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Identification of first trimester maternal serum markers predictive of spontaneous preterm birth

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Statement of Authentication

I hereby declare that the contents of this thesis consist of original work carried out by the author unless otherwise stated and duly acknowledged. To the best of my knowledge no part of this thesis has been submitted in whole or in part for the award of any other degree of the university or other institution.

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(BSc., MPhil)

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"I can do everything through him who gives me strength." (Philippians 4: 13)

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Dedication

This thesis is dedicated to Mai and Onu

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List of Abbreviations

sPTB	Spontaneous Preterm Birth
PPRoM	Preterm Prelabour Rupture of Membranes
ART	Assisted Reproductive Technology
HCA	Histologic chorioamnionitis
MIR	Maternal Inflammatory Response
FIR	Fetal Inflammatory Response
2DE	Two-Dimensional Electrophoresis
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
MW	Molecular Weight
pI	Isoelectric point
cCBB	Colloidal Coomassie Brilliant Blue
IEF	Isoelectric Focussing
LC/MS/MS	Liquid Chromatography Mass Spectrometry/Mass Spectrometry
MALDI	Matrix Assisted Laser Desorption/Ionization
MMP	Matrix Metalloproteinase
TLRs	Toll Like Receptors
IL	Interleukin
IL-6	Interleukin-6
IL-2	Interleukin 2
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-1β	Interleukin-1β
TNF	Tumor Necrosis Factor
TGF – β	Transforming Growth Factor β
CRP	C- Reactive Protein
PAPP-A	Pregnancy Associated Placental Protein
βHCG	beta Human Chorionic Gonadotropin
phIGFBP-1	Phosphorylated Insulin-like Growth Factor Binding Protein-1
CVF	Cervico-Vaginal Fluid
MRM	Multiple Reaction Monitoring
SRM	Selected Reaction Monitoring

MRM/SRM	Multiple Reaction Monitoring/ Selected Reaction Monitoring
fFN	Fetal fibronectin
QfFN	Quantitative Fetal fibronectin

Abstract

Preterm birth is the leading cause of perinatal morbidity and mortality worldwide. Despite considerable efforts, prediction and prevention of preterm birth continues to remain a challenge for obstetricians globally. Early identification of pregnancies at highest risk of preterm birth may enable the implementation of therapeutic strategies aiming to prevent preterm birth and/or the morbidities associated with early delivery.

Screening for spontaneous preterm birth is made more complex due to the heterogeneity of this condition, which has a variety of underlying aetiologies and risk factors. Even though spontaneous preterm birth is caused by several aetiologies, there appears to be a final common pathway leading to the onset of labour. There may therefore be value either in developing screening tools that screen for multiple aetiological pathways or alternatively that identify common features that develop before women become symptomatic with the onset of spontaneous labour. **Paper I** reviews recent research findings related to first trimester prediction and prevention of adverse pregnancy outcomes.

This thesis reports a body of work related to the development of a predictive test for spontaneous preterm labour. **Paper II** is focussed on the challenges of using current proteomic strategies to identify and quantify novel protein markers of disease in serum. I carried out various optimisation strategies to resolve protein species in maternal serum using a refined top-down two-dimensional gel electrophoresis method coupled with mass spectrometry. In addition to this, a process of deep imaging using third separation gel electrophoresis was adapted to effectively resolve protein species and isoforms that would not be recognised by traditional proteomic techniques as they would be masked by co-migrating protein species of higher abundance.

These techniques were applied in **Paper III** where they were used to identify protein species and post translationally modified proteoforms (phosphorylation and glycosylation) in first trimester maternal serum banked from cohorts of women who delivered spontaneously before 37 weeks' gestation. These findings were compared to serum collected from a cohort of women who delivered at term (≥ 37 weeks' gestation).

Paper IV utilised a western blot approach to determine serum concentrations of a select group of candidate protein species and proteoforms that were significantly altered in Paper II in a larger cohort of women that had delivered after spontaneous preterm labour (<37 weeks) compared to matched term (≥37 weeks) controls. A variant of Vitamin D-binding protein was found to be significantly decreased in women who delivered < 37 weeks spontaneously.

This work has shown that there is evidence of change in protein abundance as early as 11-13 weeks of gestation in women who continue on to deliver preterm after the spontaneous onset of labour. Further work is needed to determine the strength of these findings in predicting risk of preterm birth. Further work should also examine how novel biomarkers can be combined with established screening tools in larger diverse patient cohorts to validate their potential use as candidates for prediction of spontaneous preterm birth.

Thesis Structure

The work presented in this thesis describes an investigation into first trimester maternal serum biomarkers predictive of spontaneous preterm birth.

These studies are provided as a series of papers (listed below). The papers are either published (Paper I, II, III) or submitted to journals for peer-review (Paper IV).

I. D'Silva A, Fyfe R, Hyett J. First trimester prediction and prevention of adverse pregnancy outcomes related to poor placentation. Current Opinion in Obstetrics & Gynaecology. 2017; 29(6): 367-74.

II. D'Silva AM, Hyett JA, Coorssen JR. A Routine 'Top-Down' Approach to Analysis of the Human Serum Proteome. Proteomes. 2017;5(2).

III. D'Silva AM, Hyett JA, Coorssen JR. Proteomic analysis of first trimester maternal serum to identify candidate biomarkers potentially predictive of spontaneous preterm birth. Journal of Proteomics. 2018.

IV. Validation of protein biomarkers for risk of spontaneous preterm birth. (Submitted to Fetal Diagnosis and Therapy).

Conference Proceedings

This work has been disseminated at the following meetings:

D'Silva, A, Hyett Jon, Coorssen J. **Identification of Post Translational Modifications of First Trimester Maternal Serum Proteins that are Predictive for Spontaneous Preterm Birth**. Oral presentation at the 21st International Conference for Prenatal Diagnosis and Therapy (ISPD), 9-12th July, 2017, San Diego, California, USA

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Coorssen J, **D'Silva A**, Hyett J. **Post Translational Modifications of First Trimester Proteins that are Predictive for Spontaneous Preterm birth.** International Conference on Analytical Proteomics, 3rd – 6th July, 2017, Caparica, Portugal.

D'Silva A, Hyett Jon, Coorssen Jens R. **A Top-Down Proteomic Approach to the Identification of Early Serum Biomarkers Predictive of Spontaneous Preterm birth.** Poster presentation at The 21st Annual *Lorne* Proteomics Symposium, February 4th – 7th, 2016, Lorne, Victoria, Australia.

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Introduction

1.1 Background

1.1.1 Definition of preterm birth

The World Health Organization defines preterm birth as a livebirth that occurs before 37 completed weeks of pregnancy (Howson, 2012). Preterm birth can be further sub-categorised on the basis of gestational age as extremely preterm (less than 28 completed weeks of gestation), very preterm (28 to 32 completed weeks of gestation) and moderate to late preterm (32 to 36 completed weeks of gestation) (Quinn et al., 2016).

1.1.2 Characterisation of preterm birth

There are three broad clinical categories that describe preterm birth: Preterm birth may be medically indicated (iatrogenic), occur after preterm premature rupture of membranes (PPRoM) or after the spontaneous (idiopathic) onset of labour (Savitz et al., 1991, Meis et al., 1987). Whilst iatrogenic preterm birth is easy to identify, it is sometimes harder to discriminate between spontaneous preterm birth (sPTB) and labour that occurs secondary to PPRoM. Typically, PPRoM is defined in circumstances where spontaneous rupture of membranes occurs at least one hour prior to the onset of contractions, at less than 37 weeks' gestation (2018) .

1.1.3 Worldwide incidence and associated morbidity and mortality

Preterm birth is the single largest direct cause of perinatal morbidity and mortality in the developed world and has long-term adverse consequences for health (Blencowe et al., 2013). Preterm neonates have higher rates of cerebral palsy, sensory deficits, respiratory illnesses and learning disabilities as well as long term health complications resulting in enormous physical and economic costs to healthcare systems as well as emotional and financial distress to affected families (Wu and Colford, 2000, Tronnes et al., 2014, Wickremasinghe et al., 2013, Kwinta and Pietrzyk, 2010, Harris et al., 2013, Khan et al., 2015).

Worldwide, an estimated 15 million babies are born preterm each year (Blencowe et al., 2012) (Figure 1.1). Preterm birth accounts for 28% of all neonatal deaths globally (Lawn et al., 2006b). The rate of preterm birth has been reported to

range from 5% - 9% of live births in some developed countries and is estimated to be significantly higher in developing countries (Lawn et al., 2006a). Global rates of preterm birth have increased over the past two decades (Davidoff et al., 2006, Blencowe et al., 2012, Ananth et al., 2005, MacDorman et al., 2010).

About 30-35% of all preterm births are attributed to medically indicated preterm birth that occurs in the absence of PPRoM or spontaneous preterm labour (Ananth et al., 2005, Ananth and Vintzileos, 2006a). Medical indications relate to maternal complications (gestational diabetes, preeclampsia), fetal complications (growth restriction, multiple pregnancies, congenital anomalies, threatened fetal well-being) and placental complications (Xue et al., 2016, Ananth and Vintzileos, 2006b). Cases of iatrogenic prematurity are rare and occur in instances when a baby is compromised and suddenly stops moving or when a woman is induced <37 weeks due to incorrect dating (Moutquin, 2003).

Premature preterm rupture of membranes, usually followed by preterm delivery, accounts for another 30% of all preterm births (Caughey et al., 2008). Women of African-American race and those from lower socioeconomic populations are more prone to PPRoM (Shen et al., 2008). There is overwhelming evidence suggesting infection as the primary cause of labour in women affected by PPRoM (Menon and Fortunato, 2007, Goldenberg et al., 2000a, Al Riyami et al., 2013).

About 40% of all preterm births are spontaneous or ‘idiopathic’ and are more common in women without any recognized risk factors (Goldenberg et al., 2008). Early spontaneous preterm birth (<32 weeks' gestation) is linked to increased perinatal mortality (Costeloe et al., 2012, Vohr et al., 2000, Ancel, 2015). Although the risk factors associated with sPTB vary according to gestational age and are dependent on various social and environmental factors, the exact cause(s) and mechanism(s) associated with this condition remain unknown in more than 50% of the cases (Menon, 2008).

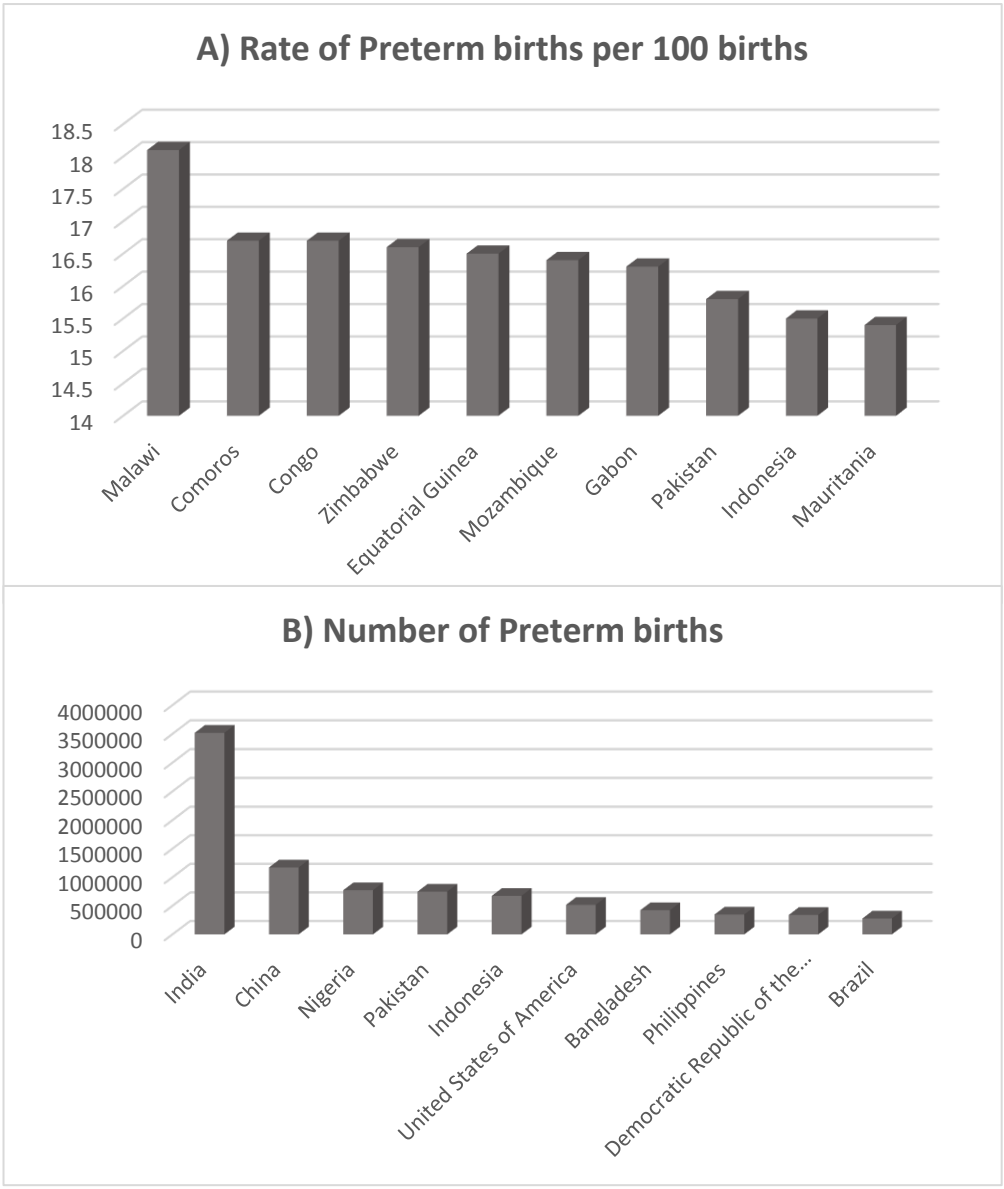

Figure 1.1: Worldwide rates of preterm birth. Estimated preterm birth rates and total number of preterm births by Millennium Development Goal region. Graphical representation of A) 10 countries with the highest rates of preterm birth per 100 live births, B) 10 countries with the greatest number of preterm births. Adapted from *Blencowe, H. et al., 2012.*

1.1.4 Preterm birth in Australia

There are 25,000 infants born prematurely in Australia each year — 8.7% of all births - seventy each day – more than one every half hour (AIHW, 2011). Almost 15% of all preterm babies need an increased level of care at birth (AIHW, 2016). Most of these preterm births (81%) belong to the late preterm birth category, followed by 11% born extremely preterm and 10% born very preterm (AIHW, 2016).

In the most recent published data, the average gestational age for all preterm birth was 33.3 weeks. The highest proportion (10.6%) of preterm births was reported in the Northern Territory while New South Wales had the lowest, at 7.4% of all births (AIHW, 2016). Among infants of Aboriginal and Torres Strait Islander mothers, preterm birth is a significant contributor to neonatal morbidity and mortality (AIHW, 2016). Preterm babies need continuous high-level support and care. Therefore, they are more likely to be admitted to a neonatal intensive care unit (72%) compared to babies delivered at term (10%) or post-term (13%) (AIHW, 2016).

Several maternal risk factors have been identified that are associated with preterm birth. In 2015, 14% of babies born to Aboriginal and Torres Strait Islander mothers were preterm, in contrast to 8.0% babies born to mothers of non-Indigenous origin (AIHW, 2016). 13% babies were born preterm to mothers who smoked during pregnancy in comparison to 8% of babies whose mothers did not smoke (AIHW, 2016). Around 64% of twins and all other multiples were born preterm in 2015 in comparison to 7% of singleton babies (AIHW, 2016). 11% and 12% of preterm babies were born to younger (< 20) and older (≥ 40) mothers compared with 8% of babies with mothers aged 20–39 (AIHW, 2016).

1.2 Pathophysiology of preterm birth

1.2.1 Causes and risk factors

Preterm labour, as described by Romero, is a 'syndrome initiated by multiple mechanisms, including infection or inflammation, uteroplacental ischaemia or haemorrhage, uterine overdistension, stress, and other immune mediated processes' (Romero et al., 2006) (Figure 1.2). The exact causative mechanism is typically not recognized in most individual cases; but risk factors, based on maternal characteristics and medical history, associated with these aetiologies can be elucidated and are often used as a basis to explain preterm labour. These risk factors are often associated with an increase in systemic inflammation involved in the common pathway to sPTB (Boyle et al., 2017). Identification of these risk factors may also be of value in predicting a population at increased risk of preterm birth.

1.2.1.1 Maternal Risk Factors

The risk of preterm birth can be directly correlated to maternal characteristics, varies between racial and ethnic groups and increases with advanced maternal age. The occurrence of preterm birth in African-American women (16–18%) is higher than that in Caucasian women (5–9%) in the United Kingdom and this is even more noticeable in the United States (MacDorman, 2011, Mohamed et al., 2014). When compared to women of other ethnic backgrounds, African-American women are three to four times more likely to have a very early preterm birth (Martin et al., 2006, Burris et al., 2011). The rate of preterm birth is typically low in women of East Asian and Hispanic origin and has not significantly increased in women from South Asia including the Indian subcontinent (Goldenberg and Culhane, 2007). The mechanism for these disparities is poorly understood, and the disparities persist even after socioeconomic factors are taken into consideration.

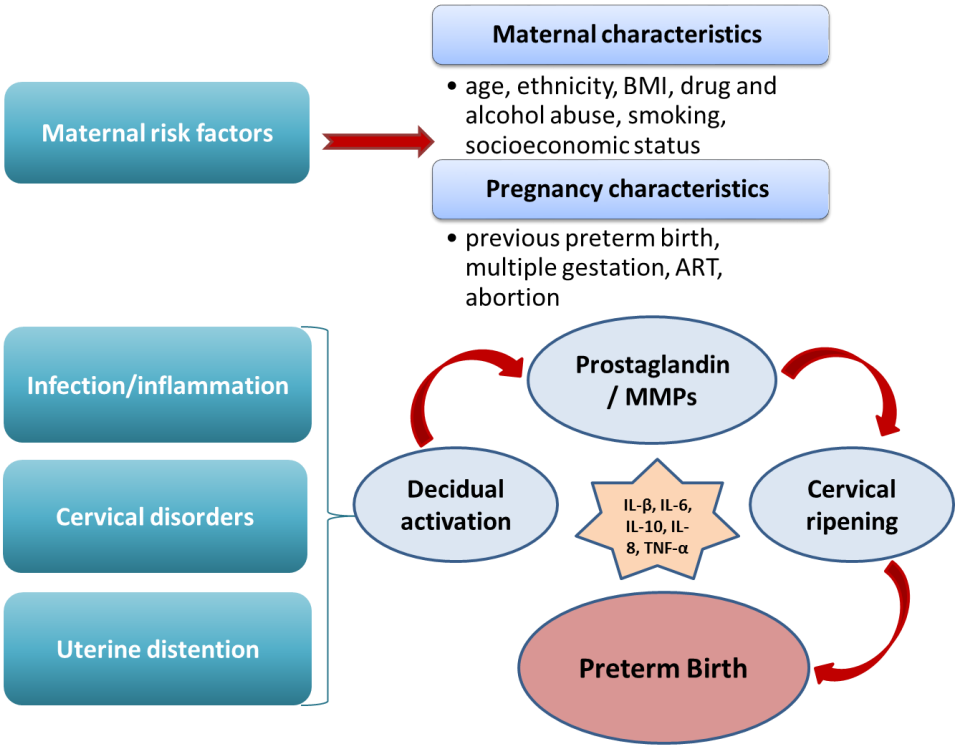

Figure 1.2: Risk factors and pathological pathways associated with preterm birth. Various maternal risk factors and proposed pathways of infection, cervical disorders and uterine distention that result in preterm birth.

1.2.1.2 Socioeconomic status

A significant socioeconomic risk factor for preterm birth is maternal smoking. Women who smoke cigarettes are twice as likely to deliver before 32 weeks' gestation as non-smoking mothers (Wisborg et al., 1996, Kyrklund-Blomberg et al., 2005). Programmes aimed at creating awareness to cease smoking during pregnancy have been found to be valuable in lowering the rate of preterm birth (Lumley et al., 2004). Preterm birth is strongly linked to lower levels of education, lower family income, access to prenatal care services and adverse neighbourhood conditions (Jansen et al., 2009, Silveira et al., 2018, Giurgescu et al., 2012). Population based studies suggest an increased risk of preterm and induced preterm birth in women with low BMI prior to pregnancy as well as in women who are overweight and obese (Han et al., 2011, Cnattingius et al., 2013) (Figure 1.3). Furthermore, alcohol and drug abuse, nutritional status, chronic diseases (such as hypertension, diabetes mellitus), and endocrinological disorders have been correlated with an elevated risk of preterm birth (Velez et al., 2008b, Xinxo et al., 2013, Catov et al., 2008, Yanit et al., 2012, Sheehan et al., 2015).

1.2.1.3 Psychosocial factors

Mothers encountering mental or social pressures during pregnancy are at a 2-fold increased risk of preterm birth (Lilliecreutz et al., 2016). Several investigations have associated stress and anxiety related to housing instability or marital hardships with risk of preterm delivery (El-Sayed et al., 2012). Although there is no known mechanism to explain the correlation of stress and risk of preterm birth, recent studies have suggested elevated levels of corticotrophin-releasing hormone (CRH) associated with stress and subsequently leading to preterm birth (Ruiz et al., 2016). During the third trimester of pregnancy, CRH is synthesized by the placenta and released in significant amounts in the maternal circulations (Sandman and Glynn, 2009). It has been labelled as a 'master stress hormone' crucial to maintain pregnancy and fetal development (Ellman et al., 2008). Similarly, high levels of C-reactive protein (CRP), an indicator of inflammation, involved in the innate immune response has been reported in women exposed to stressful conditions (Sproston and Ashworth, 2018). Together, these findings suggest possible roles of these stress related molecules in systemic inflammation pathways leading to preterm birth.

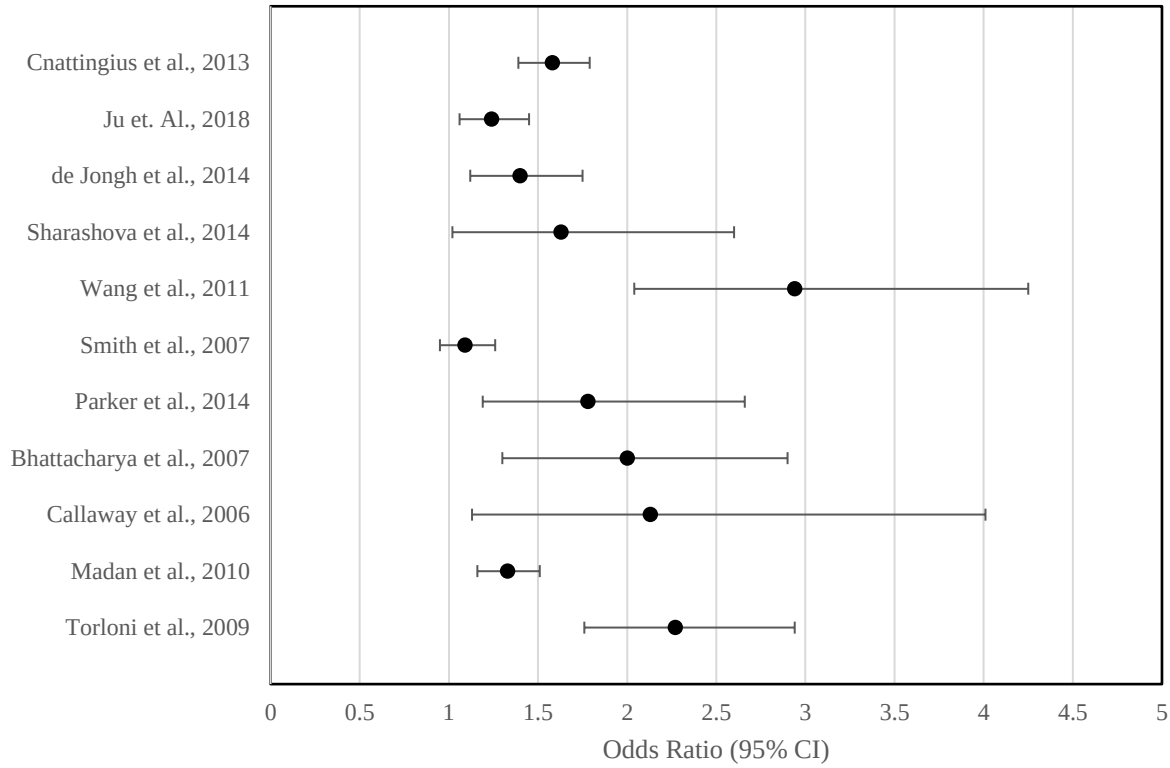

Figure 1.3: Forest plot showing studies reporting the odds ratio for preterm birth related to obesity.

1.2.1.4 Pregnancy characteristics

Pregnancy characteristics such as previous abortion, and pregnancies resulting from assisted reproductive treatment (ART) are important factors that predispose a woman to preterm birth (Freak-Poli et al., 2009, Ancel et al., 2004, Cavoretto et al., 2018) (Figure 1.4). Likewise, studies have shown a greater risk of preterm delivery (spontaneous and induced) in women undergoing invasive diagnostic procedures such as genetic amniocentesis (Medda et al., 2003).

Preterm birth is highly prevalent in multiple gestations, accounting for 15-20% of preterm births (Fuchs and Senat, 2016). Almost 60% of twins are born premature (Fuchs and Senat, 2016, National Collaborating Centre for and Children's, 2011). Nearly 40% of twins will be delivered spontaneously or their pregnancies will be interrupted by PPRoM <37 weeks' gestation, with others having an indicated preterm delivery due to maternal or fetal complications. A report from the Australian Institute of Health and Welfare states that twins and multiple gestations comprise 64% of preterm births as of 2015 (AIHW, 2017).

Women with pregnancy intervals of less than six months are at a two-fold greater risk of delivering prematurely in their following pregnancy (Smith et al., 2003). There is consistent evidence that having a previous sPTB is a strong predicting factor for recurrence in consequent pregnancies (Iams et al., 1998, Ekwo and Moawad, 1998, Kristensen et al., 1995). There is insufficient research to explain the underlying mechanism(s); a potential theory is that the uterus and uterine environment requires time to return to its quiescence state (Kota et al., 2013). Another possible theory is that a short pregnancy interval does not allow enough time to replenish essential vitamins and minerals that are depleted during the previous pregnancy (Gernand et al., 2016).

1.2.1.5 Obstetric complications

Uterine over-distension appears to play a significant role in initiating preterm labour in women with multiple gestations, polyhydramnios, and macrosomia, but the pathophysiology is yet not clearly understood (Many et al., 1996, Adams Waldorf et al., 2015) . An increased risk of preterm birth is often associated with vaginal bleeding due to placental abruption or praevia (Erez et al., 2012). However, bleeding independent of either placental abruption or praevia has also been linked with

consequent preterm birth. Women with cervical disorders such as cervical intraepithelial neoplasia have a greater risk for prematurity (Danhof et al., 2015, Kyrgiou et al., 2017). Additionally, local cervical treatment (e.g. large loop excision of transformation zone procedure) and maternal abdominal surgery predispose women to preterm delivery (Sadler et al., 2004, Jin et al., 2014) (Figure 1.5).

