The Prediction of Preterm Birth

Elizabeth Anne Bonney

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Preterm birth is a persistent and expensive global health problem accounting for almost 8% of all live births in the UK. There are some effective interventions available however, due to the heterogeneous nature of the condition; it is still difficult to tailor the correct management for each woman. A major obstacle to the development of effective treatment strategies is a limited understanding of the molecular events preceding preterm labour. Using SCOPE, a prospectively acquired global cohort, this MD investigated the three areas of clinical risk factors, biomarker discovery using proteomic technology and directed candidate cytokine analysis.

Clinical risk factor algorithms have been developed with the most clinically relevant group, those delivering less than 34 weeks, exhibiting the best predictive performance. The algorithm has an area under the ROC curve of 0.74, negative predictive value of 99%, with a positive predictive value of 33%. This is likely to be indicative of the best performance achievable using clinical data to predict preterm birth in a healthy nulliparous population.

A proteomic discovery study was performed comparing term and preterm birth. The proteins that were discovered appeared to be mainly plasma proteins related to systemic inflammation and therefore were not specific enough as predictors of spontaneous preterm birth.

As there is strong evidence to support a role for cytokines in the initiation of inflammation/infection-induced preterm labour, a panel of 27 were assessed as predictive markers for preterm birth. Of these, five cytokines (IL-4, IFN-γ, IL-6, IL-17α and MIP-1α) appeared to be the most sensitive with a predictive accuracy of 71.25%.

The data from this thesis have provided further understanding into preterm birth and provides a pathway for future investigation into the prediction and prevention of spontaneous preterm birth.

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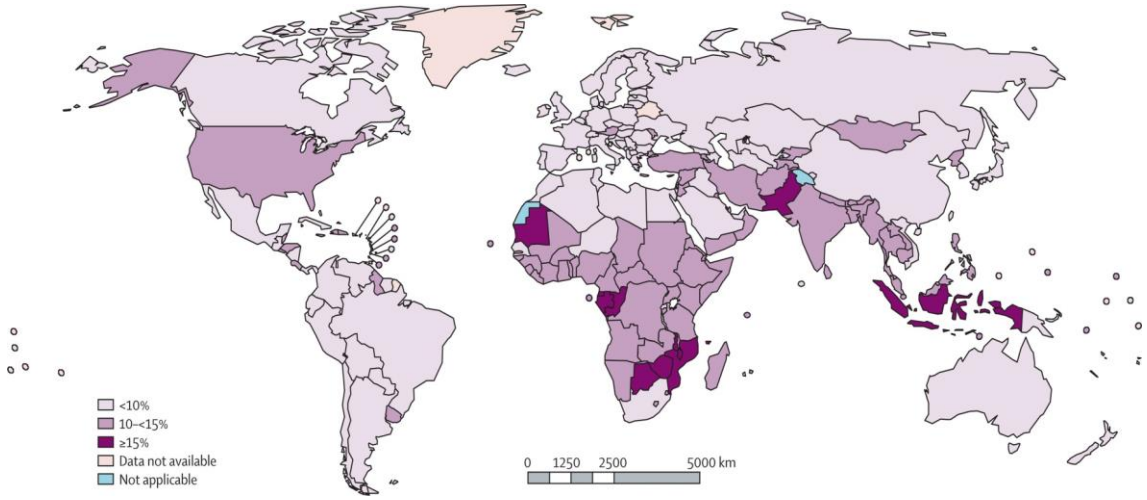
1.1 Preterm birth

1.1.1 Definition and rates

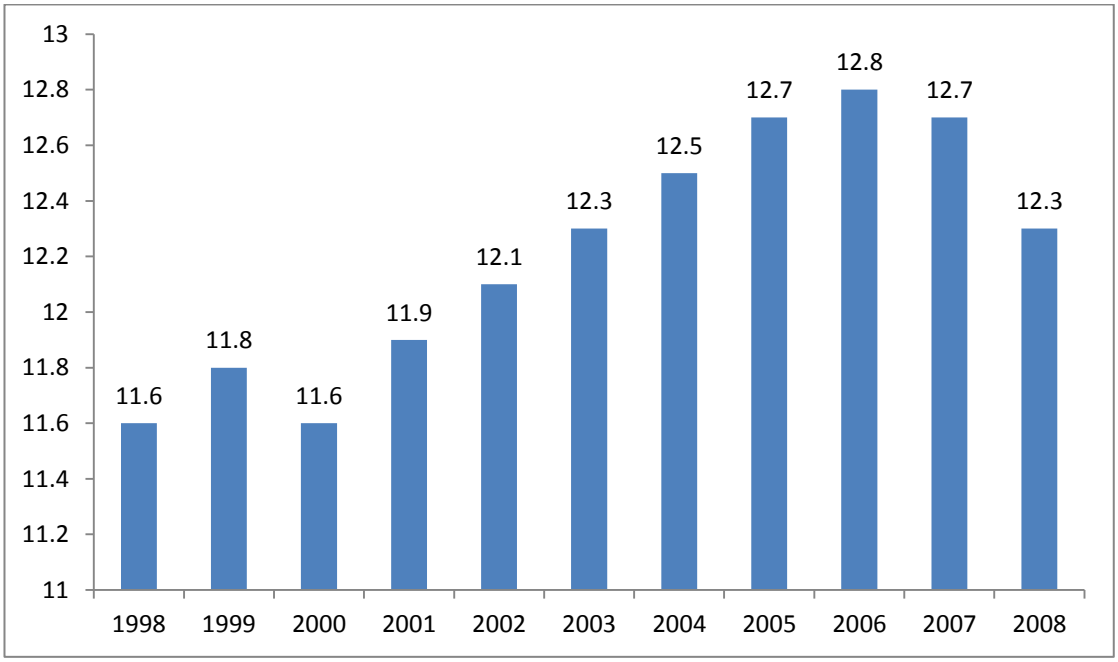
Preterm birth is defined as a delivery that occurs before 37 completed weeks (or less than 259 days) of gestation. It is a persistent and expensive global health problem. The World Health Organisation estimated the incidence to be 9.6% in 2005, equating to approximately 12.9 million preterm births worldwide each year (1). A review in 2012 reported that this had increased to 14.9 million (11.1%) in 2010 (Figure 1.1) (2).

Figure 1.1 Estimated preterm birth rates by country for the year 2010 (2)



In countries where robust data exist, preterm birth continues to be a significant adverse outcome of pregnancy. The preterm birth rate has risen in most industrialised countries, with the incidence in the USA increasing from 9.5% in 1981 to 12.8% in 2006 (Figure 1.2).

Figure 1.2 Preterm birth rates (%), USA 1998-2008 (3)

