Gardnerella vaginalis: still a prime suspect in the pathogenesis of bacterial vaginosis. Current infectious disease reports 2013;15(2):130-5.

108. Machado A, Jefferson KK, Cerca N. Interactions between Lactobacillus crispatus and bacterial vaginosis (BV)-associated bacterial species in initial attachment and biofilm formation. International journal of molecular sciences 2013;14(6):12004-12.

109. Patterson JL, Stull-Lane A, Girerd PH, Jefferson KK. Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of Gardnerella vaginalis relative to other bacterial-vaginosis-associated anaerobes. Microbiology 2010;156(2):392-9.

110. Tamrakar R, Yamada T, Furuta I, Cho K, Morikawa M, Yamada H, et al. Association between Lactobacillus species and bacterial vaginosis-related bacteria, and bacterial vaginosis scores in pregnant Japanese women. BMC infectious diseases 2007;7:128.

111. Verstraelen H, Verhelst R, Claeys G, De Backer E, Temmerman M, Vaneechoutte M. Longitudinal analysis of the vaginal microflora in pregnancy suggests that L. crispatus promotes the stability of the normal vaginal microflora and that L. gasseri and/or L. iners are more conducive to the occurrence of abnormal vaginal microflora. BMC microbiology 2009;9:116.

112. Wertz J, Isaacs-Cosgrove N, Holzman C, Marsh TL. Temporal Shifts in Microbial Communities in Nonpregnant African-American Women with and without Bacterial Vaginosis. Interdisciplinary perspectives on infectious diseases 2008;2008:181253.

113. Hillier SL, Krohn MA, Klebanoff SJ, Eschenbach DA. The relationship of hydrogen peroxide-producing lactobacilli to bacterial vaginosis and genital microflora in pregnant women. Obstetrics & Gynecology 1992;79(3):369-73.

114. Witkin SS, Mendes-Soares H, Linhares IM, Jayaram A, Ledger WJ, Forney LJ. Influence of vaginal bacteria and D-and L-lactic acid isomers on vaginal extracellular matrix metalloproteinase inducer: implications for protection against upper genital tract infections. mBio 2013;4(4):e00460-13.

115. Macklaim JM, Gloor GB, Anukam KC, Cribby S, Reid G. At the crossroads of vaginal health and disease, the genome sequence of Lactobacillus iners AB-1. Proceedings of the National Academy of Sciences 2011;108(Supplement 1):4688-95.

116. Santiago GL, Tency I, Verstraelen H, Verhelst R, Trog M, Temmerman M, et al. Longitudinal qPCR study of the dynamics of L. crispatus, L. iners, A. vaginae, (sialidase positive) G. vaginalis, and P. bivia in the vagina. PloS ONE 2012;7(9):e45281.

117. Jakobsson T, Forsum U. Lactobacillus iners: a marker of changes in the vaginal flora? Journal of clinical microbiology 2007;45(9):3145-.

118. Jespers V, Menten J, Smet H, Poradosu S, Abdellati S, Verhelst R, et al. Quantification of bacterial species of the vaginal microbiome in different groups of women, using nucleic acid amplification tests. BMC microbiology 2012;12:83.

119. Verstraelen H, Verhelst R, Vaneechoutte M, Temmerman M. The epidemiology of bacterial vaginosis in relation to sexual behaviour. BMC infectious diseases 2010;10:81.

120. Nilsson U, Hellberg D, Shoubnikova M, Nilsson S, Mårdh P-A. Sexual Behavior Risk Factors Associated With Bacterial Vaginosis andChlamydia trachomatisInfection. Sexually Transmitted Diseases 1997;24(5):241-6.

121. Allsworth JE, Peipert JF. Prevalence of bacterial vaginosis: 2001–2004 national health and nutrition examination survey data. Obstetrics and Gynecology 2007;109(1):114-20.

122. Koumans EH, Sternberg M, Bruce C, McQuillan G, Kendrick J, Sutton M, et al. The prevalence of bacterial vaginosis in the United States, 2001–2004; associations with symptoms, sexual behaviors, and reproductive health. Sexually Transmitted Diseases 2007;34(11):864-9.

123. Potter J. Should sexual partners of women with bacterial vaginosis receive treatment? British journal of general practice 1999;49(448):913-8.

124. Bailey JV, Farquhar C, Owen C. Bacterial vaginosis in lesbians and bisexual women. Sexually Transmitted Diseases 2004;31(11):691-4.

125. Swidsinski A, Doerffel Y, Loening-Baucke V, Swidsinski S, Verstraelen H, Vaneechoutte M, et al. Gardnerella biofilm involves females and males and is transmitted sexually. Gynecologic and obstetric investigation 2010;70(4):256-63.

126. Swidsinski A, Dörffel Y, Loening-Baucke V, Mendling W, Verstraelen H, Dieterle S, et al. Desquamated epithelial cells covered with a polymicrobial biofilm typical for bacterial vaginosis are present in randomly selected cryopreserved donor semen. FEMS Immunology and Medical Microbiology 2010;59(3):399-404.

127. Fethers KA, Fairley CK, Hocking JS, Gurrin LC, Bradshaw CS. Sexual risk factors and bacterial vaginosis: a systematic review and meta-analysis. Clinical Infectious Diseases 2008;47(11):1426-35.

128. Bradshaw CS, Morton AN, Hocking J, Garland SM, Morris MB, Moss LM, et al. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. Journal of Infectious Diseases 2006;193(11):1478-86.

129. Cherpes TL, Hillier SL, Meyn LA, Busch JL, Krohn MA. A delicate balance: risk factors for acquisition of bacterial vaginosis include sexual activity, absence of hydrogen peroxide-

producing lactobacilli, black race, and positive herpes simplex virus type 2 serology. Sexually Transmitted Diseases 2008;35(1):78-83.

130. Ness RB, Hillier S, Richter HE, Soper DE, Stamm C, Bass DC, et al. Can known risk factors explain racial differences in the occurrence of bacterial vaginosis? Journal of the National Medical Association 2003;95(3):201.

131. Hellberg D, Nilsson S, Mårdh P-A. Bacterial vaginosis and smoking. International journal of STD & AIDS 2000;11(9):603-6.

132. Luong ML, Libman M, Dahhou M, Chen MF, Kahn SR, Goulet L, et al. Vaginal douching, bacterial vaginosis, and spontaneous preterm birth. Journal of obstetrics and gynaecology Canada 2010;32(4):313-20.

133. Morison L, Ekpo G, West B, Demba E, Mayaud P, Coleman R, et al. Bacterial vaginosis in relation to menstrual cycle, menstrual protection method, and sexual intercourse in rural Gambian women. Sexually Transmitted Infections 2005;81(3):242-7.

134. Eschenbach DA, Thwin SS, Patton DL, Hooton TM, Stapleton AE, Agnew K, et al. Influence of the normal menstrual cycle on vaginal tissue, discharge, and microflora. Clinical Infectious Diseases 2000;30(6):901-7.

135. Smart S, Singal A, Mindel A. Social and sexual risk factors for bacterial vaginosis. Sexually Transmitted Infections 2004;80(1):58-62.

136. Marrazzo JM, Thomas KK, Agnew K, Ringwood K. Prevalence and risks for bacterial vaginosis in women who have sex with women. Sexually Transmitted Diseases 2010;37(5):335.

137. McDonald HM, Brocklehurst P, Gordon A. Antibiotics for treating bacterial vaginosis in pregnancy. Cochrane database of systematic reviews 2007;1:CD000262.

138. Nelson DB, Bellamy S, Clothier BA, Macones GA, Nachamkin I, Ruffin A, et al. Characteristics and pregnancy outcomes of pregnant women asymptomatic for bacterial vaginosis. Maternal and child health journal 2008;12(2):216-22.

139. Robinson JR, Bologna WJ. Topically applying a composition comprising water and a water-swellable, water-insoluble, crosslinked, ph buffering, bioadhesive, carboxy-containing addition polymer to acidify; microbiocides; deodorizing. Google Patents; 2000.

140. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 2006;124(4):837-48.

141. Kelly D, Conway S, Aminov R. Commensal gut bacteria: mechanisms of immune modulation. Trends in immunology 2005;26(6):326-33.

142. Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nature Reviews Immunology 2008;8(6):411-20.

143. Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proceedings of the National Academy of Sciences 2010;107(27):12204-9.

144. Mirmonsef P, Gilbert D, Zariffard MR, Hamaker BR, Kaur A, Landay AL, et al. The effects of commensal bacteria on innate immune responses in the female genital tract. American Journal of Reproductive Immunology 2011;65(3):190-5.

145. Schwebke JR. Role of vaginal flora as a barrier to HIV acquisition. Current infectious disease reports 2001;3(2):152-5.

146. Mitchell C, Marrazzo J. Bacterial vaginosis and the cervicovaginal immune response. American Journal of Reproductive Immunology 2014;71(6):555-63.

147. Atashili J, Poole C, Ndumbe PM, Adimora AA, Smith JS. Bacterial vaginosis and HIV acquisition: a meta-analysis of published studies. AIDS (London, England) 2008;22(12):1493.

148. Cherpes TL, Meyn LA, Krohn MA, Lurie JG, Hillier SL. Association between acquisition of herpes simplex virus type 2 in women and bacterial vaginosis. Clinical Infectious Diseases 2003;37(3):319-25.

149. Taylor BD, Darville T, Haggerty CL. Does bacterial vaginosis cause pelvic inflammatory disease? Sexually Transmitted Diseases 2013;40(2):117-22.

150. Soper DE. Pelvic inflammatory disease. Obstetrics & Gynecology 2010;116(2, Part 1):419-28.

151. Ness RB, Kip KE, Hillier SL, Soper DE, Stamm CA, Sweet RL, et al. A cluster analysis of bacterial vaginosis-associated microflora and pelvic inflammatory disease. American Journal of Epidemiology 2005;162(6):585-90.

152. Haggerty CL, Hillier SL, Bass DC, Ness RB. Bacterial vaginosis and anaerobic bacteria are associated with endometritis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 2004;39(7):990-5.

153. Capoccia R, Greub G, Baud D. Ureaplasma urealyticum, Mycoplasma hominis and adverse pregnancy outcomes. Current opinion in infectious diseases 2013;26(3):231-40.

154. Han YW, Shen T, Chung P, Buhimschi IA, Buhimschi CS. Uncultivated bacteria as etiologic agents of intra-amniotic inflammation leading to preterm birth. Journal of clinical microbiology 2009;47(1):38-47.

155. Rampersaud R, Randis TM, Ratner AJ. Microbiota of the upper and lower genital tract. Seminars in Fetal and Neonatal Medicine: Elsevier; 2012.

156. Combs CA, Gravett M, Garite TJ, Hickok DE, Lapidus J, Porreco R, et al. Amniotic fluid infection, inflammation, and colonization in preterm labor with intact membranes. American Journal of Obstetrics and Gynecology 2014;210(2):125 e1- e15.

157. Gravett MG, Nelson HP, Derouen T, Critchlow C, Eschenbach DA, Holmes KK. Independent associations of Bacterial vaginosis and Chlamydia trachomatis infection with adverse pregnancy outcome. JAMA: Journal of the American Medical Association 1986;256(14):1899-903.

158. Hay PE, Lamont RF, Taylorrobinson D, Morgan DJ, Ison C, Pearson J. Abnormal bacterial colonization of the genital tract and subsequent preterm delivery and late miscarriage. British Medical Journal 1994;308(6924):295-8.

159. Epstein FH, Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. New England Journal of Medicine 2000;342(20):1500-7.

160. Donders GG, Van Calsteren K, Bellen G, Reybrouck R, Van den Bosch T, Riphagen I, et al. Predictive value for preterm birth of abnormal vaginal flora, bacterial vaginosis and aerobic vaginitis during the first trimester of pregnancy. BJOG: an International Journal of Obstetrics and Gynaecology 2009;116(10):1315-24.

161. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. lancet 2008;371(9606):75-84.

162. Hendler I, Andrews WW, Carey CJ, Klebanoff MA, Noble WD, Sibai BM, et al. The relationship between resolution of asymptomatic bacterial vaginosis and spontaneous preterm birth in fetal fibronectin–positive women. American Journal of Obstetrics and Gynecology 2007;197(5):488. e1-. e5.

163. Leitich H, Kiss H. Asymptomatic bacterial vaginosis and intermediate flora as risk factors for adverse pregnancy outcome. Best Practice and Research in Clinical Obstetrics and Gynaecology 2007;21(3):375-90.

164. Boggess KA. Pathophysiology of preterm birth: emerging concepts of maternal infection. Clinics in perinatology 2005;32(3):561-9.