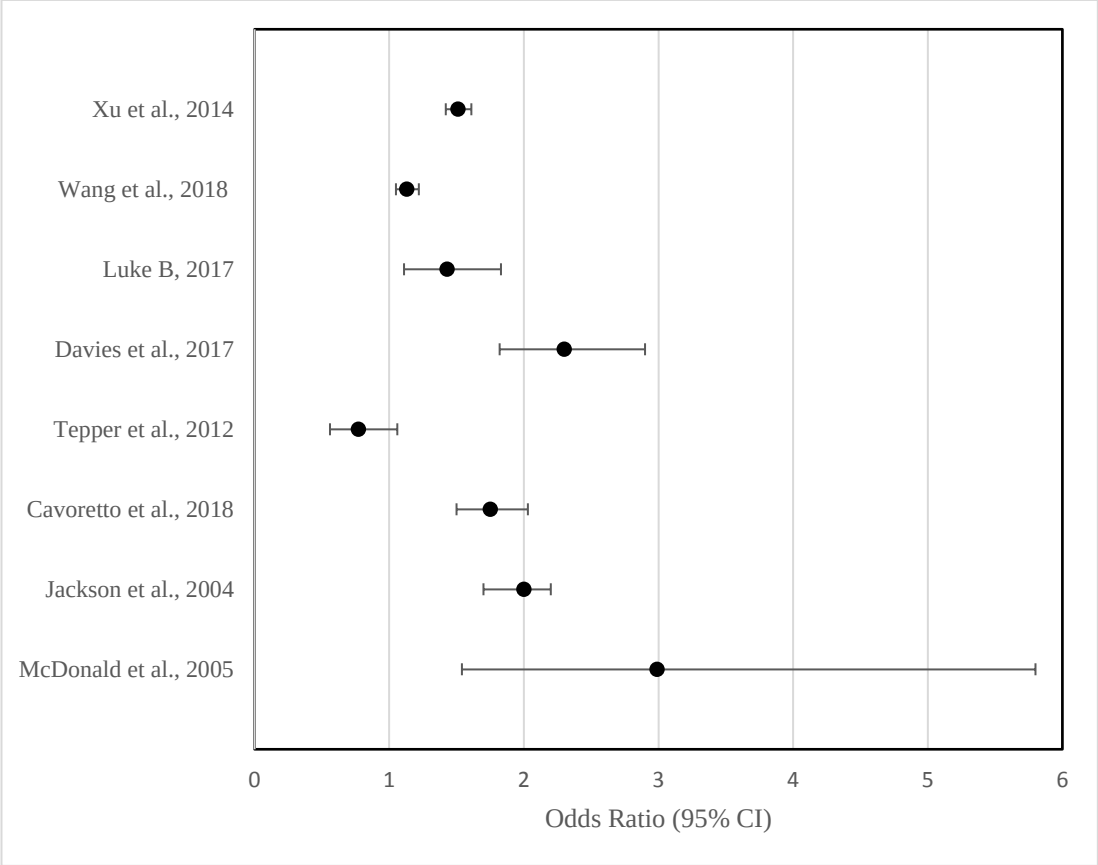

Figure 1.4: Forest plot showing studies reporting the odds ratio for preterm birth related to use of assisted reproductive technologies.

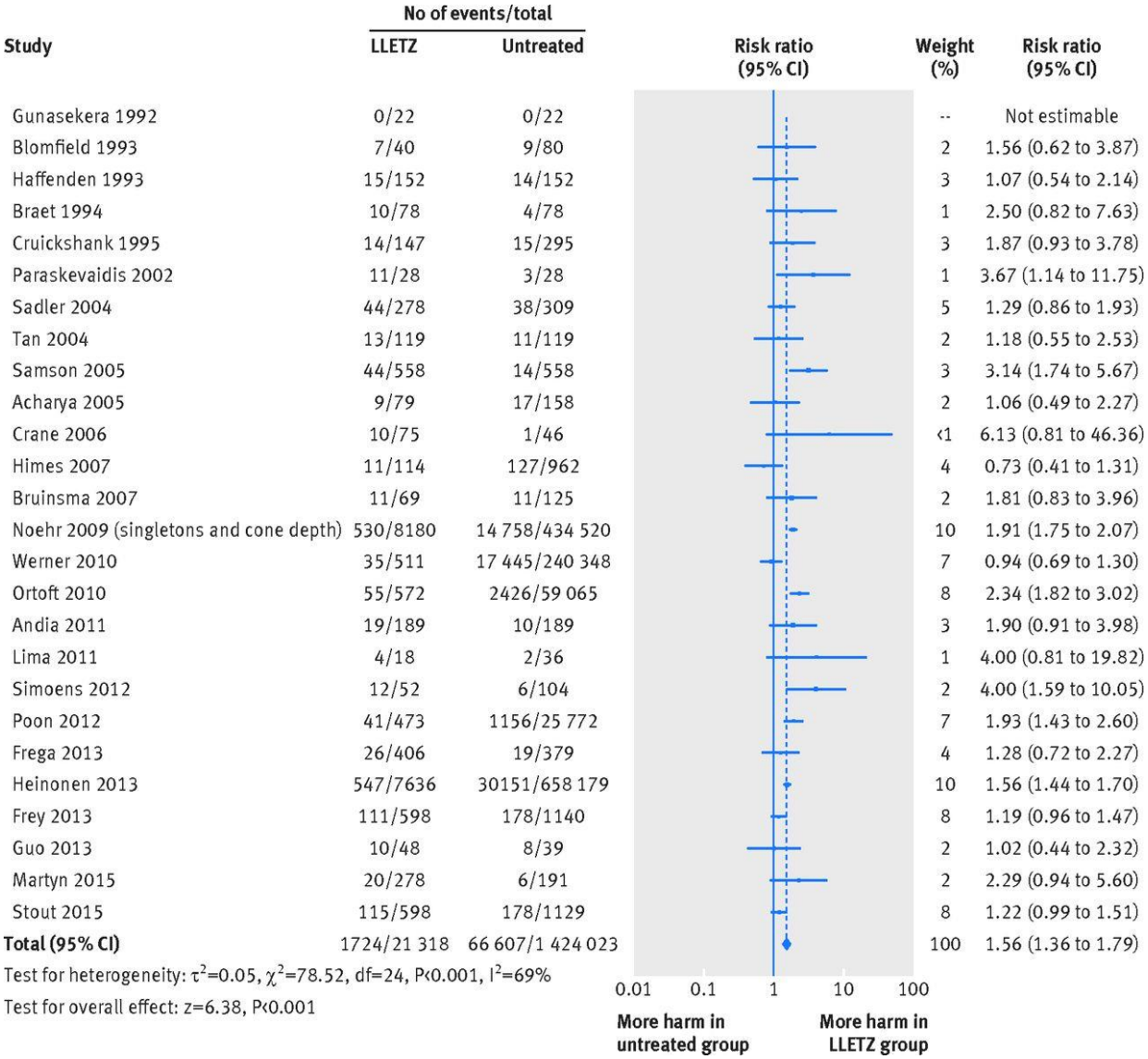

Figure 1.5: Forest plot showing studies reporting risk ratio for preterm birth in women treated with large loop excision of transformation zone versus untreated women (*Kyrgiou et al., 2016).*

1.2.1.6 Cervical insufficiency

During a normal pregnancy, the cervix remains firm, long and closed (Myers et al., 2015). The cervix shortens, softens and dilates as labour approaches (Gravett et al., 2010). Cervical insufficiency occurs when the cervix shortens and dilates before term either due to a short cervix or when there is increasing pressure on the cervix due to increasing weight of the baby as the pregnancy advances (Vink and Feltovich, 2016). Ultrasound assessments of the cervix have confirmed cervical shortening as a risk factor for preterm delivery, proving to be a valued clinical tool in asymptomatic women and in those presenting with contractions. A cervical length of less than 25 mm during the second trimester of pregnancy is used clinically as a reliable predictor of preterm birth in the asymptomatic population (McIntosh et al., 2016, Celik et al., 2008). A short cervix is directly proportional to higher risk of preterm birth (Figure 1.6). Some preterm births are caused due to cervical insufficiency related to genetic cervical weakness or trauma (Anum et al., 2009). However, there is no proven method to identify cervical insufficiency from cervical shortening due to other causes, and the exact involvement in preterm birth is not known.

1.2.1.7 Intrauterine infection

Intrauterine infection is a significant contributor to preterm birth and involves activation of the innate immune system (Keelan, 2018). Toll-like receptors (TLRs) are cell-surface proteins responsible for recognition of patterns of microorganisms (Akira et al., 2006). TLRs initiate the inflammatory cascade by the release of various inflammatory mediators including cytokines and chemokines (Mogensen, 2009). The release of these proinflammatory mediators results in production of prostaglandins that ultimately promote uterine contractility (Institute of Medicine Committee on Understanding Premature and Assuring Healthy, 2007).

Even though these risk factors are well recognised, a significant proportion of sPTB occurs without any apparent cause. Whilst preterm birth is seemingly multi-factorial, many of these risk factors appear to share aetiological mechanisms. A better understanding of these aetiological factors that operate through multiple pathophysiologic pathways may improve our ability to prevent spontaneous preterm birth.

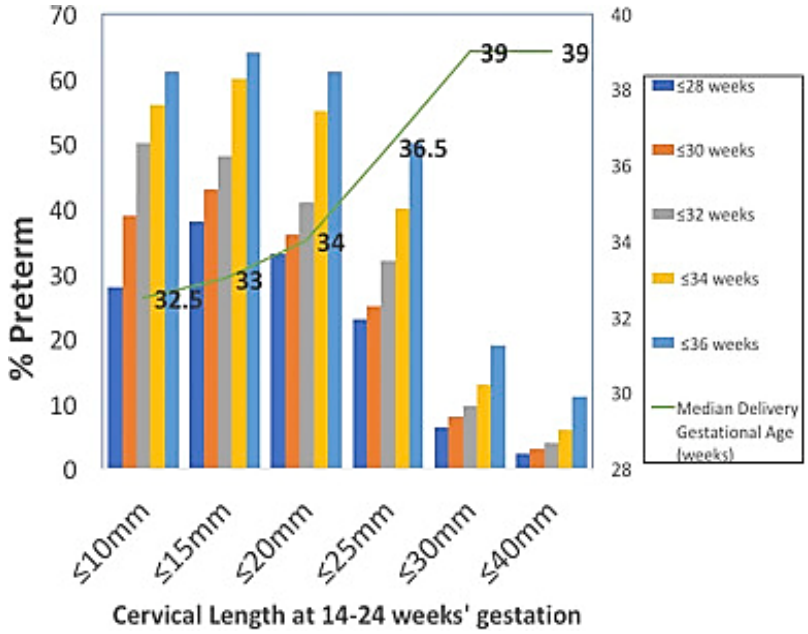

Figure 1.6: Proportion of women delivering preterm at various gestational age cut-offs according to the mid-trimester cervical length indicating that cervical length is the strongest predictor of preterm birth (*Glover and Manuck, 2018).*

1.2.2 Preterm birth: an inflammatory disorder

Conventionally, whether labour and delivery have occurred at term or preterm, they have been regarded to be driven by the same underlying pathophysiology, a process marked by production of a range of inflammatory mediators (Institute of Medicine Committee on Understanding Premature and Assuring Healthy, 2007). Labour is characterised by uterine contractility, rupture of fetal membranes and cervical remodelling (Timmons et al., 2010). This process is regulated by an increased circulation of cytokines and leukocyte activation that facilitates the transition from an inactive to a pro-inflammatory environment (Yuan et al., 2009). In normal term pregnancies, there is a withdrawal of active suppression of the decidua inducing inflammation accompanied by the release of various biologically active mediators inducing labour (Norwitz et al., 2015). Preterm birth can occur if there is dysregulation of this process earlier in gestation or if other exogenous factors activate the inflammatory cascade. This may occur, for example, if there is placental abruption or infection.

1.2.2.1 Infection and Inflammation

Infection is a significant contributor to preterm birth (Klein and Gibbs, 2005). The mechanism by which an infectious insult activates the immune system to cause inflammation remains unknown but likely involves innate immune pattern recognition receptors and their regulators. An intrauterine infection can be restricted to the decidua, spread to the amniotic sac and eventually to the fetus (DiGiulio et al., 2010) (Figure 1.7). As described above, microorganisms are recognised by the cells of the maternal-fetal interface through TLRs and nod-like receptors and activate the immuno-inflammatory cascade. These cascades may be activated by mechanisms that are not primarily caused by an infectious insult but are associated with an inflammatory response.

Although the amniotic cavity is thought to be largely sterile, accumulating evidence suggests that intra-amniotic infection can be caused by genital Mycoplasmas (Kim et al., 2015a). Microorganisms invade the amniotic cavity by various mechanisms (Goncalves et al., 2002, Goldenberg et al., 2008, Goldenberg et al., 2000b). These are by way of ascending infection, where organisms from the vagina

penetrate the cervix / cervical mucus barrier, blood borne infection – penetrating the placenta, iatrogenic infection – through an invasive procedure (e.g. amniocentesis) or spread of an intra-abdominal / peritoneal infection entering the uterus through the fallopian tubes. Most infections are attributed to the ascending (vaginal / cervical) path (Romero et al., 2014); although there is a consensus that ascent occurs during pregnancy (Koren et al., 2012), the precise timing is unknown. Infection likely precedes the onset of spontaneous labour and may therefore be detectable at an early stage of pregnancy.

1.2.2.2 Chorioamnionitis

Chorioamnionitis is responsible for 40% of all preterm births worldwide (Lamont, 2003). This rate is even higher in extreme sPTB as suggested by a study that reported a positive test for an infectious insult in 79% of patients who laboured spontaneously <28 weeks (Onderdonk et al., 2008, Watts et al., 1992). Chorioamnionitis involves inflammation of the amnion and chorion and is primarily caused by bacterial infection (Leviton et al., 1999, Hagberg et al., 2002). Multiple types of vaginal bacteria such as Ureaplasma urealyticum, Gardnerella vaginalis, Chlamydia trachomatis, Neisseria gonorrhoea, Mycoplasma hominis, Group B Streptococcus, and Trichomonas vaginalis are associated with inducing preterm labour (Nelson et al., 2009, Martius et al., 1988, Hillier et al., 1995).

The inflammatory process is generally observed as a continuum. The initial stages of the inflammatory response involve neutrophils of maternal origin migrating from the decidual vessels while the later stages involve neutrophils from fetal origin migrating from the umbilical cord (Kim et al., 2015a). Placental inflammation can be broadly classified as: (1) the maternal inflammatory response (MIR), inflammation infiltrating into the chorion, amnion or decidua (Redline et al., 2003); and (2) the fetal inflammatory response (FIR), inflammation extending to the chorionic plate, umbilical cord, and fetal vessels (Gomez et al., 1998). Both types of inflammation are associated with preterm birth and several neonatal complications including cerebral palsy, lung disorders, necrotizing enterocolitis, and intraventricular haemorrhage (Strunk et al., 2014, Claud, 2009, Ballabh, 2010).

Systemic involvement including hematologic abnormalities are found to be linked to fetuses that have a FIR (Romero et al., 2011). Histologic chorioamnionitis

(HCA) is linked with microbial invasion in the amniotic cavity and is considered as an indicator of maternal inflammation of the placenta, whereas funisitis, or inflammation of the umbilical cord, is a marker of fetal inflammation. Reports have suggested that 33% of neonates born prematurely have HCA (Menon et al., 2010, Thomas and Speer, 2011, Kim et al., 2015b, Bersani et al., 2012).

Clinically, intrauterine infection is diagnosed by examining the amniotic fluid and/or the placenta (Tita and Andrews, 2010). This presents a problem as this diagnosis is most easily made after delivery (allowing examination of the placenta) or requires an invasive test (amniocentesis) that carries its own risks to mother and fetus. If amniotic fluid is available, then this can be used to measure leuko-attractants, glucose concentration, white blood cell count and interleukin-6 (IL-6) and perform a gram-stain. Combinations of these tests have high sensitivity and specificity in identifying pregnancies that are infected (Romero et al., 1993, Gomez et al., 1994). In most clinical units, an amniocentesis in not routinely performed for all women attending with threatened preterm labour – in addition to the risks of invasive testing, this requires a skill set that is not readily available on most labour wards.

Histologic examination of the placenta, that allows accurate diagnosis of HCA, is regarded as the gold standard method for identifying infection – but is only possible post-delivery and cannot, therefore, be used to guide obstetric management. In recent years, several research groups have made attempts to develop non-invasive and rapid prenatal tests for diagnosing intrauterine inflammation by examining maternal blood. These tests include measuring maternal levels of CRP and other interleukins (ILs) to diagnose HCA (Le Ray et al., 2015, Samejima and Takechi, 2017, Oh et al., 2011). CRP is an acute phase reactant synthesized by the liver in response to inflammatory stimuli (Pepys and Baltz, 1983). CRP is essentially used when an obstetrician needs to monitor various inflammatory disorders including chorioamnionitis (Aggarwal and Pahwa, 2018). Several groups have confirmed increased CRP levels in maternal serum and plasma to be associated with HCA (Hvilsom et al., 2002, Pitiphat et al., 2005, Vogel et al., 2005). However, due to its low sensitivity, its application in a diagnostic setting is questionable (Stepan et al., 2016, Amirabi et al., 2012, Smith et al., 2012).

1.2.2.3 Vaginal microbiome

Several studies have found that preterm birth is associated with bacterial vaginosis (Manns-James, 2011, Subtil et al., 2002). Lactobacillus species dominate the vaginal microbiome in non-pregnant women (Nasioudis et al., 2017). Low abundance of lactobacilli and consistent presence of anaerobic bacteria (Gardnerella vaginalis, Mycoplasma hominis, Parvimonas micra, Aerococcus christensenii) are associated with preterm delivery (Stafford et al., 2017, Donders et al., 2009). While there is a significant body of literature describing a local inflammatory response in women with vaginitis due to trichomonas and candida, the same cannot be said for bacterial vaginosis despite the presence of extensive microbial growth (Rasti et al., 2014, Hosny et al., 2017). A recent study carried out DNA sequencing of the vaginal microbiome in an African-American women cohort (Stout et al., 2017). The researchers demonstrated that there was a decrease in the amounts and diversity of microbes in women who delivered preterm compared to those who delivered at term. The decrease was observed earlier in gestation suggesting that this period is crucial for determining subsequent term and preterm events.

Although there is convincing evidence of a causal association between intrauterine infection and sPTB, the impact of antibiotics for prevention of preterm birth has been disappointing (Nygren et al., 2008, Guise et al., 2001, Kekki et al., 2001b, Carey et al., 2000). There are several possible explanations for this, one is that preterm birth is a syndrome that involves multiple aetiological pathways and infection represents only one potential cause. Another is that clinical trials have not focused on antibiotics appropriate for the specific microbial population or the antibiotics have not been administered at an appropriate time to treat the infection.

1.2.2.4 Placental microbiome

In recent years, research has shown that the placenta, originally thought to be a sterile organ, harbors its own microbiome (Nuriel-Ohayon et al., 2016). Sequencing studies suggest temporal alterations during pregnancy are accompanied by remodelling of this microbiome (DiGiulio et al., 2015). However, the precise mechanisms by which microbes inhabit the placenta and cause the remodelling are far from understood. Aagaard et al. suggest that there are differences in the abundance of microbes such as Burkholderia, Actinomycetales, Alphaproteobacteria, Paenibacilli and Escherichia

coli in placentas obtained from term and preterm women (Aagaard et al., 2014). It is not, however, completely clear whether the alterations in microbial patterns are purely characteristic of gestational age or indicative of different pathophysiological stages of placental and/or fetal development.

Interestingly, a study has documented increased expression of human leukocyte antigen (HLA) in preterm placenta compared to term placenta (Stout et al., 2015). The findings of this study suggest that maternal factors contribute to the aetiology of preterm birth as these HLAs are involved in maternal tolerance of the fetus and regulation of maternal immune system. However, the mechanism by which levels of HLA influence the placental microbiome remains unknown.

To summarize, all pregnant women carry multiple species of bacteria within their genital tract. The interaction between their microbiota and / or with other infective pathogens can lead to an inflammatory response and this appears to play a central role in determining the timing of labour. The process may be further complicated by changes in maternal immunity that either have a genetic base or may be induced by pregnancy. Whilst most of the work attempting to define the presence of a FIR has been performed in patients who are symptomatic for preterm birth, there is potential to define changes associated with this pathway at an earlier stage of pregnancy.

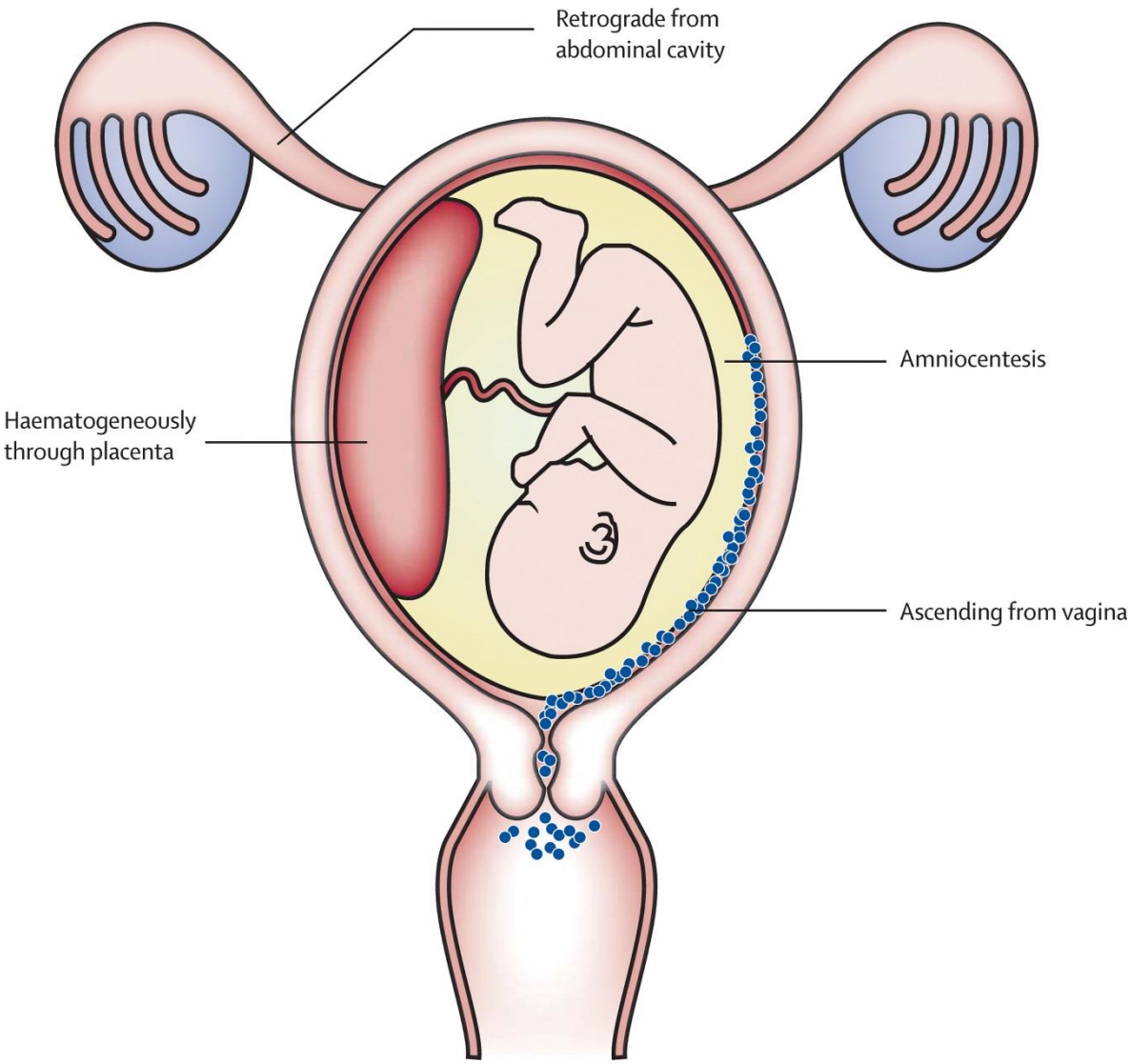

Figure 1.7: Routes of infection associated with preterm birth. Microorganisms can gain access to the amniotic cavity by: A) ascending from the vagina and the cervix; (B) haematogenous dissemination through the placenta; (C) accidental introduction at the time of invasive procedures; and (D) by retrograde spread through the fallopian tube (*Goldenberg et al., 2008).*

1.2.3 Circulating factors in preterm birth in various biological systems

Several infection-related markers including cytokines have been linked to preterm birth. A variety of bio-specimens including maternal and fetal blood, amniotic fluid, urine, cervicovaginal secretions, and placental tissue have been used to examine the role of cytokines in preterm birth. Cytokines mediate inflammation and exhibit autocrine, paracrine and endocrine functions (Zhang and An, 2007). They are involved in complex networks involved in establishment and maintenance of pregnancy (Mathialagan and Roberts, 1994).

There is overwhelming evidence confirming correlations between increased levels of circulating pro-inflammatory cytokines and preterm birth (El-Shazly et al., 2004, Fortunato et al., 2002a, Gucer et al., 2001, Nadeau-Vallee et al., 2016, Ruiz et al., 2012). The release of pro-inflammatory cytokines is followed by leucocytosis which results in apoptosis, premature rupture of membranes along with cervical remodelling and onset of premature labour. Several publications have appeared in recent years implicating Interleukin-1 (IL-1), tumor necrosis factor (TNF), and IL-6 in the onset of preterm birth (Gervasi et al., 2012). Furthermore, polymorphisms in pro- and anti-inflammatory genes have been associated with preterm birth (Romero et al., 2007, Keelan, 2011).

The inflammation cascade initiating preterm birth is mediated by several pro-inflammatory factors. Interleukin-1β (IL-1β), a major player in the inflammation process, promotes prostaglandin production when bound to TNF-α (Nadeau-Vallee et al., 2016). There is a vast body of evidence implicating TNF-α in preterm birth associated with infection (Fortunato et al., 2002b, Liang et al., 2010, Chen et al., 2003). TNF-α is produced by human decidua in response to microbial colonization (Casey et al., 1989). Furthermore, elevated levels of TNF-α have been reported in the amniotic fluid of women affected by intrauterine infection (Puchner et al., 2012). It has also been reported that there is an increased risk of preterm birth in patients with an abnormal vaginal flora who are genetically predisposed to produce an excess of TNF-α (Srinivasan et al., 2009). IL-6 and IL-8 are another two factors that are involved in the inflammation process associated with preterm birth (Wu et al., 2013, Romero et al., 1990, Velez et al., 2008a, Lee et al., 2015). IL-6 is linked to prostaglandin synthesis via the production of acute phase proteins that accompany inflammatory disease while IL-8 plays a vital role in cervical ripening (Young et al., 2002).

Whilst most ILs promote inflammation, others, such as Interleukin-10 (IL-10) help control the inflammatory response and limit associated tissue damage (Couper et al., 2008). IL-10 is highly expressed in the uterus and placenta during pregnancy and a reduction in expression has been implicated in pregnancy complications linked to inflammation (Thaxton and Sharma, 2010). IL-10 is thought to inhibit IL-1 synthesis, by which it keeps the immune response in check (Mobini et al., 2016). Transforming Growth Factor β (TGF – β) is another anti-inflammatory factor which assists in regulating progesterone, a vital hormone to maintain pregnancy (Ni and Li, 2017).

Maintaining an equilibrium of pro-inflammatory and anti-inflammatory cytokines is critical for implantation, development of the fetus and maintenance of pregnancy (Liang et al., 2015). The inflammatory response normally builds up over a period of time, reaching a point at which it triggers parturition. It may be possible to recognise this process weeks or months prior to the onset of labour – providing an opportunity to intervene and change the outcome of the pregnancy.

1.3 Current screening strategies

Clinically, women fall into one of three categories when screening for preterm birth. Some women are defined as being high-risk at the time of presentation, this is typically the case if they have a history of previous preterm birth, are known to have a short cervix (due to an operative procedure) or have a multiple gestation. Other women, who are also asymptomatic at the time of initial presentation, would be considered low-risk (generally women with singleton pregnancies without a history of a previous preterm birth or of a risk factor for cervical incompetence). The final group are women who are symptomatic for preterm birth. These women, who attend with abdominal pain and or vaginal bleeding are typically seen at a slightly later point in pregnancy. The first two categories allow screening with a view to prediction of an event that will occur sometime in the future. In contrast, symptomatic women are screened to determine whether they are at immediate risk of developing spontaneous preterm labour.