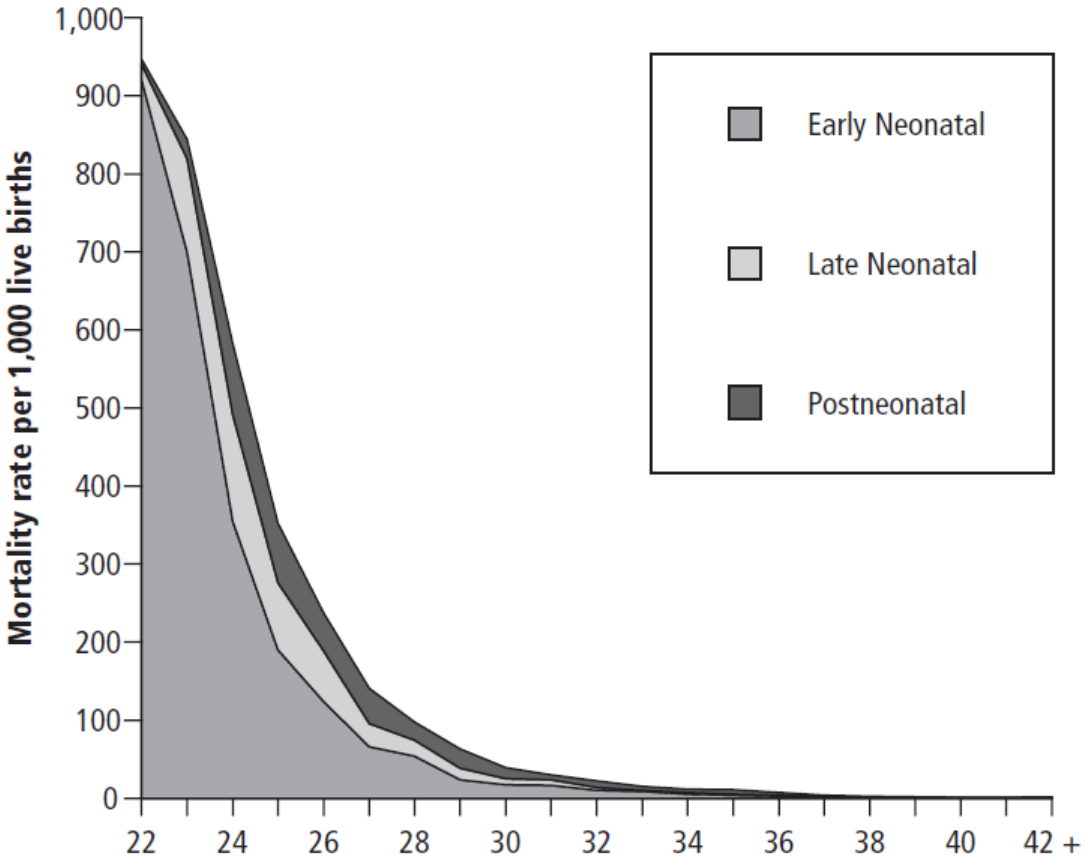


The rising trend seen in the United States of America has been mirrored in other countries such as Scotland, Australia, and Denmark (4-6). This is despite advancing knowledge of risk factors and mechanisms related to preterm labour, and the introduction of many public health and medical interventions designed to reduce preterm birth. Whilst there have been improvements in antenatal and neonatal care, the number of premature babies born each year has not significantly decreased since the 1960s (7). The United Kingdom has only recently started to collect data on preterm birth, in 2005 it was estimated to be 7.6% (8).

1.1.2 Morbidity and mortality

In developed countries, preterm birth accounts for 75% of perinatal mortality (9) and the earlier the gestation at birth, the higher the rate of mortality. In 2005 in England and Wales, there were 947 deaths per 1,000 live births at 22 weeks compared with 1.3 deaths per 1,000 live births among babies born at 40 weeks gestational age (Figure 1.3) (8).

Figure 1.3 Infant mortality rate by gestational age in England and Wales in 2005. Early neonatal, before 7 days of age; late neonatal at least 7 but under 28 days of age and post-neonatal, at least 28 days but under one year of age (taken from (8)**)**



Preterm birth is also the precursor of significant short- and long-term morbidity (see Table 1-1) (9). Children born preterm have higher rates of learning disabilities, cerebral palsy, sensory deficits and respiratory illness compared to children born at term.

Table 1-1 Complications and disabilities related to prematurity (10)

Neonatal	Short term	Long term
Respiratory distress syndrome (RDS)	Feeding and growth difficulties	Cerebral palsy
Intraventricular haemorrhage (IVH)	Infection	Sensory deficits
Periventricular leukomalacia (PVL)	Apnoea	Special health care needs
Necrotising enterocolitis (NEC)	Neurodevelopmental difficulties	Incomplete catch-up growth
Patent ductus arteriosus (PDA)	Retinopathy	Difficulties at school
Infection	Transient dystonia	Behavioural problems
Metabolic abnormalities		Chronic lung disease
Nutritional deficiencies		

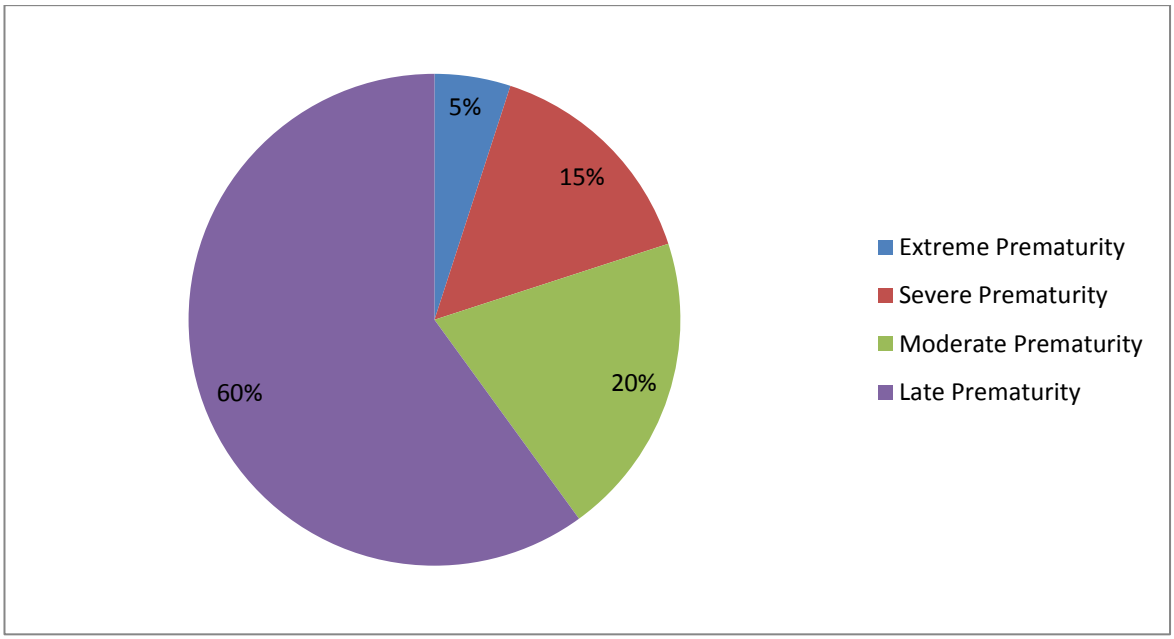
These negative health and developmental effects of preterm birth often extend into later life, resulting in significant medical, educational, psychological and social costs (11-14). Estimates indicate that within the USA, the costs in terms of medical and educational expenditure and lost productivity associated with preterm birth were more than US$26.2 billion in 2005 (15). The Epicure study (12, 16) in the UK studied outcomes for children born before 26 weeks gestation in 1995. The overall survival to discharge from hospital was 39%, rising from 20% at 23 completed weeks to 52% at 25 completed weeks. Between 54% and 75% of the population assessed at the age of six years were functioning in the normal range, or had mild disability that did not prevent them from being independent. The study was repeated in 2006 (17), with data showing that the outcomes had improved for those babies born after 24 and 25 weeks gestation in this cohort, with 34% of children having no problems at follow up at 3 years of age compared with 23% in the 1995 cohort. However, at earlier gestations,

despite the number of babies being admitted for care rising by 44%, the number of children surviving with severe disability had also risen in the 2006 cohort.

1.1.3 Types/precursors of preterm birth

Preterm birth can be subdivided according to gestational age (Figure 1.4): about 5% of preterm births occur at less than 28 weeks (extreme prematurity), about 15% between 28–31 weeks (severe prematurity), around 20% between 32–33 weeks (moderate prematurity), and 60–70% beyond 34 weeks (late prematurity) (18).

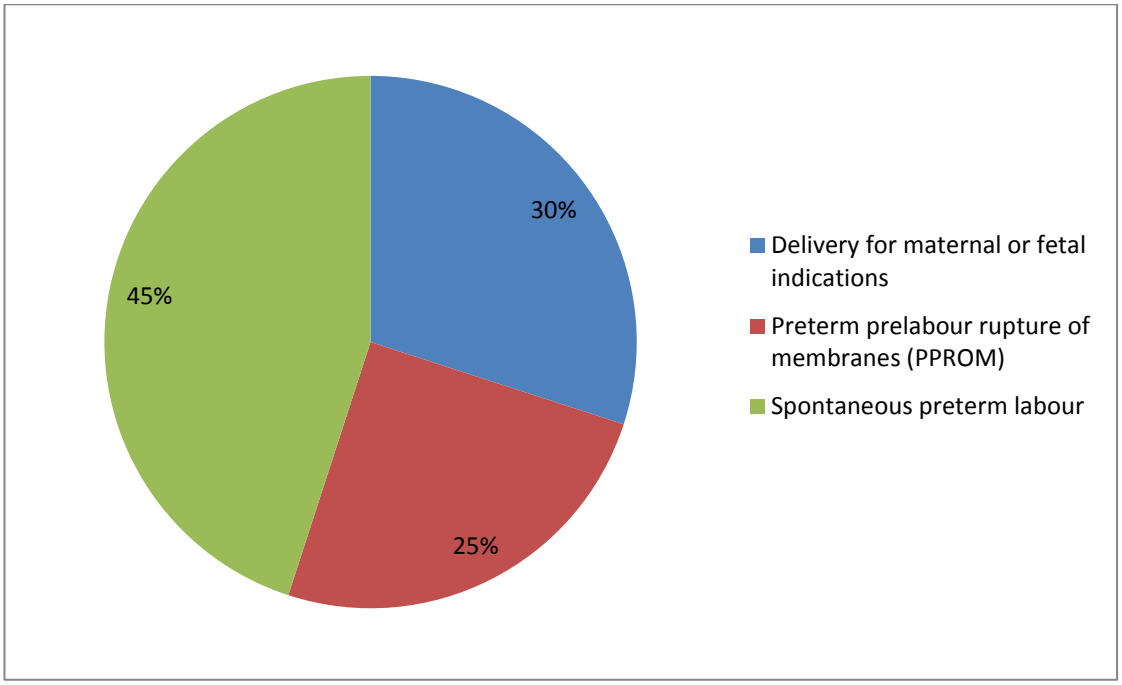
Figure 1.4 Types of preterm birth (18)