165. Romero R, Chaiworapongsa T, Kuivaniemi H, Tromp G. Bacterial vaginosis, the inflammatory response and the risk of preterm birth: a role for genetic epidemiology in the prevention of preterm birth. American Journal of Obstetrics and Gynecology 2004;190(6):1509-19.

166. Macones GA, Parry S, Elkousy M, Clothier B, Ural SH, Strauss Iii JF. A polymorphism in the promoter region of TNF and bacterial vaginosis: preliminary evidence of gene-environment interaction in the etiology of spontaneous preterm birth. American Journal of Obstetrics and Gynecology 2004;190(6):1504-8.

167. Gómez LM, Sammel MD, Appleby DH, Elovitz MA, Baldwin DA, Jeffcoat MK, et al. Evidence of a gene-environment interaction that predisposes to spontaneous preterm

birth: a role for asymptomatic bacterial vaginosis and DNA variants in genes that control the inflammatory response. American Journal of Obstetrics and Gynecology 2010;202(4):386.e1-.e6.

168. Srinivasan U, Misra D, Marazita ML, Foxman B. Vaginal and oral microbes, host genotype and preterm birth. Medical hypotheses 2009;73(6):963-75.

169. Ryckman KK, Simhan HN, Krohn MA, Williams SM. Cervical cytokine network patterns during pregnancy: the role of bacterial vaginosis and geographic ancestry. Journal of reproductive immunology 2009;79(2):174-82.

170. Simhan HN, Caritis SN, Krohn MA, de Tejada BM, Landers DV, Hillier SL. Decreased cervical proinflammatory cytokines permit subsequent upper genital tract infection during pregnancy. American Journal of Obstetrics and Gynecology 2003;189(2):560-7.

171. Manns-James L. Bacterial vaginosis and preterm birth. Journal of Midwifery & Women's Health 2011;56(6):575-83.

172. Amsel R, Totten PA, Spiegel CA, Chen KCS, Eschenbach D, Holmes KK. Nonspecific vaginitis - diagnostic criteria and microbial and epidemiologic associations. American Journal of Medicine 1983;74(1):14-22.

173. Nugent RP, Krohn MA, Hillier S. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. Journal of clinical microbiology 1991;29(2):297-301.

174. Myziuk L, Romanowski B, Johnson SC. BVBlue test for diagnosis of bacterial vaginosis. Journal of clinical microbiology 2003;41(5):1925-8.

175. Wang J. Bacterial vaginosis. Primary Care Update for Obstetricians and Gynecologists 2000;7(5):181-5.

176. Thomason J, Gelbart S, Wilcoski L, Peterson A, Jilly B, Hamilton P. Proline aminopeptidase activity as a rapid diagnostic test to confirm bacterial vaginosis. Obstetrics & Gynecology 1988;71(4):607-11.

177. Mirmonsef P, Gilbert D, Veazey RS, Wang J, Kendrick SR, Spear GT. A comparison of lower genital tract glycogen and lactic acid levels in women and macaques: implications for HIV and SIV susceptibility. AIDS research and human retroviruses 2012;28(1):76-81.

178. Graham LS, Krass L, Zariffard MR, Spear GT, Mirmonsef P. Effects of Succinic Acid and Other Microbial Fermentation Products on HIV Expression in Macrophages. BioResearch open access 2013;2(5):385-91.

179. Cook RL, Redondo-Lopez V, Schmitt C, Meriwether C, Sobel JD. Clinical, microbiological, and biochemical factors in recurrent bacterial vaginosis. Journal of clinical microbiology 1992;30(4):870-7.

180. Piot P, Van Dyck E. Isolation and identification of Gardnerella vaginalis. Scandinavian journal of infectious diseases Supplementum 1983;40:15.

181. Ison C, Easmon C, Dawson S, Southerton G, Harris J. Non-volatile fatty acids in the diagnosis of non-specific vaginitis. Journal of clinical pathology 1983;36(12):1367-70.

182. Bump RC, Buesching WJ. Bacterial vaginosis in virginal and sexually active adolescent females: evidence against exclusive sexual transmission. American Journal of Obstetrics and Gynecology 1988;158(4):935-9.

183. Hauth JC, Goldenberg RL, Andrews WW, DuBard MB, Copper RL. Reduced incidence of preterm delivery with metronidazole and erythromycin in women with bacterial vaginosis. New England Journal of Medicine 1995;333(26):1732-6.

184. Anukam K, Osazuwa E, Ahonkhai I, Ngwu M, Osemene G, Bruce AW, et al. Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14: randomized, double-blind, placebo controlled trial. Microbes and Infection 2006;8(6):1450-4.

185. Anukam KC, Osazuwa E, Osemene GI, Ehigiagbe F, Bruce AW, Reid G. Clinical study comparing probiotic Lactobacillus GR-1 and RC-14 with metronidazole vaginal gel to treat symptomatic bacterial vaginosis. Microbes and Infection 2006;8(12):2772-6.

186. Senok AC, Verstraelen H, Temmerman M, Botta GA. Probiotics for the treatment of bacterial vaginosis. Cochrane Database of Systematic Reviews 2009;4:CD006289.

187. Borges S, Silva J, Teixeira P. The role of lactobacilli and probiotics in maintaining vaginal health. Archives of gynecology and obstetrics 2014;289(3):479-89.

188. Brocklehurst P, Gordon A, Heatley E, Milan SJ. Antibiotics for treating bacterial vaginosis in pregnancy. Cochrane Database of Systematic Reviews 2013; 1:CD000262.

189. Laghi L, Picone G, Cruciani F, Brigidi P, Calanni F, Donders G, et al. Rifaximin modulates the vaginal microbiome and metabolome in women affected by bacterial vaginosis. Antimicrobial agents and chemotherapy 2014;58(6):3411-20.

190. Donders GGG, Vereecken A, Bosmans E, Dekeersmaecker A, Salembier G, Spitz B. Definition of a type of abnormal vaginal flora that is distinct from bacterial vaginosis: aerobic vaginitis. BJOG: an International Journal of Obstetrics and Gynaecology 2002;109 (1):34-43.

191. Han C, Wu W, Fan A, Wang Y, Zhang H, Chu Z, et al. Diagnostic and therapeutic advancements for aerobic vaginitis. Archives of gynecology and obstetrics 2015;291(2):251-7.

192. Vieira-Baptista P, Lima-Silva J, Pinto C, Saldanha C, Beires J, Martinez-de-Oliveira J, et al. Bacterial vaginosis, aerobic vaginitis, vaginal inflammation and major Pap smear abnormalities. European Journal of Clinical Microbiology 2016;35(4):657-64.

193. Donders GG, Van Calsteren C, Bellen G, Reybrouck R, Van den Bosch T, Riphagen I, et al. Association between abnormal vaginal flora and cervical length as risk factors for preterm birth. Ultrasound in Obstetrics and Gynecology 2010. DOI: 10.1002/uog.7568.

194. Donders G, Bellen G, Rezeberga D. Aerobic vaginitis in pregnancy. BJOG: an International Journal of Obstetrics and Gynaecology 2011;118(10):1163-70.

195. Fan A, Yue Y, Geng N, Zhang H, Wang Y, Xue F. Aerobic vaginitis and mixed infections: comparison of clinical and laboratory findings. Archives of gynecology and obstetrics 2013;287(2):329-35.

196. Beghini J, Linhares I, Giraldo P, Ledger W, Witkin S. Differential expression of lactic acid isomers, extracellular matrix metalloproteinase inducer, and matrix metalloproteinase-8 in vaginal fluid from women with vaginal disorders. BJOG: an International Journal of Obstetrics and Gynaecology 2014. DOI: 10.1111/1471-0528.13072.

197. Roberts CL, Algert CS, Rickard KL, Morris JM. Treatment of vaginal candidiasis for the prevention of preterm birth: a systematic review and meta-analysis. Systematic reviews 2015;4(1):1.

198. Rasti S, Asadi MA, Taghriri A, Behrashi M, Mousavie G. Vaginal candidiasis complications on pregnant women. Jundishapur Journal of Microbiology 2014;7(2): e10078.

199. Agrawal V, Hirsch E. Intrauterine infection and preterm labor. Seminars in Fetal and Neonatal Medicine: Elsevier; 2012; 17(1):12–19.

200. Hoffmann JN, You HM, Hedberg E, Jordan JA, McClintock MK. Prevalence of Bacterial Vaginosis and Candida among Postmenopausal Women in the United States. Journals of Gerontology Series B: Psychological Sciences and Social Sciences 2014;69(2):S205-S14.

201. Georgiou HM, Di Quinzio MK, Permezel M, Brennecke SP. Predicting Preterm Labour: Current Status and Future Prospects. Disease markers 2015;2015:435014.

202. Caughey AB, Robinson JN, Norwitz ER. Contemporary diagnosis and management of preterm premature rupture of membranes. Reviews in obstetrics and gynecology 2008;1(1):11-22.

203. Robinson JN, Regan JA, Norwitz ER. The epidemiology of preterm labor. Seminars in perinatology 2001;25(4):204-14.

204. Esplin MS, Varner MW. Genetic factors in preterm birth—the future. BJOG: an International Journal of Obstetrics and Gynaecology 2005;112:97-102.

205. Lockwood CJ. Testing for risk of preterm delivery. Clinics in Laboratory Medicine 2003;23(2):345-60.

206. Peltier MR, Klimova NG, Arita Y, Gurzenda EM, Murthy A, Chawala K, et al. Polybrominated diphenyl ethers enhance the production of proinflammatory cytokines by the placenta. Placenta 2012;33(9):745-9.

207. Donders G. Reducing infection-related preterm birth. BJOG: an International Journal of Obstetrics and Gynaecology 2015;122(2):219-.

208. Goldenberg RL, Andrews WW, Mercer BM, Moawad AH, Meis PJ, Iams JD, et al. The Preterm Prediction Study: Granulocyte colony-stimulating factor and spontaneous preterm birth. American Journal of Obstetrics and Gynecology 2000;182(3):625-30.

209. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 2011;34(5):637-50.

210. Mirmonsef P, Zariffard MR, Gilbert D, Makinde H, Landay AL, Spear GT. Short-chain fatty acids induce pro-inflammatory cytokine production alone and in combination with toll-like receptor ligands. American Journal of Reproductive Immunology 2012;67(5):391-400.

211. Goldenberg RL, Andrews WW, Hauth JC. Choriodecidual infection and preterm birth. Nutrition Reviews 2002;60(suppl 5):S19-S25.

212. Romero R, Dey SK, Fisher SJ. Preterm labor: one syndrome, many causes. Science 2014;345(6198):760-5.

213. Rahkonen L, Rutanen E-M, Unkila-Kallio L, Nuutila M, Nieminen P, Sorsa T, et al. Factors affecting matrix metalloproteinase-8 levels in the vaginal and cervical fluids in the first and second trimester of pregnancy. Human Reproduction 2009;24(11):2693-702.

214. Goldenberg RL, Hauth JC, Andrews WW. Mechanisms of disease - Intrauterine infection and preterm delivery. New England Journal of Medicine 2000;342(20):1500-7.

215. Offenbacher S. Maternal periodontal infections, prematurity, and growth restriction. Clinical obstetrics and gynecology 2004;47(4):808-21.

216. Romero BC, Chiquito CS, Elejalde LE, Bernardoni CB. Relationship between periodontal disease in pregnant women and the nutritional condition of their newborns. Journal of periodontology 2002;73(10):1177-83.

217. Romero R, Espinoza J, Gonçalves L, Kusanovic JP, Friel L, Hassan S. The role of inflammation and infection in preterm birth. Seminars in reproductive medicine; 2007; 25(1): 021-039.

218. Lamont RF. The role of infection in preterm labour and birth. Hospital medicine (London, England: 1998) 2003;64(11):644-7.

219. Nelson DB, Bellamy S, Nachamkin I, Ness RB, Macones GA, Allen-Taylor L. First trimester bacterial vaginosis, individual microorganism levels, and risk of second trimester pregnancy loss among urban women. Fertility and sterility 2007;88(5):1396-403.

220. Romero R, Espinoza J, Goncalves LF, Kusanovic JP, Friel LA, Nien JK. Inflammation in preterm and term labour and delivery. Seminars in fetal & neonatal medicine 2006;11(5):317-26.

221. Andrews WW. The preterm prediction study: Association of mid-trimester genital chlamydia infection and subsequent spontaneous preterm birth (SPTB). American Journal of Obstetrics and Gynecology 1997;176(1 PART 2):S52-S.