1.3.1 Screening women who are high risk but asymptomatic for preterm birth

As discussed previously (Section 1.2.1), several risk factors for preterm birth are recognised and at the time of the first clinical visit in pregnancy, maternal characteristics and previous obstetric history can be used to define women as high or low risk for preterm birth. The strongest historical risk factor for subsequent preterm birth is a previous preterm birth – and the risk is highest for women who laboured the earliest in gestation (Carr-Hill and Hall, 1985, Ekwo et al., 1993, Mercer et al., 1999, Ekwo and Moawad, 1998, Adams et al., 2000). Other women who are recognised as high risk for preterm birth are those that have had previous cervical surgery (large loop excision of the transformation zone or cone biopsy) and these women are also typically considered to be high risk (Chandiramani et al., 2011, Poon et al., 2012).

Use of this binary form of risk assessment (in which women either have these risks factors or they do not) is not a very sensitive screening tool; for instance, 50% of the population will not have a prior pregnancy history. This screening strategy is however typically used to inform models of care – and women at risk of preterm birth will often be placed in a high-risk, medicalised model. The accuracy of this historical form of assessment could be checked using a number of investigative tools which have higher screening efficacy – and which can be applied to this high-risk population. The

findings of these investigations may then be used to define the most appropriate management pathway.

If a woman is deemed high risk, then it is possible to immediately assess the length of the cervix with ultrasound. Cervical length is known to be a good predictor of risk of preterm delivery. If a woman who has a high-risk - on the basis of previous preterm delivery – has a short cervix (< 25mm) – then this will increase the risk of subsequent preterm delivery even further – and will often lead the clinician to instigate some preventative treatment – such as provision of vaginal progesterone therapy or cervical cerclage (Romero et al., 2018, Althuisius et al., 2000, Alfirevic et al., 2017).

Fetal fibronectin (fFN) is another valuable tool to define the risk of sPTB in a high-risk asymptomatic group (Lockwood et al., 1991). fFN is an adhesive protein, found at the maternal–fetal interface and acts like a glue between the amniotic fluid and the inner lining of the uterus. The release of this glycoprotein in the vaginal discharge is an indicator of inflammation caused by placental/membrane damage (Zhang et al., 2014). Typically, in a normal pregnancy fFN is very low (<50 ng/mL) in cervico-vaginal secretions (Lockwood et al., 1991). Levels of fFN are found to be elevated before 22 weeks and after 35 weeks' gestation (Kiefer and Vintzileos, 2008). A concentration of ≥50 ng/mL at ≥22 weeks' gestation is associated with a greater risk of sPTB (Levine et al., 2018). Interestingly, fFN has been found to be most informative when the result is negative. Subsequently, several systematic reviews and meta-analyses have indicated a negative fFN to identify women who are unlikely to deliver within seven to ten days, and do not require intervention in the form of antenatal corticosteroids or tocolytic agents (Revah et al., 1998, Honest et al., 2002).

In reality, only 5% of pregnant women will have had a prior preterm birth and a surveillance program that is based purely on identifying this high-risk cohort will not detect >60% of preterm births that are actually seen in a 'low risk' cohort (Greco et al., 2012). The only way that these cases can be successfully identified is to change the screening strategy so that all women are offered assessment using either ultrasound (cervical length assessment) or fFN testing – so that the high-risk group can be determined using this assessment tool rather than history alone.

1.3.2 Screening low risk women who are asymptomatic of preterm birth

95% of women who have their first clinic visit during pregnancy are low-risk for preterm birth based on their previous obstetric history. Several studies have shown that cervical length is a predictive tool for preterm birth in low-risk as well as high-risk patients (Iams et al., 1996, Taipale and Hiilesmaa, 1998, Heath et al., 1998). Furthermore, there is good evidence that risk of preterm birth can be reduced by treating these women with progesterone (Romero et al., 2012). fFN is not routinely used to screen low-risk asymptomatic women due to lack of evidence for improved perinatal outcomes.

There is evidence of an elevated risk of spontaneous preterm delivery in women that have a short cervix (<25mm) (Iams et al., 1996), and this is supported by the fact that sonographic measurement of cervical length at 23 weeks is highly reproducible (Heath et al., 1998); a mean cervical length of 38 mm was reported, that was comparable to other studies that assessed low-risk populations by measuring cervical length at 20-24 weeks. A number of other groups have made similar observations and these data were collated in a meta-analysis that reported transvaginal cervical sonography as an effective tool to identify women at an increased risk of sPTB (Honest et al., 2002).

Good normative ranges for cervical length have been established across gestational age (Salomon et al., 2009). If assessment is made at 18 - 23 weeks, the shorter the cervix is, the more likely it will be associated with early delivery (Celik et al., 2008). Rather than using these likelihood ratios to give individualised risks of preterm birth, most groups consider a fixed cut-off (25 mm) to be associated with an increased risk of preterm birth. Screening using this cut-off will identify ~3% of women to be at risk and this cohort will include 80% of women that deliver spontaneously before 34 weeks (Salomon et al., 2009, Celik et al., 2008).

Women that have a short cervix can be offered progesterone, cerclage or a cervical pessary (Alfirevic et al., 2013). A meta-analysis reporting the impact of progesterone showed a 45% reduction in rates of sPTB in women that were identified as being at risk and given treatment (Hassan et al., 2011). The data to support use of a suture or pessary in this situation appears to be slightly weaker. According to a meta-analysis that included three studies, the application of cervical pessary has not proven to be effective in lowering the risk of sPTB (risk ratio, 0.71; 95% confidence interval,

0.21-2.43; p = 0.59) (Conde-Agudelo et al., 2013). Furthermore, there is no evidence that identifies whether a combination of these treatments is more useful than one intervention alone.

Cervical assessment is traditionally performed at 18-23 weeks and there will, therefore, be a small cohort of pregnancies that are identified too late to prevent spontaneous preterm labour occurring. There is no data currently available in the literature that proves cervical length to be effective in improving outcomes in asymptomatic women who are screened after 24 weeks' gestation. If it were possible to identify women earlier, then preventative strategies may be more effective. The development of a simple blood test for screening would also negate the need for transvaginal cervical assessment in all pregnancies – a procedure that requires expensive ultrasound equipment, trained staff, image interpretation, and is inconvenient for women.

1.3.3 Screening women symptomatic of preterm birth

This cohort is different as women typically attend with symptoms of preterm birth later in pregnancy rather than at the time of their antenatal booking appointment or their 20-week scan. The purpose of screening to define a high-risk cohort is also different: If women are defined as being at high-risk in early pregnancy then it is possible to provide a prophylactic intervention that will prevent progression and clinical development of the disorder. If women are identified after they have become symptomatic, then the focus of care is on ensuring they are in an appropriate environment of delivery, have appropriate neonatal support available and are given drugs (steroids and magnesium sulphate) that will reduce mortality and morbidity for the infant.

Cervical examination through routine ultrasound is not associated with a significant reduction of sPTB (Iams et al., 1996, Heath et al., 1998, Honest et al., 2003). Digital cervical evaluation using the Bishop Score is an effective parameter for the prediction of preterm birth (Newman et al., 2008). A high Bishop score is predictive of greater risk of preterm birth. It is not possible to recommend the use of a tool more than another (e.g. cervix ultrasound versus vaginal examination) in women experiencing preterm labour.

Screening for fFN in the genital tract of patients with preterm labour has an excellent negative predictive value for sPTB at 48 hours and 7 days (Goldenberg et al., 1996, Honest et al., 2002, Peaceman et al., 1997). Nevertheless, its use in symptomatic women is not associated with a reduction of preterm birth rate and is therefore not recommended. However, the rationale for screening women with symptoms of preterm labour is different from that of primary prediction and prevention and is not the focus of the current work.

1.3.4 Current predictive tests for spontaneous preterm birth

1.3.4.1 Ultrasonographic assessment of cervical length

Typically, risk assessments for predicting preterm birth include examining the cervix, maternal demographics, obstetric history and biochemical tests (Table 1.1). Currently, a short cervical length in mid-trimester, irrespective of prior pregnancy history is one of the strongest indicators of spontaneous preterm birth. As noted in the above

sections, previous studies have documented that the risk of sPTB is inversely proportional to cervical length. Transvaginal ultrasound measurement is regarded as the 'gold standard' for cervical length as it is a reliable, safe and highly reproducible method when performed by trained providers (McIntosh et al., 2016). A threshold range of 20 to 30 mm is regarded as "short" for cervical length depending on the gestational age and the study population (Lim et al., 2011). Various studies across different populations show similar results. Typically, a cervical length less than 30 mm is regarded as "short" in the mid-trimester, as this corresponds to the 10th percentile for this gestational age (Hibbard et al., 2000). Additionally, there is a higher risk of sPTB if the cervix is found to be short at 18 weeks' gestation compared to 22 weeks (Owen et al., 2004).

1.3.4.2 Fetal fibronectin

The quantitative fFN (QfFN) assay, on the other hand, is in use in several countries for screening women to predict sPTB. Many studies have confirmed improved clinical performance for sPTB screening when fFN is combined with other risk factors such as cervical length (Hincz et al., 2002, Gomez et al., 2005, Schmitz et al., 2006, Ness et al., 2007). Nonetheless, additional research is still required to determine the accuracy of the QfFN assay, in combination with other screening modalities, in optimising neonatal outcomes for women at risk of sPTB, regardless of previous history of preterm birth.

Table 1.1: An overview of risk assessment approaches for preterm birth. Adapted from *Haque et al., 2016.*

Risk scoring	Physical examination	Biochemical tests
Medical history	Cervical length	Pregnancy Associated Placental Protein (PAPP-A), serum Alpha-fetoprotein
Demographic factors	Fetal crown rump length	Placental protein 13
Psychological and lifestyle factors	Uterine artery pulsatility index	Cervicovaginal IL-6/8, phIGFBP1, fFN

1.3.5 Current preventive measures / intervention strategies for spontaneous preterm birth

In the last few years there has been growing interest in treating low risk women with a short cervix in mid-trimester with progesterone or cervical cerclage. Progesterone is the most widely studied medications for prevention of preterm birth in women with a prior history of preterm birth. Several randomized clinical trials have documented a significant decrease in the rate of sPTB in women with a prior history of preterm birth who received vaginal progesterone commencing in the mid-trimester to 37 weeks' gestation (Karbasian et al., 2016, El-refaie et al., 2016). These findings have further been confirmed in systematic reviews and a meta-analysis of asymptomatic women with a short cervix and multiple gestation in the mid-trimester (Romero et al., 2018). Moreover, administration of vaginal progesterone has proven to be effective in decreasing the risk of sPTB even in those women without a prior history of preterm birth who are incidentally found to have a short cervix (Norwitz and Caughey, 2011). Progesterone has anti-inflammatory properties and thus an important role in maintaining pregnancy by decreasing the production of prostaglandins and subsequently limiting uterine contractility and cervical dilation and effacement; however, its exact mechanism of action in preventing sPTB is not known (Siiteri et al., 1977).

Cervical cerclage is a well know surgical technique employed to prevent recurrent sPTB in women known to have cervical insufficiency (Wang et al., 2016). The cervix acts as a physical barrier and protects the uterus and growing fetus from bacterial infections ascending from the vaginal canal (Myers et al., 2015). A cerclage is thought to be beneficial in high-risk women as it provides structural support to the cervix (Nott et al., 2016). Nevertheless, some women fail treatment and still deliver a very preterm infant. In current clinical practice, women with a prior sPTB <34 weeks may be offered the option of prophylactic cerclage - normally performed after the 11-13-week scan has demonstrated normal fetal anatomy - or cervical length screening with serial transvaginal scans through the second trimester. If serial transvaginal ultrasound assessments indicate shortening of the cervix without symptoms of preterm labour, cerclage placement may be considered.

Prescription of vaginal progesterone or cervical cerclage have been reported to be equally effective in preventing preterm birth in women with a history of previous

preterm birth and a short cervix at 16-20 weeks (Conde-Agudelo et al., 2018). In the absence of clear benefit of cerclage over vaginal progesterone in otherwise low risk women with a short cervix, progesterone is generally the preferred treatment due to the lower risk of surgical complications.

1.4 Identification of specific proteins associated with preterm birth using -Omics approaches

Currently, there is a substantial gap in the knowledge base concerning molecular mechanisms underlying spontaneous preterm birth. Exploring and defining the proteome (i.e. the entire set of proteins expressed in an organism, cell or tissue including all isoforms and posttranslational modifications (PTMs)) of the first trimester maternal serum in women who have delivered preterm spontaneously should allow improved insight into the evolution and progression of this devastating condition (Wilkins et al., 1996, Blakeley et al., 2010). Proteomics is thus likely to provide new targets for more effective medical management and potentially a novel method of minimally-invasively providing a prognosis and/or tracking paradigm for women suffering from this complex and debilitating condition. One of the primary goals of clinical functional proteomics is to characterise the cross-talk within biological samples, such as serum, through the various molecular pathways and interactions (e.g. cell signalling, apoptosis, angiogenesis, cellular proliferation, oxidative stress, etc.) in healthy and diseased states (Wu et al., 2014).

Notably, PTMs have been recognised to play crucial roles in signalling mechanisms as they regulate the activity, localization and interaction of proteins with one another and with other molecules (Beltrao et al., 2013). Identifying consistent changes in the proteome, and specific PTMs to proteins (e.g. phosphorylation and glycosylation) is likely to provide avenues for early intervention and prediction of sPTB at an earlier stage of pregnancy. This should improve research outcomes with progressively wider realization that proteomes are composed of proteoforms.

1.4.1 Post translational modifications

Alterations to proteoforms may indicate different biological activities (Godovac-Zimmermann et al., 2005). These proteoforms may include PTMs by phosphorylation, acetylation, methylation, or glycosylation (Duan and Walther, 2015). Protein PTMs contribute to the complexity that arises from the genome to the proteome level.

Depending on the nature and type, PTMs can be reversible/irreversible and can occur at any stage in the "life cycle" of a protein (Boyer, 2006). For instance, some proteins are modified shortly after the process of translation to either facilitate protein

folding, maintain protein integrity or to help the protein traverse to the nucleus or membrane (Jenkins et al., 2008). Other proteins are modified after the completion of folding to stimulate catalytic/biological activity of the protein. (Boyer, 2006).

Although only 5% of the proteome comprises enzymes, more than 200 types of PTMs are due to specific enzymatic activities (Duan and Walther, 2015). These enzymes modify proteins by catalysing the addition or removal of functional groups therefore altering protein activity and biological function. For example, kinases that add phosphate groups to proteins and proteases which cleave peptide bonds and therefore remove a specific sequence from a protein (Knorre et al., 2009).

The investigation of proteoforms is particularly significant in understanding the molecular basis of preterm birth. Identification of consistent changes in specific PTMs (e.g. phosphorylation, glycosylation) defining select proteoforms is likely to provide better understanding of protein-protein interactions, signalling pathways, and ultimately provide opportunities for intervention and prediction of preterm birth at an earlier stage of pregnancy. My focus in the current research, is on PTM such as phosphorylation and glycosylation and their potential role in defining early biomarkers predictive of preterm birth.

1.4.1.1 Role of protein phosphorylation in preterm birth

Phosphorylation plays fundamental roles in the regulation of cell function, apoptosis and signalling cascades (Ardito et al., 2017). In recent years, research on role of protein phosphorylation in the pathogenesis of various disease states has gained attention (Harsha and Pandey, 2010, Hanahan and Weinberg, 2011, Javidi-Sharifi et al., 2015, Zhu et al., 2015). One rapid test involves the detection of a post-translationally modified species of insulin-like growth factor binding protein-1 (IGFBP-1) in cervical secretions in women who are already symptomatic for preterm birth (i.e. in 2nd trimester) (Kekki et al., 2001a). The phosphorylated species of this protein i.e. phIGFBP-1 is mainly synthesized in the decidual tissues during pregnancy (Akercan et al., 2004, Lembet et al., 2002). The presence of phIGFBP-1 in cervical secretions may be an indicator of tissue damage at the choriodecidual interface. The fetal membrane begins to separate from the decidua in the earlier stages of labour. At this

stage, the presence of phIGFBP-1 in cervicovaginal secretions has been suggested as a predictor of preterm delivery but no testing has been done during the first trimester of pregnancy.

1.4.1.2 Role of protein glycosylation in preterm birth

Glycosylation is recognised as one of the most common and major PTMs responsible for regulation of anti-inflammatory immune responses (Caramelo and Parodi, 2007, Helenius and Aebi, 2004, Lowe and Marth, 2003). Glycosylation describes the process whereby sugar-moieties attach to the primary protein. A diverse group of sugar complexes and protein species may be involved; ranging from simple monosaccharide to highly complex polysaccharide modifications (Stowell et al., 2015). Abnormal glycosylation states have been associated with pathological conditions related to human implantation (Clark, 2015). Previous studies indicate alterations in glycosylated serum proteins such as IgG and α1-antitrypsin during pregnancy (Bondt et al., 2013, Ruhaak et al., 2014). Increased levels of pregnancy associated anti-inflammatory IgG Fc-linked N-glycan galactosylation and sialylation has also been demonstrated by several groups (Bondt et al., 2013, Raju and Lang, 2014, van de Geijn et al., 2009).

Due to a high amount of glycoconjugates in the human uterus, not only the sperm, the implanting fetus and embryo can bind effectively, but also any pathogen that may gain entry to the uterus (Jones and Aplin, 2009, Carson, 2002). During the luteal phase of the menstrual cycle, secretions of the endometrial glands increase markedly (Jones et al., 2001). These secretions are supplemented with nutrients and growth factors that maintain the implantation of the embryo and the subsequent development of a viable fetus (Clark, 2015). It is still unknown whether there is a link between defective placental or uterine glycosylation and adverse obstetric outcomes including preeclampsia, intrauterine growth restriction and preterm birth. Furthermore, the role of glycans linked to uterine and trophoblast glycoconjugates also remains unknown.

Recent studies, however, have reported the presence of altered glycosylation in trophoblasts obtained from patients that develop preeclampsia and preterm birth (Marini et al., 2011). Alterations in the pattern of β1 integrin glycosylation in placentas

obtained from women that had early spontaneous miscarriage relative to matched controls have also been identified (Zhang et al., 2015). The functional significance of shifts in glycosylation and its potential role in the development of preterm birth is yet not well-defined.

1.4.2 Omic approaches

Until now, most of the research focussed in the field of molecular biology is related to DNA (genomics) analysis and thus on the message (i.e. mRNA or transcriptomics) rather than the translation product of this message (i.e. the protein or proteomics). The rapidly expanding knowledge relating to altered cellular molecular processes in diseased states has brought about a "systems biology" approach and combined the use of the various complementary "-omics" (e.g. genomics, transcriptomics, proteomics, metabolomics, lipidomics) methods.

To date, most of the research focused on understanding the pathophysiology of preterm birth has involved the assessment of potential genetic markers. However, application of these as clinical markers, targets for drug intervention, or for the development of therapeutic agents is likely to be limited. Genomic data will provide a basis on which protein studies may be built. However, genomics alone will not provide the functional and biochemical information on messenger or protein abundance or speciation as the genome is relatively static when compared to the proteome that evolves in response to myriad intra- and extracellular environmental signals (Graves and Haystead, 2002).

Transcriptomic analyses, on the other hand, focus on identifying critical mRNAs as they are directly coded by the genome (Han et al., 2015). Proteomic investigation offers molecular and cellular information relating to protein abundance and the diversity of proteoforms present (Larochelle, 2016). Identifying changes in copy numbers or altered levels of DNA or RNA respectively does not necessarily identify changes at the protein level and therefore might not be directly relevant to the biological nature of a physiological state. In contrast, systems biology approaches, including proteomics and lipidomics provide direct information concerning a given state, and thus opportunities for study of both healthy and adverse pregnancy outcomes such as preterm birth.

Omics strategies offer much in terms of biomarker discovery, as multiple molecules can be assessed simultaneously. There has been an increasing trend in the use of omics approaches in reproductive medicine. In the last decade, omics (genomics, transcriptomics or proteomics) research related to preterm birth has attracted much attention. (McPherson and Manuck, 2016, Sheikh et al., 2016, Paquette

et al., 2018, Bukowski et al., 2017, Law et al., 2015, Bradford et al., 2017, Horgan and Kenny, 2011). However, most of this research has focussed on single omics approaches and have failed to be translated into clinical practice mainly due to lack of reproducibility.

1.4.3 Proteomics

As defined above, the proteome comprises of all protein species including splice variants and PTMs present in an organism at a given point in time and proteomics refers to the large scale study of protein species and the applications of technologies for their identification and quantification (Aslam et al., 2017, Aebersold and Mann, 2016). Understanding the proteome is crucial for biomarker discovery because protein species are most likely to be impacted by the disease or disease response (Kavallaris and Marshall, 2005).

The impact of proteomic research within the last decade in attempting to understand the molecular biology of reproductive medicine and gynaecological diseases has been enormous (Kolialexi et al., 2008, Kosteria et al., 2017, De Rose et al., 2018, Hernández-Núñez and Valdés-Yong, 2015, Butt et al., 2006). Specifically, in preterm birth, the application of proteomics has not only attempted to define the aetiology, great emphasis has also been placed on the search for discriminatory biomarkers during the early phases of pregnancy (Kacerovsky et al., 2014, Law et al., 2015, Buhimschi et al., 2008, Bujold et al., 2008, Bradford et al., 2017).

However, there continues to be a substantial gap in the knowledgebase concerning molecular mechanisms underlying preterm birth. Exploring, defining and understanding the maternal serum proteome should allow improved insight into the evolution and progression of this disease in women from various ethnic backgrounds. Proteomics may also provide new targets for more effective medical management and potentially a novel method for rapid, cost-effective prognosis and/or diagnostic tracking of women suffering from this condition (Figure 1.8).

The following sections will describe the predominant techniques for comparative proteomics employed in the current research and the bioinformatics methods used to extract significant quantitative information from these techniques.

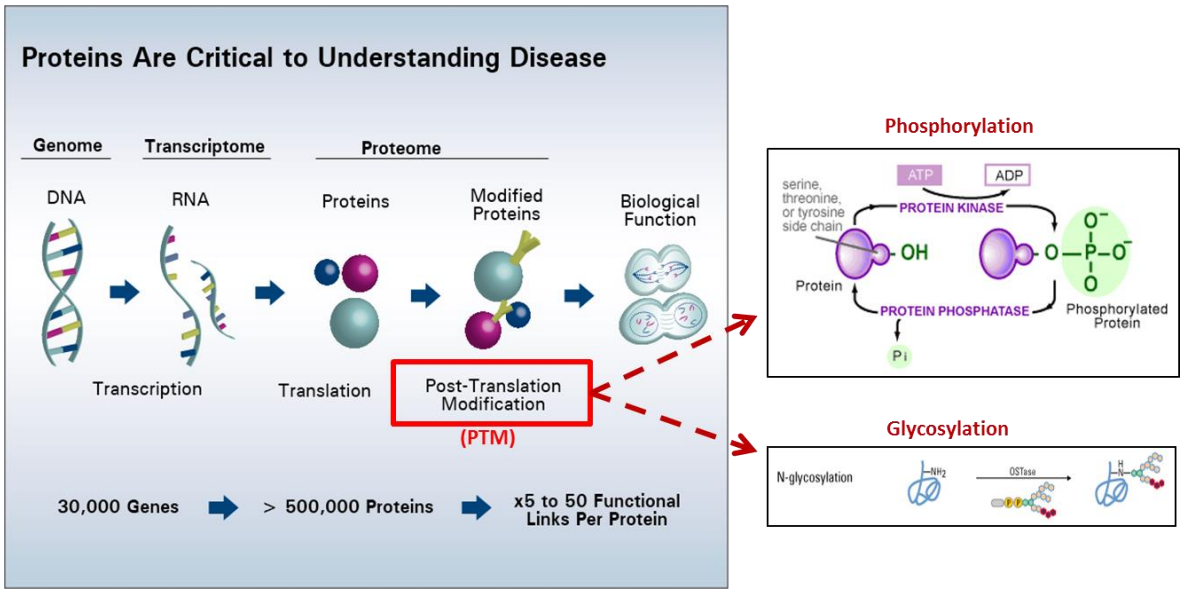

Figure 1.8: The role of proteins and post translational modification in understanding disease. Adapted from *Bernstein, 2014.*

1.4.4 Proteomics methods

One of the two major approaches for profiling protein species and peptides is "Bottom-up" proteomics which refers to the identification of amino acid sequences by analysis of peptides in proteolytic digests, and the assessment of these to determine which proteins are potentially present in a sample (Figure 1.9). The term bottom-up thus indicates that evidence about the potential constituent proteins is reassembled from individually identified fragment peptides (Wolters et al., 2001, Yates, 2004, Link et al., 1999). The major drawback of this approach is that only a small fraction of the total peptide population of a protein can be identified so there is low percentage sequence coverage (Tuli and Ressom, 2009). This poses a major problem in identifying all proteins of interest. The "top-down" approach enables separation of intact proteoforms and their subsequent identification and is therefore considered to be superior to the bottom up approach (Kelleher, 2004, Smith et al., 2013). Two-dimensional gel electrophoresis (2DE) has been one of the mostly commonly used approaches to resolve proteoforms (Oliveira et al., 2014a) (Figure 1.9).

A top-down proteomic approach utilizing 2DE coupled with liquid chromatography mass spectrometry/mass spectrometry (LC/MS/MS) was used in the current study to investigate first trimester serum protein species from women delivering spontaneously prior to 37 weeks' gestation. This method of serum protein analysis was chosen because it is the only electrophoretic technique that can be routinely applied to quantitative expression profiling of large sets of complex protein mixtures such as serum. Additionally, 2DE facilitates a more complete total profiling including intact proteoforms reflecting the changes in abundance of the protein species present and thus retaining this critical biological/molecular information (Gorg et al., 2000, Corbett et al., 1994, Oliveira et al., 2014b, Coorssen and Yergey, 2015).

The technique of 2DE involves the separation of proteins from complex samples in accordance with two unique protein properties, namely iso-electric point (pI) and size (i.e. molecular weight; MW). The first dimension of separation is isoelectric focusing (IEF), which involves separation of proteins according to their pI on an immobilised pH gradient gel (O'Farrell, 1975). First dimension IEF is then coupled with second dimension sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), to separate proteins by their MW resulting in the creation of a 2D proteome reference map of the proteoforms present (Laemmli, 1970).

After staining with colloidal Coomassie Brilliant Blue (cCBB) for total protein detection, the individual protein spots can be quantified and further analysed by MS (Gauci et al., 2013).

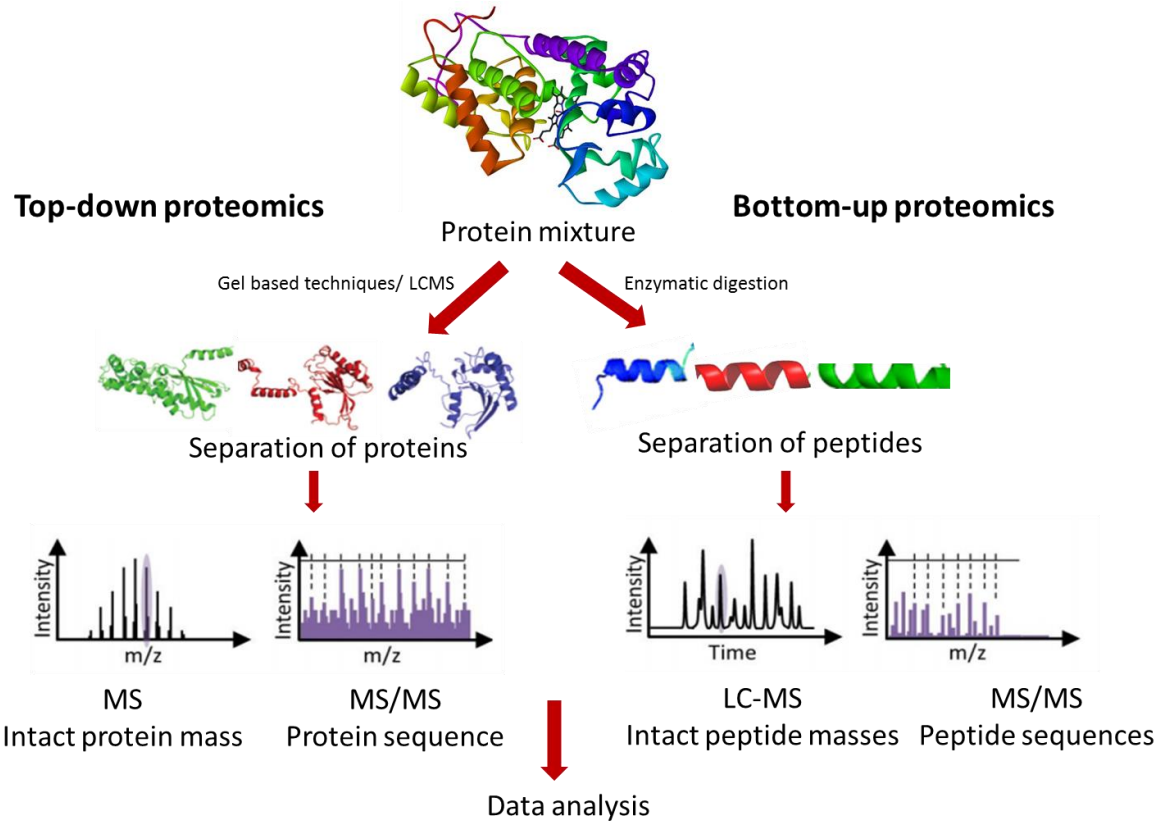

Figure 1.9: Schematic representation of top-down and bottom-up proteomic approaches. Adapted *from Switzart et al.,2013.*

PTMs such as phosphorylation and glycosylation are identified using the fluorescent stains Pro-Q Diamond and Pro-Q Emerald, respectively (Orsatti et al., 2009, Hart et al., 2003). Pro-Q Diamond and Pro-Q Emerald use a novel fluorophore to generate fluorescent signals by reacting with the phosphate and periodate-oxidized carbohydrate groups respectively. N-linked and O-linked processes represent the two major types of protein glycosylation. Reversible phosphorylation of serine, threonine or tyrosine residues, represents one of the most well-studied mechanisms of PTM (Lodish H, 2000, Humphrey et al., 2015).