Alternatively, it may be classified according to factors governing birth. In this way about 30-35% of preterm births are *indicated*, which means delivery for maternal or fetal indications, in which labour is either induced or the infant is delivered by prelabour caesarean section. The remainder of deliveries are regarded as *spontaneous*, referring to preterm birth with intact membranes (40-45%) or that consequent upon preterm prelabour rupture of the membranes (PPROM) (25-30%), irrespective of whether delivery is vaginal or by caesarean section (Figure 1.5) (19). PPROM is defined as spontaneous rupture of

membranes at less than 37 weeks' gestation at least one hour before the onset of contractions with risk factors generally similar to those for spontaneous preterm labour. Most women with PPROM begin labour spontaneously within several days, but a small proportion of women remain undelivered for weeks or months. Since the membranes generally form a barrier to ascending infection, a common complication of PPROM is the development of intrauterine infection and preterm labour (20).

Figure 1.5 Precursors of preterm birth (adapted from (18))

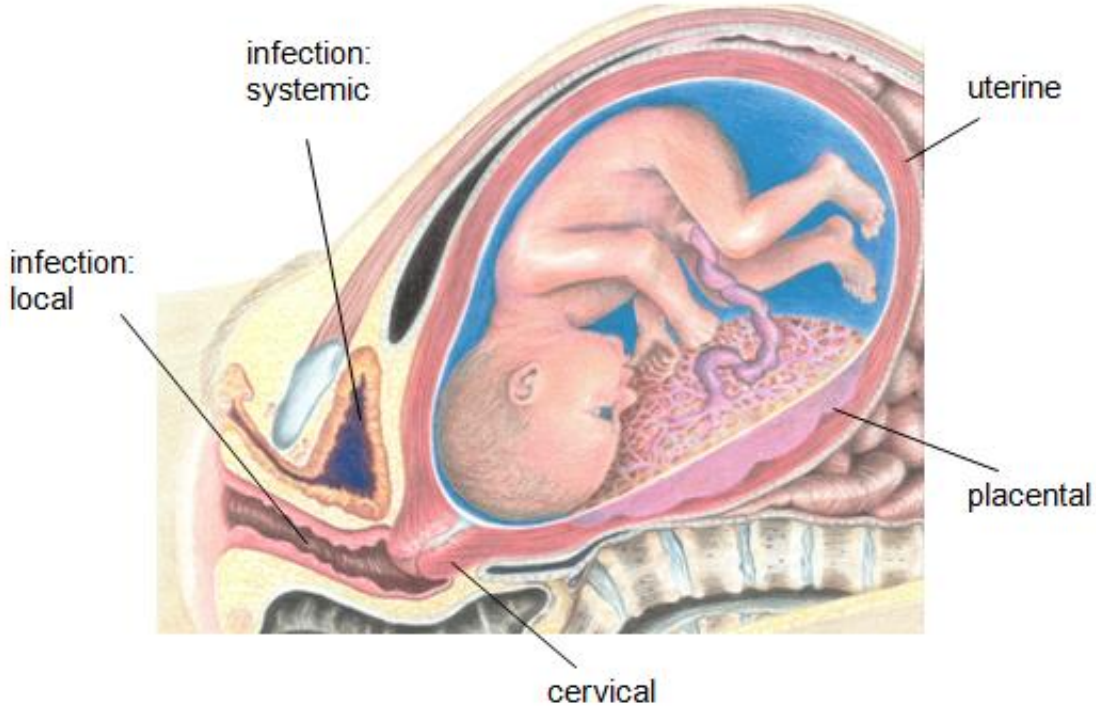


Much of the increase in the singleton preterm birth rate has been attributed to rising numbers of indicated late preterm births (21). The higher number of preterm multiple gestations associated with assisted reproductive technologies is an important contributor to the overall increase in preterm births in this group; however, even singleton pregnancies following *in-vitro* fertilisation are at increased risk of preterm birth (22). Accurate estimation of preterm birth rates and their proper characterisation (e.g. spontaneous or indicated) is essential for accurate determination of global incidence in order to determine and implement appropriate interventions to reduce the risk of preterm birth.

1.1.4 Causes of preterm birth

A major obstacle to the development of effective treatment strategies for preterm labour is a limited understanding of the molecular events required to initiate and maintain term and preterm labour. The common manifestations of term and preterm parturition include increased myometrial contractility, cervical ripening (dilatation and effacement) and decidual/membrane activation. Romero *et al.*(23) proposed the term 'Preterm Parturition Syndrome' in view of the combination of signs and/or symptoms that form a distinct clinical picture indicative of premature parturition. Preterm labour is now thought to be initiated by multiple mechanisms, including local and systemic infection or inflammation, placental factors, uterine factors and cervical factors (Figure 1.6).

Figure 1.6 Causes of preterm birth (taken from (24))



1.1.4.1 Local and Systemic Infection

Intrauterine colonisation and concomitant infection/inflammation has emerged as a major pathogenic mechanism to explain preterm birth. Histopathologic and microbiological studies suggest that infection may account for 25-40% of preterm birth (25). Furthermore, the earlier the delivery, the higher the frequency of intrauterine infection (25, 26). Inflammatory infiltration of the fetal membranes and/or maternal decidua in early preterm labour provokes a large increase in prostaglandin output by these tissues, which may trigger delivery (27). There is evidence to support this causal link from animal studies, where intrauterine infection or the systemic administration of microbial products results in spontaneous preterm birth (28-30). The most common microbial isolates from women with preterm labour are *Ureaplasma urealyticum*, *Fusobacterium* species and *Mycoplasma hominis* (31).

Microorganisms may gain access to the uterine cavity and fetus by ascending from the vagina and cervix, through haematogenous dissemination through the placenta, through retrograde seeding from the peritoneal cavity via the fallopian tube, or by accidental introduction at the time of invasive procedures such as amniocentesis. The most common pathway for intrauterine colonisation is the ascending route from the lower genital tract to the choriodecidual space and subsequently to the amniotic cavity and fetus (23). It is believed that term delivery is associated with an endocrine-mediated local inflammatory process leading to the release of uterotonic agents (32), in the course of an ascending infection, this inflammatory cascade may be prematurely activated. Microorganisms produce enzymes such as proteases and mucinases, which weaken the cervical mucus plug barrier and allow further ascent of bacteria. Bacteria also release phospholipases, which promote the formation of arachidonic acid, from which prostaglandins are produced. Prostaglandins have a fundamental role in contraction of the smooth muscle of the uterus and the biophysical changes associated with cervical ripening. Bacteria also release endotoxins, which cause a release of proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor (TNF). The proinflammatory cytokines in turn stimulate the expression of enzymes in the prostaglandin biosynthetic pathway. Microorganisms also enhance the production of matrix metalloproteinases, leading to the breakdown of the fetal

membranes, ripening of the cervix and uterine contractions (23). Antibiotic treatment of ascending uterine infections can prevent preterm birth in experimental models for chorioamnionitis (33).

Bacterial vaginosis (BV) is a disorder defined by a change in the microbial ecosystem of the vagina. It is diagnosed clinically by the presence of clue cells, a vaginal pH greater than 4.5, a profuse white discharge, and a fishy odour when the vaginal discharge is exposed to potassium hydroxide. In the laboratory, BV is defined by the Nugent criteria in which Gram-stained smears are scored on the basis of numbers of lactobacilli, which tend to be low, and the presence of organisms resembling *Gardnerella, Mycoplasma, Mobiluncus* and *Bacteroides,* the numbers of which tend to be high. A score of 7–10 is used to diagnose BV, and has been associated with a 1.5-fold to 3-fold increase in the rate of preterm birth (34, 35). Black women in both the USA and the UK are three times more likely to have BV than are white women, and this difference might in part explain 50% of the excess preterm births in black women (36, 37). The mechanisms by which bacterial vaginosis is associated with preterm birth are unknown.