222. Andrews WW, Goldenberg RL, Mercer B, Iams J, Meis P, Moawad A, et al. The Preterm Prediction Study: association of second-trimester genitourinary chlamydia infection with subsequent spontaneous preterm birth. American Journal of Obstetrics and Gynecology 2000;183(3):662-8.

223. Gencay M, Koskiniemi M, Ammala P, Fellman V, Narvanen A, Wahlstrom T, et al. Chlamydia trachomatis seropositivity is associated both with stillbirth and preterm delivery. APMIS: acta pathologica, microbiologica, et immunologica Scandinavica 2000;108(9):584-8.

224. Kataoka S, Yamada T, Chou K, Nishida R, Morikawa M, Minami M, et al. Association between preterm birth and vaginal colonization by mycoplasmas in early pregnancy. Journal of clinical microbiology 2006;44(1):51-5.

225. Bittar RE, Da Fonseca EB, De Carvalho MHB, Martinelli S, Zugaib M. Predicting preterm delivery in asymptomatic patients with prior preterm delivery by measurement of cervical length and phosphorylated insulin-like growth factor-binding protein-1. Ultrasound in Obstetrics and Gynecology 2007;29(5):562-7.

226. Owen J, Hankins G, Iams JD, Berghella V, Sheffield JS, Perez-Delboy A, et al. Multicenter randomized trial of cerclage for preterm birth prevention in high-risk women with shortened midtrimester cervical length. American Journal of Obstetrics and Gynecology 2009;201(4):375 e1-8.

227. Szychowski JM, Owen J, Hankins G, Iams J, Sheffield J, Perez-Delboy A, et al. Timing of mid-trimester cervical length shortening in high-risk women. Ultrasound in obstetrics and gynecology 2009;33(1):70-5.

228. Andrews WW, Klebanoff MA, Thom EA, Hauth JC, Carey JC, Meis PJ, et al. Midpregnancy genitourinary tract infection with Chlamydia trachomatis: Association with subsequent preterm delivery in women with bacterial vaginosis and Trichomonas vaginalis. American Journal of Obstetrics and Gynecology 2006;194(2):493-500.

229. Silveira MF, Ghanem KG, Erbelding EJ, Burke AE, Johnson HL, Singh RH, et al. Chlamydia trachomatis infection during pregnancy and the risk of preterm birth: a case-control study. International journal of STD & AIDS 2009;20(7):465-9.

230. Blas MM, Canchihuaman FA, Alva IE, Hawes SE. Pregnancy outcomes in women infected with Chlamydia trachomatis: a population-based cohort study in Washington State. Sexually Transmitted Infections 2007;83(4):314-8.

231. Rours GI, de Krijger RR, Ott A, Willemse HF, de Groot R, Zimmermann LJ, et al. Chlamydia trachomatis and placental inflammation in early preterm delivery. European journal of epidemiology 2011;26(5):421-8.

232. Kalinka J, Laudanski T, Hanke W, Krzeminski Z, Wasiela M. The evaluation of prevalence and the impact of pathological microflora of the lower genital tract among women at early pregnancy on the risk of preterm delivery. Medycyna Doswiadczalna i Mikrobiologia 2003;55(3):277-84.

233. AbeleHorn M, Wolff C, Dressel P, Pfaff F, Zimmermann A. Association of Ureaplasma urealyticum biovars with clinical outcome for neonates, obstetric patients, and gynecological patients with pelvic inflammatory disease. Journal of clinical microbiology 1997;35(5):1199-202.

234. Abrahams VM, Schaefer TM, Fahey JV, Visintin I, Wright JA, Aldo PB, et al. Expression and secretion of antiviral factors by trophoblast cells following stimulation by the TLR-3 agonist, Poly(I : C). Human Reproduction 2006;21(9):2432-9.

235. Onderdonk AB, Delaney ML, DuBois AM, Allred EN, Leviton A, Investigators ELGANS. Detection of bacteria in placental tissues obtained from extremely low gestational age neonates. American Journal of Obstetrics and Gynecology 2008;198(1):110. e1-. e7.

236. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. Science Translational Medicine 2014;6(237):237ra65-ra65.

237. Steel JH, Malatos S, Kennea N, Edwards AD, Miles L, Duggan P, et al. Bacteria and inflammatory cells in fetal membranes do not always cause preterm labor. Pediatric research 2005;57(3):404-11.

238. Romero R, Gotsch F, Pineles B, Kusanovic JP. Inflammation in pregnancy: Its roles in reproductive physiology, obstetrical complications, and fetal injury. Nutrition Reviews 2007;65(12):S194-S202.

239. Andrade Ramos B, Kanninen TT, Sisti G, Witkin SS. Microorganisms in the Female Genital Tract during Pregnancy: Tolerance versus Pathogenesis. American Journal of Reproductive Immunology 2014.

240. Witkin S, Linhares I, Bongiovanni A, Herway C, Skupski D. Unique alterations in infection-induced immune activation during pregnancy. BJOG: an International Journal of Obstetrics and Gynaecology 2011;118(2):145-53.

241. Taylor BD, Holzman CB, Fichorova RN, Tian Y, Jones NM, Fu W, et al. Inflammation biomarkers in vaginal fluid and preterm delivery. Human Reproduction 2013;28(4):942-52.

242. Pavcnik-Arnol M, Lucovnik M, Kornhauser-Cerar L, Premru-Srsen T, Hojker S, Derganc M. Lipopolysaccharide-binding protein as marker of fetal inflammatory response syndrome after preterm premature rupture of membranes. Neonatology 2014;105(2):121-7.

243. Holst D, Garnier Y. Preterm birth and inflammation—The role of genetic polymorphisms. European Journal of Obstetrics & Gynecology and Reproductive Biology 2008;141(1):3-9.

244. Roberts AK, Monzon-Bordonaba F, Van Deerlin PG, Holder J, Macones GA, Morgan MA, et al. Association of polymorphism within the promoter of the tumor necrosis factor α gene with increased risk of preterm premature rupture of the fetal membranes. American Journal of Obstetrics and Gynecology 1999;180(5):1297-302.

245. Menon R, Merialdi M, Betrán AP, Dolan S, Jiang L, Fortunato SJ, et al. Analysis of association between maternal tumor necrosis factor-α promoter polymorphism (–308), tumor necrosis factor concentration, and preterm birth. American Journal of Obstetrics and Gynecology 2006;195(5):1240-8.

246. Park KH, Chaiworapongsa T, Kim YM, Espinoza J, Yoshimatsu J, Edwin S, et al. Matrix metalloproteinase 3 in parturition, premature rupture of the membranes, and microbial invasion of the amniotic cavity. Journal of perinatal medicine 2003;31(1):12-22.

247. Tency I. Inflammatory response in maternal serum during preterm labour. Facts, views & vision in Obstetrics and Gynecology 2014;6(1):19-30.

248. Yoon BH, Oh SY, Romero R, Shim SS, Han SY, Park JS, et al. An elevated amniotic fluid matrix metalloproteinase-8 level at the time of mid-trimester genetic amniocentesis is a

risk factor for spontaneous preterm delivery. American Journal of Obstetrics and Gynecology 2001;185(5):1162-7.

249. Roberts AK, Monzon-Bordonaba F, Van Deerlin PG, Holder J, Macones GA, Morgan MA, et al. Association of polymorphism within the promoter of the tumor necrosis factor alpha gene with increased risk of preterm premature rupture of the fetal membranes. American Journal of Obstetrics and Gynecology 1999;180(5):1297-302.

250. Moore S, Ide M, Randhawa M, Walker JJ, Reid JG, Simpson NA. An investigation into the association among preterm birth, cytokine gene polymorphisms and periodontal disease. BJOG : an international journal of obstetrics and gynaecology 2004;111(2):125-32.

251. Menon R, Fortunato SJ, Edwards DR, Williams SM. Association of genetic variants, ethnicity and preterm birth with amniotic fluid cytokine concentrations. Annals of human genetics 2010;74(2):165-83.

252. Ferrand PE, Parry S, Sammel M, Macones GA, Kuivaniemi H, Romero R, et al. A polymorphism in the matrix metalloproteinase-9 promoter is associated with increased risk of preterm premature rupture of membranes in African Americans. Molecular human reproduction 2002;8(5):494-501.

253. Fujimoto T, Parry S, Urbanek M, Sammel M, Macones G, Kuivaniemi H, et al. A single nucleotide polymorphism in the matrix metalloproteinase-1 (MMP-1) promoter influences amnion cell MMP-1 expression and risk for preterm premature rupture of the fetal membranes. Journal of biological chemistry 2002;277(8):6296-302.

254. Kalish RB, Vardhana S, Gupta M, Perni SC, Witkin SS. Interleukin-4 and -10 gene polymorphisms and spontaneous preterm birth in multifetal gestations. American Journal of Obstetrics and Gynecology 2004;190(3):702-6.

255. Kalish RB, Nguyen DP, Vardhana S, Gupta M, Perni SC, Witkin SS. A single nucleotide A>G polymorphism at position -670 in the Fas gene promoter: relationship to preterm premature rupture of fetal membranes in multifetal pregnancies. American Journal of Obstetrics and Gynecology 2005;192(1):208-12.

256. Choi SJ, Park SD, Jang IH, Uh Y, Lee A. The Prevalence of Vaginal Microorganisms in Pregnant Women with Preterm Labor and Preterm Birth. Annals of Laboratory Medicine 2012;32(3):194-200.

257. Gibbs RS, Romero R, Hillier SL, Eschenbach DA, Sweet RL. A review of premature birth and subclinical infection. American Journal of Obstetrics and Gynecology 1992;166(5):1515-28.

258. Mathews TJ, MacDorman MF. Infant mortality statistics from the 2006 period linked birth/infant death data set. National vital statistics reports : from the Centers for Disease Control and Prevention, National Center for Health Statistics, National Vital Statistics System 2010;58(17):1-31.

259. Hagberg H, Mallard C, Jacobsson B. Role of cytokines in preterm labour and brain injury. BJOG: an international journal of obstetrics and gynaecology 2005;112 Suppl 1:16-8.

260. Simhan HN, Caritis SN, Krohn MA, Hillier SL. Elevated vaginal pH and neutrophils are associated strongly with early spontaneous preterm birth. American journal of obstetrics and gynecology 2003;189(4):1150-4.

261. Hillier SL, Martius J, Krohn M, Kiviat N, Holmes KK, Eschenbach DA. A case-control study of chorioamnionic infection and histologic chorioamnionitis in prematurity. New England journal of medicine 1988;319(15):972-8.

262. Watts DH, Krohn MA, Hillier SL, Eschenbach DA. The association of occult amniotic fluid infection with gestational age and neonatal outcome among women in preterm labor. Obstetrics and gynecology 1992;79(3):351-7.

263. Shah PS, Shah V. Influence of the maternal birth status on offspring: a systematic review and meta-analysis. Acta obstetricia et gynecologica Scandinavica 2009;88(12):1307-18.

264. Plunkett J, Muglia LJ. Genetic contributions to preterm birth: Implications from epidemiological and genetic association studies. Annals of Medicine 2008;40(3):167-79.

265. Anum EA, Springel EH, Shriver MD, Strauss JF. Genetic contributions to disparities in preterm birth. Pediatric research 2009;65(1):1-9.

266. Hyman RW, Fukushima M, Jiang H, Fung E, Rand L, Johnson B, et al. Diversity of the Vaginal Microbiome Correlates With Preterm Birth. Reproductive Sciences 2014;21(1):32-40.

267. Fettweis JM, Brooks JP, Serrano MG, Sheth NU, Girerd PH, Edwards DJ, et al. Differences in vaginal microbiome in African American women versus women of European ancestry. Microbiology 2014;160(10):2272-82.

268. Vrachnis N, Karavolos S, Iliodromiti Z, Sifakis S, Siristatidis C, Mastorakos G, et al. Impact of mediators present in amniotic fluid on preterm labour. in vivo 2012;26(5):799-812.

269. Menon R, Velez DR, Morgan N, Lombardi SJ, Fortunato SJ, Williams SM. Genetic regulation of amniotic fluid TNF-alpha and soluble TNF receptor concentrations affected by race and preterm birth. Human genetics 2008;124(3):243-53.

270. Menon R, Thorsen P, Vogel I, Jacobsson B, Morgan N, Jiang L, et al. Racial disparity in amniotic fluid concentrations of tumor necrosis factor (TNF)-α and soluble TNF receptors in spontaneous preterm birth. American Journal of Obstetrics and Gynecology 2008;198(5):533.e1-.e10.