There is consistent evidence in the literature supporting the critical roles of glycoproteins in biological mechanisms and cellular processes (Stadlmann et al., 2017, Chen et al., 2013). Characterization of the glycome and glycoproteome can be challenging due to their complex nature (Jensen et al., 2012). Furthermore, the glycosylation machinery is sensitive to disease manifestations; this has, for example led to several groups suggesting that changes in glycan assembly could be promising candidate biomarkers (Ruhaak et al., 2013, Zhu et al., 2017, Thaysen-Andersen et al., 2016). Even subtle changes in sugar structure such as a N-glycan core substitution by a single sugar moiety can have adverse functional consequences and be of potential value from a clinical perspective (Shah et al., 2015, Zhang et al., 2016, Haakensen et al., 2016, Brown et al., 2018).

1.4.4.1 Deep imaging and third-dimension electrophoresis (3DE)

Third dimension separation (3DE; 'postfractionation') is used to further resolve co-migrating proteins that appear as hyper-abundant spots after initial resolution by 2DE, using a gradient gel customized to provide optimal resolution within the target molecular weight range (Butt and Coorssen, 2005, Wright et al., 2014b, D'Silva et al., 2017). Deep imaging involves excising saturating spots/regions and re-imaging the gel at a higher photon-multiplier tube setting of 750 V, thus enabling detection of lower abundance proteins, that were originally below the level of detection (Wright et al., 2014b, Wright et al., 2014a, Partridge et al., 2016, D'Silva et al., 2017).

1.4.4.2 Mass spectrometry

Protein species of interest are excised from cCBB stained 2D gels and subjected to proteolytic cleavage with trypsin to generate peptides in solution. Peptides are ionized within a mass spectrometer to produce a measurement of their masses. A number of ionization methods are used; peptides can be co-crystallized with a matrix for matrix assisted laser desorption/ionization (MALDI) (Duncan et al., 2008), or the liquid peptide mixture can be subjected to electrospray ionization (ESI) (Liuni and Wilson, 2011). After ionization, peptides are detected within the spectrometer using time-of-flight (TOF), quadrupole, ion trap, or fourier transform ion cyclotron resonance mass analysers (Signor and Boeri Erba, 2013). Mass spectrometers are designated by their combination of ionization method and mass analysers. MS/MS methods refer to multiple peptide fragmentation steps occurring within the mass spectrometer, generally within a single run, and provide a greater level of detail about peptide amino acid sequence not available from single fragmentation approaches like MALDI-TOF (Wysocki et al., 2005). This sequence information can be key in identifying the specific protein isoform of interest (Henzel et al., 2003).

1.4.4.3 Quantitative image analysis

The most important objective of proteomics is to identify differences in protein abundances (increase or decrease as assessed by spot intensity) between control and test samples. Once these protein spots have been identified using spot analysis software, the proteins of interest can be excised for identification using MS. Specialized software packages such as PDQuest (Bio-Rad), Melanie (GenBio), Delta2D (Decodon), Decyder 2D (GE Health) are used to analyse and quantify the changes in abundance of every protein spot by defining spot area boundaries and matching spots between gel images, and then comparing spot densities (i.e. via fluorescence or densitometry) (Rosengren et al., 2003, Kang et al., 2009).

The image analysis for this study was performed using Delta2D V4.0 (DECODON Gmbh, Greifswald, Germany), which is an alignment-based approach matching all preterm cases and control samples between the different gels (Millioni et al., 2010). Delta2D generates normalised average protein abundance levels for each individual spot from a warped gel image. Spots were deemed differentially abundant

and significant (one-way analysis of variance; ANOVA), when a > 1.5-fold change in abundance was detected with a significance of $p < 0.05$.

1.4.4.4 Bioinformatics

Bioinformatics is the application of analytics and computational databases – for proteomics or any of the complementary omics - to assess and understand biological processes (Kumar and Mann, 2009). The most common protein database resources available to researchers for protein identification from MS data include the United Protein Database (UniProt, http://www.uniprot.org), which centralises three existing protein sequencing databases - SwissProt, Translational European Molecular Biology Laboratory (TrEMBL) and the Protein Information Resource - into one global resource. Other key protein database and analysis resources include the Mascot Daemon search algorithm. All these resources are aimed to provide free and unrestricted access to the most comprehensive and non-redundant information regarding protein structure and function. However, limitations of these latter resources must be considered when interpreting the results relative to the proteins experimentally identified. One such instance is the possibility of one spot containing 40-60 proteins and the ability of databases to identify the top-most hit i.e. protein of high abundance (Zhan et al., 2018). However, it is possible that apparent changes in protein abundance may be related to changes in other, lower abundance proteins associated with that spot.

Additionally, pathway analysis software such as PANTHER (Protein Analysis Through Evolutionary Relationships) classification system, Reactome Pathway Database, STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database and DAVID (the database for annotation, visualization and integrated discovery) enable at least an initial, provisional functional interpretation of large protein datasets, providing some first-pass guidance as to their biological context, involvement in various physiological pathways and association with disease pathophysiology.

1.4.5 The dynamic range challenge of serum

Human serum proteins serve as a rich source of biomarkers as they reflect various physiological conditions at a given time point (Anderson et al., 2004). However, one of the greatest challenges in analysing the serum proteome is its complexity and wide dynamic range of concentration of individual proteins that extends from several milligrams to less than one pictogram per millilitre (Chandramouli and Qian, 2009). Furthermore, the high-abundance of certain protein species tends to mask proteins of lower abundance, some of which might potentially be relevant to disease states (Millioni et al., 2011).

Amongst the other highly abundant proteins, human serum albumin alone constitutes approximately 60% of the total serum proteome causing an overwhelming signal in detection assays (Guizado, 2014). Because of its high abundance, it plays a vital role in facilitating drug delivery (Larsen et al., 2016, Elsadek and Kratz, 2012). Human serum albumin can also undergo PTMs including glycation, S-guanylation and dehydroalanine conversion – the nature of the PTMs affects the ability of albumin to interact with drugs (Lee and Wu, 2015). Current research aims to utilize human serum albumin to improve efficiency of drug delivery and subsequent treatment of diseases (Lomis et al., 2016, Qi et al., 2016, Quan et al., 2011).

Past research has focused on strategies to deplete human serum albumin for discovery of potential biomarkers that are often found in low abundance (Gianazza et al., 2016). However, studies have noted the presence of interesting molecules such as cytokines in the albumin depleted fractions (Granger et al., 2005). In general, the albumin-binding peptides and proteins are collectively called the albuminome (Liu et al., 2017). The albuminome has been suggested to be of value in identifying disease biomarkers when combined with other protein components (Lowenthal et al., 2005, Camaggi et al., 2010).

Besides albumin, human serum also contains other highly abundant protein species: IgG, IgA, transferrin, haptoglobin, and antitrypsin. Collectively, these six abundant proteins constitute over 85% of the human serum proteome (Echan et al., 2005). Depletion methods for highly abundant proteins have been widely investigated and a number of these have become commercially available (Polaskova et al., 2010, Bjorhall et al., 2005). These techniques include use of classical methods (Cibracon

Blue and protein A/G chromatography methods), immuno-affinity subtraction chromatography as well as various affinity spin tube filter, resins, and columns. The depletion strategies, however, are disadvantageous for two reasons: a) removal of the initially most abundant proteins merely exposes a second cohort that is highly abundant in comparison to other species; and b) these depletion methods lead to the concomitant removal of non-targeted protein species of interest (Bellei et al., 2011).

Several critical assessments and refinements to better enable the resolution and comparison of serum proteomes were carried out as part of the research described in this thesis. The methods were then applied to identify first trimester maternal serum proteoforms between women who delivered spontaneously and those who delivered at term.

1.5 Previous attempts to identify protein biomarkers for preterm birth using proteomic approaches

To date, many studies have documented the use of various proteomic approaches to predict preterm birth. Several biospecimens including cervico-vaginal fluid (CVF), amniotic fluid, saliva, plasma and placenta have been investigated to identify markers for preterm birth. However, none of the identified markers were found to be clinically effective for prediction of preterm delivery.

A study examined the CVF to identify markers for preterm birth by using a gel based fluorescence 2D differential gel electrophoresis approach as well as a bottom-up technique (Multidimensional Protein Identification Technology (MudPIT)) (Pereira et al., 2007). Among the identified protein species, calgranulins, annexins, S100 calcium-binding protein A7, and epidermal fatty acid binding protein were differentially abundant in sPTB and control samples. Interestingly, the group identified serum proteins (R-1-antitrypsin, R1-acid glycoprotein, haptoglobin, serotransferrin, and vitamin D binding protein) that were also differentially abundant in the CVF.

The same group employed mass spectrometry and ELISA techniques to identify biomarkers of recurrent preterm birth in a larger cohort of at-risk women who underwent serial CVF sampling (Lo et al., 2014). The group identified 784 proteins in total, of which four proteins (Cysteine-rich secretory protein 3, Heat Shock Protein Beta-1, Protein S100-A7 and Alpha Enolase-1) were involved in the inflammatory cascade. However, ELISA analysis was unable to confirm the apparent altered expression of the candidate proteins.

Another study cultured human columnar epithelial endocervical-1 and vaginal cells and utilized stable isotope labelling technology to generate labelled CVF proteomes in vitro (n = 5 preterm birth and n = 5 controls). The labelled secreted proteins from both cell lines were combined and characterized by LC/MS/MS. The group identified 1211 proteins in total and shortlisted 15 candidate proteins as potential preterm birth biomarkers previously linked to mechanisms underlying preterm birth. Furthermore, the group quantified 15 candidate proteins by employing multiple reaction monitoring (MRM) MS assay and demonstrated significantly increased levels of three proteins (desmoplakin isoform 1, stratifin, and thrombospondin 1 precursor) in women who delivered preterm (Shah et al., 2009).

An Australian group used 2D DIGE and MS to analyse the CVF proteome in symptomatic women who delivered preterm spontaneously and identified 12 proteins. Based on fold change and previous observations relating to human parturition, four proteins (albumin, IL-1ra, thioredoxin and VDBP) were validated in a larger independent cohort of symptomatic women. ELISA analysis revealed significantly altered levels of albumin and VDBP in women who delivered preterm compared to term controls. When compared to fFN, a combined model of albumin/VDBP was found to be more effective to predict sPTB in symptomatic women within 7 days (Liong et al., 2015).

Inflammatory biomarkers characteristic to HCA and funisitis (neutrophil defensins 2 and 1 and calgranulins C and A) have been identified in amniotic fluid using a proteomics approach (Buhimschi et al., 2009). Based on these findings, researchers sought to determine the relationship between identified inflammation associated biomarkers and cord blood IL-6 to assess the likelihood and severity of developing FIR. A direct correlation was seen between the AF and cord blood IL-6 levels; however, severity of infection could not be used to measure the intensity of FIR.

The results obtained by Romero at al. suggest that mass spectrometric signatures of the amniotic fluid can be used to distinguish women delivering preterm due to intrauterine infection from women delivering without inflammation/infection at term (Romero et al., 2008). Another study used amniotic fluid in the mid-trimester of pregnancy to identify biomarkers via an LC/MS/MS approach in the exploratory phase. They identified six dysregulated proteins and suggested CRP as a marker to predict sPTB at 14-19 weeks' gestation. However, the group was not able to validate their findings through ELISA (Hallingstrom et al., 2016).

In recent years, research into microparticles has attracted much attention. Microparticles are a group of small bioactive vesicles released into the circulation due to stress, cellular damage or infection (Herring et al., 2013). Pregnancy associated microparticles are involved in cross-talk between maternal and fetal tissues and play crucial roles in various immune related pathways. Analysis of circulating microparticles in first trimester plasma samples identified several clinically significant protein mediators involved in the complement system and associated inflammatory

pathways (Cantonwine et al., 2016). Another study examined circulating microparticles in second trimester maternal serum and identified proteins associated with inflammation and cell injury to be predictive of sPTB (Ezrin et al., 2015).

It has been hypothesized that even prior to the onset of clinical signs, there are differences in the maternal serum proteomic profile in women destined to deliver a premature baby (Esplin, 2014). A study utilizing an Electrospray-ionization, time-of-flight mass spectrometry (EOI-TOFMS) technique to assess serum identified three peptides arising from inter-alpha-trypsin inhibitor heavy chain 4 protein to be significantly decreased in asymptomatic women at 24-28 weeks having subsequent sPTB. Nonetheless, the results do not allow for any confident differentiation of the three proposed biomarkers according to a potential aetiology for sPTB (Esplin et al., 2011).

Another study, employing shotgun proteomic approache, indicated that elevated levels of serpin B7 in maternal serum can be used as a predictive marker for sPTB in symptomatic women (Parry et al., 2014). A panel of 31 proteins were found to be in low abundance and only serpin B7 was found to be increased in serum samples from women who delivered preterm compared to women who delivered at term. The role of serpin B7 in the context of sPTB has not yet been studied. Consequently, validation studies in asymptomatic women to understand the mechanism of serpin B7 in sPTB are warranted.

1.6 Hypothesis and Aims

Hypothesis

Differences in the serum proteomes of preterm or term pregnancies are present as early as 11-13^{+6} weeks' gestation. These differences can be identified through proteomics and will likely prove to be of substantial value in predicting risk of spontaneous preterm birth, thus enabling early interventions that reduce or eliminate this risk in the future.

Aims and Objectives

- To test and refine methods for characterization of maternal serum protein profile using a top-down proteomic approach.

- To characterise differential protein profiles and post translational modifications of the first trimester maternal serum proteome using a highly refined top-down proteomic approach based on 2DE coupled with mass spectrometry.

- To validate candidate protein biomarkers by use of a western blot approach in a larger cohort of affected pregnancies and therefore identify protein markers that will be of value in the prediction of preterm labour.

Paper I

Published in Obstetrics and Gynaecology

Contributions

AMD carried out literature review and write first draft of manuscript

Paper II

Published in Proteomes

Contributions

AMD carried out all experimentation, analysed the data and wrote first draft of manuscript

proteomes

MDPI

Article

A Routine 'Top-Down' Approach to Analysis of the Human Serum Proteome

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Abstract: Serum provides a rich source of potential biomarker proteoforms. One of the major obstacles in analysing serum proteomes is detecting lower abundance proteins owing to the presence of hyper-abundant species (e.g., serum albumin and immunoglobulins). Although depletion methods have been used to address this, these can lead to the concomitant removal of non-targeted protein species, and thus raise issues of specificity, reproducibility, and the capacity for meaningful quantitative analyses. Altering the native stoichiometry of the proteome components may thus yield a more complex series of issues than dealing directly with the inherent complexity of the sample. Hence, here we targeted method refinements so as to ensure optimum resolution of serum proteomes via a top down two-dimensional gel electrophoresis (2DE) approach that enables the routine assessment of proteoforms and is fully compatible with subsequent mass spectrometric analyses. Testing included various fractionation and non-fractionation approaches. The data show that resolving 500 µg protein on 17 cm 3–10 non-linear immobilised pH gradient strips in the first dimension followed by second dimension resolution on 7–20% gradient gels with a combination of lithium dodecyl sulfate (LDS) and sodium dodecyl sulfate (SDS) detergents markedly improves the resolution and detection of proteoforms in serum. In addition, well established third dimension electrophoretic separations in combination with deep imaging further contributed to the best available resolution, detection, and thus quantitative top-down analysis of serum proteomes.

Keywords: deep Imaging; Lithium Dodecyl Sulfate; prefractionation; postfractionation; proteomics; proteoforms; three-dimensional gel electrophoresis; two-dimensional gel electrophoresis

1. Introduction

Detailed analyses of the serum proteome are important as they provide a source of diagnostic or prognostic biomarkers as well as insight into the mechanisms underlying disease development and progression [1,2]. Due to the heterogeneity of disease, single protein markers are frequently not sufficiently predictive of a condition to be of significant clinical value. A panel of candidate biomarkers is typically needed to improve diagnostic efficacy [3,4]. Although fitness-for-purpose must be considered in deciding between the use of bottom-up or top-down proteomic approaches [5], quantification of disease-associated alterations is often best achieved by the latter, in which intact proteoforms (i.e., protein species) are resolved from complex biological samples using techniques such as 2-dimensional gel electrophoresis (2DE) coupled with mass spectrometry (MS) [6]. 2DE is the only available proteomic technique that can simultaneously resolve hundreds-to-thousands of

proteoforms in a single analytical run, while also enabling multiple parallel analyses. As the only such routine top-down analytical protocol, it is thus the only available approach that enables quantitative profiling of large sets of complex protein mixtures; that is, as part of the routine analytical protocol, this approach resolves protein isoforms, splice variants, and the vast range of post-translationally modified protein species that define biological functionality.

Serum contains one of the most complex proteomes that has thus far been researched. The dynamic range of protein characteristics (e.g., isoelectric point, mass, hydrophobicity, concentration, and post-translational modifications) makes effective coverage of the serum proteome very challenging as it is difficult to resolve such a diverse range of macromolecules [7]. High-abundance proteins tend to mask those of lower abundance and have typically been removed to allow better resolution of other species [8]. However, removal of this fraction risks removal of non-targeted proteins that may impact on our understanding of the mechanisms underlying disease as well as on discovery and quantification of novel biomarkers [9]. In addition, removal of the most abundant proteins merely exposes a second cohort that is highly abundant in comparison to other species—so this intervention fails to resolve the fundamental problem of dynamic range and largely obviates the objective of quantitative analysis [5].

As with other complex samples, methods used to reduce the complexity of the serum proteome are based on the physicochemical and structural characteristics of the constituent proteins, including solubility, hydrophobicity, molecular weight and isoelectric point. Ultracentrifugation provides a simple approach for the separation of high molecular weight proteins but is non-selective and thus also does not address the issue of protein-protein binding and non-specific losses [10,11]. Similarly trichloroacetic acid (TCA) has been used to precipitate high abundance proteins such as albumin by forming a TCA-albumin complex [12]. Phase separation of detergents such as Triton X-114 (TX-114) distinguishes between proteins on the basis of hydrophobicity and is relatively cheap and versatile but the partitioning behaviour depends on the properties of the proteins being resolved (e.g., molecular weight and surface exposure of different amino acid residues) and may still not fully address the issue of non-specific protein losses to one fraction or the other [13]. Other methods are mainly used to target the removal of hyper-abundant proteins and are based on affinity phases, ion-exchange and antigenic activity [14]; these processes are similarly compromised by a lack of specificity and/or by the potential of complex protein-protein interactions leading to the unintended removal of (lower abundance) species bound to the highly abundant fraction. Whilst these techniques facilitate identification of some less abundant proteins there is a risk that others will not be recognised and that any attempts at quantification do not in fact represent the native state of the proteome.

In preparation for assessment of the serum proteomes of pregnant women who laboured preterm, we have developed an analytic technique that does not remove protein species but nonetheless enables improved differentiation of both high and low abundance proteoforms, of both high and low molecular weight. As the whole serum proteome is conserved, the technique also allows quantification of species, and for further future improvements as detection methods continue to improve in sensitivity and selectivity [15–19]. In addition to the various techniques discussed and tested in order to optimize efficient resolution of the native serum proteome, we have also combined a robust and well-established 2DE protocol [20,21] with (i) a new, high sensitivity staining and detection protocol [15,17,18]; (ii) postfrationation or third dimension electrophoresis (3DE) [22,23]; and (iii) Deep Imaging [23–25]—as well as selective staining to assess phospo- and glycoprotein subproteomes (i.e., proteoforms) in order to extract as much information as possible from each gel [9,24] (Figure 1). 3DE is used to further resolve co-migrating proteins that appear as hyper-abundant spots after initial resolution by 2DE, using a gradient gel customized to provide optimal resolution within the target molecular weight range [22,23]. Deep imaging involves excising saturating spots/regions and imaging the gel at 750 V, thus enabling detection of lower abundance proteins [23–25]. We thus report the development of an efficient, sensitive and reproducible technique that substantially improves the quantitative protein profiling of native human serum, and that should prove widely applicable to a range of comparable sample types including plasma and urine.

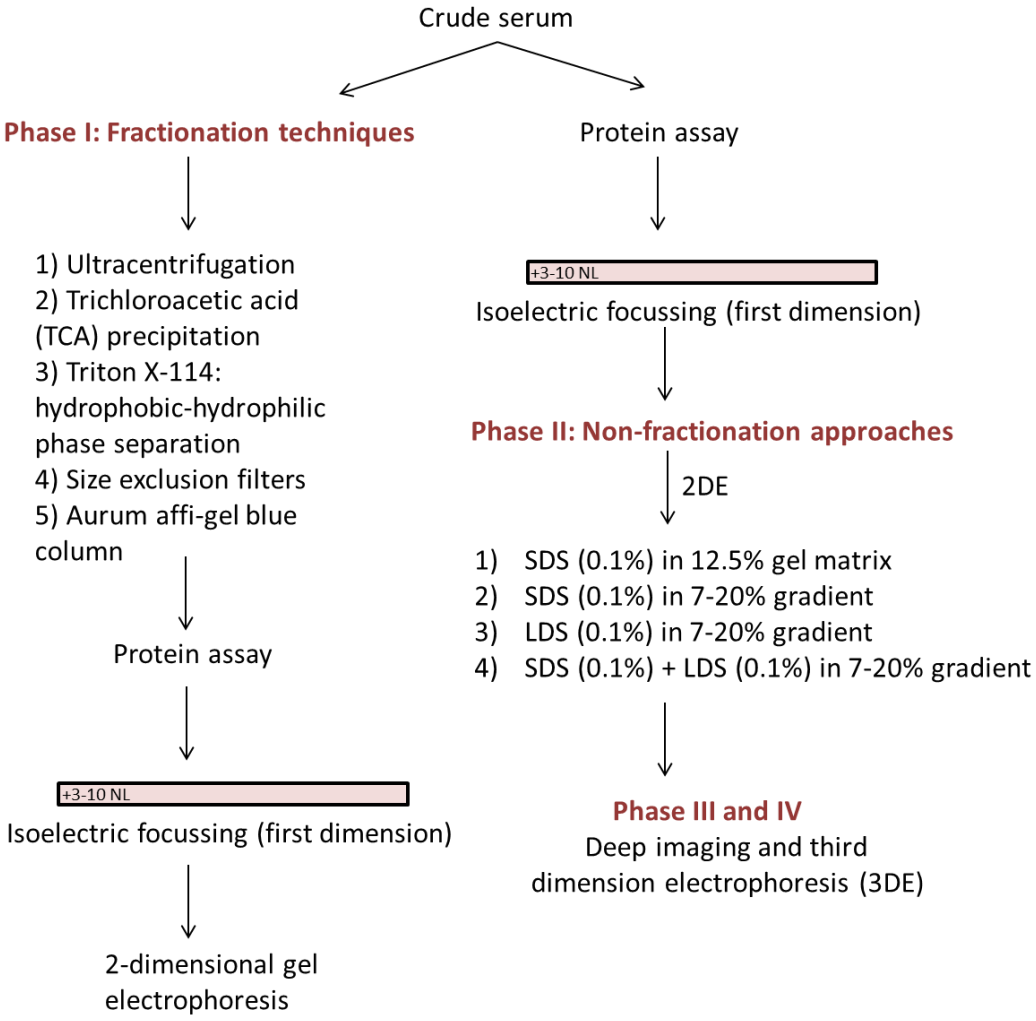

Figure 1. A simplified flow chart of various phases used during optimization of serum two-dimensional gel electrophoresis (2DE).

2. Materials and Methods

Banked serum samples were analysed from a cohort of women attending for combined first trimester screening, a screening test for Down syndrome at $11–13^{+6}$ weeks' gestation. Samples were collected between 28 June 2011 and 15 April 2013, and were initially centrifuged for 10 min at $2000\times g$ in a NATA approved clinical laboratory within four hours of collection. These samples were used to determine serum PaPP-A and free βhCG levels, while the residual serum was immediately stored at −80 °C. The subsequent pregnancy outcomes have been recorded and the samples selected for this work were from uncomplicated pregnancies. Sample use was approved by the Royal Prince Alfred Hospital ethics committee (X11-0305/HREC/11/RPAH/472). A reference proteome was created using serum pooled from three samples.

2.1. Protein Assay

Protein estimation was performed using the EZQ Protein Quantitation Kit with BSA standards according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). A baseline native serum profile was prepared by solubilising crude serum in 2DE buffer containing 8 M urea, 2 M thiourea, 4% (*w*/*v*) CHAPS and a cocktail of protease, kinase and phosphatase inhibitors (referred to as PI) at a ratio of 1× conc of PI (initial conc of 500×): 2 mL of serum [26].

2.1.1. 2-Dimensional Gel Electrophoresis (2DE)

Proteins were resolved using a well-established protocol and further detail is provided in the supplementary methods section [24]. Several methods of sample preparation prior to the first dimension (termed phase I) and at the 2DE stage (termed phase II) were tested for potential improvements in resolution whilst ensuring minimal loss of low abundant species and preservation of proteoform integrity for quantitative assessment.

Phase I: Testing of fractionation techniques involved use of Ultracentrifugation, Trichloroacetic acid (TCA) precipitation, Triton X-114 phase separation, Size exclusion filters, and the Aurum Affi-Gel Blue column prior to the first dimension of resolution (Figure 1).

2.1.2. Ultracentrifugation

Considering the previously identified complement of membranous material and membrane protein in serum and plasma [27,28], ultracentrifugation was carried out according to Churchward et al., (2005) (supplementary methods) [29]. 2 mL of thawed serum with an added 1× conc of PI was centrifuged at 146,542× *g* for three hours at 4 °C using a Beckman Coulter Optima L-100 XP ultracentrifuge; the separate supernatant and pellet fractions were collected.

2.1.3. Trichloroacetic Acid (TCA) Precipitation

500 µL TCA (100% (*w*/*v*)) was added to 500 µL of crude serum and incubated at −30 °C overnight (supplementary methods) [12]. The sample was centrifuged at 15,000× *g* for 30 min at 4 °C.

2.1.4. Triton X-114: Hydrophobic-Hydrophilic Phase Separation

TX-114 phase separation was carried out using a modification of the Bordier protocol [30]. In brief, a cushion of 2000 µL of 6% (*w*/*v*) sucrose, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.5% TX-114 was placed at the bottom of a Falcon 15 mL conical centrifuge tube. 500 µL of crude serum sample with 1 × PI was then overlaid on this cushion and the tube was incubated for 3 min at 30 °C to effect condensation. The tube was centrifuged at RT for 3 min at 300× *g* to effect phase separation, yielding a clear, viscous lower detergent phase (DP) and an upper aqueous phase (AP). After phase separation, the DP and AP were analysed separately as described in the supplementary material.