It was initially thought that prophylactic antibiotics for the prevention of preterm birth in women at risk may have no effect on the preterm birth rate (38). However, a more recent metaanalysis (39) of randomized clinical trials showed that when clindamycin is administered to pregnant women with evidence of BV before 22 weeks of gestation the rate of PTB before 37 weeks of gestation and spontaneous late miscarriage is significantly lower than in the control group. The reduction in the risk of PTB before 37 weeks of gestation was only statistically significant for oral clindamycin but not for vaginal clindamycin.

Extrauterine infections such as malaria (40) and pyelonephritis (41, 42) have also been associated with preterm birth and treatment of asymptomatic bacteriuria has been shown to reduce low birth weight but has conflicting evidence for the reduction of preterm birth (43, 44).

Periodontal disease (45-48) may allow organisms within gingival crevices to cause a maternal bacteraemia and transplacental passage of bacteria, resulting in an intrauterine infection. Oral bacteria has been demonstrated to be present in the amniotic fluid of women who develop preterm birth and/or low birth weight (49).The biological pathways underlying the relationship between periodontal disease and preterm birth remains elusive.

1.1.4.2 Placental factors and haemorrhage

Evidence to support that poor placentation, consequent upon defective remodelling of spiral arteries leading to their atherosis or thrombosis, may cause preterm birth is increasing. Arias *et al*. (50) reported vascular lesions in decidual vessels attached to the placenta in 34% of women in spontaneous preterm labour, in 35% of those with PPROM, and in only 12% of control women with term pregnancies. Placental abruption is more frequent in women who deliver preterm than those who deliver at term (50). Women who develop spontaneous preterm birth have a higher percentage of failure of physiological transformation in the myometrial segment of the spiral arteries than women who deliver at term (51, 52). It has also been shown that the frequency of small-for-gestational-age infants is increased in women who have spontaneous preterm birth (53, 54). The precise mechanisms for the onset of preterm labour in women with uteroplacental ischaemia are unknown.

Vaginal bleeding caused by placental abruption or placenta praevia is associated with a very high risk of preterm delivery, but bleeding in the first and second trimesters that is not associated with abruption or praevia is also related to subsequent preterm birth (55). The risk factors for placental abruption include maternal cigarette smoking and cocaine use, chronic hypertension and pre-eclampsia, maternal trauma, IUGR, and hereditary coagulopathies. Each of these conditions is associated with damage to the spiral arteries and the bleeding is closely related to thrombin generation. Thrombin acts to stimulate coagulation and clot formation; however, it also stimulates the production of proteases capable of ripening the cervix and damaging fetal membranes, leading to PPROM. Thrombin may also exert an indirect uterotonic effect on the myometrium and stimulate contractions (56).

1.1.4.3 Uterine factors

Women with Mullerian duct variants (57-59), polyhydramnios (60) and multiple pregnancy (23) are at increased risk of preterm birth. Nahum (61) established that uterine abnormalities are found in 1 in 201 (0.5%) of the general female population. The distribution was 7% arcuate, 34% septate, 39% bicornuate, 11% didelphic, 5% unicornuate and 4% hypoplastic/aplastic/solid and other forms. A systematic review by Chan *et al.* in 2011 (62), suggested that there was no significant increased risk of preterm birth in women with arcuate uteri, often considered an incidental benign finding. There was however, a significant increased risk with canalization defects (RR 2.14; 95% CI, 1.48-3.11; P<0.001), more specifically, subseptate (RR, 2.01; 95% CI, 1.16-3.51; P=0.01), and septate uteri (RR, 2.30; 95% CI, 1.46-3.62; P<0.001). Whilst the exact aetiology is unknown, it has been suggested that the endometrium overlying the septum is abnormal and therefore a poor site for implantation (63-65). Unification defects, such as the bicornuate, unicornuate and didelphic uterus (RR, 2.97; 95% CI, 2-08-4.23; P<0.001) also showed significantly increased risks of preterm birth.

Intraamniotic pressure remains relatively stable throughout gestation despite the growing fetus and placenta (66, 67). This has been attributed to progressive myometrial relaxation due to the effects of progesterone and endogenous myometrial relaxants such as nitric oxide. However, accelerated stretching due to polyhydramnios or multiple pregnancy can induce increased myometrial contractility, prostaglandin release and upregulate oxytocin receptors in the myometrium. The effect of stretch increases in late gestation and is maximal during labour due to reduction of uterine stretch and decrease in concentrations of progesterone (23).

1.1.4.4 Cervical factors

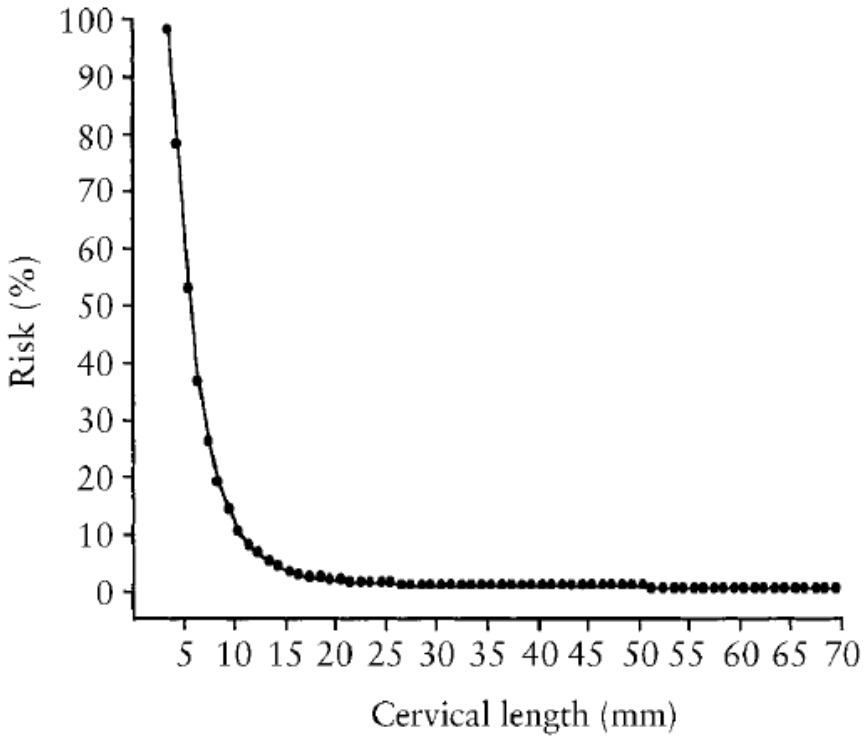
The spectrum of consequences manifest in cervical-related pathology includes recurrent pregnancy loss in the mid-trimester, varying presentations of preterm labour (i.e. presenting with bulging membranes with no apparent uterine activity), and probably precipitate labour at term. Cervical disease may be the result of a congenital disorder (e.g. DES exposure in utero), surgical trauma

(e.g. cone biopsy or LLETZ/LEEP procedures), or traumatic damage to the integrity of the cervix (e.g. multiple dilations of the cervix).

In low-risk women, cervical length is a normally-distributed variable with a mean of 35-40mm from 14 to 30 weeks. The lower 10^{th} percentile is 25mm. A progressive shortening is noticed after 30 weeks (68). Studies (68-70) in asymptomatic singleton pregnancies demonstrated that the shorter the cervical length, the higher the risk of preterm birth (Figure 1.7). However, in the low risk population the sensitivity is low, with 82% of women found to have a short cervical length at 24 weeks delivering at or after 35 weeks (68).

In contrast, cervical length is a good predictor in women at high risk, such as those who have had a previous preterm birth (71). The sensitivity is around 60-80% and a positive predictive value of 70% can be obtained when a cervical length of less than 25mm is seen between 14 and 18 weeks (72). Conversely, high-risk patients with a normal cervical measurement between 14 and 18 weeks only have a 4% risk of delivering preterm. The presence of internal os collapse (funnelling) (73) and of amniotic fluid particulate matter ('sludge') (74) increases the risk of preterm birth and chorioamnionitis. After 30 weeks of gestation, the cervix progressively shortens physiologically in preparation for labour and as a result, it is not recommended to rely on cervical length measurement at this gestation and beyond for the prediction of spontaneous preterm birth in asymptomatic women.