271. Usynina AA, Postoev VA, Grjibovski AM, Krettek A, Nieboer E, Odland JØ, et al. Maternal Risk Factors for Preterm Birth in Murmansk County, Russia: A Registry-Based Study. Paediatric and perinatal epidemiology 2016;30:462–472.

272. Ahumada-Barrios ME, Alvarado GF. Risk Factors for premature birth in a hospital. Revista Latino-Americana de Enfermagem 2016;24:e2750.

273. Hollier LM. Preventing preterm birth: What works, what doesn't. Obstetrical and Gynecological Survey 2005;60(2):124-31.

274. Fredricks DN, Fiedler TL, Marrazzo JM. Molecular identification of bacteria associated with bacterial vaginosis. New England Journal of Medicine 2005;353(18):1899-911.

275. Gupta K. Inverse association of H_2O_2-producing lactobacilli and vaginal Escherichia coli colonization in women with recurrent urinary tract infections. Journal of Infectious Diseases 1998;178:446-50.

276. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and environmental microbiology 2007;73(16):5261-7.

277. Drell T, Lillsaar T, Tummeleht L, Simm J, Aaspollu A, Vaein E, et al. Characterization of the Vaginal Micro- and Mycobiome in Asymptomatic Reproductive-Age Estonian Women. PloS ONE 2013;8(1).

278. Kroes I, Lepp PW, Relman DA. Bacterial diversity within the human subgingival crevice. Proceedings of the National Academy of Science of the United States of America 1999;96(25):14547-52.

279. Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, et al. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Applied and environmental microbiology 1999;65(11):4799-807.

280. Burton JP, Reid G. Evaluation of the bacterial vaginal flora of 20 postmenopausal women by direct (Nugent score) and molecular (polymerase chain reaction and denaturing gradient gel electrophoresis) techniques. Journal of Infectious Diseases 2002;186(12):1770-80.

281. Hold GL, Pryde SE, Russell VJ, Furrie E, Flint HJ. Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. FEMS Microbiology Ecology 2002;39(1):33-9.

282. Kazor CE, Mitchell PM, Lee AM, Stokes LN, Loesche WJ, Dewhirst FE, et al. Diversity of bacterial Populations on the tongue dorsa of patients with halitosis and healthy patients. Journal of clinical microbiology 2003;41(2):558-63.

283. Schmidt TM, Relman DA. Phylogenetic identification of uncultured pathogens using ribosomal RNA sequences. In: Clark VL, Bavoil PM, eds. Methods in Enzymology; Bacterial pathogenesis, Part A: Identification and regulation of virulence factors; 1994.

284. Brett PJ, DeShazer D, Woods DE. Burkholderia thailandensis sp. nov., a Burkholderia pseudomallei-like species. International Journal of Systematic Bacteriology 1998;48 Pt 1:317-20.

285. Kolbert CP, Persing DH. Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. Current Opinion in Microbiology 1999;2(3):299-305.

286. Patel JB. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. Molecular Diagnosis 2001;6(4):313-21.

287. Pereira F, Carneiro J, Matthiesen R, van Asch B, Pinto N, Gusmao L, et al. Identification of species by multiplex analysis of variable-length sequences. Nucleic Acids Research 2010;38(22):e203.

288. Van de Peer Y, Chapelle S, De Wachter R. A quantitative map of nucleotide substitution rates in bacterial rRNA. Nucleic Acids Research 1996;24(17):3381-91.

289. Lu JJ, Perng CL, Lee SY, Wan CC. Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. Journal of clinical microbiology 2000;38(6):2076-80.

290. Schmalenberger A, Schwieger F, Tebbe CC. Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. Applied and environmental microbiology 2001;67(8):3557-63.

291. Baker GC, Smith JJ, Cowan DA. Review and re-analysis of domain-specific 16S primers. Journal of microbiological methods 2003;55(3):541-55.

292. Munson MA, Banerjee A, Watson TF, Wade WG. Molecular analysis of the microflora associated with dental caries. Journal of clinical microbiology 2004;42(7):3023-9.

293. Chakravorty S, Helb D, Burday M, Connell N, Alland D. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. Journal of microbiological methods 2007;69(2):330-9.

294. Engelbrektson A, Kunin V, Wrighton KC, Zvenigorodsky N, Chen F, Ochman H, et al. Experimental factors affecting PCR-based estimates of microbial species richness and evenness. ISME Journal 2010;4(5):642-7.

295. Bai G, Gajer P, Nandy M, Ma B, Yang H, Sakamoto J, et al. Comparison of Storage Conditions for Human Vaginal Microbiome Studies. PloS ONE 2012;7(5).

296. Hutter G, Schlagenhauf U, Valenza G, Horn M, Burgemeister S, Claus H, et al. Molecular analysis of bacteria in periodontitis: evaluation of clone libraries, novel phylotypes and putative pathogens. Microbiology 2003;149(Pt 1):67-75.

297. Moawad AH, Goldenberg RL, Mercer B, Meis PJ, Iams JD, Das A, et al. The Preterm Prediction Study: the value of serum alkaline phosphatase, α-fetoprotein, plasma corticotropin-releasing hormone, and other serum markers for the prediction of spontaneous preterm birth. American Journal of Obstetrics and Gynecology 2002;186(5):990-6.

298. Goldenberg RL, Goepfert AR, Ramsey PS. Biochemical markers for the prediction of preterm birth. American Journal of Obstetrics and Gynecology 2005;192(5):S36-S46.

299. Fanos V, Atzori, L., Makarenko, K., Melis, G.B., Ferrazi, E. Metabolomics application in maternal-fetal medicine. BioMed research international 2013;2013(720514):http://dx.doi.org/10.1155/2013/720514.

300. Genc MR, Ford CE. The clinical use of inflammatory markers during pregnancy. Current opinion in obstetrics and gynecology 2010;22(2):116-21.

301. Abbott DS, Radford SK, Seed PT, Tribe RM, Shennan AH. Evaluation of a quantitative fetal fibronectin test for spontaneous preterm birth in symptomatic women. American Journal of Obstetrics and Gynecology 2013;208(2):122.e1-.e6.

302. DeFranco EA, Lewis DF, Odibo AO. Improving the screening accuracy for preterm labor: is the combination of fetal fibronectin and cervical length in symptomatic patients a useful predictor of preterm birth? A systematic review. American Journal of Obstetrics and Gynecology 2013;208(3):233. e1-. e6.

303. Heng YJ, Liong S, Permezel M, Rice GE, Di Quinzio MK, Georgiou HM. Human cervicovaginal fluid biomarkers to predict term and preterm labor. Frontiers in physiology 2015;6:151.

304. Berghella V, Hayes E, Visintine J, Baxter JK. Fetal fibronectin testing for reducing the risk of preterm birth. Cochrane Database of Systematic Reviews 2008;4: CD006843.

305. Chandiramani M, Di Renzo GC, Gottschalk E, Helmer H, Henrich W, Hoesli I, et al. Fetal fibronectin as a predictor of spontaneous preterm birth: a European perspective. Journal of Maternal-Fetal and Neonatal Medicine 2011;24(2):330-6.

306. Malak TM, Sizmur F, Bell SC, Taylor DJ. Fetal fibronectin in cervicovaginal secretions as a predictor of preterm birth. British journal of obstetrics and gynaecology 1996;103(7):648-53.

307. Goldenberg RL, Iams JD, Mercer BM, Meis PJ, Moawad A, Das A, et al. The Preterm Prediction Study: Toward a multiple-marker test for spontaneous preterm birth. American Journal of Obstetrics and Gynecology 2001;185(3):643-51.

308. Goepfert AR, Goldenberg RL, Mercer B, Iams J, Meis P, Moawad A, et al. The preterm prediction study: quantitative fetal fibronectin values and the prediction of spontaneous preterm birth. The National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. American Journal of Obstetrics and Gynecology 2000;183(6):1480-3.

309. Roman AS, Koklanaris N, Paidas MJ, Mulholland J, Levitz M, Rebarber A. "Blind" vaginal fetal fibronectin as a predictor of spontaneous preterm delivery. Obstetrics and Gynecology 2005;105(2):285-9.

310. Riboni F, Vitulo A, Dell'avanzo M, Plebani M, Battagliarin G, Paternoster D. Biochemical markers predicting pre-term delivery in symptomatic patients: phosphorylated insulin-like growth factor binding protein-1 and fetal fibronectin. Archives of gynecology and obstetrics 2011;284(6):1325-9.

311. Goldenberg RL, Mercer BM, Meis PJ, Cooper RL, Das A, McNellis D. Preterm prediction study: Fetal fibronectin testing and spontaneous preterm birth. Obstetrics and Gynecology 1996;87(5):643-8.

312. Leitich H, Egarter C, Kaider A, Hohlagschwandtner M, Berghammer P, Husslein P. Cervicovaginal fetal fibronectin as a marker for preterm delivery: a meta-analysis. American Journal of Obstetrics and Gynecology 1999;180(5):1169-76.

313. Honest H, Bachmann LM, Gupta JK, Kleijnen J, Khan KS. Accuracy of cervicovaginal fetal fibronectin test in predicting risk of spontaneous preterm birth: systematic review. British Medical Journal 2002;325(7359):301-4C.

314. Morrison JC, Naef RW, Botti JJ, Katz M, Belluomini JM, McLaughlin BN. Prediction of spontaneous preterm birth by fetal fibronectin and uterine activity. Obstetrics and Gynecology 1996;87(5):649-55.

315. Ramsey PS, Andrews WW. Biochemical predictors of preterm labor: fetal fibronectin and salivary estriol. Clinics in perinatology 2003;30(4):701-33.

316. Goldenberg RL. The management of preterm labor. Obstetrics and Gynecology 2002;100(5, Part 1):1020-37.

317. Moore ML. Biochemical markers for preterm labor and birth: what is their role in the care of pregnant women? American Journal of Maternal/Child Nursing 1999;24(2):80-6.

318. Sadovsky Y, Friedman SA. Fetal fibronectin and preterm labor. New England journal of medicine 1992;326(10):709.

319. Shimoya K, Hashimoto K, Shimizu T, Saji F, Murata Y. Effect of sexual intercourse on fetal fibronectin concentration in cervicovaginal secretions. American Journal of Obstetrics and Gynecology 1998;179(1):255-6.

320. Bolt LA, Chandiramani M, De Greeff A, Seed PT, Kurtzman J, Shennan AH. The value of combined cervical length measurement and fetal fibronectin testing to predict spontaneous preterm birth in asymptomatic high-risk women. Journal of maternal-fetal and neonatal medicine 2011;24(7):928-32.

321. Fox NS, Rebarber A, Roman AS, Klauser CK, Peress D, Saltzman DH. Combined fetal fibronectin and cervical length and spontaneous preterm birth in asymptomatic triplet pregnancies. Journal of maternal-fetal and neonatal medicine 2012;25(11):2308-11.

322. Hincz P, Wilczynski J, Kozarzewski M, Szaflik K. Two-step test: the combined use of fetal fibronectin and sonographic examination of the uterine cervix for prediction of preterm delivery in symptomatic patients. Acta obstetricia et gynecologica Scandinavica 2002;81(1):58-63.

323. Schmitz T, Maillard F, Bessard-Bacquaert S, Kayem G, Fulla Y, Cabrol D, et al. Selective use of fetal fibronectin detection after cervical length measurement to predict spontaneous preterm delivery in women with preterm labor. American Journal of Obstetrics and Gynecology 2006;194(1):138-43.

324. Dutta D, Norman JE. The efficacy of fetal fibronectin testing in minimising hospital admissions, length of hospital stay and cost savings in women presenting with symptoms of pre-term labour. Journal of obstetrics and gynaecology 2010;30(8):768-73.

325. Brik M, HernÁNdez ANAIM, Pedraz CC, Perales A. Phosphorylated insulin-like growth factor binding protein-1 and cervical measurement in women with threatening preterm birth. Acta obstetricia et gynecologica Scandinavica 2010;89(2):268-74.

326. Vogel I, Thorsen P, Curry A, Sandager P, Uldbjerg N. Biomarkers for the prediction of preterm delivery. Acta obstetricia et gynecologica Scandinavica 2005;84(6):516-25.

327. Hee L. Likelihood ratios for the prediction of preterm delivery with biomarkers. Acta obstetricia et gynecologica Scandinavica 2011;90(11):1189-99.

328. Tanir HM, Sener T, Yildiz Z. Cervical phosphorylated insulin-like growth factor binding protein-1 for the prediction of preterm delivery in symptomatic cases with intact membranes. Journal of Obstetrics and Gynaecology Research 2009;35(1):66-72.