2.1.5. Size Exclusion Filters

100 kDa and 50 kDa Amicon ultra-centrifugal low protein binding filter units (Merck Millipore, Billerica, MA, USA) were briefly rinsed with 200 µL of 0.9% NaCl before use. 2 mL of crude serum was mixed with an equal volume of saline containing 1× PI, and centrifuged in two stages (using the 100 kDa and 50 kDa filters, respectively) to produce three fractions of nominally > 100 kDa (fraction A), 50–100 kDa (fraction B) and < 50 kDa (fraction C); both centrifugation steps were carried out at 1008× *g* for 20 min at 4 °C. Desalting and the estimation of protein concentration (both as above) were carried out and all three fractions were then analysed by 2DE.

2.1.6. Aurum Affi-Gel Blue Column

The effect of albumin removal on analysis of the serum proteome was also tested using Aurum affi-gel blue mini columns (Bio-Rad, Hercules, CA, USA). This protocol has been explained in the supplementary material.

Phase II: Optimisation of 'non-fractionation' approaches involved using serum in the native form for the first dimension while replacing or supplementing SDS with LDS for the second dimension (Figure 1).

2.1.7. Lithium Dodecyl Sulfate (LDS) vs. Sodium Dodecyl Sulfate (SDS)

We explored an alternative strategy to improving the resolution of protein species by resolving native serum on large (i.e., 20 cm × 20 cm) 7–20% gradient acrylamide gels which enabled a larger amount of protein to be resolved. LDS alone as well as a combination of LDS and SDS were tested by first resolving native serum without any fractionation on 2D gels. LDS was added in the equilibration buffer (6 M Urea, 20% glycerol, 375 mM Tris; pH 8.8, 0.1% LDS or a combination of 0.1% LDS and 0.1% SDS) when reducing and alkylating the IPG strip after IEF, as well as in the gel matrix (7% and 20% acrylamide, 375 mM Tris, 0.1% LDS or a combination of 0.1% LDS and 0.1% SDS) used in the second resolving dimension (see supplementary material).

Phase III: Deep imaging and third-dimension electrophoresis (3DE).

Here, 2DE was combined with 3DE (a postfractionation approach) and integrated with a well-established 'deep imaging' protocol [23–25]. For each 2DE analysis, 500 µg of protein was resolved on 17 cm, 3–10 NL IPG strips, as described above; areas of near-saturating signal (i.e., hyper-abundant proteins) were manually excised from the cCBB stained gels using a scalpel. Imaging was repeated at 750 V for higher sensitivity protein detection. All excised spots were further resolved using a standardized 3DE protocol. Briefly, those gels designated for 3DE were initially stained using the reversible zinc-imidazole protocol [24], and saturating spots/regions were excised, the zinc fixation reversed on both the main gel and the excised regions, and the latter were turned 90° relative to the initial path of resolution and then subjected to a third round of electrophoresis, on narrow gradient gels customized to the corresponding molecular weight region; 10–15% for heavy cut spots corresponding to 60–200 kDa and 15–18% for light cut spots corresponding to 10–50 kDa. Once electrophoresis was completed, gels were stained with cCBB, destained, and imaged as described above.

2.2. Phospho and Glyco Staining

Pro-Q Diamond and Pro-Q Emerald stains were used to identify post translation modifications (PTM), specifically, phosphorylation and glycosylation, respectively. Pro-Q Emerald 488 Glycoprotein and Pro-Q Diamond Phosphoprotein stains were obtained from Life Technologies (Carlsbad, CA, USA). Following 2DE, phospho- and glyco- staining was carried out on control serum sample according to manufacturer's protocols.

2.3. Image and Statistical Analyses

The resulting resolved protein spots from all 2DE and 3DE gels were quantitatively analysed using automated spot detection in Delta 2D (version 4.08; DECODON GmbH, Greifswald, Germany). For fractionation techniques, all images of replicate gels of both fractions were fused to create a representative image. When serum was resolved, without any fractionation, mean spot counts have been reported. Only protein species consistently and reproducibly detected across a given set of replicate gels were considered for the spot count. These spot counts were obtained excluding the gel edges and the protein ladder [24]. In order to examine the variation in protein spot numbers following fractionation and alternate methods, the analysis of variance (ANOVA) with Tukey's Multiple Comparison Test was carried out.

3. Results

The mean spot count for the preliminary reference proteomes of native serum was 367 ± 2 for mini gels and 709 ± 15 for large gels (Tables 1 and 2); this is 'standard' SDS-PAGE (2% SDS in equilibration buffer and 0.1% SDS in resolving gel matrix) in the second dimension [29]. As well established in the literature, larger gels with larger protein loads resulted in better resolution of proteoforms ($p \leq 0.001$).

Table 1. Total protein species resolved by 2DE after various methods of fractionation.

Methods	Type of Gel	Gel %	Protein Conc.	Protein Species Detected
Native serum (Baseline for statistical comparison)	Mini	12.5%	100 µg	† 367 ± 2
Ultra 3 h		12.5%	100 µg	424 ± 21
Trichloroacetic acid (TCA)		12.5%	100 µg	358 ± 6
Tx-114		12.5%	100 µg	415 ± 3
Size exclusion filters		Frac A and Frac B (7–10%) Frac C (12–20%)	100 µg	† 392 ± 24
Aurum Affi-Gel Blue column		12.5%	100 µg	285 ± 7
Tx-114	Large	7–20%	500 µg	† 779 ± 51 *

Values given are average for total spot counts; all mean values were derived from combining fractions; $n = 6$ gels, except † $n = 4$, Statistical significance indicated as * $p \leq 0.001$; One way analysis of variance (ANOVA), Tukey's multiple comparison test.

Table 2. A comparison of the total number of protein species resolved using different 2DE and deep imaging techniques.

Method	Type of Gel	Gel %	Protein Concentration	Number of Protein Species Identified
Sodium dodecyl sulfate (SDS) (0.1%) No gradient (Baseline for statistical comparison)	Large gel	12.5%	500 µg	709 ± 15
SDS (0.1%)		7–20%	500 µg	864 ± 11 *
Lithium dodecyl sulfate (LDS) (0.1%)		7–20%	500 µg	870 ± 12 *
SDS (0.1%) + LDS (0.1%)		7–20%	500 µg	919 ± 15 *
Deep imaging SDS (0.1%) + LDS (0.1%)		7–20%	500 µg	942 ± 7 *

Values given are mean ± SEM for total spot counts; all mean values were derived from three technical replicates. Statistical significance indicated as * $p \leq 0.001$; One way ANOVA, Tukey's multiple comparison test.

For the purpose of presentation, total protein species detected using each approach are given as mean ± SEM (standard error of the mean) of the technical replicates. Individual mean ± SEM for every fraction type are reported as supplementary data.

The spot counts and representative 2DE gels for the different methods of fractionation carried out in phase I optimisation are shown in Table 1 and Figures 2–6, respectively. All fractionation methodologies showed poor protein separation in terms of the molecular properties being targeted. That is, there was considerable overlap in the distribution of protein species between fractions that would have been expected to be far more distinct or defined based on the fractionation technique used. The overlap between the fractions has been represented in the form of fusion images in the supplementary material section. Ultracentrifugation (either 3 h or 16 h) was used to pellet a denser protein fraction. The resolved 2DE gels indicated that a substantial portion of albumin was found in the pellet fraction but most protein species were distributed across both the soluble and pellet fractions (Figure 2). Following TCA/acetone precipitation of serum, the resulting 2DE gels of the pellet fraction were better resolved to a certain extent; there was less streaking and a lower background than seen after ultracentrifugation (Figure 3) and some less abundant species previously masked by albumin were better resolved. However, the total number of protein species detected was reduced compared to the native serum proteome (Table 1), and gels of the organic supernatant fraction suggested that substantial amounts of proteins and charge variants other than albumin were lost using this protocol.

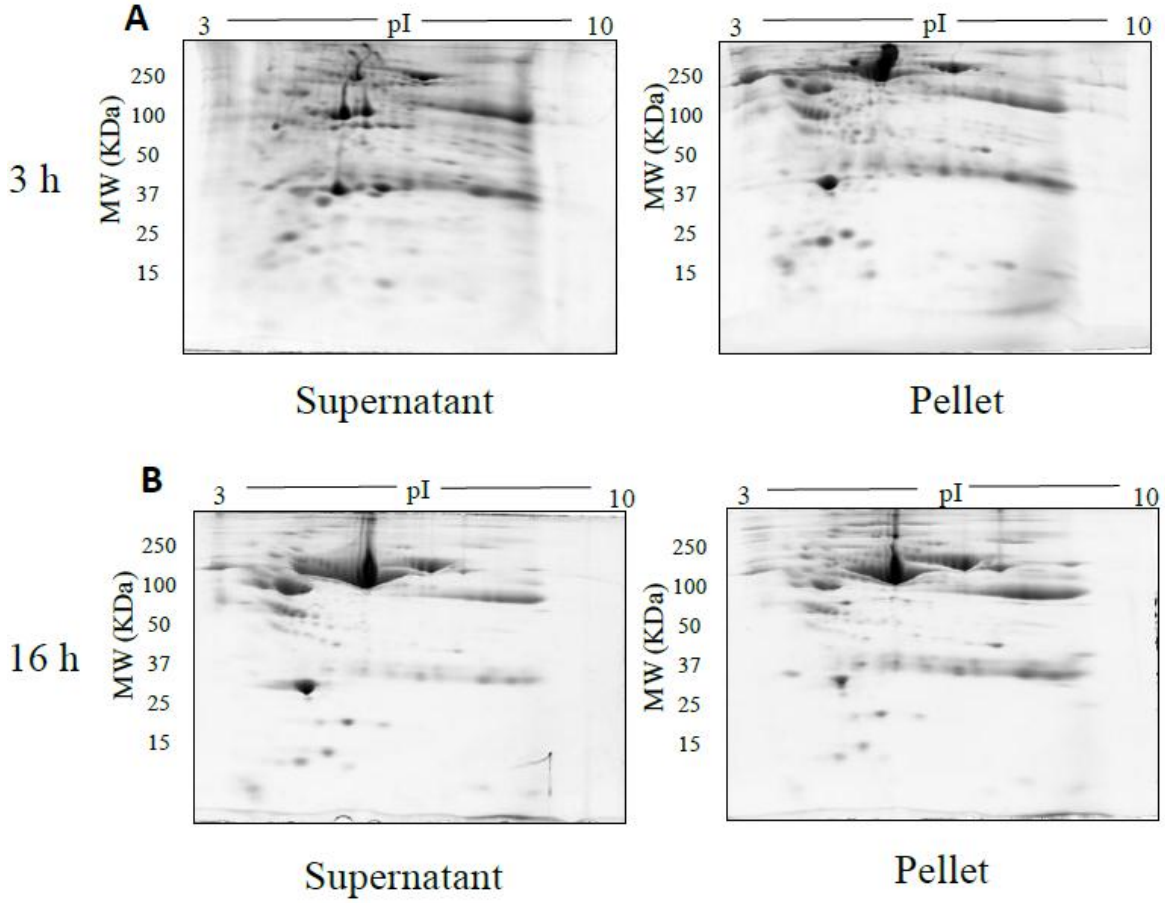

Figure 2. Representative gel images of supernatant and pellet fractions obtained after 3 h (**A**), 16 h (**B**) ultracentrifugation respectively. 100 µg of supernatant and pellet fractions was loaded onto a 7 cm 3–10 NL IPG strip, following 2DE on 12.5% acrylamide gel.

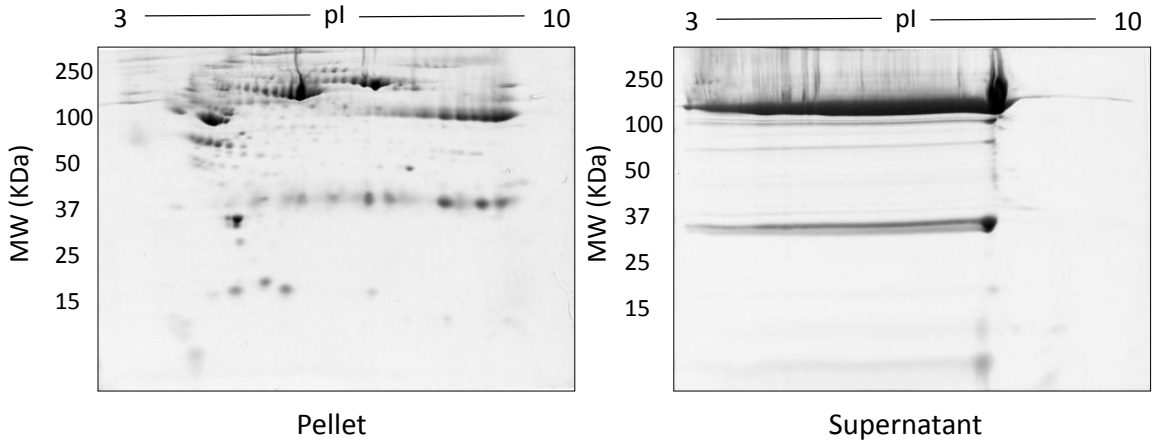

Figure 3. Representative gel images following TCA/acetone precipitation: Shown are the pellet and supernatant fractions resolved on 12.5% acrylamide gels using 100 µg of fractionated serum.

In TX-114 phase separation, the AP did not provide effective separation of proteins, apparently due to residual TX-114 (Figure 4). Incorporating multiple wash steps of the AP helped to remove remaining TX-114 (Figure 4). The number of proteins resolved in AP and DP using TX-114 phase separation was found to be significantly increased ($p \leq 0.001$) compared to native serum (Table 1). Though size exclusion filters performed well in promoting the overall resolution of proteins species relative to the native serum gel, they did not appear to enable effective separation of protein species of different size ranges, nor in limiting albumin to a single fraction. There was thus also substantial evidence that low molecular weight proteins were retained in the high MW (>100 kda) fraction (Figure 5). The commercial affinity columns also proved to be poor discriminators, removing a large number of non-specifically bound proteins together with albumin (Figure 6). Overall, for these initial 12.5% mini gel tests, 3 h ultracentrifugation and TX-114 precipitation resulted in a significant increase in protein species detected compared to native serum. Moreover, when resolved on a large gradient gel of 7–20%, prior TX-114 phase separation resulted in the subsequent detection of 779 ± 51 spots in comparison to 709 ± 15 ($p \leq 0.001$) detected when analysing unfractionated native serum (Table 1).

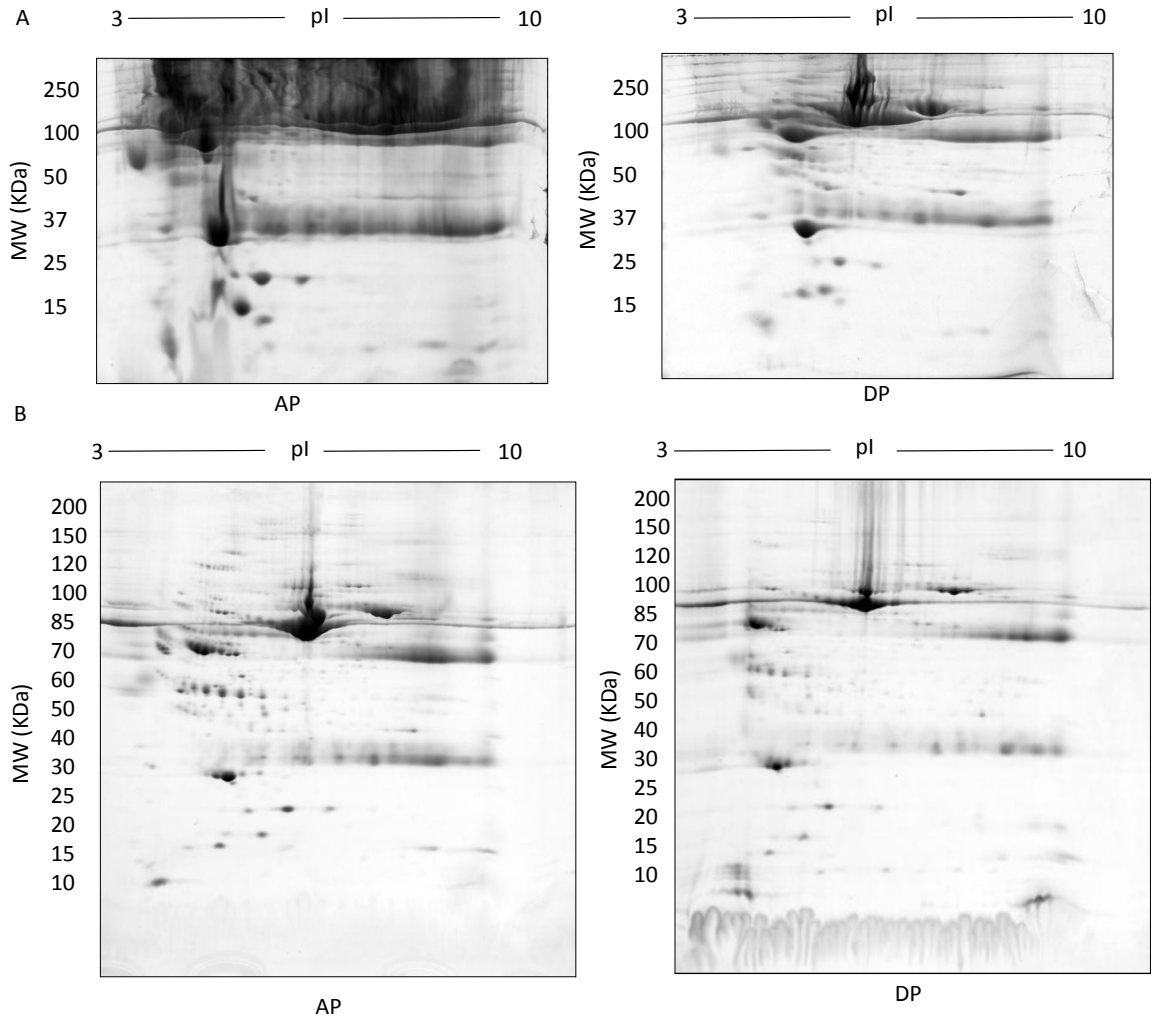

Figure 4. TX-114: Representative images of gels of the detergent phase (DP) and aqueous phase (AP) (**A**), 500 µg fractionated serum resolved on large 7–20% gradient gels after multiple wash steps to ensure removal of TX-114 detergent (**B**).

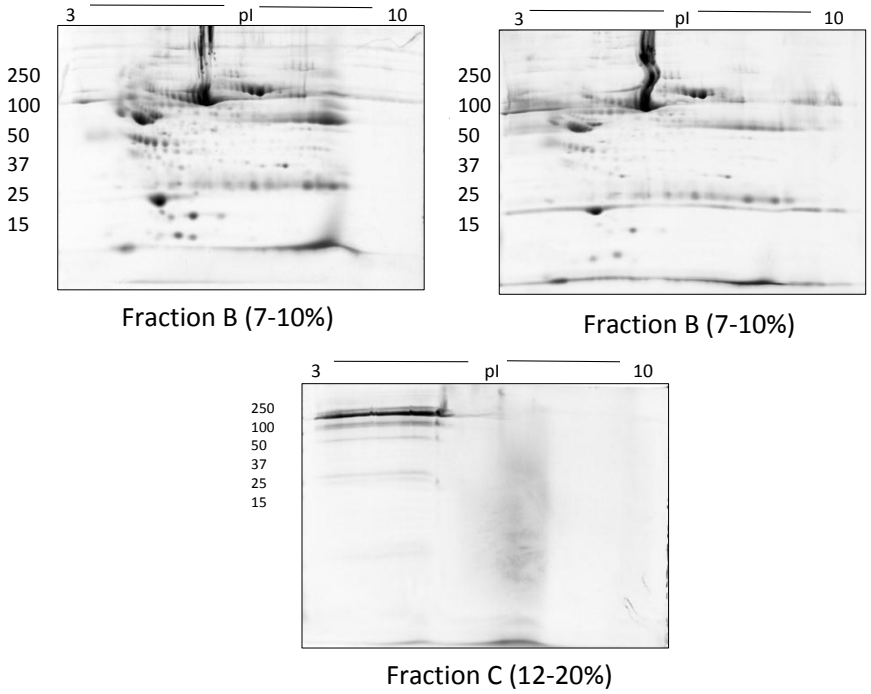

Figure 5. Representative gel images of > 100 kda (fraction A) and flow through fractions i.e., 50–100 kda (fraction B) and < 50 kda (fraction C) following the use of Amicon size exclusion filters.

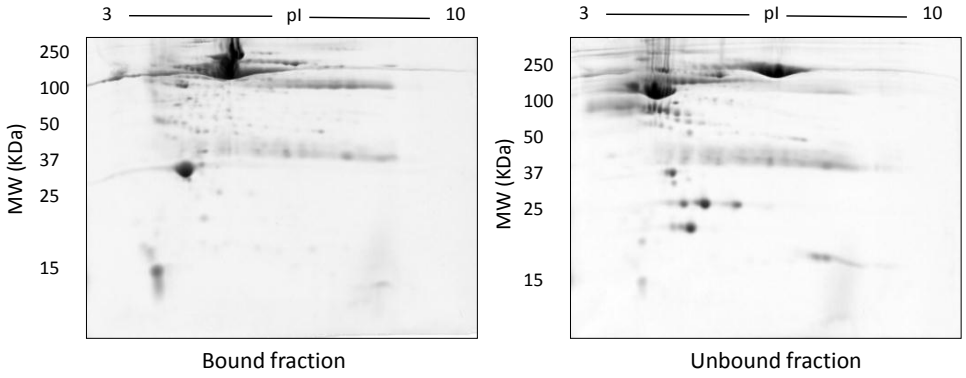

Figure 6. Representative gel images of bound and unbound fractions collected following use of the Bio-Rad Affi-Gel kit. Following manufacturer's instructions, 100 µg of both bound and unbound fractions were resolved on 12.5% acrylamide gels after undergoing isoelectric focusing on a 3–10 NL IPG strip.

In this early stage of optimisation (i.e., phase I), most of the 2DE was carried out on mini gels. However, there was a significant increase in detectable protein species resolved on 7–20% gradient gels rather than 12.5% gels in the second dimension (Tables 1 and 2, and Figure 7). Hence phase II optimisation was carried out on large, 7–20% gradient gels. Phase II optimisation involved resolving native serum without any fractionation step. Table 2 documents a significant increase in the number of protein spots resolved by using a combination of SDS (0.1%) and LDS (0.1%) as compared to the classical SDS (0.1%) in the second dimension (Figure 7). Furthermore, deep imaging of the 0.1% SDS + 0.1% LDS gels resulted in a greater than 2-fold increase of resolved protein species compared to the standard SDS alone ($p \leq 0.001$) (Table 2, Figure 8). Third dimension separation of hyper-abundant protein spots (particularly those known to correspond to albumin, immunoglobin heavy and light chains, and serotransferrin) from serum proteomes initially resolved by 2DE enabled resolution of

additional protein species from these more abundant co-migrating proteins (Figure 9). When fully resolved by 3DE separation, the largest 2DE 'spot' (i.e., more of an irregular shaped, saturating 'blotch') was found to consist of more than one protein, clearly signifying that multiple species are present in large and/or poorly resolved 'spots' (i.e., those not relatively small and tightly circular; Figure 9) [22,24].

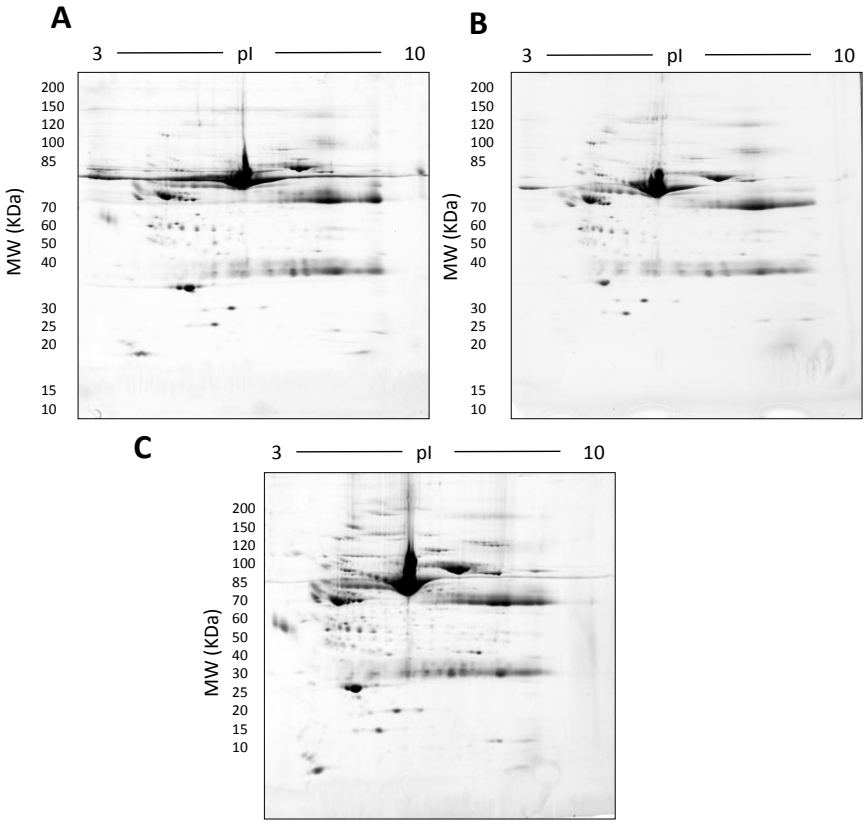

Figure 7. Representative gel images showing the effect of various detergents on the resolution of unfractionated serum: 500 µg total protein, 17 cm, 3–10 NL IPG, and 7–20% second dimension gradient gel using 0.1% SDS (**A**), 0.1% LDS (**B**) and 0.1% SDS + 0.1% LDS (**C**).

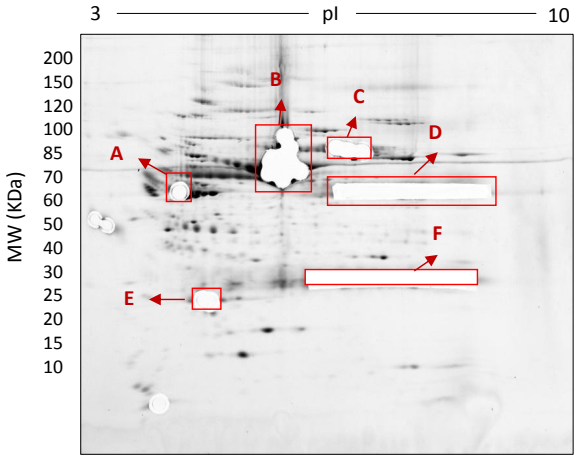

Figure 8. Deep imaged serum proteome after excision of high abundance proteins (i.e., saturating spots). 500 µg total protein, 17 cm, 3–10 NL IPG, and 7–20% second dimension gradient gel using 0.1% SDS + 0.1% LDS.