Figure 1.7 Risk of spontaneous delivery at ≤ 32 weeks according to cervical length at 23 weeks of gestation (70)



History of cervical cone biopsy or loop electrocautery excision procedures (LEEP) secondary to premalignant cervical disorders have also been associated with an increase in spontaneous preterm birth (75, 76). A recent systematic review (77) demonstrated this increased risk (RR 2.19, 95% CI 1.93-2.49), with cold knife cone biopsy (RR 3.41, 95% CI 2.38-4.88) and laser conisation (3.58, 95% CI 1.93-6.61) being more pronounced than LEEP (RR 1.85, 95% CI 1.59-2.15). Repeat LEEP procedures increase the risk almost five-fold (RR 5.15, 95% CI 2.45-7.84) (75) and increasing depth of excised tissue further increases risk, with an estimated 6% increase in risk for each additional millimetre (OR 1.06, 95% CI 1.03-1.09) (78). There is increasing evidence that the presence of CIN itself (even without treatment) is associated with an increased risk of preterm birth, perhaps through shared risk factors for preterm birth (79-81). In most cases it will be the combination of pathological processes, such as cervical disease and infection, which will cause the preterm birth. Intrauterine infection has been shown to be present in nearly 50% of women with acute cervical insufficiency (82).

A history of one induced termination of pregnancy is associated with an increased risk of spontaneous preterm birth (OR 1.36, 95% CI 1.24-1.50), which increases as the number of terminations increases (OR 1.93, 95% CI 1.28-2.71) (83). Suspected mechanisms causing spontaneous preterm birth in this scenario include alterations of the cervical immunological environment; the cervicovaginal flora and the host antimicrobial response to ascending infection; mechanical trauma to the cervix; and scarring following repeated dilatation and curettage resulting in altered tissue quality. Interestingly, a recent paper has speculated that recently the risk may have lessened in preterm birth following termination of pregnancy, and that this may be due to a change in termination practice such as increasing use of medical abortion and cervical pre-treatment prior to surgical termination (84).

1.1.5 Clinical risk factors

Precise mechanisms cannot always be established, therefore risk factors associated with preterm birth have been sought to explain or predict preterm birth. An increasing number of risk factors are thought to interact to cause a transition from uterine quiescence towards preterm labour or PPROM. As preterm birth is a heterogeneous condition, it is very likely that several different clinical (11-14) and biochemical (85, 86) parameters confer an increased risk.

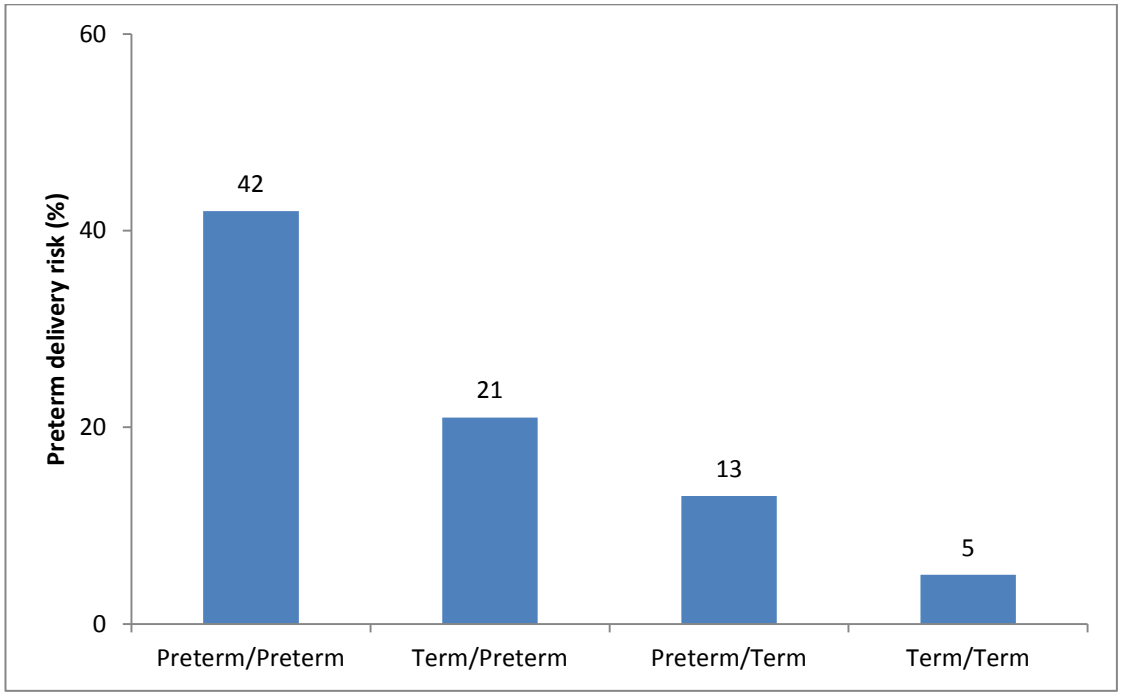
Defining risk factors for the prediction of preterm birth is a reasonable goal for several reasons. First, identification of an at-risk woman allows initiation of risk-specific treatment (87). Second, the risk factors might define a population useful for studying specific interventions. Finally, identification of risk factors might provide important insights into mechanisms leading to preterm birth.

1.1.5.1 Previous pregnancy history

Previous preterm labour and delivery is the most significant risk factor for preterm birth (88, 89). This association is modified by three factors: the frequency of prior preterm deliveries, the gestational age at which the previous birth occurred, and the order in which the prior preterm birth occurred (90). The incidence of subsequent recurrent spontaneous preterm birth is increased twofold in women with a history of one spontaneous preterm birth (91, 92).

McManemy *et al.* (Figure 1.8) demonstrated that the recurrence risk ranged from 42% in women with two prior preterm births, down to 21% (term/preterm), 13% (preterm/term), and 5% (term/term). The recurrence risk was highest (57%) for women with two prior very preterm deliveries (21-31 weeks) and lowest (33%) for those with two prior moderate preterm deliveries (32-36 weeks) (90).

Figure 1.8 Preterm delivery risk in third births (taken from (90))



1.1.5.2 Race

In the UK and USA, women classified as black, African-American, and Afro-Caribbean are consistently reported to be at higher risk of preterm delivery: preterm rates are in the range 16-18% in black women compared with 5-9% for white women. Black women are also three or four times more likely to have an extreme preterm birth than women from other racial or ethnic groups (93, 94). Furthermore the causes of preterm birth differ by ethnic group. Preterm birth is most commonly preceded by preterm labour in white women, whereas PPROM is the most frequent antecedent in black women (19). Over time, the disparity in

preterm birth rates between black and white women has remained largely unchanged and unexplained, and contributes to a cycle of reproductive disadvantage with far-reaching social and medical consequences (95). East Asian and Hispanic women typically have lower preterm birth rates. Women from South Asia, including the Indian subcontinent, may have higher rates of low birth weight caused by decreased fetal growth, but preterm delivery does not seem to be substantially increased. Other maternal demographic characteristics associated with preterm birth include low socioeconomic and educational status, low and high maternal age, and single marital status (96-98). The mechanisms by which these maternal demographic characteristics are related to preterm birth are unknown. Few population based studies have looked at the immigration effect of pregnancy outcome. Friedman *et al* (99) found that infants delivered to Caribbean-born black mothers had a significantly lower low birth rate (LBW) rate than did infants delivered to US-born black mothers. A further study (100) looking at the women of Illinois supported these findings. This suggests secondary social factors such as social evaluative threat may play a significant role in adverse pregnancy outcome.

1.1.5.3 Low socioeconomic status

Socioeconomic status (SES) (98, 101-104) is generally used to define social inequality, and is usually measured by income and/or educational attainment. Education is the dimension of SES that most strongly and consistently predicts health (104). A low level of education limits a person's access to jobs and other social resources, which in turn limits his/her capacity to integrate within society and thereby increases the risk of subsequent poverty. Socio-economic disadvantage is unlikely to be a direct, independent determinant of preterm birth; instead, it probably leads to unhealthy lifestyle choices, exposure to stress, and psychological reactions to stress that shorten gestation (105).

As discussed previously, the potential role of genital tract colonisation, infection and inflammation has been identified as a cause of preterm birth (26, 34, 106-109). BV is more common among the socially disadvantaged (34, 110), and it therefore seems reasonable to speculate that a genital tract infection could be

an important mediating variable in explaining the high risk of preterm birth among the socially disadvantaged.

It has been shown that women living in low SES areas experience more stressful life events during their pregnancy and the chronic stressors often result from the environment they live in (101, 111-113). These include financial insecurities, poor and crowded housing conditions, living without a partner, unsatisfying marital relationships, domestic violence, and stressful working conditions (101, 114, 115). They also often have a more restricted social support network (116) which is often less available during their pregnancy which can increase the impact of those stressors.

1.1.5.4 Stress

Mothers experiencing high levels of psychological or social stress are at an increased risk of preterm birth even after adjustment for the effects of sociodemographic, medical, and behavioural risk factors (117, 118). Furthermore, exposure to objectively stressful conditions, such as housing instability and severe material hardship, has also been associated with preterm birth (119). Fetal stress may be due to problems in placental function.