329. Azlin MN, Bang HK, An LJ, Mohamad S, Mansor N, Yee BS, et al. Role of phIGFBP-1 and ultrasound cervical length in predicting pre-term labour. Journal of Obstetrics and Gynaecology 2010;30(5):456-9.

330. Bittar R, Da Fonseca E, De Carvalho M, Martinelli S, Zugaib M. Predicting preterm delivery in asymptomatic patients with prior preterm delivery by measurement of cervical length and phosphorylated insulin-like growth factor-binding protein-1. Ultrasound in Obstetrics and Gynecology 2007;29(5):562-7.

331. Khambay H, Bolt L, Chandiramani M, De Greeff A, Filmer J, Shennan A. The Actim Partus test to predict pre-term birth in asymptomatic high-risk women. Journal of Obstetrics and Gynaecology 2012;32(2):132-4.

332. Rahkonen L, Unkila-Kallio L, Nuutila M, Sainio S, Saisto T, Rutanen EM, et al. Cervical length measurement and cervical phosphorylated insulin-like growth factor binding protein-1 testing in prediction of preterm birth in patients reporting uterine contractions. Acta obstetricia et gynecologica Scandinavica 2009;88(8):901-8.

333. Kwek K, Khi C, Ting H, Yeo G. Evaluation of a bedside test for phosphorylated insulin-like growth factor binding protein-1 in preterm labour. Annals Academy of Medicine Singapore 2004;33(6):780-3.

334. Balic D, Latifagic A, Hudic I. Insulin-like growth factor-binding protein-1 (IGFBP-1) in cervical secretions as a predictor of preterm delivery. Journal of Maternal-Fetal and Neonatal Medicine 2008;21(5):297-300.

335. Kekki M, Kurki T, Karkkainen T, Hiilesmaa V, Paavonen J, Rutanen EM. Insulin-like growth factor-binding protein-1 in cervical secretion as a predictor of preterm delivery. Acta obstetricia et gynecologica Scandinavica 2001;80(6):546-51.

336. Maria Paternoster D, Muresan D, Vitulo A, Serena A, Battagliarin G, Dell'Avanzo M, et al. Cervical phIGFBP-1 in the evaluation of the risk of preterm delivery. Acta obstetricia et gynecologica Scandinavica 2007;86(2):151-5.

337. Paternoster D, Riboni F, Vitulo A, Plebani M, Dell'Avanzo M, Battagliarin G, et al. Phosphorylated insulin-like growth factor binding protein-1 in cervical secretions and sonographic cervical length in the prediction of spontaneous preterm delivery. Ultrasound in Obstetrics and Gynecology 2009;34(4):437-40.

338. Conde-Agudelo A, Papageorghiou AT, Kennedy SH, Villar J. Novel biomarkers for the prediction of the spontaneous preterm birth phenotype: a systematic review and meta-analysis. BJOG: an International Journal of Obstetrics and Gynaecology 2011;118(9):1042-54.

339. Ting HS, Chin PS, Yeo GS, Kwek K. Comparison of bedside test kits for prediction of preterm delivery: phosphorylated insulin-like growth factor binding protein-1 (pIGFBP-1) test and fetal fibronectin test. Annals Academy of Medicine Singapore 2007;36(6):399-402.

340. Liong S, Di Quinzio M, Fleming G, Permezel M, Rice G, Georgiou H. New biomarkers for the prediction of spontaneous preterm labour in symptomatic pregnant women: a comparison with fetal fibronectin. BJOG: an International Journal of Obstetrics & Gynaecology 2015;122(3):370-9.

341. Yoneda S, Sakai M, Sasaki Y, Shiozaki A, Hidaka T, Saito S. Interleukin-8 and glucose in amniotic fluid, fetal fibronectin in vaginal secretions and preterm labor index based on clinical variables are optimal predictive markers for preterm delivery in patients with intact membranes. Journal of Obstetrics and Gynaecology Research 2007;33(1):38-44.

342. Saade GR, Boggess KA, Sullivan SA, Markenson GR, Iams JD, Coonrod DV, et al. Development and validation of a spontaneous preterm delivery predictor in asymptomatic women. American Journal of Obstetrics and Gynecology 2016;214:633.e1-24.

343. Holst RM, Mattsby-Baltzer I, Wennerholm UB, Hagberg H, Jacobsson B. Interleukin-6 and interleukin-8 in cervical fluid in a population of Swedish women in preterm labor: relationship to microbial invasion of the amniotic fluid, intra-amniotic inflammation, and preterm delivery. Acta obstetricia et gynecologica Scandinavica 2005;84(6):551-7.

344. Yoneda S, Shiozaki A, Yoneda N, Shima T, Ito M, Yamanaka M, et al. Prediction of exact delivery time in patients with preterm labor and intact membranes at admission by amniotic fluid interleukin-8 level and preterm labor index. Journal of Obstetrics and Gynaecology Research 2011;37(7):861-6.

345. Liong S, Di Quinzio MK, Fleming G, Permezel M, Rice GE, Georgiou HM. Prediction of spontaneous preterm labour in at-risk pregnant women. Reproduction 2013;146(4):335-45.

346. Kumar S, Palaia T, Hall CE, Ragolia L. Role of Lipocalin-type prostaglandin D2 synthase (L-PGDS) and its metabolite, prostaglandin D2, in preterm birth. Prostaglandins and Other Lipid Mediators 2015;118–119:28-33.

347. Kim A, Lee ES, Shin JC, Kim HY. Identification of biomarkers for preterm delivery in mid-trimester amniotic fluid. Placenta 2013;34(10):873-8.

348. Nakatsuka M, Habara T, Kamada Y, Tada K, Kudo T. Elevation of total nitrite and nitrate concentration in vaginal secretions as a predictor of premature delivery. American Journal of Obstetrics and Gynecology 2000;182(3):644-5.

349. Giannella L, Beraldi R, Giulini S, Cerami LB, Mfuta K, Facchinetti F. Nitric oxide metabolite levels and assessment of cervical length in the prediction of preterm delivery among women undergoing symptomatic preterm labor. International Journal of Gynecology and Obstetrics 2012;116(3):223-7.

350. Spencer K, Cowans NJ, Molina F, Kagan KO, Nicolaides KH. First-trimester ultrasound and biochemical markers of aneuploidy and the prediction of preterm or early preterm delivery. Ultrasound in Obstetrics and Gynecology 2008;31(2):147-52.

351. Heine RP, McGregor JA, Goodwin TM, Artal R, Hayashi RH, Robertson PA, et al. Serial salivary estriol to detect an increased risk of preterm birth. Obstetrics and Gynecology 2000;96(4):490-7.

352. Priya B, Mustafa M, Guleria K, Vaid N, Banerjee B, Ahmed R. Salivary progesterone as a biochemical marker to predict early preterm birth in asymptomatic high-risk women. BJOG: an International Journal of Obstetrics and Gynaecology 2013;120(8):1003-11.

353. Lachelin GCL, McGarrigle HHG, Seed PT, Briley A, Shennan AH, Poston L. Low saliva progesterone concentrations are associated with spontaneous early preterm labour

(before 34 weeks of gestation) in women at increased risk of preterm delivery. BJOG: an International Journal of Obstetrics and Gynaecology 2009;116(11):1515-9.

354. Maged AM, Mohesen M, Elhalwagy A, Abdelhafiz A. Salivary progesterone and cervical length measurement as predictors of spontaneous preterm birth. Journal of maternal-fetal and neonatal medicine 2014;28(10):1147-51.

355. Shah SJ, Yu KH, Sangar V, Parry SI, Blair IA. Identification and Quantification of Preterm Birth Biomarkers in Human Cervicovaginal Fluid by Liquid Chromatography/Tandem Mass Spectrometry. Journal of Proteome Research 2009;8(5):2407-17.

356. Chan RL. Biochemical markers of spontaneous preterm birth in asymptomatic women. BioMed research international 2014;2014:164081.

357. Romero R, Mazaki-Tovi, S., Vaisbuch, E., Kusanovic, J.P., Chaiworapongsa, T., Gomez, R., Nien, J.K., Yoon, B.H., Mazor, M., Luo, J., Banks, D., Ryals, J., Beecher, C. Metabolomics in premature labor: a novel approach to identify patients at risk for preterm delivery. J Matern Fetal Neonatal Med 2010;23(12):1344-59.

358. Ghartey J, Bastek JA, Brown AG, Anglim L, Elovitz MA. Women with preterm birth have a distinct cervicovaginal metabolome. American Journal of Obstetrics and Gynecology 2015;212(6):776. e1-. e12.

359. Auray-Blais C, Raiche E, Gagnon R, Berthiaume M, Pasquier J-C. Metabolomics and preterm birth: What biomarkers in cervicovaginal secretions are predictive of high-risk pregnant women? International Journal of Mass Spectrometry 2011;307(1-3):33-8.

360. Maitre L, Fthenou E, Athersuch T, Coen M, Toledano MB, Holmes E, et al. Urinary metabolic profiles in early pregnancy are associated with preterm birth and fetal growth restriction in the Rhea mother–child cohort study. BMC medicine 2014;12(1):110.

361. Pitiphat W, Gillman MW, Joshipura KJ, Williams PL, Douglass CW, Rich-Edwards JW. Plasma C-reactive protein in early pregnancy and preterm delivery. American Journal of Epidemiology 2005;162(11):1108-13.

362. Goel A, Jain V, Gupta I, Varma N. Serial serum ferritin estimation in pregnant women at risk of preterm labor. Acta obstetricia et gynecologica Scandinavica 2003;82(2):129-32.

363. Thomakos N, Daskalakis G, Papapanagiotou A, Papantoniou N, Mesogitis S, Antsaklis A. Amniotic fluid interleukin-6 and tumor necrosis factor-α at mid-trimester genetic amniocentesis: Relationship to intra-amniotic microbial invasion and preterm delivery. European Journal of Obstetrics and Gynecology and Reproductive Biology 2010;148(2):147-51.

364. Wiberg-Itzel E, Cnattingius S, Nordstrom L. Lactate determination in vaginal fluids: a new method in the diagnosis of prelabour rupture of membranes. BJOG : an international journal of obstetrics and gynaecology 2005;112(6):754-8.

365. Hyman RW, Fukushima M, Jiang H, Fung E, Rand L, Johnson B, et al. Diversity of the vaginal microbiome correlates with preterm birth. Reproductive Sciences 2013:1933719113488838.

366. Mella MT, Berghella V. Prediction of Preterm Birth: Cervical Sonography. Seminars in perinatology 2009;33(5):317-24.

367. Moroz LA, Simhan HN. Rate of sonographic cervical shortening and the risk of spontaneous preterm birth. American Journal of Obstetrics and Gynecology 2012;206(3):234.e1-.e5.

368. Peng C-R, Chen C-P, Wang K-G, Wang L-K, Chen C-Y, Chen Y-Y. The reliability of transabdominal cervical length measurement in a low-risk obstetric population: Comparison with transvaginal measurement. Taiwanese Journal of Obstetrics and Gynecology 2015;54(2):167-71.

369. Owen J, Iams JD, Natl Inst Child Hlth Human Dev M. What we have learned about cervical ultrasound. Seminars in perinatology 2003;27(3):194-203.

370. Berghella V, Roman A, Daskalakis C, Ness A, Baxter JK. Gestational age at cervical length measurement and incidence of preterm birth. Obstetrics and Gynecology 2007;110(2 Pt 1):311-7.

371. Yost NP, Bloom SL, Twickler DM, Leveno KJ. Pitfalls in ultrasonic cervical length measurement for predicting preterm birth. Obstetrics and Gynecology 1999;93(4):510-6.

372. Owen J, Yost N, Berghella V, MacPherson C, Swain M, Dildy GA, et al. Can shortened midtrimester cervical length predict very early spontaneous preterm birth? American Journal of Obstetrics and Gynecology 2004;191(1):298-303.

373. Yost NP, Owen J, Berghella V, Macpherson C, Swain M, Dildy GA, 3rd, et al. Number and gestational age of prior preterm births does not modify the predictive value of a short cervix. American Journal of Obstetrics and Gynecology 2004;191(1):241-6.

374. Berghella V, Odibo AO, To MS, Rust OA, Althuisius SM. Cerclage for short cervix on ultrasonography: meta-analysis of trials using individual patient-level data. Obstetrics and Gynecology 2005;106(1):181-9.

375. Martin JA, Hamilton BE, Sutton PD, Ventura SJ, Menacker F, Kirmeyer S, et al. Births: final data for 2005. National vital statistics reports : from the Centers for Disease Control and Prevention, National Center for Health Statistics, National Vital Statistics System 2007;56(6):1-103.