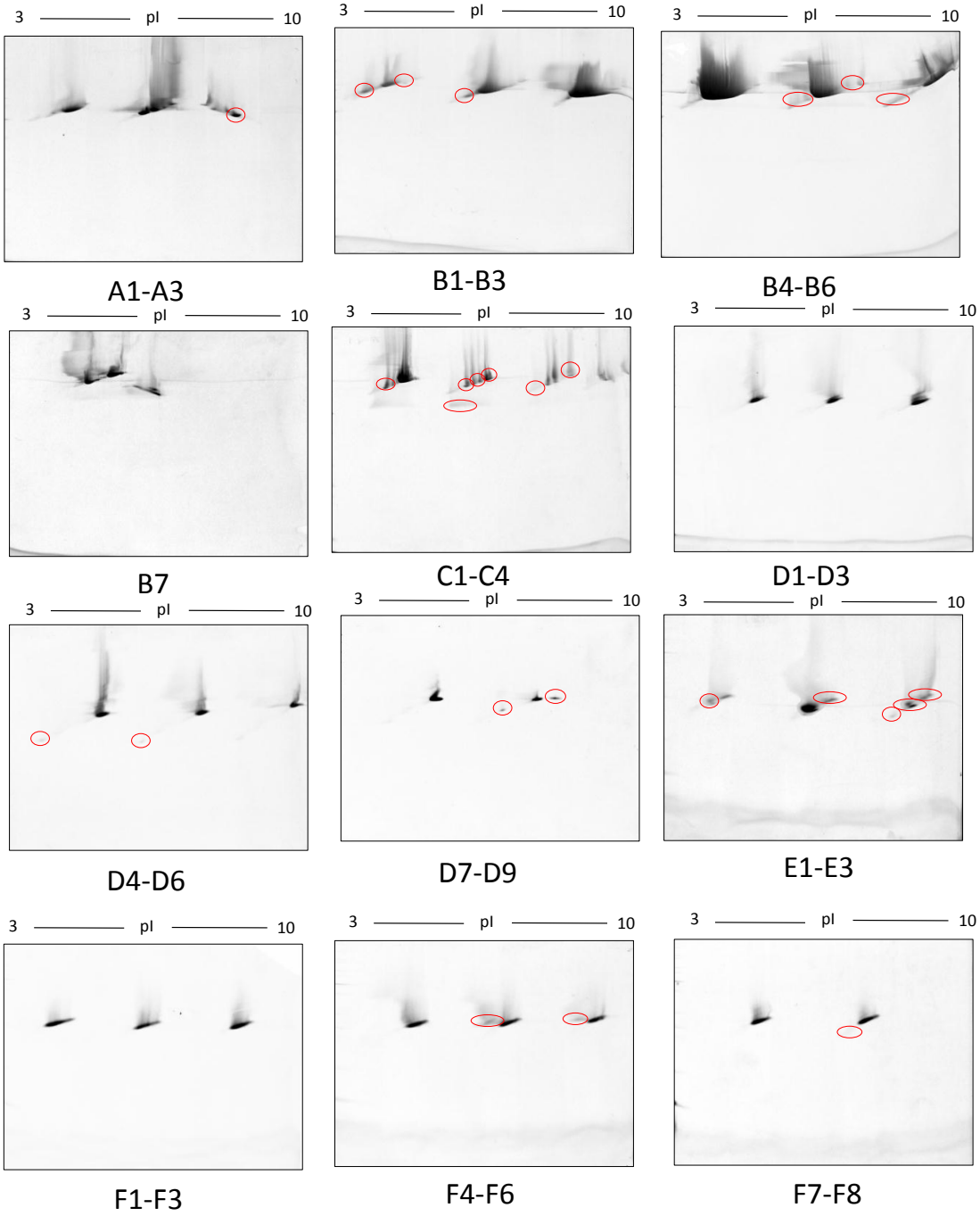

Figure 9. Third-dimension separations of high-density (i.e., hyper-abundant) protein regions excised from serum proteomes resolved by 2DE; red circles indicate protein species resolved from co-migrating hyper-abundant proteins. Designations A-F refer to excised gel regions (see Figure 8), and in each case the associated numbers refer to specific subsections of those excised gel pieces (i.e., A1-A3 means excised region A was subdivided into three approximately equal sized gel pieces that were then resolved in parallel on third gels (see Materials and Methods).

In addition to the general optimisation of serum analyses, we were also interested in PTM. For confirmation at this stage, we thus also trialled the use of phospho- and glyco- staining as a first analysis of select proteoforms (Figure 10).

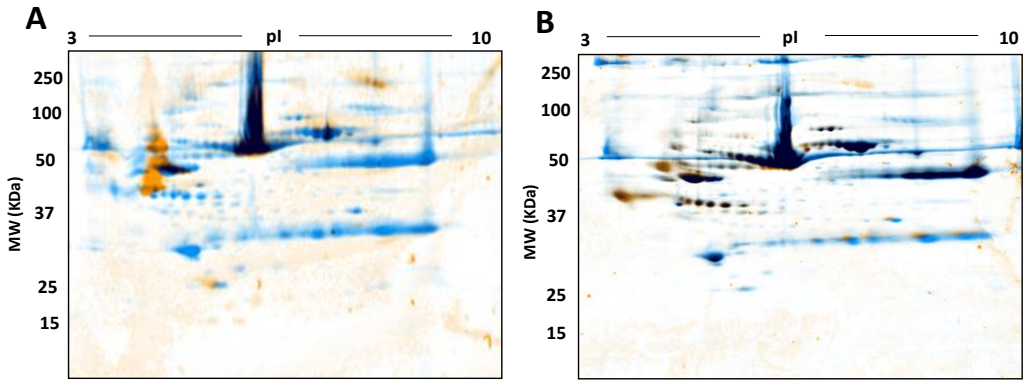

Figure 10. Phospho- and Glyco- proteome assessment: Control serum proteomes resolved by 2DE, stained for phosphoproteins (**A**) and glycoproteins (**B**) respectively followed by total staining by cCBB. In A and B, orange represents phosphoproteins and glycoproteins identified by Pro-Q Diamond Phosphoprotein and Pro-Q Emerald 488 Glycoprotein stains, blue represents total proteins identified by cCBB and black represents the overlap of phosphoproteins and glyco proteins with the total proteins.

4. Discussion

A variety of prefractionation methods have been developed to remove albumin and other high abundance proteins from serum samples prior to proteomic analysis thereby presumably enhancing the detection of lower abundance species of potential interest. However, this approach could lead to the concomitant removal of some non-targeted proteins of potential scientific and clinical interest. We have developed a routine technique for analysing the proteome of crude serum samples that does not involve fractionation and therefore retains the native complement and stochiometry of protein species, and fully enables quantitative analysis. Through systematic comparison of a variety of techniques, we have shown that relatively heavy loading with 500 µg total serum protein on 17 cm 3–10 NL IPG strips in the first dimension followed by second dimension resolution on 7–20% gradient gels with a combination of LDS and SDS detergents provides the optimal current top-down methodology to resolve the human serum proteome. Our optimisation process was carried out in three phases (Figure 1). Phase I optimisation (fractionation) was carried out to reduce the complexity of serum by restricting highly abundant proteins to one fraction. All of the commonly used prefractionation techniques (phase I) that we tested showed substantial overlap in terms of protein content in the separate fractions. This overlap indicates insufficient resolving power of these methods and increases concerns that quantitative analysis using any of these methods is unlikely to yield satisfactory outcomes. In phase II, we were able to obtain significantly increased numbers of resolved protein species by using crude serum itself (Table 2 and Figure 7); this involved optimising 2DE without any fractionation and hence no loss of proteins, in order to facilitate subsequent quantitative assessment. In addition to using standard 2DE techniques for analysis we found that phase III, 3DE of highly abundant proteins, enabled further resolution of co-migrating proteoforms, and Deep Imaging enabled further detection of lower abundance species (Table 2, Figures 8 and 9) [17,23,24]. Use of selective phospho- and glycoprotein stains confirmed the well-established ability of 2DE to routinely resolve proteoforms (Figure 10).

Whilst serum markers play an important role in medical screening, many have seemingly been discovered somewhat serendipitously rather than through a systematic process of biomarker discovery. The subsequent translation of biomarkers from discovery to clinical practice involves multiple stages with many potential pitfalls. One major challenge is the inherent biological complexity of the serum proteome [7]. To date, the majority of research directed to identifying serum biomarkers through a top-down 2DE approach has involved the use of depletion columns to remove the most abundant proteins. The traditional depletion strategy involves the use of a hydrophobic dye, Cibacron blue, which has a high affinity for albumin. This strategy for removing albumin is frequently used in proteomic analyses of serum because of its relatively low cost [31]. The use of immunoaffinity media,

which consist of matrices with covalently attached antibodies to the most abundant proteins has also become commonplace [32,33]. Antibody-based affinity chromatography techniques to remove albumin have been established to isolate and investigate albumin-bound proteins [34]. Immunoglobulins G represent the second most abundant protein species in serum and some methods have been established to remove not only albumin, but also this class of proteins [35,36]. Methodologies based on the depletion of high abundance proteins followed by liquid chromatography-mass spectrometry have been used as well [37,38]. Combinatorial hexapeptide ligand libraries have also been used to enable detection of low abundance proteins of interest [39,40]. This approach also works on the principle of affinity chromatography and has been coupled with antibody based depletion methods to treat human serum and other complex biological extracts [32,33,41,42]. However, a major a disadvantage of this method is that in spite of the large number of ligands, if none has affinity for a given protein, the latter will not be captured [39]. Furthermore, quantitative removal of any given species is not ensured, and nonselective loss of proteins has also been documented, indicating similar selectivity issues and concerns for later quantitative analyses as noted for other affinity-based fractionation approaches [8,43,44]. Overall, the methods above are based on depletion of proteins to 'reduce' the complexity of the serum proteome, usually with the aim of qualitatively detecting more proteins rather than quantitatively identifying and confirming any given species as a biomarker.

However, these depletion strategies can lead to the concomitant non-specific removal of proteins that may be of potential interest [8]. As a transport protein, albumin binds to various compounds including hormones, lipids and amino acids so the loss of low-abundance peptides or small proteins of interest, such as cytokines, is inevitable [45]. Stempfer et al., (2008) quantified the effectiveness of human high-abundance serum and plasma protein depletion using 2DE and bottom-up shotgun MS (i.e., 2D capillary chromatography with MS/MS). The data indicate that some low-abundance proteins were still identified following the depletion protocol; nevertheless, on resolving the depleted fractions, several proteins were found to adhere to the depletion matrix and were thus completely lost to analysis [46]. In brief, these methods have a clear shortcoming in terms of the loss of potentially critical protein species that could be of translational significance in a clinical setting and/or to understanding disease mechanisms, and thus also hamper or even obviate the quantitative analyses necessary to establish the importance of such disease markers or effectors.

Sample preparation is a critical step in the proteomics workflow as the quality and reproducibility of protein extraction and handling significantly impact coverage and quantitative analysis of the native proteome. Minimising sample preparation avoids proteoform degradation and modification. Most of the prefractionation methods we assessed involved several steps and resulted in unexplained variations in analyses of the same samples, including substantial overlap of protein species (i.e., poor separation) between fractions (Figures 2–6). In contrast, starting with 500 µg serum protein on 3–10 NL IPG strips in the first dimension followed by second dimension resolution on a large 7–20% gradient gel by SDS/LDS PAGE improved the resolution and detection of protein species (Table 2). Little is known about the mechanism by which LDS and SDS in combination contribute to protein resolution, although it is well documented that LDS promotes the solubilization and resolution of certain hydrophobic proteins, particularly under the temperature conditions used in our established protocol (i.e., 4°C) [47–49]. In this study, in particular, low molecular mass protein species were strongly enriched using a combination of SDS and LDS confirming what has been previously noted in the literature [50]. Furthermore, adding LDS pre- and post-second dimension of electrophoresis (i.e., in the equilibrating buffer, the gel matrix, and the 2D buffer system) enabled resolution of certain proteins not detectable when using SDS or LDS alone (Table 2). We also confirm the resolution of proteoforms using selective staining for phospho- and glycoproteins (Figure 10).

5. Conclusions

When selecting a prefractionation method to assist in sample preparation, it is imperative to assess the potential for loss of low abundance proteins; the possibility of carrier protein interactions with

critical low molecular weight biomarkers is likely a serious issue hampering further advances. Most studies that have investigated the serum proteome in various disease conditions have not been able to successfully characterize potential biomarkers nor validate their results in a larger cohort. Simply, altering the native stoichiometry of the proteome components may yield a more complex series of issues than dealing directly with the inherent complexity of the sample. This is the fundamental basis for our focus on top-down proteomic analyses of native samples.

In terms of the current investigation, maternal serum samples were pooled from three individual patients in order to evaluate methods and develop an analytical process. The method developed in this study is now being routinely applied to clinical samples. We will be applying the approach defined here to further characterize and quantify protein species in a larger cohort of subjects to provide a better understanding of the maternal serum proteome. One of the potential limitations of the results presented here was the need to 'over'-load the gel with 500 µg protein in order to assess lower abundance species (although this only amounts to an average of ~10 µL of serum). While this would not be an issue for several standard clinical samples (e.g., plasma, urine, saliva), it may not be feasible with regard to other health complications [5]. Importantly, the advantages of the approach defined here cannot be overstated: quantitative analysis of protein species in their native melieu (i.e., without exposure to fractionation and other chemical manipulations). It is expected that this represents an opportunity for broader use of this critical top-down approach to proteomic analyses of serum and other important clinical samples. We anticipate that this top-down 2DE approach will prove to be a powerful tool for quantitative, reproducible and thorough analyses of proteoforms, and thus imperative to assessing health, disease state and progression, as well as the identification of critical biomarkers.

Supplementary Materials: Supplementary Materials are available online.

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Author Contributions: J.R.C. and A.M.D. conceived and designed the experiments; J.A.H. collected serum samples; A.M.D. performed the experiments; J.R.C. and A.M.D. analysed the data; J.R.C., J.A.H. and A.M.D. wrote the paper. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Contributions

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First trimester protein biomarkers for risk of spontaneous preterm birth: identifying a critical need for more rigorous approaches to biomarker identification and validation

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Short title: Early biomarkers for preterm birth

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Abstract

Spontaneous preterm birth is the leading cause of perinatal morbidity and mortality worldwide and continues to present a major clinical dilemma. We previously reported that a number of protein species were dysregulated in maternal serum collected at 11-13^{+6} weeks' gestation from pregnancies that continued to labour spontaneously and deliver preterm. In this study, we aimed to validate changes seen in four candidate protein species: Alpha-1-antitrypsin, Vitamin D-binding protein, Alpha-1 beta-glycoprotein and Apolipoprotein A-1 in a larger cohort of women using a western blot approach. Serum levels of all four proteins were reduced in women who laboured spontaneously and delivered preterm. This reduction was significant for Vitamin D-binding protein ($p=0.04$), which has been shown to be involved in a plethora of essential biological functions, including actin scavenging, fatty acid transport, macrophage activation and chemotaxis. The decrease in select proteoforms of Vitamin D-binding protein may result in an imbalance in the optimal intrauterine environment for the developing fetus as well as to a successful uncomplicated pregnancy. Thus, certain (phosphorylated) species of Vitamin D-binding protein may be of value in developing a targeted approach to the early prediction of spontaneous preterm labour. Importantly, this study raises the importance of a focus on proteoforms and the need for any biomarker validation process to most effectively take these into account rather than the more widespread practice of simply focussing on the primary amino acid sequence of a protein.

Introduction

Spontaneous preterm birth (sPTB), defined as spontaneous birth before 37 weeks' gestation, is the most significant problem affecting contemporary obstetric practice and is recognised as a major health concern worldwide (1-4). 15 million babies are born preterm each year and prematurity is described as the cause of death in 10% of stillbirths and 40% of neonatal deaths (5). Preterm infants are ≥20 times more likely to require neonatal admission than term babies and these infants suffer significant short and long term morbidity (6). Despite considerable effort, the incidence of preterm birth is still rising (7). Given the complex and poorly characterized pathophysiological pathways associated with preterm birth, its prediction has been a challenge. As a result, almost every preterm event is an emergency, adding further stress to mother and fetus, as well as healthcare systems.

Current strategies for prediction and prevention of preterm birth focus on identification of, and intervention in, a high-risk cohort in the second or third trimester of pregnancy (8-10). Screening tools such as ultrasound assessment of cervical length require significant capital investment and the availability of trained staff but have modest screening efficacy (11). Women deemed to be at high-risk of spontaneous preterm birth may be prescribed preventative therapies such as progesterone, but this does not prevent all cases of prematurity and there is some controversy about the impact of preventative interventions at this relatively late stage (12, 13). There are far fewer data describing first trimester models for risk prediction and these are currently less effective than second / third trimester counterparts (14). Cervical length is often used in these models but is harder to assess earlier in gestation due to apparent continuity with the lower uterine segment. Multivariate algorithms that involve combinations of maternal history and other investigational tools have shown only moderate levels of efficacy in screening for sPTB (15, 16). Given that current screening strategies have not made a significant impact on preterm delivery rates, there is an urgent need to improve our understanding of the molecular mechanisms underlying preterm birth and to identify and develop other biomarkers that can be assessed early in pregnancy, enabling more timely and effective preventative action.

Current proteomic approaches enable comprehensive surveys of low- and high-abundance proteins in tissues and various biological fluids. Notably, as protein species or proteoforms (i.e. isoforms, splice variants, and post-translationally modified forms) are generally the effectors of biological mechanisms, it is critical to employ an analytical approach that can effectively resolve proteomes without loss of the important information embodied by this diverse group of molecules (17-19). Thus, in order to identify protein species that may be of

value in identifying pregnancies at risk of preterm birth, we used a refined top-down proteomic approach coupling two-dimensional gel electrophoresis (2DE) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) to characterise differential proteoform profiles in first trimester maternal serum (20, 21). In contrast to other analytical approaches, the 2DE/LC/MS/MS approach can simultaneously resolve hundreds-to-thousands of proteoforms in a single analytical run and enables identification of species undergoing changes in abundance. In an initial analysis of serum (20), thirty proteoforms were found to be significantly altered at 12 weeks of gestation in a group of women who subsequently experienced sPTB relative to a matched group who experienced normal term delivery. The distinguishing proteoforms included Alpha-1-antitrypsin (A1AT), Vitamin D-binding protein (VDBP), Alpha-1beta-glycoprotein (A1BG) and Apolipoprotein A-1 (ApoA1) that had previously been described in pathways associated with preterm birth. In the current targeted analysis, we sought to validate whether these protein species were similarly altered in a second, larger cohort of patients that subsequently delivered preterm after spontaneous onset of labour.

Materials and methods

Study design

Proteomic analyses were carried out on a cohort of maternal serum samples prospectively collected and stored during first trimester screening at $11\text{-}13^{+6}$ weeks' gestation. The samples were collected between 2011 and 2014 and serum was separated within four hours of collection. Aliquots of residual serum, available after measurement of free βhCG and PaPP-A, were stored immediately at -80°C. Details of pregnancy outcome were collated at the end of pregnancy allowing identification of a cohort of women who laboured spontaneously and delivered before 37 weeks' gestation (n = 44). These were matched (one to one) to a cohort of women who laboured spontaneously and delivered at term (>37 weeks' gestation) (n = 44). Note, this total cohort also included serum samples from the original 10 sPTB and 10 term delivery cases analysed using 2DE/LC/MS/MS (20). Control samples were matched by maternal age, gestational age, BMI, parity, smoking status, sex of the fetus and sample storage time. Samples were excluded if preterm delivery was elective, had occurred after spontaneous rupture of membranes or involved a multiple pregnancy or a pregnancy affected by chromosomal or structural abnormality. The study was approved by the local hospital Human Ethics Committee (Protocol No: X11-0305).

Differences in abundance of select serum proteins identified in an earlier initial quantitative proteomic analysis (20) were validated further using a well-established, high sensitivity

western blotting approach. Selection of the proteins for validation was based on fold-changes in abundance (from the original 2DE assessments) and possible functional association of the proteins with PTB as documented in the literature (20). Western blot analyses of four differentially expressed target proteins — Alpha 1-antitrypsin, Vitamin D-binding protein, Alpha-1beta-glycoprotein and Apolipoprotein A-1 — were carried out on serum samples from the 44 sPTB patients and 44 matched term controls described above.

Antibodies

Primary antibodies: Monoclonal rabbit [EPR17087-50] to Alpha-1-antitrypsin (ab207303), monoclonal rabbit [EP2923Y] to Vitamin D-binding protein (ab81307) and monoclonal rabbit [EPR9830(B)] to Alpha-1beta-glycoprotein (ab156009) were purchased from Abcam Australia Pty Ltd, Melbourne, Australia. Monoclonal mouse Apolipoprotein A-1 (B-10) (SC-376818) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). These antibodies are hereafter referred to as ab207303, ab81307, ab156009 and SC-376818, respectively.

Secondary Antibodies: Goat Anti-Rabbit IgG heavy and light (H&L) conjugated to Horseradish Peroxidase (HRP) (ab97051) was purchased from Abcam Australia and was used to detect ab207303, ab81307 and ab156009. Mouse IgG kappa binding protein (m-IgGκ BP) conjugated to HRP (SC-516102) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and was used to detect SC-376818. All antibodies were stored and handled according to the manufacturer's instructions.

1-Dimensional gel electrophoresis (1D SDS-PAGE)

Crude maternal serum with added protease, kinase and phosphatase inhibitors (21) at a ratio of 1 × conc of inhibitors (initial conc of 500 ×) : 2 mL of serum was diluted in 1 x Phosphate buffered saline (PBS) and solubilized in 2 x sample buffer (Laemmli 2 x concentrate (Tris 0.5 M, pH 6.8, Glycerol 20% (v/v), EGTA 6 mM (w/v), SDS 4% (w/v), bromophenol blue 0.001% (w/v), β-mercaptoethanol 0.2% (v/v), added 1:1 just prior to use)) and heated at 100ºC for 5 min. The total protein concentration of each sample was determined using the EZQ Protein Quantitation Kit with BSA standards according to the manufacturer's instructions (Molecular Probes, Oregon, USA). A final concentration of 5 µg total protein in 20 µL was loaded onto each lane of 8 – 16 % SDS polyacrylamide mini electrophoresis gels (10 x 8 cm SDS-PAGE) for Alpha-1-antitrypsin; 10 % mini SDS-PAGE was used to resolve Vitamin D-binding protein, Alpha-1beta-glycoprotein and Apolipoprotein A-1, respectively. The samples were also randomized to ensure that replicates of any given sample were not resolved on the same gel.

The gels were resolved at 100 V in SDS running buffer at 4ºC. All gels and thus subsequent immunoblots were replicated at least in triplicate.

Western blot

Western blotting was carried out essentially as previously described (22). Briefly, after completion of SDS-PAGE, proteins were electrophorectically wet-transferred using 1 x transfer buffer (Glycine 192 mM, Tris 25 mM, methanol 20%) at 100V to a 0.2 µm pore size polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) for 2 hrs at 4ºC, thereby immobilizing the separated proteins. Post transfer, the PVDF membrane was blocked in TBST buffer (Tris 10 mM pH 7.4, NaCl 150 mM, 0.1% Tween 20) containing 5% skim milk powder (Woolworths brand) and 1% polyvinylpyrrolidone (PVP-40) for 1 hr at room temperature (RT) on a shaker, and subsequently washed with TBST 3 x 15 min. Primary antibodies [(ab207303 1:5000 dilution), (ab81307 1: 5000 dilution), (ab156009 1:5000), (SC-376818 1: 500 dilution)] were applied to the blots and incubated overnight at 4ºC (~16 hrs). The following day, blots were washed with TBST buffer 3 x 5 min and incubated with HRP conjugated secondary antibodies [(ab97051 1: 25,000 dilution), (SC-516102 1: 1000 dilution)] for one hour at RT on a shaker. After secondary incubation, the blots were washed with TBST 3 x 10 min followed by TBS for 10 min and protein bands were developed using the Enhanced chemiluminescence (ECL) Plus detection kit (GE Healthcare). The immunoblots were scanned and florescence signals measured using the LAS 4000 Biomolecular Imager, and the integrated pixels was quantified using Multi Gauge software v3.0 ((FUJIFILM, Tokyo, Japan). Images were captured using high resolution sensitivity and 5 s 'precision' exposure, at tray positions TP 1 – 4, for ab207303, ab81307, ab156009 and 2 s exposures for SC-376818, at tray positions 1 – 4.

Each patient sample was resolved in triplicate for every antibody. All western blots were imaged individually. A series of prior optimizations were carried out for each antibody. This included optimizing protein load (i.e. ensuring that signal did not saturate and thus changes in signal intensity (i.e. protein abundance) could be readily and consistently detected), percentage of acrylamide gel, transfer time, primary and secondary antibody dilutions and incubation time for probing with the primary antibodies (Figure 1). Additionally, secondary antibody controls were carried out in parallel for each antibody and, after electrophoretic blotting, SDS-PAGE gels were stained and assessed using a protocol providing low-to-sub-femtomole detection of proteins (Figure 2) (23, 24); relative to identical control gels that had not undergone blotting, these confirmed a consistent transfer efficiency of ~95% for each of

the target proteins (Figure 3). Total lane scans of random control gels (i.e. not blotted) also confirmed consistency of total protein load per lane and thus overall normalization of loading.

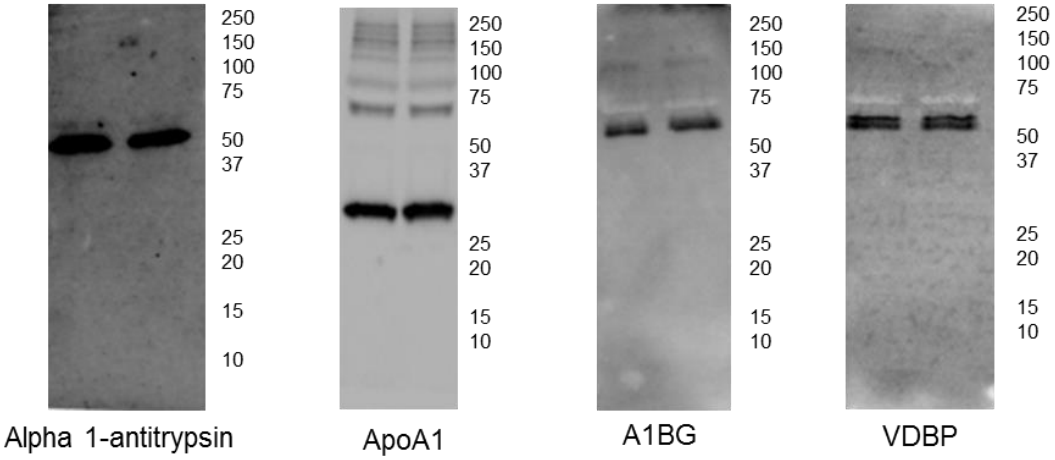

Figure 1: Representative immunoblot images of full lanes of Alpha 1-antitrypsin, Vitamin D-binding protein, Alpha-1beta-glycoprotein and Apolipoprotein A-1 to indicate selectivity of bands.