Although the mechanism underlying the association between psychological or social stress and increased risk of preterm birth is unknown, a role for corticotrophin-releasing hormone (CRH) has been proposed. CRH mediates pituitary adrenocorticotropin (ACTH) secretion in both the maternal and fetal pathways, which in turn increases maternal and fetal cortisol secretion. Increased cortisol levels rapidly increase the amount of circulating CRH. These elevated CRH levels induce prostaglandin production (120-122). Prostaglandins cause contractions, cervical ripening and sensitise the myometrium to oxytocin. CRH also enhances placental oestrogen production by stimulating the secretion of its precursor from the fetal adrenal gland. Oestrogens interact with the myometrium leading to contractions and cervical changes.

Women exposed to stressful conditions also have increased serum concentrations of inflammatory markers, such as C-reactive protein; an observation not accounted for by other established risk factors for inflammation (123). These findings suggest that systemic inflammation might be a pathway by which stress could increase the risk of preterm birth.

1.1.5.5 Nutritional status/BMI

Nutritional status during pregnancy can be quantified by assessments of body size such as body mass index (BMI), nutritional intake, and serum assessments of various analytes (124-126). A low pre-pregnancy BMI is associated with a high risk of preterm birth, whereas moderate obesity can be protective (126). Women with low serum concentrations of iron, folate, or zinc have more preterm births than those with measurements in the normal range (124, 127). There are many potential mechanisms by which maternal nutritional status might affect preterm birth.

1.1.5.6 Smoking

Tobacco use increases the risk of preterm birth (<2-fold) after adjustment for other factors (128-130). The mechanisms by which smoking is related to preterm birth are unclear. There are more than 3000 chemicals in cigarette smoke and the biological effects of most are unknown (131); however, both nicotine and carbon monoxide are powerful vasoconstrictors, and are associated with placental damage and decreased uteroplacental blood flow. Both of these pathways lead to fetal growth restriction and indicated preterm births, however, interestingly, smoking reduces the risk of developing pre-eclampsia, another cause of indicated preterm birth (132). Smoking is associated with a systemic inflammatory response and may increase spontaneous preterm birth through that pathway (133, 134).

1.1.5.7 Interpregnancy interval

Both short and long interpregnancy intervals have been associated with an increased risk of adverse perinatal outcomes (135). Smith *et al.*(136) found an interpregnancy interval of less than 6 months conferred a greater than two-fold

increased risk of preterm birth after adjustment for maternal age, marital status, height, socioeconomic deprivation, smoking, previous birth weight and previous caesarean section. The association was specific to preterm birth and neonatal death, as no association existed between a short interpregnancy interval and the risk of delivering a growth-restricted infant.

1.1.5.8 Multiple Pregnancy

Multiple gestations account for only 2-3% of all infants, but they carry a substantial risk of preterm delivery, and so account for 15-20% of all preterm births. Nearly 60% of twins are born preterm. About 40% of twins will spontaneously labour or rupture their membranes before 37 weeks, with others requiring an indicated preterm delivery because of preeclampsia, or other maternal or fetal complications (18). Nearly all higher multiple gestations will result in preterm delivery. Uterine overdistension, resulting in contractions and PPROM, is believed to be one of the causative mechanisms for the rate of increased spontaneous preterm births (23).

1.1.5.9 Familial risk factors

A study in Norway has shown that mothers who themselves were born preterm had a significantly increased risk of having preterm offspring (137). This followed a dose-response pattern such that increasing severity of prematurity was associated with an increasing risk of preterm birth in their offspring. Compared with 6.4% of index women born at term having preterm offspring, the incidence of having preterm offspring was 14.0% among women who had been born at 22 to 27 weeks, 9.2% for those born at 28 to 32 weeks, and 8.8% for those born at 33 to 36 weeks (137). Porter *et al.*(138) also established that a woman who herself was delivered preterm was more likely to suffer spontaneous preterm labour and preterm birth, and it has been demonstrated that mothers with an older sister who had given birth to a preterm infant also had an 80% higher risk of giving birth to a preterm infant (139).

1.1.6 Why are nulliparous women so important?

In low risk nulliparous women the most pertinent clinical risk indicator, that of a previous preterm delivery, is not applicable and therefore in routine clinical practice we have no means of identifying which healthy nulliparous women will later deliver preterm. A test which would allow a proportion of these women to be identified as high risk in mid gestation would allow a targeted programme of cervical surveillance and the potential for prophylactic treatments such as progesterone (140-142).

Development of a successful predictive test for PTB will probably require the combined assessment of multiple biochemical, clinical and biophysical risk factors. To date, this strategy has not been possible due to the necessity for a large pregnancy biobank of appropriate samples collected, in conjunction with detailed pregnancy metadata. The recent creation of unique pregnancy biobanks such as SCOPE (Screening for Pregnancy Endpoints - www.medscinet.net/scope) have now made this work achievable. Furthermore, recent advances in analytical techniques have resulted in technologies capable of quantifying multiple analytes simultaneously. As a consequence studies of the proteome, genome, and metabolome are now feasible which themselves may offer further insights into the multiplicity of pathways which lead to preterm birth.

1.1.7 Why Screen?

The World Health Organisation produced guidelines detailing the principles of a screening test in 1968 (143). These guidelines are still applicable today and preterm birth fulfils some of the WHO criteria for screening. It is an important health problem for the individual and the community; screening would probably be economically balanced in relation to possible expenditure on medical care as a whole; there is evidence that treatment started at an early stage would be of more benefit than treatment started later. There are, however, some aspects which are still lacking: the natural history of the disease is not yet adequately understood, there is no universally accepted treatment or useful intervention for patients with the disease, and a suitable and acceptable screening test or examination is not yet established.

1.1.8 Traditional methods of screening

The traditional methods of screening are based on a range of epidemiological risk factors such as previous preterm birth, maternal age, race and smoking status. Several risk scoring systems have been developed. The most commonly evaluated scoring system is the Creasy score (144, 145), which was developed by ascribing a numerical value to individual risk factors and by weighting risk factors differently depending on their importance. This predictive method, however, lacks sufficient sensitivity and specificity for routine clinical use. Most women who deliver preterm are not identified by the risk-scoring system and the majority of women identified as high risk will not deliver preterm. Furthermore, because a traditional risk factor score is based largely on previous obstetric history, its accuracy is particularly poor among women expecting their first child, who comprise half of those affected by PTB. A systematic review (145) showed a lack of studies evaluating clinically important outcomes such as birth before 34 weeks' gestation, and suggested there is a need for better quality information about accuracy of risk scoring systems.

1.1.9 Screening of symptomatic women

The symptoms associated with preterm labour are often varied and non-specific. Of patients who present with symptoms of preterm labour (i.e., uterine contractions), 50-80% will deliver at term without any intervention (146, 147). It is therefore important for the clinician to be able to distinguish between non-specific abdominal pain or uterine activity and true preterm labour. This will prevent unnecessary admission and treatment of patients who are not at risk of premature delivery. Once a woman presenting with symptoms has been highlighted as being at risk of preterm birth, with signs such as cervical dilatation, it is often too late to make a significant difference. Tocolysis can be administered to allow administration of corticosteroids, to improve lung maturation, and to permit transfer to a facility with an appropriate neonatal unit, however, there is no evidence it will prolong pregnancy in any other way which will improve outcome (148-150). Non-invasive tests are commercially available to identify those women at greatest risk of preterm birth and include cervicovaginal fetal fibronectin (fFN) and phosphorylated insulin-like growth factor binding protein-1 (phIGFBP-1).

1.1.9.1 Fetal Fibronectin

Fetal fibronectin (fFN) is a glycoprotein found in the amniotic fluid, placental tissue, and the extracellular component of the decidua basalis adjacent to the placental intervillous space. It should not normally be present in cervicovaginal fluids between 22 and 35 weeks, however, it may be released after mechanical- or inflammatory-mediated damage to the membranes or placenta before birth and therefore may be present in women at risk of preterm birth (151). Cervicovaginal fluid fFN has a negative predictive value of approximately 99% for predicting delivery in symptomatic women within 2 weeks of testing (147, 152). Positive prediction, however, is modest (<20%), and because most women have a good outcome, many are overtreated (147). The current qualitative test (Rapid fFN TLI_{IQ}; Hologic, Marlborough, MA) provides a positive or negative result based on a threshold of 50ng/mL (153).

Studies that have used enzyme-linked immunosorbent assay (ELISA)-based quantification of fFN suggest that concentrations of fFN within cervicovaginal secretions correlate with the risk of spontaneous preterm birth. Knowledge of the fFN concentration appears to improve prediction and this is now commercially available as a bedside test (Hologic 10Q system, Hologic, Marlborough, MA) (154, 155). This bedside test has been evaluated in a recent study of 300 patients which demonstrated that the quantitative information adds further value to the qualitative test (156).