376. Hassan SS, Romero R, Berry SM, Dang K, Blackwell SC, Treadwell MC, et al. Patients with an ultrasonographic cervical length <= 15 mm have nearly a 50% risk of early spontaneous preterm delivery. American Journal of Obstetrics and Gynecology 2000;182(6):1458-64.

377. Crane JMG, Hutchens D. Transvaginal sonographic measurement of cervical length to predict preterm birth in asymptomatic women at increased risk: a systematic review. Ultrasound in Obstetrics and Gynecology 2008;31(5):579-87.

378. Crane JMG, Hutchens D. Use of transvaginal ultrasonography to predict preterm birth in women with a history of preterm birth. Ultrasound in Obstetrics and Gynecology 2008;32(5):640-5.

379. Honest H, Bachmann LM, Coomarasamy A, Gupta JK, Kleijnen J, Khan KS. Accuracy of cervical transvaginal sonography in predicting preterm birth: a systematic review. Ultrasound in Obstetrics and Gynecology 2003;22(3):305-22.

380. Durnwald CP, Walker H, Lundy JC, Iams JD. Rates of recurrent preterm birth by obstetrical history and cervical length. American Journal of Obstetrics and Gynecology 2005;193(3):1170-4.

381. Chao A-S, Chao A, Hsieh PC-C. Ultrasound assessment of cervical length in pregnancy. Taiwanese Journal of Obstetrics and Gynecology 2008;47(3):291-5.

382. Owen J, Yost N, Berghella V, Thom E, Swain M, Dildy GA, et al. Mid-trimester endovaginal sonography in women at high risk for spontaneous preterm birth. Journal of the American Medical Association 2001;286(11):1340-8.

383. Vaisbuch E, Romero R, Mazaki-Tovi S, Erez O, Kusanovic JP, Mittal P, et al. The risk of impending preterm delivery in asymptomatic patients with a nonmeasurable cervical length in the second trimester. American journal of obstetrics and gynecology 2010;203(5):446.e1-9.

384. Vaisbuch E, Hassan SS, Mazaki-Tovi S, Nhan-Chang C-L, Kusanovic JP, Chaiworapongsa T, et al. Patients with an asymptomatic short cervix (<= 15 mm) have a high rate of subclinical intraamniotic inflammation: implications for patient counseling. American Journal of Obstetrics and Gynecology 2010;202(5): 433.e1–433.e8.

385. Hassan S, Romero R, Hendler I, Gomez R, Khalek N, Espinoza J, et al. A sonographic short cervix as the only clinical manifestation of intra-amniotic infection. Journal of perinatal medicine 2006;34(1):13-9.

386. Mancuso MS, Figueroa D, Szychowski JM, Paden MM, Owen J. Midtrimester bacterial vaginosis and cervical length in women at risk for preterm birth. American Journal of Obstetrics and Gynecology 2011;204(4):342.e1-.e5.

387. Ramaeker DM, Simhan HN. Sonographic cervical length, vaginal bleeding, and the risk of preterm birth. American journal of obstetrics and gynecology 2012;206(3):224.e1-4.

388. Guzman ER, Walters C, Ananth CV, O'Reilly-Green C, Benito CW, Palermo A, et al. A comparison of sonographic cervical parameters in predicting spontaneous preterm birth

in high-risk singleton gestations. Ultrasound in Obstetrics and Gynecology 2001;18(3):204-10.

389. Celik E, To M, Gajewska K, Smith GCS, Nicolaides KH, Fetal Medicine Fdn S. Cervical length and obstetric history predict spontaneous preterm birth: development and validation of a model to provide individualized risk assessment. Ultrasound in Obstetrics and Gynecology 2008;31(5):549-54.

390. Conde-Agudelo A, Romero R, Hassan SS, Yeo L. Transvaginal sonographic cervical length for the prediction of spontaneous preterm birth in twin pregnancies: a systematic review and metaanalysis. American journal of obstetrics and gynecology 2010;203(2):128.e1-.e12.

391. Eroglu D, Yanik F, Oktem M, Zeyneloglu HB, Kuscu E. Prediction of preterm delivery among women with threatened preterm labor. Gynecologic and obstetric investigation 2007;64(2):109-16.

392. Sotiriadis A, Papatheodorou S, Kavvadias A, Makrydimas G. Transvaginal cervical length measurement for prediction of preterm birth in women with threatened preterm labor: a meta-analysis. Ultrasound in Obstetrics and Gynecology 2010;35(1):54-64.

393. Gomez R, Romero R, Medina L, Nien JK, Chaiworapongsa T, Carstens M, et al. Cervicovaginal fibronectin improves the prediction of preterm delivery based on sonographic cervical length in patients with preterm uterine contractions and intact membranes. American journal of obstetrics and gynecology 2005;192(2):350-9.

394. van Baaren G-J, Vis JY, Wilms FF, Oudijk MA, Kwee A, Porath MM, et al. Predictive value of cervical length measurement and fibronectin testing in threatened preterm labor. Obstetrics and Gynecology 2014;123(6):1185-92.

395. Berghella V, Baxter JK, Hendrix NW. Cervical assessment by ultrasound for preventing preterm delivery. Cochrane database of systematic reviews 2013;1: CD007235.

396. Lim K, Crane J. Ultrasonographic cervical length assessment in predicting preterm birth in singleton pregnancies. SOGC Clinical Practice Guideline. Journal of Obstetrics and Gynaecology Canada 2011:486-9.

397. Mancuso MS, Owen J. Prevention of preterm birth based on a short cervix: cerclage. Seminars in perinatology: Elsevier; 2009;33(5):325-333.

398. Orzechowski KM, Boelig R, Nicholas SS, Baxter J, Berghella V. Is universal cervical length screening indicated in women with prior term birth? American Journal of Obstetrics and Gynecology 2015;212(2):234.e1-.e5.

399. Iams JD, Romero R, Culhane JF, Goldenberg RL. Primary, secondary, and tertiary interventions to reduce the morbidity and mortality of preterm birth. Lancet;371(9607):164-75.

400. Wang Y, Cao Z, Peng Z, Xin X, Zhang Y, Yang Y, et al. Folic acid supplementation, preconception body mass index, and preterm delivery: findings from the preconception cohort data in a Chinese rural population. BMC pregnancy and childbirth 2015;15(1):1.

401. Thinkhamrop J, Hofmeyr GJ, Adetoro O, Lumbiganon P, Ota E. Antibiotic prophylaxis during the second and third trimester to reduce adverse pregnancy outcomes and morbidity. Cochrane database of systematic reviews 2015; 1: CD002250.

402. Kenyon S, Boulvain M, Neilson JP. Antibiotics for preterm rupture of membranes. Cochrane database of systematic reviews 2013;12: CD001058.

403. Flenady V, Hawley G, Stock OM, Kenyon S, Badawi N. Prophylactic antibiotics for inhibiting preterm labour with intact membranes. Cochrane database of systematic reviews 2013; 12:CD000246.

404. Conde-Agudelo A, Romero R. Vaginal progesterone to prevent preterm birth in pregnant women with a sonographic short cervix: clinical and public health implications. American Journal of Obstetrics and Gynecology 2016;214(2):235-42.

405. Romero R, Nicolaides K, Conde-Agudelo A, O'brien J, Cetingoz E, Da Fonseca E, et al. Vaginal progesterone decreases preterm birth≤ 34 weeks of gestation in women with a singleton gestation and a short cervix: an updated meta-analysis including data from the opptimum study. Ultrasound in Obstetrics and Gynecology 2016. DOI: 10.1002/uog.15953.

406. van Baaren G-J, Bruijn MMC, Vis JY, Wilms FF, Oudijk MA, Kwee A, et al. Risk factors for preterm delivery: do they add to fetal fibronectin testing and cervical length measurement in the prediction of preterm delivery in symptomatic women? European Journal of Obstetrics and Gynecology and Reproductive Biology 2015;192:79-85.

407. Holst RM, Jacobsson B, Hagberg H, Wennerholm UB. Cervical length in women in preterm labor with intact membranes: relationship to intra-amniotic inflammation/microbial invasion, cervical inflammation and preterm delivery. Ultrasound in Obstetrics and Gynecology 2006;28(6):768-74.

408. Rafael TJ, Berghella V, Alfirevic Z. Cervical stitch (cerclage) for preventing preterm birth in multiple pregnancy. Cochrane database of systematic reviews 2014;9: CD009166.

409. Iams JD. Prevention of preterm parturition. New England Journal of Medicine 2014;370(3):254-61.

410. Alfirevic Z, Stampalija T, Roberts D, Jorgensen AL. Cervical stitch (cerclage) for preventing preterm birth in singleton pregnancy. Cochrane database of systematic reviews 2012; 4:CD008991.

411. Illia R, Leveque R, Mayer H, de Anchorena M, Uranga Imaz M, Habich D. Role of cervical cerclage and prolonged antibiotic therapy with azithromycin in patients with previous perinatal loss amnionitis. Journal of Maternal-Fetal and Neonatal Medicine 2016:1-4.

412. Kemp M, Newnham J, Challis J, Jobe A, Stock S. The clinical use of corticosteroids in pregnancy. Human reproduction update 2015:dmv047.

413. Brownfoot FC, Gagliardi DI, Bain E, Middleton P, Crowther CA. Different corticosteroids and regimens for accelerating fetal lung maturation for women at risk of preterm birth. Cochrane database of systematic reviews 2013; 8: CD006764.

414. Vogel JP, Nardin JM, Dowswell T, West HM, Oladapo OT. Combination of tocolytic agents for inhibiting preterm labour. Cochrane database of systematic reviews 2014;7: CD006169.

415. Reinebrant HE, Pileggi-Castro C, Romero C, Dos Santos R, Kumar S, Souza JP, et al. Cyclo-oxygenase (COX) inhibitors for treating preterm labour. Cochrane database of systematic reviews 2015;6:CD001992.

416. Flenady V, Reinebrant HE, Liley HG, Tambimuttu EG, Papatsonis DN. Oxytocin receptor antagonists for inhibiting preterm labour. Cochrane database of systematic reviews 2014;6:CD004452.

417. Flenady V, Wojcieszek A, Papatsonis D, Stock O, Murray L, Jardine L, et al. Calcium ch flannel blockers for inhibiting preterm labour and birth. Cochrane database of systematic reviews 2014;6:CD002255.

418. McNamara HC, Crowther CA, Brown J. Different treatment regimens of magnesium sulphate for tocolysis in women in preterm labour. Cochrane database of systematic reviews 2015;12:CD011200.

419. Chatham JC, Blackband SJ. Nuclear magnetic resonance spectroscopy and imaging in animal research. IlAR Journal 2001;42(3):189-208.

420. Hayes AW, Kruger CL. Hayes' principles and methods of toxicology: CRC Press; 2014.

421. Eisenreich W, Bacher A. Advances of high-resolution NMR techniques in the structural and metabolic analysis of plant biochemistry. Phytochemistry 2007;68(22–24):2799-815.

422. Schneider B. Nuclear magnetic resonance spectroscopy in biosynthetic studies. Progress in Nuclear Magnetic Resonance Spectroscopy 2007;51(3):155-98.

423. Shah N, Sattar A, Benanti M, Hollander S, Cheuck L. Magnetic resonance spectroscopy as an imaging tool for cancer: a review of the literature. Journal of the American Osteopathic Association 2006;106(1):23-7.

424. Bainbridge A. Nuclear magnetic resonance spectroscopy overview. In: Poole PWT, ed. Encyclopedia of Analytical Science (Second Edition). Oxford: Elsevier; 2005.

425. Chalbot M-CG, Kavouras IG. Nuclear magnetic resonance spectroscopy for determining the functional content of organic aerosols: A review. Environmental Pollution 2014;191:232-49.

426. Grutzner JB. Nuclear magnetic resonance spectroscopy overview principles. In: Poole PWT, ed. Encyclopedia of Analytical Science (Second Edition). Oxford: Elsevier; 2005.

427. Claridge TD. High-resolution NMR techniques in organic chemistry: Newnes; 2008.

428. Harris RK, Becker ED, Cabral de Menezes SM, Goodfellow R, Granger P. NMR nomenclature: Nuclear spin properties and conventions for chemical shifts (IUPAC recommendations 2001). Concepts in Magnetic Resonance 2002;14(5):326-46.

429. Monasterio O. Nomenclature for the applications of nuclear magnetic resonance to the study of enzymes. Perspectives in Science 2014;1(1–6):88-97.