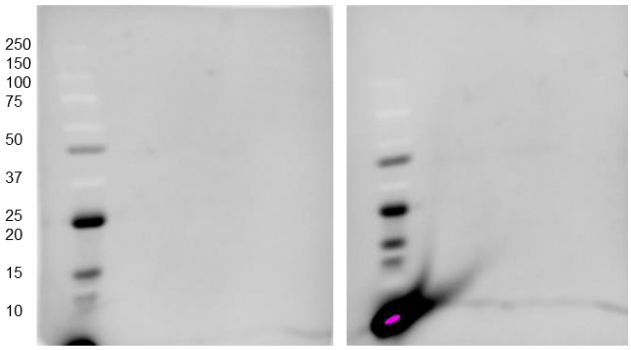

Figure 2: Representative images for secondary antibody; Goat Anti-Rabbit IgG and Mouse IgG kappa binding protein

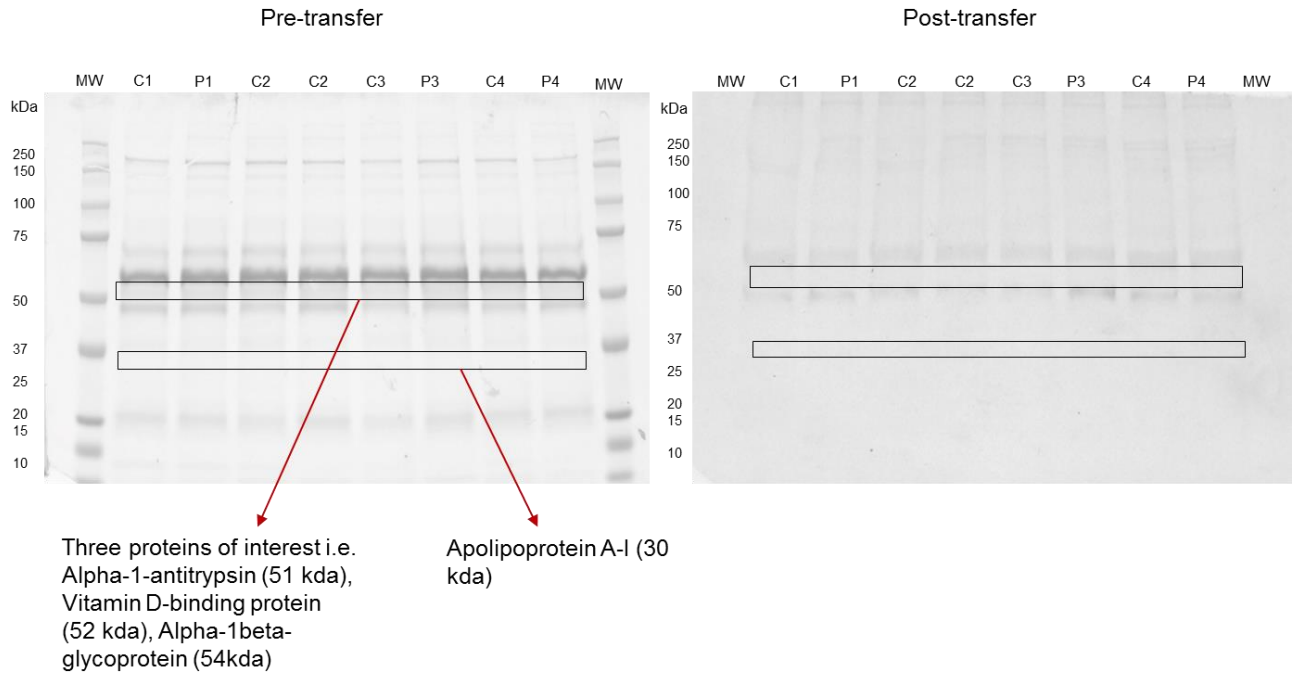

Figure 3: A stained gel before and after transfer with the areas of interest indicated

Image analysis

Multi Gauge software was used for densitometric analyses of western blots. A rectangular box was created for each band of interest using the 'rectangular select tool'. Per band, background measurements of equivalent total area were taken from 'clear' or 'blank' regions of the blot, beneath the band of interest. Resulting raw data were analysed in Excel (Microsoft office, 2016) to obtain background subtracted final values for every protein.

Statistical analysis

Comparisons between the term and preterm groups were made in Graph Pad Prism v7.03 using Paired t-tests. A value of $p < 0.05$ (*) was considered statistically significant, and $p < 0.01$ (**) was considered highly statistically significant. Data are presented as the mean ± standard error mean (SEM). Each result represents data from three independent western blots for each sample. The groups were tested for normal distribution using Wilcoxon's signed ranked test. Pairwise comparisons were carried out using SPSS statistics software v24.0 after adjusting for maternal age, BMI, ethnicity and parity.

Results

88 first trimester serum samples (44 from pregnancies that delivered after the spontaneous onset of labour <37 weeks gestation and 44 from pregnancies that delivered ≥37 weeks gestation) were analysed here using a high sensitivity western blot approach. This included the 10 sPTB and 10 terms samples used in our discovery phase (20). Demographic and clinical characteristics of these samples are presented in Table 1. The only significant difference between sPTB cases and controls were the gestational age at delivery (p=0.0001) and birthweight (p=0.0001), as was the case in the initial biomarker discovery study (20).

Table 1: Baseline characteristics of original and validation cohorts; sPTB vs term control pregnancies.

Demographic feature	sPTB (n = 44)	Controls (> 37 wks.) (n = 44)	Significance (p value)
Maternal characteristics			
Age (years)	31.45 ± 0.56	32.57 ± 0.43	0.06
Gestational age at delivery (weeks)	32.66 ± 0.51	39.48 ± 0.15	0.0001*
Parity: = 0	43%	45%	
> 0	56%	54%	NA
Ethnicity: White	40%	40%	
East Asian	43%	43%	NA
South east asian	11%	11%	
Black	4%	4%	
BMI (kg/m^2)	24.53 ± 0.75	23.71 ± 0.66	0.07
Smoking status: Yes	-	-	
No	44	44	NA
Outcome characteristics			
Birth weight (g)	2236 ± 92.7	3514 ± 63.3	0.0001*
Sex: Male	65%	61%	
Female	34%	38%	NA

Values have been represented as mean ± SEM or %, statistical analysis involved an unpaired *t* test. * Denotes statistical significance

Representative western blot images for Alpha-1-antitrypsin, Vitamin D-binding protein, Alpha-1beta-glycoprotein and Apolipoprotein A-1 immuno-reactive bands are shown in Figure 4. Statistical analyses of these data indicated a significant reduction in levels of Vitamin D-binding protein in the sPTB cohort compared to the term controls (27.8 ± 1.7 vs. 30.8 ± 2; p = 0.04 (Table 2; Figures 4 and 5). There were no significant differences in the serum concentrations of the remaining three candidate proteins (Table 2; Figures 4 and 5).

Table 2: Western blot analyses of selected proteins in women who subsequently had a sPTB vs. full term birth controls

	sPTB n = 44	**Controls n = 44**	**Significance (p value)**
Alpha 1 antitrypsin	80.3 ± 2.9	80.9 ± 2.8	0.81
ApoA1	49.2 ± 4	48.4 ± 4.9	0.87
A1BG	30.7 ± 2.0	32.1 ± 2.2	0.25
VDBP	27.8 ± 1.7	30.8 ± 2	0.04

Intensities (arbitrary units) of immuno-blot bands; (mean ± SEM)

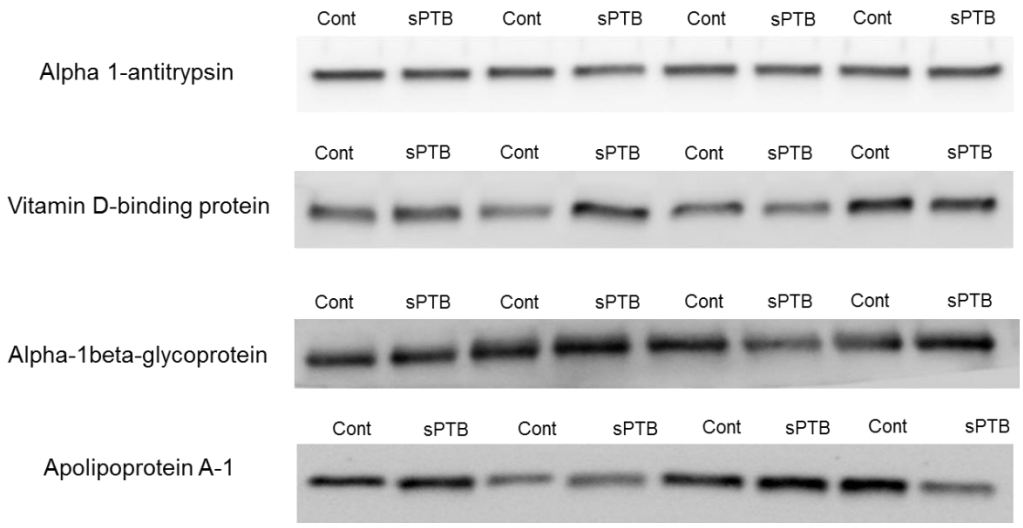

Figure 4: Representative immunoblot images of Alpha 1-antitrypsin, Vitamin D-binding protein, Alpha-1beta-glycoprotein and Apolipoprotein A-1 in control and sPTB. 5 µg of total protein was loaded per lane.

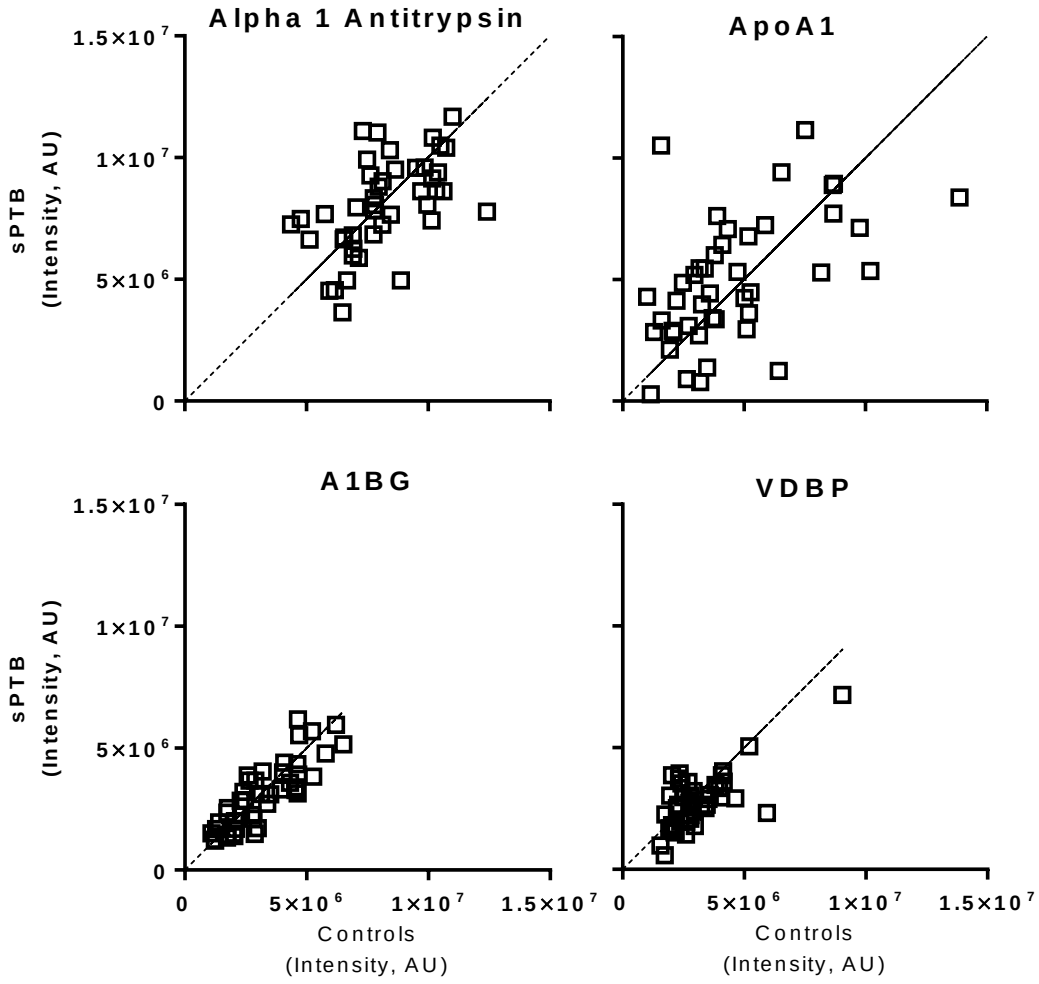

Figure 5: Representative graphs showing levels of Alpha 1-antitrypsin, Vitamin D-binding protein, Alpha-1beta-glycoprotein and Apolipoprotein A-1 in serum from sPTB and matched controls.

Further analysis of the data, allowing for differences in maternal age, BMI, parity, ethnicity and sex of fetus did not reveal any significant differences in protein levels between preterm cases and controls (Table 3). Statistical analysis accounting for maternal factors identified significant differences of Alpha-1-antitrypsin relative to ethnicity (Table 3). Women of South East Asian background had the highest levels of Alpha-1-antitrypsin, followed by Caucasians and East Asians (Table 3). Levels of Vitamin D-binding protein were significantly different comparing Caucasian and East Asian women (p=0.017); Caucasians had a 33% higher level. Maternal age appeared to be inversley correlated with Apolipoprotein A-1, but this did not reach significance (p = 0.056). Serum concentrations of Alpha-1beta-glycoprotein were reduced in nulliparous women (p=0.058) and there was an inverse relationship between Alpha-1beta-glycoprotein and BMI (Table 3; p=0.044). Assessment of combinations of protein species after adjusting for maternal age, BMI, parity and ethnicity, did not identify any significant differences in protein levels between sPTB cases and matched term controls (Table 4).

Table 3: Parameter estimates of Alpha-1-antitrypsin, Vitamin D-binding protein, Alpha-1beta-glycoprotein and Apolipoprotein A-1 in relation to maternal characteristics

	Parameter	Mean difference	Significance
Alpha 1-antitrypsin	Ethnicity: Caucasian	27.6	0.005
	East Asian	22.0	0.024
	South East Asian	30.3	0.006
Apolipoprotein A-1	Maternal age	-44.83	0.056
Alpha-1beta-glycoprotein	Parity	-.203	0.058
	BMI	-.023	0.044
Vitamin D-binding protein	Ethnicity: Caucasian	-2.85	0.017
	East Asian	2.85	0.017

*For the purpose of this presentation, only findings that reached significance or close to significance are reported.

Table 4: The effect of case status on ***combined*** protein expression, while adjusting for maternal age, BMI, parity and ethnicity

Combination of protein species	coef_for_case_status	pv_for_case_status
Alpha.1.antitrypsin+VDBP	-983276	0.067
Alpha.1.antitrypsin+ApoA1	-1046846	0.234
Alpha.1.antitrypsin+A1BG	-841040	0.214
VDBP+ApoA1	-792428	0.289
VDBP+A1BG	-586622	0.154
ApoA1+A1BG	-650192	0.384
Alpha.1.antitrypsin+VDBP+ApoA1+A1BG	-1633468	0.106

pv = p-value, coef = the effect of being a case on the sum of given proteins

Discussion

We carried out a larger-scale validation study concerning previous findings identifying altered abundance of four protein species that appeared to be strong candidates as first trimester serum biomarkers for subsequent spontaneous preterm labour. Only one of these four proteins, Vitamin D-binding protein, showed a significant change in abundance in this validation cohort. Interestingly, this involved a decrease in the abundance of Vitamin D-binding protein whereas our smaller discovery data set had suggested an increase in the amount of some proteoforms and a decrease in a phosphorylated species. The broader implications of these findings to biomarker identification and validation studies are discussed.

Vitamin D-binding protein, a 56–58 kDa plasma alpha-globulin, binds vitamin D and promotes actin clearance during tissue remodelling following the augmentation of the pro-inflammatory response (25). Serum concentrations of both vitamin D and Vitamin D-binding protein are typically increased in pregnancy (26). Dysregulation of both total serum Vitamin D levels and of Vitamin D-binding protein have been associated with adverse pregnancy outcomes such as pre-eclampsia, intrauterine growth restriction, gestational diabetes and preterm birth (27-31).

Evidence regarding associations between plasma concentrations of Vitamin D-binding protein and pregnancy complications is conflicting. Kolialexi *et al*. (2017) analysed first trimester

maternal plasma using 2DE and MALDI-TOF-TOF MS/MS to identify biomarkers for early onset preeclampsia (ePET). They identified 12 differentially expressed proteins, including Vitamin D-binding protein, which showed 3.4 fold up-regulation (32). In contrast, Tannetta *et al*. (2014) who investigated the actin scavenging system in pre-eclampsia, showed that plasma levels of actin-free Vitamin D-binding protein (measured using an ELISA immunoassay) tended to be lower in ePET pregnancies (33). The authors suggested that these findings were associated with depleted actin clearance and could be associated as a cause or a consequence of preeclampsia pathophysiology. A third study, by Powe *et al.* (2010), showed no significant difference in first trimester concentrations of Vitamin D-binding protein (measured using ELISA) between preeclamptic and control cohorts (29).

Although there are no studies reporting changes in Vitamin D-binding protein concentrations in maternal blood specifically related to preterm labour, analysis of the proteome of cervical secretions has shown an increase in Vitamin D-binding protein in women that deliver preterm (34). In a second study, Liong *et al.* (2013) concluded that assessment of cervical swabs for Vitamin D-binding protein could be a useful predictor in women presenting with threatened preterm labour with sensitivity and specificity of 58.5% and 98.6% respectively (35). As Vitamin D-binding protein protects against vascular dysfunction and modulates the inflammatory response it is not unreasonable to anticipate that it will be altered in pregnancies that deliver preterm. The precise nature of the metabolic effect(s) mediated by VDBP require further elucidation and there needs to be more work to identify its importance in this field.

There are several reasons why we may not have been able to validate apparent changes in protein abundance associated with subsequent onset of sPTB in the remaining three proteins examined in this study, and why the validation of VDBP does not fully correlate with our previous findings. The cohort of women included in this study delivered a mean of three weeks later than those included in the original cohort. Similarly, this cohort included women from a wider range of ethnic backgrounds than the original cohort. There is also increasing evidence indicating that 2DE gel spots do not necessarily contain a single protein species; more sensitive mass spectrometry may identify 40-60 proteins from one spot (36). The proteins assessed in this paper were originally identified through a 2DE/LC/MS/MS approach which included the criteria of high mascot scores, unique peptides and acceptable sequence coverage. It is however possible that apparent changes in protein abundance may have been related to changes in other, lower abundance proteins associated with that spot. Alternatively, like perhaps all previous studies in this and other areas, the immunodetection analysis used (e.g. western blot here; ELISA in many other studies) may have failed to adequately identify critical proteoforms (e.g. specific post-translationally modified species) as the antibodies used

in these assays may or may not differentiate between species and/or may be differentially selective depending on the type and location of different modifications (e.g. phosphorylated or glycosylated moieties).

Thus, using VDBP as an example, the antibody used here clearly detects a prominent band on 1D gels at a MW consistent with the amino acid sequence of the protein. Small chemical modifications such as phosphorylation will not significantly change the MW of the protein as it resolves on a 1D gel (although will shift the pI on a 2D gel and will be particularly detectable if there is more than one phosphate group attached); potentially larger PTM such as large sugar moieties may however also result in detectable shifts in MW on 1D gels. However, depending on the location of any particular PTM (and likely its size as well), these may interfere with antibody binding. It is thus noteworthy that in our original study, VDBP was identified from three separate spots following high resolution 2DE (20). In the first instance VDBP was detected based on total protein staining, but this species was substantially more acidic than what is theoretically expected based on the amino acid sequence alone, implying substantial PTM such as multiple phosphorylations (20). Indeed, there are multiple phosphorylation sites as well as other PTM associated with VDBP, including a known glycosylation, and at least two isoforms of VDBP are produced by alternative splicing. Thus, it is not surprising that our high resolution 2DE protocol also identified both phosphorylated and glycosylated proteoforms of VDBP and found changes associated with sPTB: while the glycosylated proteoform and those detected with total protein staining were found to increase in sPTB cases, at least one (or more) phosphorylated species was found to decrease significantly in the first trimester serum of women who subsequently experienced a case of sPTB (20).

Taking all this information into consideration, it would seem that at least some phosphorylated proteoforms of VDBP (which would be unlikely to resolve as separate bands on a 1D gel) are already sufficiently decreased in abundance in the first trimester serum of women who subsequently experience sPTB to yield detectable differences in the high sensitivity western blot assay used here. That is, these phosphorylated proteoforms are of sufficient abundance that their detection outweighs the detection of other, unphosphorylated VDBP species that undoubtedly also resolve in the same gel band. This suggests the existence of one or more phosphorylated VDBP species that may well prove to be highly sensitive and selective early serum biomarkers for sPTB. This also raises the concern that lack of validation of the other three potential biomarker proteins assessed in this study may well rest with the antibodies and choice of assay used (i.e. lower resolution 1D gels). Are some proteoforms that are not

selectively associated with sPTB simply of higher abundance than some that are, and the result in an overall 'average' 1D western blot signal that is not significantly different between the control and sPTB study cohorts? The same result would also occur using commercial ELISA assays since most commercial antibodies are selected for their ability to detect a protein of interest rather than a specific proteoform (although there are of course exceptions, including antibodies to specific phosphorylated variants of proteins). Would an antibody selective for a PTM modified species of these proteins and/or the use of higher resolution 2DE rather than 1DE have resulted in confirmation of another potential biomarker or at least clear-cut data eliminating a candidate? These are specific directions that will be addressed in future studies.

Importantly, for this and any other biomarker studies in any field, these findings raise critical issues regarding the quality of up-front (i.e. initial) analyses and thus detection of meaningful molecular species, as well as the need for rigour in the validation process, with far more stringent attention to potentially critical proteoforms rather than simply the amino acid sequence of a protein in a database. Post-translational modifications may well hold the key to more successful identification and validation of critical biomarkers. Thus, spontaneous preterm birth continues to be a significant public health problem and the development of a first trimester predictive test that allows modification of pregnancy outcome remains a research priority. The abundance of different proteoforms of Vitamin D-binding protein, in particular phosphorylated species, appear to be significantly altered in these pregnancies and these may prove to be a valuable biomarker for the prediction of spontaneous preterm birth. Further identification and validation studies to define critical proteoforms across different ethnic groups are needed to most fully investigate the regulatory and immune functions of Vitamin D-binding protein species and which could serve as sensitive and selective early biomarkers for spontaneous preterm birth.

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Statement of Ethics:

- Subjects have given their written informed consent.

- The study protocol has been approved by the Human Ethics Committee (Protocol No: X11-0305).

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Discussion

Summary of this work

Preterm birth is the commonest adverse pregnancy outcome, affecting 8% of Australian pregnancies (Li Z, 2013). It is associated with increased rates of fetal mortality and morbidity. Rates of preterm birth have not reduced significantly over the last 20-30 years. In some countries rates have, in fact, increased (Blencowe et al., 2012, Liu et al., 2016). One approach to reducing the prevalence of preterm birth involves early prediction of high-risk pregnancies that would potentially benefit from preventative intervention. Such a process, involving screening at 12 weeks' gestation, has been successful in predicting and preventing other pregnancy complications such as preeclampsia (Anderson et al., 2015, Poon and Nicolaides, 2014, Rolnik et al., Rolnik et al., 2017b). The primary aim of this thesis was to identify biomarkers that may be of value in screening for sPTB – measured at a similar early gestational age.

Whilst there may be multiple aetiological pathways that lead to spontaneous preterm birth, these pathways appear to converge such that there is a final common process triggering labour. Given that these aetiological processes are often chronic, we hypothesised that it would be possible to identify alterations in protein abundance as early as 11-13^{+6} weeks' gestation in pregnancies that go on to labour spontaneously. We therefore conducted a series of experiments designed to identify candidate protein markers and to validate the findings of these proteomic studies.

The initial focus of this work was the development of a sensitive quantitative top-down analytical technique that would allow detailed assessment of the first trimester maternal serum proteome. The process needed a large dynamic range to be able to resolve both high and low abundance proteoforms (D'Silva et al., 2017). This approach was needed as traditional proteomic techniques remove high abundant proteins to resolve less abundant counterparts (Echan et al., 2005, Gong et al., 2006, Bellei et al., 2011). This traditional approach may remove other important species, which are bound to more highly abundant proteins such as albumin; this can therefore prevent accurate quantification of serum proteoforms.

The methods used to resolve the serum proteome in this study were based on the physicochemical and structural characteristics of the constituent proteins, including solubility, hydrophobicity, molecular weight and isoelectric point. A series of optimisation techniques followed by comparative analyses were undertaken to

determine the most efficient and optimal method of sample handling/prefractionation, resolution and identification of native protein species. I was able to thus, establish a 2DE/LC/MS/MS approach to resolve and identify native protein species from first trimester maternal serum (D'Silva et al., 2017).

In addition to the various techniques tested to optimize efficient resolution of the native serum proteome, we also combined the top-down 2DE protocol with a highly sensitive staining and detection protocol, third dimension electrophoresis and deep imaging - as well as selective staining to assess phospho- and glycoprotein sub proteomes (i.e. proteoforms) (Gauci et al., 2013, Wright et al., 2014b, Wright et al., 2014a). This enabled me to maximise extraction of data from each gel. In summary, I have developed a technique that can be routinely used for analysing the proteome of crude serum samples, that does not involve fractionation and therefore retains the native complement and stoichiometry of protein species, and fully enables quantitative analysis (D'Silva et al., 2017).

Optimisation of this 2DE/LC/MS/MS method for examining the first trimester maternal serum proteome enabled further investigation and comparison of first trimester maternal protein profiles from pregnancies that subsequently delivered preterm (spontaneously) and those that delivered at term. In our second paper, we identified 30 proteoforms that were altered in the sPTB group in comparison to the full-term cohort. This included 9 phosphoproteins and 11 glycoproteins. Notably, key findings of this manuscript included identification of serum albumin, Vitamin D-binding protein and Apolipoprotein A-1 across three different assessments: namely total protein profiles, as well as in the phosphoproteome and the glycoproteome (D'Silva et al., 2018).

Several of the identified proteins were present in the 2DE gels as more than one proteoform, with MW and/or pI differing from the theoretical values that are based only on amino acid sequences. It is interesting to note that amongst the differentially abundant protein species, three (i.e. Alpha-1-antitrypsin, Vitamin D-binding protein, Apolipoprotein A-1) were found to be both phosphorylated and glycosylated. Complement C4-A was identified across total protein profiles and phospho analyses whilst Ig gamma-1 chain C region and Complement C3 were identified across total protein profiles and glyco analyses.

Notably, this study included a heterogeneous group of women with diverse ethnic backgrounds and pregnancy characteristics. Most identified proteins were

involved in clinically relevant biological processes, including interrelated biological networks linked to regulation of the complement cascade and coagulation pathways, immune modulation, metabolic processes and cell signalling (D'Silva et al., 2018).

To the best of our knowledge, this is the first study to investigate PTMs as early as the first trimester of pregnancy (D'Silva et al., 2018). The findings from this study suggested that these proteins had potential as important early biomarkers of sPTB and possible disease mediators. Extending to validation of the proteomic analyses, we selected four proteins of interest to be assessed in a larger cohort of patients. The four proteins (Alpha-1-antitrypsin, Vitamin D-binding protein, Alpha-1 beta-glycoprotein and Apolipoprotein A-1) were selected due to substantial changes in their abundance in the sPTB group when compared to the term cohort, as well as their association with other pregnancy complications including preterm birth (Madar et al., 2013, Sorensen et al., 2016, Feng et al., 2012, Timur et al., 2016).

Western blot analysis confirmed increased abundance of Alpha-1-antitrypsin and Vitamin D-binding protein in sPTB cohort of the original sample set (n=10) and generally, the overall pattern of expression did reflect the observations from the initial 2DE observations. Quantitative immunoblot analyses of the selected candidate proteins were also carried out to determine their expression levels in a larger independent cohort of women. The analysis determined that Vitamin D-binding protein decreased ($p = 0.04$) in the sPTB cohort when compared to the control. The reason for this shift in the trend of abundance and why the validation of Vitamin D-binding protein does not fully correlate with our previous 2DE findings may well be because Vitamin D-binding protein was identified by total protein staining but also as clearly separate phospho- and glyco-proteoforms. Vitamin D-binding protein contains multiple phosphorylation sites as well as other PTM. It is likely that a one-dimensional immunodetection analysis, such as the western blotting protocol used here (Paper 3), would not have adequately resolved nor thus identified these critical proteoforms.

However, analyses of the other three proteins did not demonstrate sufficient sensitivity or specificity to offer promise as screening tools for sPTB but may well require further testing in a larger population cohort, particularly with regard to specific proteoforms as opposed to (presumably) assessing the total complement of a given protein rather than specific species (i.e. using immunodetection methods such as

western and ELISA, without species-selective antibodies). There are several reasons why we may not have been able to validate apparent changes in protein abundance associated with subsequent onset of sPTB in the remaining three proteins examined in this study. This has been discussed in some detail in Paper IV. Thus, the predictive usefulness of the current potential biomarkers to detect subtypes of sPTB as well as other gestational disorders (e.g. preeclampsia) also warrants more detailed study.