1.1.9.2 Actim Partus (phIGFBP-1)

Insulin-like growth factor is a protein synthesised by the liver and is involved in the control of fetal growth and development. Its binding protein (IGFBP-1) is found in different forms and the phosphorylation status varies in different body fluids and tissues (157, 158). The non-phosphorylated isoform predominates in amniotic fluid and its detection in cervical and vaginal samples is diagnostic of membrane rupture (159). The highly phosphorylated isoform (phIGFBP-1), which is produced by the decidua, is not present in the amniotic fluid, but may be an indicator of tissue damage at the choriodecidual interface. During the events preceding labour, fetal membranes start to detach from the decidua and phIGFBP-1 leaks into cervical secretions (160). A bedside kit called Actim

Partus (Alere, UK) has been developed for the detection of phIGFBP-1 in these secretions. One of the largest studies (301 women) has calculated the sensitivity, specificity, PPV and NPV to be 69.2%, 90.5%, 50% and 95.5% respectively for delivery before 37 weeks' gestation in symptomatic women (160).

1.1.9.3 Transvaginal ultrasound of the cervix

Transvaginal scanning of the cervix in women who are symptomatic of preterm labour may also help to detect those who will deliver preterm. Unfortunately, the availability of equipment and skills needed to perform a TVS are not commonly found on a labour ward in the United Kingdom. Several studies have found that the shortening of the uterine cervix as assessed by transvaginal sonography had sensitivity ranging between 73–100% and specificity between 44–94% for symptomatic patients (161-163). Most importantly, it has an excellent NPV of about 80%, meaning that it can identify a group of women who are at low risk for preterm delivery, allowing a reduction in the number of tocolytic treatments. According to Iams *et al*. (163), a canal length >30 mm indicates that significant effacement has not occurred and the likelihood of preterm birth is low.

1.1.10 Screening of asymptomatic women

Identifying women at high risk of preterm birth before their symptoms appear would allow initiation of early and targeted interventions to try and modify the outcome. At present there is no commercial test to fulfil that need. An ideal predictive tool for preterm birth would identify women who present with various subtypes of the same condition (i.e. preterm birth attributable to several different aetiologies).

1.1.10.1 Transvaginal ultrasound of the cervix

The most accurate and reproducible method of cervical evaluation is transvaginal ultrasound, and cervical length assessment is an established predictor for preterm birth. Data indicates that the shorter the cervical length, the higher the risk of spontaneous preterm birth (164). As a result, cervical length measurement is currently the gold standard for prediction of spontaneous

preterm birth in the high risk population. Table 1-2 illustrates the predictive ability of cervical length measurement in identifying those who will deliver spontaneously preterm.

In low risk women, the mean cervical length at 14-30 weeks of gestation is 35-40mm, with the lower 10^{th} percentile being 25mm and the upper 10^{th} percentile being 50mm (68). Prior to 14 weeks of gestation, it is less easy to define the cervical length. After 30 weeks, cervical length measurement is not recommended as the cervix progressively shortens physiologically in preparation for labour. An ultrasound-indicated cerclage in the low risk population does not seem to prevent PTB (165, 166) therefore it is not recommended that cervical length is used as a routine screening predictor of PTB in low-risk women.

Cervical length assessment is most sensitive in screening singleton pregnancies with a previous preterm birth, as over two-thirds of women destined to deliver preterm can be detected early whilst asymptomatic (167). Additionally, sensitivity remains over 50% in singleton pregnancies with other risk factors for preterm birth, such as a previous cone biopsy (168), Mullerian anomaly (58) or previous multiple dilatations and curettage (169).

Despite all these data, there still remains a lack of understanding regarding the association between cervical length and spontaneous preterm birth and this is demonstrated by the fact that some women with a short cervix will have a preterm birth, whilst others will have uncomplicated term deliveries (164). Ideally, determining the risk status of a woman would be done in early pregnancy to allow appropriate targeted antenatal care. However, accurate prediction of symptomatic and asymptomatic women in later pregnancy is equally important in order to guide the administration of antenatal corticosteroids for fetal lung maturation, which is of greatest benefit in the first week following administration (149), hospital admission and appropriate transfer to neonatal facilities. Whilst TVS of the cervix may aid this the newer prediction tests such as fibronectin are more superior.

Table 1-2 Prediction of preterm birth in singletons by cervical length. (Adapted from (170))

	n	PTB (%)	Primary outcome (GA)	GA	CL	Sens	Spec	PPV	NPV	RR
Low risk (68)	2915	4.3	<35	22-25	25	47	84	35	90	3.4
Prior preterm birth (167)	183	26	<35	16-24	25	69	80	55	88	4.4
Prior LEEP (168)	55	6	<35	16-24	25	67	87	22	98	10.2
Prior cone biopsy (168)	45	22	<35	16-24	25	60	69	35	86	2.5
Prior multiple D&Cs (169)	131	30	<35	14-24	25	53	75	48	78	2.2
Uterine anomalies (58)	64	11	<35	$14\text{-}23^{+6}$	25	71	91	50	95	13.5

PTB, preterm birth; GA, gestational age in weeks; CL, cervical length; Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value; RR' relative risk; LEEP, loop electrical excision procedure; D&C, dilatation and curettage.

1.1.10.2 Biochemical markers

1.1.10.2.1 Fetal fibronectin

Although the use of fFN for identifying symptomatic women at risk of preterm labour is now widely accepted, the role of fFN in high risk asymptomatic women has been less clear (85, 171). This is mainly due to the limited specificity and positive predictive value of the qualitative bedside test, however, the newer quantitative test appears to improve its predictive performance (154, 172). A recent study (172) of fFN levels taken at 24 weeks in asymptomatic women with a prior preterm birth demonstrated that as the fibronectin concentrations increased, the risk of spontaneous preterm birth became progressively greater (Table 1-3). The numbers in this study were small however, so these findings need to be confirmed in larger studies.

Table 1-3 Relative risk of spontaneous preterm delivery by fetal fibronectin level (relative to fetal fibronectin = 0ng/mL) (taken from (172))

	sPTD <28wk RR (95% CI)	sPTD <32 wk RR (95% CI)	sPTD <34wk RR (95% CI)	sPTD <37wk RR (95% CI)
1-49ng/mL	0 (0)	1.21 (0.05-7.00)	2.42 (0.76-5.66)	1.21 (0.60-2.12)
50-199ng/mL	9.75 (0.38-91.41)	8.77 (1.94-27.02)	4.68 (1.28-10.95)	2.27 (1.07-3.81)
≥200ng/mL	41.41 (4.13-228.7)	18.64 (4.12-50.58)	9.94 (2.90-19.67)	3.45 (1.44-5.29)

sPTD, spontaneous preterm delivery; RR, relative risk

1.1.10.2.2 Other predictive markers

Although many candidate-driven approaches to studying biomarkers have shown promise, this has not resulted in the identification of predictive PTB biomarkers for the general pregnant population. A recent systematic review by Conde-Aguedelo *et al* (173) investigated the accuracy of novel biomarkers to predict spontaneous preterm birth in women with singleton pregnancies and no symptoms of preterm labour. Table 1-4 shows the range of novel biomarkers for the prediction of preterm birth identified in the literature.

None of the biomarkers evaluated met the criteria to be considered a clinically useful test to predict spontaneous preterm birth. This could be due to bias in the methodology as the majority of studies are involving small numbers and investigate specific populations rather than a range of women from different backgrounds. This along with the complex heterogeneity of preterm birth may be some of the reasons why discovering novel biomarkers have proved difficult.