430. Parvizi J. Metabolomics: key to understanding human individuality. Journal of bone and joint surgery American volume 2011;93(21):1.

431. Piñero-Sagredo E, Nunes S, de los Santos M, Celda B, Esteve V. NMR metabolic profile of human follicular fluid. NMR in biomedicine 2010;23(5):485-95.

432. Hasim A, Ali M, Mamtimin B, Ma JQ, Li QZ, Abudula A. Metabonomic signature analysis of cervical carcinoma and precancerous lesions in women by (1)H NMR spectroscopy. Experimental and therapeutic medicine 2012;3(6):945-51.

433. Romick-Rosendale LE, Goodpaster AM, Hanwright PJ, Patel NB, Wheeler ET, Chona DL, et al. NMR-based metabonomics analysis of mouse urine and fecal extracts following oral treatment with the broad-spectrum antibiotic enrofloxacin (Baytril). Magnetic resonance in chemistry : MRC 2009;47 (1):S36-46.

434. Himmelreich U, Somorjai RL, Dolenko B, Lee OC, Daniel HM, Murray R, et al. Rapid identification of Candida species by using nuclear magnetic resonance spectroscopy and a statistical classification strategy. Applied and environmental microbiology 2003;69(8):4566-74.

435. Marcone MF, Wang S, Albabish W, Nie S, Somnarain D, Hill A. Diverse food-based applications of nuclear magnetic resonance (NMR) technology. Food Research International 2013;51(2):729-47.

436. Vlahov G, Rinaldi G, Del Re P, Giuliani AA. 13C nuclear magnetic resonance spectroscopy for determining the different components of epicuticular waxes of olive fruit (Olea europaea) Dritta cultivar. Analytica Chimica Acta 2008;624(2):184-94.

437. Shah PS. Parity and low birth weight and preterm birth: a systematic review and meta-analyses. Acta obstetricia et gynecologica Scandinavica 2010;89(7):862-75.

438. Kozuki N, Lee AC, Silveira MF, Sania A, Vogel JP, Adair L, et al. The associations of parity and maternal age with small-for-gestational-age, preterm, and neonatal and infant mortality: a meta-analysis. BMC public health 2013;13(3):1.

439. Vitali B, Cruciani F, Picone G, Parolin C, Donders G, Laghi L. Vaginal microbiome and metabolome highlight specific signatures of bacterial vaginosis. European Journal of Clinical Microbiology 2015;34(12):2367-76.

440. Bertini I, Calabro A, De Carli V, Luchinat C, Nepi S, Porfirio B, et al. The metabonomic signature of celiac disease. Journal of proteome research 2008;8(1):170-7.

441. Hendriks MM, van Eeuwijk FA, Jellema RH, Westerhuis JA, Reijmers TH, Hoefsloot HC, et al. Data-processing strategies for metabolomics studies. Trends in Analytical Chemistry 2011;30(10):1685-98.

442. Picone G, Mezzetti B, Babini E, Capocasa F, Placucci G, Capozzi F. Unsupervised principal component analysis of NMR metabolic profiles for the assessment of substantial equivalence of transgenic grapes (Vitis vinifera). Journal of agricultural and food chemistry 2011;59(17):9271-9.

443. Madhu B, Narita M, Jauhiainen A, Menon S, Stubbs M, Tavaré S, et al. Metabolomic changes during cellular transformation monitored by metabolite–metabolite correlation analysis and correlated with gene expression. Metabolomics 2015;11(6):1848-63.

444. Akobeng AK. Understanding diagnostic tests 1: sensitivity, specificity and predictive values. Acta paediatrica 2007;96(3):338-41.

445. Akobeng AK. Understanding diagnostic tests 3: receiver operating characteristic curves. Acta paediatrica 2007;96(5):644-7.

446. Akobeng AK. Understanding diagnostic tests 2: likelihood ratios, pre-and post-test probabilities and their use in clinical practice. Acta paediatrica 2007;96(4):487-91.

447. Altman DG, Bland JM. Statistics Notes: Diagnostic tests 2: predictive values. British Medical Journal 1994;309(6947):102.

448. Boris S, Barbés C. Role played by lactobacilli in controlling the population of vaginal pathogens. Microbes and Infection 2000;2(5):543-6.

449. Linhares IM, Summers PR, Larsen B, Giraldo PC, Witkin SS. Contemporary perspectives on vaginal pH and lactobacilli. American Journal of Obstetrics and Gynecology 2011;204(2):120. e1-. e5.

450. Dieterle F, Ross A, Schlotterbeck G, Senn H. Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in ^{1}H NMR metabonomics. Analytical chemistry 2006;78(13):4281-90.

451. Thomas MM, Sulek K, McKenzie EJ, Jones B, Han T-L, Villas-Boas SG, et al. Metabolite Profile of Cervicovaginal Fluids from Early Pregnancy Is Not Predictive of Spontaneous Preterm Birth. International journal of molecular sciences 2015;16(11):27741-8.

452. Aldunate M, Tyssen D, Latham C, Ramsland P, Perlmutter P, Moench T, et al. Vaginal Concentrations of Lactic Acid Potently Inactivate HIV-1 Compared to Short Chain Fatty Acids Present During Bacterial Vaginosis. AIDS research and human retroviruses 2014;30(S1):A228-A.

453. Aldunate M, Tyssen D, Johnson A, Zakir T, Sonza S, Moench T, et al. Vaginal concentrations of lactic acid potently inactivate HIV. Journal of Antimicrobial Chemotherapy 2013;68(9):2015-25.

454. Griffin C, Harding J, Sutton C. Re: The vaginal microbiome, vaginal anti-microbial defence mechanisms and the clinical challenge of reducing infection-related preterm birth. BJOG: an International Journal of Obstetrics and Gynaecology 2015;122(7):1033-.

455. Chaudry AN, Travers PJ, Yuenger J, Colletta L, Evans P, Zenilman JM, et al. Analysis of vaginal acetic acid in patients undergoing treatment for bacterial vaginosis. Journal of clinical microbiology 2004;42(11):5170-5.

456. Aroutcheva A, Gariti D, Simon M, Shott S, Faro J, Simoes JA, et al. Defense factors of vaginal lactobacilli. American Journal of Obstetrics and Gynecology 2001;185(2):375-9.

457. Mendes-Soares H, Suzuki H, Hickey RJ, Forney LJ. Comparative functional genomics of Lactobacillus spp. reveals possible mechanisms for specialization of vaginal lactobacilli to their environment. Journal of Bacteriology 2014;196(7):1458-70.

458. Díaz J, Chedraui P, Hidalgo L, Medina M. The clinical utility of fetal fibronectin in the prediction of pre-term birth in a low socio-economic setting hospital in Ecuador. Journal of Maternal-Fetal and Neonatal Medicine 2009;22(2):89-93.

459. Zhou M-X, Zhou J, Bao Y, Chen Y-Q, Cai C. Evaluation of the ability of cervical length and fetal fibronectin measurement to predict preterm delivery in asymptomatic women with risk factors. Journal of Maternal-Fetal and Neonatal Medicine 2014;28(2):153-7.

460. Boots AB, Sanchez-Ramos L, Bowers DM, Kaunitz AM, Zamora J, Schlattmann P. The short-term prediction of preterm birth: a systematic review and diagnostic metaanalysis. American Journal of Obstetrics and Gynecology 2014;210(1):54.e1-.e10.

461. Hermans FJ, Bruijn M, Vis JY, Wilms FF, Oudijk MA, Porath MM, et al. Risk stratification with cervical length and fetal fibronectin in women with threatened preterm labor before 34 weeks and not delivering within 7 days. Acta obstetricia et gynecologica Scandinavica 2015.

462. Bookstaver PB, Bland CM, Griffin B, Stover KR, Eiland LS, McLaughlin M. A Review of Antibiotic Use in Pregnancy. Pharmacotherapy: Journal of Human Pharmacology and Drug Therapy 2015;35(11):1052-62.

463. Atkins P, De Paula J. Physical chemistry for the life sciences: Oxford University Press; 2011.

464. Davies PG, Venkatesh B, Morgan TJ, Presneill JJ, Kruger PS, Thomas BJ, et al. Plasma acetate, gluconate and interleukin-6 profiles during and after cardiopulmonary bypass: a comparison of Plasma-Lyte 148 with a bicarbonate-balanced solution. Critical Care 2011;15(1):R21.

465. Zozaya-Hinchliffe M, Lillis R, Martin DH, Ferris MJ. Quantitative PCR assessments of bacterial species in women with and without bacterial vaginosis. Journal of clinical microbiology 2010;48(5):1812-9.

466. Fredricks DN, Fiedler TL, Thomas KK, Oakley BB, Marrazzo JM. Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. Journal of clinical microbiology 2007;45(10):3270-6.

467. Ling Z, Kong J, Liu F, Zhu H, Chen X, Wang Y, et al. Molecular analysis of the diversity of vaginal microbiota associated with bacterial vaginosis. BMC genomics 2010;11:488.

468. Kusters J, Reuland E, Bouter S, Koenig P, Dorigo-Zetsma J. A multiplex real-time PCR assay for routine diagnosis of bacterial vaginosis. European Journal of Clinical Microbiology 2015;34(9):1779-85.

469. Nelson DB, Hanlon A, Nachamkin I, Haggerty C, Mastrogiannis DS, Liu C, et al. Early Pregnancy Changes in Bacterial Vaginosis-Associated Bacteria and Preterm Delivery. Paediatric and perinatal epidemiology 2014;28(2):88-96.

470. Fredricks DN. Molecular methods to describe the spectrum and dynamics of the vaginal microbiota. Anaerobe 2011;17(4):191-5.

471. Zariffard MR, Saifuddin M, Sha BE, Spear GT. Detection of bacterial vaginosis-related organisms by real-time PCR for Lactobacilli, Gardnerella vaginalis and Mycoplasma hominis. FEMS immunology and medical microbiology 2002;34(4):277-81.

472. Walter J, Margosch D, Hammes WP, Hertel C. Detection of Fusobacterium species in human feces using genus-specific PCR primers and denaturing gradient gel electrophoresis. Microbial Ecology in Health and Disease 2002;14(3):129-32.

473. Bernhard AE, Field KG. A PCR assay To discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. Applied and environmental microbiology 2000;66(10):4571-4.

474. Ke D, Menard C, Picard FJ, Boissinot M, Ouellette M, Roy PH, et al. Development of conventional and real-time PCR assays for the rapid detection of group B streptococci. Clinical Chemistry 2000;46(3):324-31.

475. Spiegel C, Amsel R, Holmes K. Diagnosis of bacterial vaginosis by direct gram stain of vaginal fluid. Journal of clinical microbiology 1983;18(1):170-7.

476. Giacomini G, Calcinai A, Moretti D, Cristofani R. Accuracy of cervical/vaginal cytology in the diagnosis of bacterial vaginosis. Sexually Transmitted Diseases 1998;25(1):24-7.

477. Sodhani P, Garg S, Bhalla P, Singh MM, Sharma S, Gupta S. Prevalence of bacterial vaginosis in a community setting and role of the pap smear in its detection. Acta cytologica 2005;49(6):634-8.

478. Morgan XC, Huttenhower C. Chapter 12: human microbiome analysis. PLoS Computational Biology 2012;8(12):e1002808.

479. Meltzer MC, Desmond RA, Schwebke JR. Association of Mobiluncus curtisii with recurrence of bacterial vaginosis. Sexually Transmitted Diseases 2008;35(6):611.

480. Menard JP, Fenollar F, Henry M, Bretelle F, Raoult D. Molecular quantification of Gardnerella vaginalis and Atopobium vaginae loads to predict bacterial vaginosis. Clinical infectious diseases 2008;47(1):33-43.

481. Nelson DB, Hanlon A, Hassan S, Britto J, Geifman-Holtzman O, Haggerty C, et al. Preterm labor and bacterial vaginosis-associated bacteria among urban women. Journal of perinatal medicine 2009;37(2):130-4.

482. Menard JP, Mazouni C, Salem-Cherif I, Fenollar F, Raoult D, Boubli L, et al. High vaginal concentrations of Atopobium vaginae and Gardnerella vaginalis in women undergoing preterm labor. Obstetrics and Gynecology 2010;115(1):134-40.

483. Obata-Yasuoka M, Ba-Thein W, Hamada H, Hayashi H. A multiplex polymerase chain reaction-based diagnostic method for bacterial vaginosis. Obstetrics and Gynecology 2002;100(4):759-64.