A strength of the 2DE technique employed throughout this thesis is its resolution of proteoforms. This led to one of the key results of this research: the detection of two differentially glycosylated isoforms - Vitamin D-binding protein and Alpha-1beta-glycoprotein whose abundance levels changed between women with sPTB and healthy pregnant women. Glycosylation is one of the most common and vital PTM involved in modifying protein function (Apweiler et al., 1999). It is therefore not surprising that so many proteins identified in this thesis research are glycoproteins. Amongst the identified glycosylated proteins were Complement C3 and Antithrombin-III in sPTB (D'Silva et al., 2018). Complement C3 has been extensively studied in the literature for its role in the innate immune response while Antithrombin-III has been associated with the coagulation cascade (Regal et al., 2015, Ramalakshmi et al., 1995). Although traditionally viewed as separate entities, studies have suggested that functional relationships exist between coagulation and innate immunity and that exploration of these integrated systems may further develop our understanding of responses to tissue injury and subsequent inflammation (Delvaeye and Conway, 2009, Opal and Esmon, 2002). Despite its critical role in several disease mechanisms, the application of glycoproteomics to the discovery of biomarkers is still in its infancy. Significant improvements in technology will certainly advance this field rapidly in the coming years.

Review of additional / recent research findings

Since the inception of this project and completing the initial literature review that facilitated study design, a few additional research papers have been published which are relevant to this work.

Additional work on the inflammatory cascade/inflammation and infection

Previous research has documented that obesity is related to chronic inflammation (Djelantik et al., 2012, Wise et al., 2010). To understand the effect of obesity on preterm birth, a study examined second trimester serum samples from obese women who delivered prematurely and obese women who delivered at term using regression tree techniques. The study found that soluble vascular endothelial growth factor receptor-3, soluble interleukin-2 (IL-2) receptor alpha-chain, and soluble tumour necrosis factor receptor 1 were associated to preterm birth in obese women (Wallenstein et al., 2016). The finding of these inflammatory biomarkers in this pilot study is generally consistent with previous work that implicates inflammatory mediators in the common pathways of preterm birth and obesity.

Additional studies for single biomarkers

Much research on the utility of single biomarkers such as alpha-fetoprotein, fFN, C-reactive protein, IL-6 in the prediction of preterm birth has been done (Table 1.2). Increased levels of IL-6, but not pIGFBP1 in second trimester cervical secretions has been linked with threatened preterm labour and intact membranes (Brik et al., 2014). In another study, IL-6 and fFN in CVF have been suggested to be effective in the prediction of preterm delivery in symptomatic patients (Hadži-Lega et al., 2015). Using an IL-6 cut-off of 1305 pg/mL performed best in prediction of preterm delivery with sensitivity of 69.4% and specificity of 68.2%. A positive fFN test had an OR of 6.4 (95% CI: 2.0 - 20.8) to deliver prematurely. The combination of both tests performed better than the individual tests.

The same group conducted another study to determine the relationship between cervical length, fFN, phIGFBP-1, cytokines (IL-6, IL-2 receptor and TNF-α) and sPTB (which may have occurred up to 14 days from the point of sampling). The biochemical markers assessed were only moderately successful in the prediction of preterm delivery (Hadzi-Lega et al., 2015). Another study suggested alternative risk thresholds (less than 10 ng/mL and greater than 200 ng/mL) to improve the accuracy of fFN to define risk of sPTB for asymptomatic women with a short cervix (Abbott et al., 2015).

Table 1.2: Predictive accuracy for the different biomarkers of preterm birth (*Lucaroni et al., 2017)*

Key proteins	Author	Results
fFN	Boots et al., 2014 Sanchez-Ramos et al., 2009	Delivery within 48 h: Sensitivity: 62% (43–68); specificity: 81% (74–86); LR+3.3 (95%CI 2.1–5); LR 0.47 (95%CI 0.29–0.76); DOR 7 (95%CI 3–17) Delivery within 7 days: Sensitivity: 79% (69–80); specificity: 79% (76–83); LR+3.6 (95%CI 3.1–4.3) LR 0.31 (95%CI 0.25–0.39) DOR 11.5 (95%CI 8–16) Delivery within 7 days: Sensitivity: 76% (69–82); specificity: 82% (79–84); LR+4.2 (95%CI 3.5–5) LR 0.29 (95%CI 0.22–0.38); DOR: 14.4 (95%CI 9.7–21.3)
TNF- α	Liu, 2015 Conde-Agudelo, 2011	OR : 0.84 (95%CI 0.65–1.07) LR+: 3.9 (2.6–5.9); LR: 0.2 (0.1–0.4) (amniotic fluid)
IL – 1 **IL – 6** **IL – 8** **IL – 18** **IFN – γ**	Hee, 2011 Wu, 2013 Hee, 2011 Hee, 2011 Liu, 2015	LR+: 2.3 (0.8–5.7); LR: 0.6 (0.3–1.3) (amniotic fluid) OR : 0.98 (95%CI 0.72–1.33) LR+: 9.7 (3.2–29); LR: 0.2 (0.1–0.4) (amniotic fluid) LR+: 4.4 (1.4–15); LR: 0.7 (0.6–0.9) (serum) OR : 1.14 (95%CI 1.11–1.73)
CRP	Hee, 2011 Wei, 2010	LR+: 2.8 (1.5–5.4); LR: 0.6 (0.4–0.8) OR 1.53 (95%CI 1.22–1.90) (plasma) OR 7.85 (95%CI 3.88–15.87) (amniotic fluid)
MMP – 8 **MMP – 9**	Conde-Agudelo, 2011 Hee, 2011	LR+: 40.0 (5.3–301.4); LR: 0.6 (0.4–0.9) LR+: 6.0 (0.9–41); LR: 0.8 (0.6–1.1) (urine) LR+: 7.3 (1.1–50); LR: 0.4 (0.1–0.9) (plasma)
phIGFBP-1	Conde-Agudelo, 2015 Hee, 2011 Conde-Agudelo, 2011	Sensitivity: 38 (32–44); specificity: 77 (76–78); LR+: 1.6(1.3–2.0); LR: 0.8 (0.7–0.9) (cervicovaginal fluid) LR+: 5.7 (2.3–14); LR: 0.3 (0.1–0.9) Sensitivity: 38 (31–45); specificity: 58 (56–61); LR+: 0.9 (0.8–1.1); LR: 1.1 (1.0–1.2) (blood)
PAPP-A	Conde-Agudelo, 2011	Sensitivity: 11 (10–12); specificity: 93 (93–93); LR+: 1.6 (1.4–1.8); LR: 1.0 (0.9–1.0) (blood)
Placental protein 13	Conde-Agudelo, 2011	Sensitivity: 28 (17–43); specificity: 90 (86–93); LR+: 2.8 (1.6–5.0); LR: 0.8 (0.6–1.0) (blood)

*LR: likelihood ratio, OR: odds ratio

Additional studies using multivariate models

Various groups have made attempts to establish models for accurate prediction of preterm birth. These models, however, have only been effective in the symptomatic and high-risk asymptomatic populations. One such example is a model combining fFN and previous sPTB/PPRoM to predict sPTB in symptomatic women (Kuhrt et al., 2016a). Another study by the same group has proposed combining fFN, cervical length and past obstetric history to predict sPTB in asymptomatic high-risk women (Kuhrt et al., 2016b). These models have been incorporated into an App (QUiPP) to determine a woman's risk of sPTB that is freely available online.

In a more recent study, the QUiPP App was used to identify those at risk of sPTB, relative to a treat-all strategy in symptomatic women at 24 to 29^{+6} weeks' gestation. The study showed that the QUiPP App can accurately guide management at risk thresholds for sPTB of 1%, 5% and 10%. While this study provides useful insight, this approach may not be practical in all situations as the model assumes that every clinician and patient accept the findings of the App. Furthermore, data from only a minority of women was included to develop the App and this needs to be tested in an external dataset to test its utility (Watson et al., 2017)

Another European group constructed two modified prediction models for assessment of individualized risk and accurate prediction of preterm birth (within the next 48hr, and before a 32 completed weeks of gestation) (Mailath-Pokorny et al., 2015). The models incorporated the presence of PPRoM and/or vaginal bleeding, cervical length, gestational age, fFN, and serum CRP. Development of such models can be used to counsel patients and might help to individualize patient care. Nevertheless, future studies are necessary to investigate the external validity of the models and whether additional validated markers should be incorporated.

Nomograms are models designed to help clinical decision making when assessing patient risk and outcome (Dabi et al., 2017, Kattan et al., 2004). A nomogram with a high positive predictive value and low false-negative rate was developed for preterm delivery (Allouche et al., 2011). Prospective validation of the model was carried out by another group to demonstrate the efficiency of the model in predicting individual risk of preterm delivery. The tested nomogram assessed the probability of delivery within 48 hours and the probability of delivery before 32 weeks of gestation.

Several parameters including number of fetuses, cervical length was used to calculate individual scores for the risk of delivery. The authors conclude that a threshold set at 15% would help minimize the risk of preterm deliveries in singleton pregnancies and should reduce unnecessary, costly and stressful *in utero* transfer (Dabi et al., 2017).

Additional work about post-translational modifications

As noted previously, elevated levels of phIGFBP-1 in mid-trimester cervical secretions have been reported to be a good predictor of preterm birth in symptomatic women (Kekki et al., 2001, Kurkinen-Raty et al., 2001). Several other groups have confirmed these findings and have shown phIGFBP-1 to be advantageous when compared to cervicovaginal fFN (Goyal et al., 2016, Tripathi et al., 2016). A recent systematic review and meta-analysis has demonstrated the ability of cervical phIGFBP-1 to identify patients with an episode of preterm labour who will not deliver within 48 hours (Conde-Agudelo and Romero, 2016). Currently, many countries including Canada, United Kingdom, Germany, France, Italy, Australia and New Zealand are using cervical phIGFBP-1 test for predicting predict preterm birth in the symptomatic cohort. Nevertheless, the overall predictive value of cervical phIGFBP-1 for identification of preterm birth has been challenged in some reports.

Common findings

A large-scale bottom-up proteomic analysis was carried out to identify biomarkers associated with preterm birth using first trimester plasma samples (Lynch et al., 2016). The study identified complement factors B and H and the coagulation factors IX and IX ab as the highest-ranking proteins distinguishing cases of preterm birth from term controls. The top three pathways associated with preterm birth were the complement cascade, the immune system, and the clotting cascade. As shown in Paper III, the results are consistent with previous investigations suggesting that inflammatory and immune related events in early pregnancy are part of the pathogenic mechanisms of sPTB. Our study demonstrated elevated levels of complement factors B and H in the sPTB cohort when compared to the full-term cohort (D'Silva et al., 2018). These complement factors are likely elevated as part of an effort to self-regulate in response

to inflammatory events originating in the placenta in early pregnancy (D'Silva et al., 2018). My study identified proteins associated with the complement and clotting pathways in women that had no signs of intra-amniotic infection and delivered spontaneously preterm, implying that activation of these pathways individually or in a combined manner in the first trimester is prompted by inflammatory events that may not necessarily be infectious in origin.

First trimester prediction and prevention of preterm birth

The first trimester of pregnancy provides a valuable opportunity for screening and preventative intervention, enabling critical, earlier streamlining of care. It is equally important to identify women at low risk for adverse pregnancy outcomes – as these women can be offered simpler forms of supervision through pregnancy without over-medicalization of their care. Despite the vast amount of knowledge available, the clinical utility of biomarkers and risk assessments for accurate prediction of adverse pregnancy outcomes is questionable.

Nonetheless, in the last decade, first trimester tests to identify women at risk of developing adverse pregnancy complications including preeclampsia, gestational diabetes and fetal growth restriction has attracted much attention from research teams. Most of the research directed toward screening high-risk women relied upon the hypothesis that adverse pregnancy outcome was related to poor placentation. One of the strongest examples that illustrates the success of first trimester screening is the ASPRE trail; >90% of preeclampsia cases leading to delivery <34 weeks were predicted through a multivariate $11\text{-}13^{+6\text{-week}}$ screening test. Prophylactic prescription of aspirin then prevented 80% of disease affecting this cohort (Rolnik et al., 2017a).

Current screening algorithms have poor predictive efficacy for preterm birth, particularly for nulliparous women. For several years, great effort has been devoted to study several proteins including PAPP-A, placental growth factor and CRP. Although reduced PAPP-A levels have been observed in women who deliver <34 weeks spontaneously, its predictive capacity is no greater than maternal characteristics. In an unselected population, the situation is even poorer, with no predictive capacity for a variety of proposed first trimester biomarkers and ultrasound measurements. Hence,

there is a need for large prospective studies to ascertain the combination of different biochemical markers for improvement of this condition.

Difficulties of using proteomics to investigate / identify novel serum markers for T1 screening

MS-based proteomic analysis is a powerful tool for identifying disease specific proteins in sera and to aid in understanding complex molecular networks and pathways. Many studies have successfully utilised various proteomic methods in order to determine some aspects of the underlying molecular and functional pathways associated with preterm birth. However, there are many discrepancies in the reported results. A major reason for these possible discrepancies in protein data relates to the proteomic technology selected. Even in studies utilising similar methodologies, different research groups and laboratories have also found variations in the identified proteins or peptides of interest.

These differences could be due to variabilities introduced during sample collection and processing (time and temperature), sample storage and number of freeze thaw cycles introduced (McLerran et al., 2008, Lygirou et al., 2015). In order to reduce experimental variability, it is essential for all case and control samples to be handled in the exact same manner throughout the study (Timms et al., 2007). Even minor differences in specimen handling could impact the stability of protein species and eventually affect reproducibility of the experiment (Enroth et al., 2016, Tuck et al., 2009, Guest and Rahmoune, 2017). There is a need for stringent standard operating procedures specimen collection and handling to facilitate proteomic work for biomarker discovery and subsequent validation. One of the advantages of our work was that all samples had been managed through a formal clinical quality assurance process and both subjects and controls were managed in the same way.

Traditional 2DE techniques do not allow precise analysis of co-migrating spots; other validation techniques must be applied to confirm the presence of and quantitative changes in protein species of interest. Specific immuno-detection in western blot analysis provides a semi-quantitative assessment of changes in protein species. Multiple Reaction Monitoring (MRM), a promising new MS quantitation technique, uses triple quadrupole mass spectrometers to target and quantify proteins of interest, (Anderson and Hunter, 2006). This approach enables some degree of enhanced specificity and does not suffer from the same extent of issues related to cross-

reactivity with other molecules, and hence may likely replace some immunoassay measurements in the near future.

Nevertheless, MRM-MS presents its own challenges. One major issue with conducting MRM-MS is its sensitivity. The MS signal produced by a peptide or protein is negatively affected by the complexity of the sample. To overcome this, complex samples such as serum would require steps such as fractionation to reduce its complexity or enrichment for targeting specific proteins prior to MRM-MS analysis (Mehaffy et al., 2017). This step introduces experimental variability and further decreases throughput. The results of MRM-MS are dependent on effective coverage i.e. the more chosen peptides, the better the coverage. Typically, three peptides unique to the full target protein is preferred, adding to the costs of the assay (Liebler and Zimmerman, 2013). MRM-MS is also limited to the analysis of one sample at a time, and the only way of substantially increasing throughput is to install multiple mass spectrometers, which represents a major capital expense (Parker and Borchers, 2014).

Multiple proteins were identified from each of the 2D gel spots examined in this study. Until quite recently, it has been common to assume in 2DE analysis that only a single proteoform is present (or identifiable) within a single gel spot. If multiple proteoforms are present, and differential regulation of *at least one* species result in apparent changes on gel images, which species is responsible? The phenomenon of protein co-migration, where multiple proteins are present and identified within a single gel spot, has not been well discussed in the older 2DE literature. Our study employed refinement approaches (3DE and deep imaging) to further resolve highly abundant and associated species as an approach to address the issue of co-migration. The large amount of protein data derived from MS analysis of each gel spot in the current study required its own dataset filtering method, similar to the large volume of 2D gel data initially leading to those spots.

Although conveniently available, human serum is one of the most challenging biospecimens to work with. The valuable information obtainable from serum probably closely reflects the specific and changing physiological and pathological states of the whole human body. Nevertheless, the two major complications in discovery proteomics of serum is that the dynamic range of proteins complicates the

identification of candidate proteoforms. Another important question concerns sample size and the associated biological disparity within any cohort.

Most serum proteome studies are carried out by comparing protein profiles in pooled sample sets from healthy and diseased individuals. This approach has two major disadvantages: a) it does not account for individual variation and b) it can lead to false positive and false negative results as some biomarkers display low inter-individual variation while others exhibit high inter-individual variation. It is therefore imperative to take biological variation into account at, preferentially, an early point in the biomarker discovery pipeline. For this very reason, the proteomic and immunoassay methods employed in this thesis involved analysis of individual samples in technical replicates (minimum of triplicates).

Only a limited number of studies rely on the quantitative proteomic analysis of a number of individual samples, which gives some insight into biological variation, and also insight into how the scientific method is bypassed in many studies that seek high throughput over high quality data. In any case, follow-up studies, for quantification of potential candidates by well-designed orthogonal methods (e.g. immuno assays or multiplexed MRM) are necessary to ensure that selected candidate biomarker proteins can be fully validated. For effective biomarker discovery, collection and storage of high-quality samples is critical. Furthermore, various environmental influences may affect the serum proteome due to which accurate disease-specific factors are difficult to identify by simply comparing different individuals. Therefore, using proteomic methods to identify sensitive biomarkers specific to preterm birth has been challenging. To screen for preterm birth-related proteins, in this study we obtained paired sera from patients that delivered spontaneously <37 weeks' gestation and those that delivered at full-term >37 weeks' gestation.

Although the sample size in our initial study was small, we were able to obtain high quality well characterised sPTB samples after applying stringent inclusion and exclusion criteria. To date, many studies have investigated the serum proteome, attempting to characterise the protein expression within this complex biological fluid using a variety of proteomic methodologies. However, the choice of methods

employed to address this complex dynamic sample has not proven to be effective in reproducing the results across different populations.

Traditionally, analyses using 2DE posed challenges including low dynamic range of proteins and the visualization of faint protein spots (i.e. of low abundance) resolved on 2DE gels (Magdeldin et al., 2014). To overcome these issues, my study capitalized on further refinement of an established sensitive 2DE method that was not only able to resolve native serum proteins but was able to further resolve proteoforms by a 3DE approach enabling detection of co-migrating species. Furthermore, a deep imaging protocol developed for serum involved excision of a range of selected highly abundant proteins after an initial round of imaging, to enable the visualisation of lower abundance proteins. I was thus able to develop an efficient, sensitive and reproducible technique that substantially improved the quantitative protein profiling of native human serum, and that should prove widely applicable to a range of comparable sample types including plasma and urine.

Difficulties in assessing PTMs

Due to its complex and diverse nature, PTM identification continues to remain a challenge in the field of proteomics. To date, more than 300 PTMs known to occur physiologically in humans have been identified (Liddy et al., 2013, Witze et al., 2007). Typically, PTM analysis is based on single peptide species. PTM identification is further complicated as they generate complex fragmentation patterns in tandem mass spectrometry (Wang et al., 2014). Several groups have made attempts to develop methods for identification of common protein PTMs (phosphorylation and acetylation); however, identification of the uncommon protein PTMs (glypiation, sumoylation, cholesteroylation) continues to remain a challenge and these methods cannot be automated (Basak et al., 2016).

Furthermore, for the methods that can be automated, prior enrichment such as immunoprecipitation is necessary. Affinity based enrichment techniques are typically employed for low abundance PTMs. However, affinity based enrichment methods are only applicable for known well-characterised protein targets and, furthermore can cause concomitant removal of non-targeted protein species (Xie et al., 2009). The enriched proteins are then analysed by downstream methods like western blot with a

target PTM antibody (after being separated by SDS-PAGE) or by mass spectrometry to determine if a protein of interest is post-translationally modified (Farley and Link, 2009, Young et al., 2010).

Choosing an appropriate method is critical to study low abundance PTM modifications. PTM site determination is very dependent on the particular peptide sequence (Parker CE, 2010). A further challenge in PTMs is not only to accurately identify them but also to validate them due to lack of commercially available PTM specific antibodies (Tyther and Jenkins, 2011). Additionally, previous research has shown that PTM modifications may block the antibody binding site on the protein of interest, generating false negative results (Hattori and Koide, 2018).

One of the most common methods for identifying PTM is tandem mass spectrometry combined with searching a protein sequence database (Guthals and Bandeira, 2012). This method has several drawbacks, as the user needs to specify all anticipated modifications before the search engine feeds this information considering various combinations of these modifications for all peptide sequences. If several potential modifications are considered, the search can take much longer than the data acquisition, creating a bottleneck in high-throughput analysis (Savitski and Savitski, 2010).

Various targeted approaches employing selected reaction monitoring/multiple reaction monitoring (SRM/MRM) have been employed for the study of several enzymatic and non-enzymatic PTMs in cardiac proteomic research (Holman et al., 2013, Gianazza and Banfi, 2018). Further research on development of techniques to accurately identify and quantify PTM for preterm birth in clinical samples would help to determine whether a pathway is critically modulated in the disease state and how this activity is affected by treatment/intervention. Currently the community is still at the stage of proof of principle investigations of these concepts (Lima et al., 2018).

My study applied MS-based proteomics after resolving the serum proteome using highly sensitive phosphor- and glycol-staining approaches as an initial screen to identify globally altered proteoforms in the maternal serum (as discussed in paper 2). Though advantageous as a screening approach, bottom-up MS-based proteomics provide tens of thousands of sites, raising the question of their biological relevance, and imposing the challenge of how to select a very small number of relevant sites from the vast number of hits generated, and how to perform functional follow-up on these.

At a minimum, data collected through this approach requires validation by orthogonal methods before any statement about their validity can be made. Obvious prioritization criteria used in this thesis include high identification and quantification accuracy (based on mascot scores, unique peptides and sequence coverage).

In this thesis, a western blot approach was used as a validation tool to quantify candidate proteins - namely Alpha-1-antitrypsin, Vitamin D-binding protein, Alpha-1 beta-glycoprotein and Apolipoprotein A-1 in a larger independent cohort of women. The candidate proteins were found to be present as phosphorylated and glycosylated proteoforms. However, most antibodies used in classical immunodetection approaches such as western blot do not account for specific proteoforms (i.e. phosphorylation and glycosylation).

Although the sample size in our validation study was small (i.e. 44 sPTB and 44 matched controls), the western blot results obtained after extensive optimisation were highly reproducible. As emerging evidence suggests a role for combinations of PTMs in the identification of disease markers, (Gajjala et al., 2015, Duan and Walther, 2015) it likely would be valuable to investigate other phosphorylated and glycosylated proteoforms from the original list of identified proteins (Table 4, paper 3). For example, phosphorylated vitronectin was found to be significantly increased in the sPTB cohort by 2-fold (D'Silva et al., 2018).

Vitronectin is involved in immune defence and enhances the inflammatory process during infection (Hadders et al., 2012). Previous studies have shown a correlation between vitronectin and preeclampsia demonstrating an increase in the level of vitronectin in placenta and plasma of patients with preeclampsia, consistent with my findings (Shen et al., 2013, Blumenstein et al., 2009, Balci Ekmekci et al., 2015). As both preterm birth and preeclampsia are apparently related via inflammation, it would be worthwhile examining whether phosphorylated vitronectin is involved in the mechanistic pathway associated with preterm delivery.

As PTMs are so diverse and serve different purposes in cellular processes, studies must focus their attention on identifying different combination patterns of PTMs in order to understand their roles in disease mechanisms and biological outcomes. Further research into developing monoclonal antibodies that recognize biologically important PTMs is desirable.

It is impractical to hope that established immunoassays (based on antibodies primarily targeting canonical proteoforms) can validate more than a small fraction of the proteoforms / PTM sites being identified. Whilst MS-based proteomic techniques such as SRM/MRM have proven to be successful in identifying and measuring specific post-translationally modified proteoforms, the limitation associated with these methods should be carefully considered (Percy et al., 2013). It is, however, important to recognise that these techniques have enormous potential to revolutionize biomarker measurement techniques (Ozcan et al., 2017, Cordeiro et al., 2017).

Future Directions

The most immediate aim of future investigation is to quantitatively validate other 'hits' arising from this first trimester maternal serum proteome investigation. A multidisciplinary approach to preterm birth research should be considered, as the molecular pathophysiology, aetiology and symptomatology are highly complex and poorly understood. Combining an omics-based Systems Biology approach to the investigation of pathophysiological process underlying diseased states will bring about more personalised, predictive and preventive medical care.

Considerable effort is being made to integrate the large datasets generated from each of the complementary omic methods (i.e. genomics, transcriptomics, proteomics, epigenetics, metabolomics, glycomics) to understand disease mechanisms. Due to the multifactorial nature of preterm birth, it is very likely that a combination of markers will be necessary to effectively detect this condition. To look for such "fingerprints" of preterm birth and understand its pathophysiology, will require not only in-depth proteomic profiling, but also linkage of the proteomic data to its genomic expression and sophisticated bioinformatics tools for complex data analysis and pattern recognition.

Furthermore, it would be interesting to study the epigenetic modifications (DNA methylation and histone modifications etc.) associated with preterm birth. Epigenetic modifications and genetic mechanisms work hand in hand to regulate transcriptional activity. These epigenetic changes can precede disease pathology and thus could prove to be prognostic indicators for risk of preterm birth and may also serve as indicators of disease progression. This may provide the potential for developing a minimally-invasive prognostic or screening tool for preterm birth.

More specifically, one of the challenges I encountered in this study included finding commercially available monoclonal antibodies that were tested in serum. In order to test other candidate proteins, it would be worthwhile investing resources to raise in-house antibodies (with high specificity and sensitivity) against a select group of candidate proteins. An ideal candidate would not only show a change in protein abundance but would also have a small standard deviation – allowing more effective discrimination between control and preterm groups.

As my study identified significantly altered levels of VDBP, future work should include use of a target-based approach (such as MRM) to validate this finding in a larger independent cohort. Ideally this would be tested in a population-based sample so that relative distributions in control and preterm cases can be calculated, which will allow the development of likelihood ratios to describe the risk of preterm delivery. This type of population would also allow the efficacy of this screening tool (in terms of sensitivity and specificity) to be assessed. As most of the proteoforms identified in my study were linked to the inflammation cascade, it would be worthwhile reviewing whether these markers are also significantly altered in women who have PPRoM later in their pregnancy. There are very few identified biomarkers for this condition – which causes significant morbidity.

While this study only focussed on first trimester samples and did not include serial evaluation of markers, the identification of inflammatory biomarkers as early as the first trimester of pregnancy suggests that serial sampling could be beneficial for two reasons. First, to follow subjects with symptoms of preterm labour and determine when interventions such as antenatal steroids should be administered and second to determine mechanisms by which these biomarkers impact outcomes.

There are challenges in establishing a reliable minimally-invasive diagnostic method. Ideal requirements include high sensitivity and specificity, satisfactory positive and negative predictive values for detecting sPTB, and for the test to be cost effective and readily available. Additional considerations when developing a reliable and valid diagnostic or screening tool include the need to account for natural individual variations within the population. Nevertheless, rapid advances in proteomic technology will build upon the knowledge currently available to understand the molecular events and pathways that lead to the initial implantation and consequent development of disease. The introduction of advanced protein analysis platforms into clinical trial usage for generating biomarker profiles will offer improved diagnostic and prognostic efficacy.

Without a doubt, proteomic technologies are providing valuable information to further understand the molecular biochemistry behind the initiation of preterm labour, as evidenced by the steadily expanding body of literature. There is an urgent need for improved clinically well-characterised samples (both biological tissues and fluids) and

joint collaborations with obstetricians and scientists to appropriately characterise different subtypes of preterm birth.

The proteins identified from amniotic fluid, serum, plasma, urine and CVF are all contributing significant information to the pathway puzzles that are the biology of preterm birth. It is evident that with advancement of proteomic technologies, new molecules will continually be found to have specific associations with aspects of functional networks underlying preterm birth.

Summary

In summary, the proteomic methodology utilised in this thesis has enabled identification of dysregulated protein species and post translational modifications that may have future application in the diagnosis of preterm birth. Finding differences in key molecular processes has pointed toward the likely disruption of pathways associated with the complement cascade, immune modulation, metabolic processes and cell signalling all being able to play roles in the pathogenesis of spontaneous preterm birth.

There are still so many uncertainties about our knowledge of prematurity that this will remain a critical field for research for many years to come. A multidisciplinary approach that not only involves clinicians and scientific researchers, but also brings together complementary omic-based methods will be critical to understanding these underlying disease processes. Our ultimate aim should be to devise a more personalised, predictive, preventive and effective healthcare model for this challenging disease.

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