484. De Backer E, Verhelst R, Verstraelen H, Alqumber MA, Burton JP, Tagg JR, et al. Quantitative determination by real-time PCR of four vaginal Lactobacillus species, Gardnerella vaginalis and Atopobium vaginae indicates an inverse relationship between L. gasseri and L. iners. BMC microbiology 2007;7:115.

485. Nejad VM, Shafaie S. The association of bacterial vaginosis and preterm labor. Journal-Pakistan Medical Association 2008;58(3):104-106.

486. Vouga M, Greub G, Prod'hom G, Durussel C, Roth-Kleiner M, Vasilevsky S, et al. Treatment of genital mycoplasma in colonized pregnant women in late pregnancy is associated with a lower rate of premature labour and neonatal complications. Clinical Microbiology and Infection 2014;20(10):1074-9.

487. Heath PT, Jardine LA. Neonatal infections: group B streptococcus. Clinical evidence 2010;2010.

488. Bretelle F, Rozenberg P, Pascal A, Favre R, Bohec C, Loundou A, et al. High Atopobium vaginae and Gardnerella vaginalis Vaginal Loads Are Associated With Preterm Birth. Clinical Infectious Diseases 2015;60(6):860-7.

489. Machado A, Cerca N. Influence of Biofilm Formation by Gardnerella vaginalis and Other Anaerobes on Bacterial Vaginosis. Journal of Infectious Diseases 2015:jiv338.

490. Gilbert NM, Lewis WG, Lewis AL. Clinical features of bacterial vaginosis in a murine model of vaginal infection with Gardnerella vaginalis. PloS ONE 2013;8(3).

491. Sobel JD. Bacterial vaginosis-an ecologic mystery. Annals of internal medicine 1989;111(7):551-3.

492. Spiegel CA, Amsel R, Eschenbach D, Schoenknecht F, Holmes KK. Anaerobic bacteria in nonspecific vaginitis. New England Journal of Medicine 1980;303(11):601-7.

493. Forsum U, Holst E, Larsson P-G, Vasquez A, Jakobsson T, Mattsby-Baltzer I. Bacterial vaginosis–a microbiological and immunological enigma. Acta pathologica, microbiologica, et immunologica Scandinavica 2005;113(2):81-90.

494. Marrazzo JM. A persistent (ly) enigmatic ecological mystery: bacterial vaginosis. Journal of Infectious Diseases 2006;193(11):1475-7.

495. Pybus V, Onderdonk AB. Evidence for a commensal, symbiotic relationship between Gardnerella vaginalis and Prevotella bivia involving ammonia: potential significance for bacterial vaginosis. Journal of infectious diseases 1997;175(2):406-13.

496. Holst E, Goffeng AR, Andersch B. Bacterial vaginosis and vaginal microoganisms in idiopathic premature labour and association with pregnancy outcome. Journal of clinical microbiology 1994;32(1):176-86.

497. Gatti M. Isolation of Mobiluncus species from the human vagina. Zentralblatt für Bakteriologie 2000;289(8):869-78.

498. Roberts MC, Hillier SL, Schoenknecht FD, Holmes KK. Comparison of gram stain, DNA probe, and culture for the identification of species of Mobiluncus in female genital specimens. Journal of Infectious Diseases 1985;152(1):74-7.

499. Holst E, Wathne B, Hovelius B, Mårdh P-A. Bacterial vaginosis: microbiological and clinical findings. European journal of clinical microbiology 1987;6(5):536-41.

500. Schwebke JR, Lawing LF. Prevalence of Mobiluncus spp among women with and without bacterial vaginosis as detected by polymerase chain reaction. Sexually Transmitted Diseases 2001;28(4):195-9.

501. Menon R, Conneely KN, Smith AK. DNA methylation: an epigenetic risk factor in preterm birth. Reproductive Sciences 2012;19(1):6-13.

502. Gracie SK, Lyon AW, Kehler HL, Pennell CE, Dolan SM, McNeil DA, et al. All Our Babies Cohort Study: recruitment of a cohort to predict women at risk of preterm birth through the examination of gene expression profiles and the environment. BMC pregnancy and childbirth 2010;10(1):87.

503. Iams JD, Goldenberg RL, Meis PJ, Mercer BM, Moawad A, Das A, et al. The length of the cervix and the risk of spontaneous premature delivery. New England Journal of Medicine 1996;334(9):567-72.

504. Jespers V, Crucitti T, Menten J, Verhelst R, Mwaura M, Mandaliya K, et al. Prevalence and correlates of bacterial vaginosis in different sub-populations of women in sub-Saharan Africa: a cross-sectional study. PLoS ONE 2014;9(10): e109670.

505. Jespers V, van de Wijgert J, Cools P, Verhelst R, Verstraelen H, Delany-Moretlwe S, et al. The significance of Lactobacillus crispatus and L. vaginalis for vaginal health and the negative effect of recent sex: a cross-sectional descriptive study across groups of African women. BMC infectious diseases 2015;15(1):115.

506. Suhag A, Berghella V. Short Cervical Length Dilemma. Obstetrics and Gynecology Clinics of North America 2015;42(2):241-54.

507. Dodd JM, Jones L, Flenady V, Cincotta R, Crowther CA. Prenatal administration of progesterone for preventing preterm birth in women considered to be at risk of preterm birth. Cochrane database of systematic reviews 2013; 7: CD004947.

508. Gonzalez Garay AG, Reveiz L, Velasco Hidalgo L, Solis Galicia C. Ambroxol for women at risk of preterm birth for preventing neonatal respiratory distress syndrome. Cochrane database of systematic reviews 2014;10:CD009708.

509. Foroozanfard F, Tabasi Z, Mesdaghinia E, Sehat M, Mahdian M. Cervical length versus vaginal PH in the second trimester as preterm birth predictor. Pakistan Journal of Medical Sciences 2015; 31(2): 374–378.

510. Berthelot-Ricou A, Tur S, Guidicelli B, Capelle M, Gamerre M, Courbiere B. Pronostic risk assessment of delivery, in patients admitted for threatened preterm birth, in a tertiary

care maternity center. Journal de gynecologie, obstetrique et biologie de la reproduction 2014;43(8):600-9.

511. Markova I, Nikolov A, Markov P. Preliminary results of fetal fibronectin testing in twin pregnancies. Akusherstvo i ginekologiia 2014;53(6):4-8.

512. Hadži-Lega M, Markova AD, Stefanovic M, Tanturovski M. Correlation of cervical length, fetal fibronectin, phIGFBP-1, and cytokines in spontaneous preterm birth up to 14 days from sampling. Journal of perinatal medicine 2014;43(5):545-551.

513. Lockwood CJ. Risk factors for preterm birth and new approaches to its early diagnosis. Journal of perinatal medicine 2015;43(5):499-501.

514. Deplagne C, Maurice-Tison S, Coatleven F, Vandenbossche F, Horovitz J. Sequential use of cervical length measurement before fetal fibronectin detection to predict spontaneous preterm delivery in women with preterm labor. Journal de gynecologie, obstetrique et biologie de la reproduction 2010;39(7):575-83.

515. Deshpande S, van Asselt A, Tomini F, Armstrong N, Allen A, Noake C, et al. Rapid fetal fibronectin testing to predict preterm birth in women with symptoms of premature labour: a systematic review and cost analysis. Health Technology Assessment 2013; 17.40

516. Di Stefano L, Carta G, Di Paolantonio L, Palermo P, Moscarini M. Preterm delivery: predictive value of cervico-vaginal fetal fibronectin. Clinical and experimental obstetrics and gynecology 1999;26(3-4):187.

517. Audibert F, Fortin S, Delvin E, Djemli A, Brunet S, Dubé J, et al. Contingent use of fetal fibronectin testing and cervical length measurement in women with preterm labour. Journal of obstetrics and gynaecology Canada 2010;32(4):307-12.

518. Dutta D, Norman J. The efficacy of fetal fibronectin testing in minimising hospital admissions, length of hospital stay and cost savings in women presenting with symptoms of pre-term labour. Journal of Obstetrics and Gynaecology 2010;30(8):768-73.

519. Sen T, Dutta A, Maity G, Chatterjee A. Fibronectin induces matrix metalloproteinase-9 (MMP-9) in human laryngeal carcinoma cells by involving multiple signaling pathways. Biochimie 2010;92(10):1422-34.

520. Esparza J, Vilardell C, Calvo J, Juan M, Vives J, Urbano-Márquez A, et al. Fibronectin upregulates gelatinase B (MMP-9) and induces coordinated expression of gelatinase A (MMP-2) and its activator MT1-MMP (MMP-14) by human T lymphocyte cell lines. A process repressed through RAS/MAP kinase signaling pathways. Blood 1999;94(8):2754-66.

521. Thant AA, Nawa A, Kikkawa F, Ichigotani Y, Zhang Y, Sein TT, et al. Fibronectin activates matrix metalloproteinase-9 secretion via the MEK1-MAPK and the PI3K-Akt pathways in ovarian cancer cells. Clinical and experimental metastasis 2000;18(5):423-8.

522. Han S, Ritzenthaler JD, Sitaraman SV, Roman J. Fibronectin increases matrix metalloproteinase 9 expression through activation of c-Fos via extracellular-regulated kinase and phosphatidylinositol 3-kinase pathways in human lung carcinoma cells. Journal of Biological Chemistry 2006;281(40):29614-24.

523. Das S, Banerji A, Frei E, Chatterjee A. Rapid expression and activation of MMP-2 and MMP-9 upon exposure of human breast cancer cells (MCF-7) to fibronectin in serum free medium. Life sciences 2008;82(9):467-76.

524. Gonzalez JM, Dong Z, Romero R, Girardi G. Cervical remodeling/ripening at term and preterm delivery: the same mechanism initiated by different mediators and different effector cells. PloS ONE 2011;6(11):e26877.

525. Gonzalez JM, Franzke C-W, Yang F, Romero R, Girardi G. Complement activation triggers metalloproteinases release inducing cervical remodeling and preterm birth in mice. American journal of pathology 2011;179(2):838-49.

526. Gonzalez Velez JM. Cervical Remodeling/ripening At Term And Preterm Delivery: The Same Mechanism Initiated By Different Mediators And Different Effector Cells. Wayne State University Dissertations 2014. Paper 1135. http://digitalcommons.wayne.edu/oa_dissertations/1135

527. Sendag F, Kazandi M, Akercan F, Kazandi A, Karadadas N, Sagol S. Vaginal fluid pH, cervicovaginitis and cervical length in pregnancy. Clinical and experimental obstetrics and gynecology 2009;37(2):127-30.

528. Simhan HN, Caritis SN, Krohn MA, Hillier SL. The vaginal inflammatory milieu and the risk of early premature preterm rupture of membranes. American Journal of Obstetrics and Gynecology 2005;192(1):213-8.

529. Matijevic R, Grgic O, Knezevic M. Vaginal pH versus cervical length in the mid-trimester as screening predictors of preterm labor in a low-risk population. International Journal of Gynecology and Obstetrics 2010;111(1):41-4.

530. Hoyme U, Huebner J. Prevention of preterm birth is possible by vaginal pH screening, early diagnosis of bacterial vaginosis or abnormal vaginal flora and treatment. Gynecologic and obstetric investigation 2010;70(4):286-90.

531. Krauss-Silva L, Almada-Horta A, Alves MB, Camacho KG, Moreira MEL, Braga A. Basic vaginal pH, bacterial vaginosis and aerobic vaginitis: prevalence in early pregnancy and risk of spontaneous preterm delivery, a prospective study in a low socioeconomic and multiethnic South American population. BMC pregnancy and childbirth 2014;14(1):107.

532. Hamilton BE, Hoyert DL, Martin JA, Strobino DM, Guyer B. Annual summary of vital statistics: 2010–2011. Pediatrics 2013:peds. 2012-3769.

533. Beck S, Wojdyla D, Say L, Betran AP, Merialdi M, Requejo JH, et al. The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. Bulletin of the World Health Organization 2010;88(1):31-8.

534. Werner EF, Hamel MS, Orzechowski K, Berghella V, Thung SF. Cost-effectiveness of transvaginal ultrasound cervical length screening in singletons without a prior preterm birth: an update. American Journal of Obstetrics and Gynecology 2015;213(4):554. e1-. e6.

535. Orzechowski KM, Nicholas SS, Baxter JK, Weiner S, Berghella V. Implementation of a universal cervical length screening program for the prevention of preterm birth. American journal of perinatology 2014;31(12):1057-62.

536. Donders GG, Zodzika J, Rezeberga D. Treatment of bacterial vaginosis: what we have and what we miss. Expert opinion on pharmacotherapy 2014;15(5):645-57.