Table 1-4 Novel biomarkers for the prediction of the spontaneous preterm birth phenotype identified in the literature (taken from (173))

1. Inflammation-related biomarkers Interleukins-2, -6, -8 and -10 Tumour necrosis factor-α Granulocyte colony–stimulating factor Stromal cell-derived factor-1α Interferon-γ Matrix metalloproteinase-8 A disintegrin and metalloproteinase-8 and -12 Elastase Secretory leukocyte proteinase inhibitor Soluble vascular cell adhesion molecule-1 Soluble intercellular adhesion molecule-1 C-reactive protein Ferritin Alkaline phosphatase
2. Placental proteins/hormone-related biomarkers Human chorionic gonadotropin Phosphorylated insulin-like growth factor binding protein-1 Prolactin Relaxin Pregnancy-associated plasma protein A Placental protein 13 Activin A Pregnancy-specific β-1-glycoprotein
3. Angiogenesis-related biomarkers Angiogenin Soluble endoglin
4. Coagulation-related biomarkers Thrombin-antithrombin III complex
5. Proteomics-related biomarkers Desmoplakin isoform-1 Stratifin Thrombospondin-1 precursor
6. Genetic biomarkers TNF-alpha (G-308>A)

1.1.11 Prevention of preterm birth

Many proposed interventions to prevent delivery or delay and improve neonatal outcome remain unsatisfactory or unproven. Strategies to reduce morbidity and mortality in infants born preterm have been limited to the administration of antenatal corticosteroids for fetal lung maturation and advances in neonatal intensive care (149). Repeated corticosteroid doses are no longer common practice following randomised controlled trials which have shown alterations in fetal growth and impaired neurodevelopment outcomes without long term benefit on pulmonary function (174, 175). However a recent Cochrane Systematic Review (176) has now refuted this claim and suggested that repeat doses of glucocorticoids should be considered in women at risk of preterm birth seven or more days after an initial course, in view of the neonatal benefits.

1.1.11.1 Traditional recommendations

Bed rest in home or hospital has been widely recommended in the past for the prevention of preterm birth. This advice is based on the observation that hard work and physical activity during pregnancy could be associated with preterm birth and that bed rest could reduce uterine activity. There is however, no evidence, either supportive or refuting, for the use of bed rest to prevent preterm birth, however, jobs requiring heavy lifting/upright posture have been shown to be associated with preterm birth (177, 178). This has even been demonstrated in women with a short cervix (179). When women are offered bed rest, there are many potential adverse effects on women and their families and, increased costs for the healthcare system (180).

1.1.11.2 Cervical Cerclage

The traditional intervention for women with recurrent preterm deliveries or second trimester losses is cervical cerclage. Although reported by Shirodkar (181) in 1955, cervical cerclage remains one of the more controversial surgical interventions in obstetrics (182). Its use has expanded to include the management of women with other risk factors of preterm birth, such as multiple pregnancy, uterine anomalies, a history of cervical trauma (e.g. cone biopsy) and cervical shortening on ultrasound examination. The use in these differing groups is highly controversial. Whist cerclage may provide a degree of structural support to a ‘weak’ cervix; its more important role may be in

maintaining the cervical length and the endocervical mucus plug as a mechanical barrier to ascending infection.

Cervical cerclage is not without risk. Reported adverse events shortly after suture insertion include abdominal pain, vaginal bleeding, PPROM and bladder injury. Late complications can include infections such as chorioamnionitis or miscarriage.

Three different categories of patients who may benefit from cerclage can be defined. History-indicated or elective cerclage is performed on women with a recurrent history of mid-trimester loss or early preterm birth. The second category is ultrasound-indicated or emergent cerclage, that is performed on asymptomatic women where ultrasound examination reveals a short cervix with or without collapse of the internal os (also known as 'funnelling'). The third is rescue cerclage, where there are clear signs of cervical dilatation and significant effacement with exposed fetal membranes at the external cervical os or within the vagina but with no uterine contractions.

Although there is little evidence as to the efficacy of elective cervical cerclage, it is widely accepted practice (183). A large trial comparing history-indicated cerclage with expectant management (184) showed there were fewer deliveries before 33 weeks gestation in the cerclage group compared with controls (13% v 17%; RR 0.75; 95% CI 0.58-0.98). There were no significant differences between the two groups in fetal/neonatal outcome. In the subgroup analyses, only women who had had a history of three or more pregnancies ending before 37 weeks benefited from cerclage (delivery before 33 weeks 15% v 32%, P<0.05).

When the history is less conclusive, some clinicians choose to insert a cervical cerclage only when a short cervix is found on ultrasound (164, 166, 183, 185). An RCT (186) reporting on women with a history of spontaneous preterm birth between 17^{+0} and 33^{+6} weeks gestation who were found to have a cervix <25mm on ultrasound scan, demonstrated that when cerclage was compared to expectant management, it led to a reduction in preterm birth <24 weeks gestation (6.1% v 14%; *P*=0.03), but did not prevent birth <35 weeks gestation (32% v 42%; OR 0.67, 95% CI 0.42-1.07) unless the cervical length was less

than 15mm (OR 0.23; 95% CI 0.08-0.66). Table 1-5 illustrates evidence on the use of cerclage for prevention of spontaneous preterm birth in women with a short cervix.

Rescue cerclage has a success rate of approximately 50% (187-189) and is employed after careful consideration due to the extremely poor prognosis once fetal membranes are visible. The evidence for benefit is not substantial as there are a lack of controlled trials in this area. Nonetheless, under ideal conditions, rescue cerclage may significantly prolong pregnancy and increase the likelihood of a viable pregnancy outcome (187, 190, 191). As sub-clinical infection is likely to be implicated in the aetiology of bulging membranes, some clinicians will perform amniocentesis prior to cerclage insertion to exclude infection (192).

To *et al* (165) screened low risk women using transvaginal ultrasound to measure cervical length between 22-24 weeks gestation. Women who had a cervix of 15mm or less were randomised to cerclage or expectant management. There was no difference in the number of preterm deliveries between these groups (22% v 26%; RR 0.84; 95% CI 0.54-1.3, *P*=0.44). Other studies have since confirmed this and it is therefore not standard practice to insert cerclage into low risk women with a cervix of 25mm or less. (166) The Royal College of Obstetricians and Gynaecologists green-top guideline suggests insertion of a cerclage after 3 or more preterm births/mid-trimester losses or when the cervix is <25mm in a woman who has previously had a preterm birth/mid-trimester loss (193)

Table 1-5 Summary of evidence for the use of cerclage to prevent spontaneous preterm birth in women with a short cervix

Study	Population screened	Singletons with prior PTB (%)	GA at screening (week)	Cervical length (mm)	Cerclage type	Primary outcome	Relative risk PTB <35 weeks (95% CI)	Relative risk PTB <35 weeks in singletons with prior PTB 16-36 weeks (95% CI)
Rust *et al.* (2001)(194)	All singleton, twins, triplets	102/207 (49)	16-24	<25	McDonald	PTB<34 weeks	0.37 (0.13-1.03)	0.07 (0.00-1.07)
Althuisius *et al.* (2001)(195)	Singletons with suspected cervical incompetence ; twins	26/35 (74)	14-27	<25	McDonald	PTB<34 weeks	0.98 (0.69-1.39)	0.75 (0.40-1.40)
To *et al.* (2004)(165)	All singletons	44/253 (17)	22-24	≤15	Shirodkar	PTB<33 weeks	0.97 (0.56-1.67)	0.70 (0.33-1.46)
Berghella *et al.* (2004)(196)	All singletons with risk factors for PTB; twins	36/57 (63)	14-24	<25	McDonald	PTB<35 weeks	0.76 (0.51-1.15)	0.68 (0.27-1.77)
Owen *et al.* (2009) (186)	All singletons with a prior PTB < 34 weeks	1014/1014 (100)	$16\text{-}22^{+6}$	<25	McDonald	PTB<35 weeks	0.67 (0.42-1.07)	0.67 (0.42-1.07)

PTB, preterm birth; GA, gestational age; CI, confidence interval

1.1.11.3 Progesterone

Progesterone has been administered for the prevention of preterm birth for many years. The first trial using progesterone for the prevention of preterm birth in women at increased risk was published in 1970 (197). The exact mechanism/s by which progesterone confers benefit is/are not fully understood but it is thought to be essential for pregnancy maintenance (198). It is postulated to maintain myometrial quiescence, inhibit cervical ripening, increase the efficacy of the cervical mucus barrier, and decrease the production of proinflammatory cytokines which are thought to be key to decidual/membrane activation (199, 200). This has in part been challenged by Sexton *et al* (201) as their study suggested that the benefits of 17-alpha hydroxyprogesterone caproate (17P) for preterm labour prevention are not achieved, even partially, by a direct utero-relaxant effect (*in vitro*, on myometrial strips). They postulated that their findings opened the possibility that genomic effects of 17P, achieved over long periods of administration, are required for its reported therapeutic benefits.

It is known that inhibition of progesterone action can result in parturition for example, administration of a progesterone antagonist (RU486, Mifepristone) to pregnant women, can induce labour (198). In many species, a fall in serum progesterone concentration occurs prior to spontaneous parturition, however this has not been demonstrated in humans (202).

Randomised clinical trials indicate that progesterone administration to women with a previous preterm birth reduces the rate of spontaneous preterm birth (140, 141, 203). A Cochrane meta-analysis assessing the benefits and harms of progesterone during pregnancy to prevent spontaneous preterm birth showed that progesterone is associated with a reduction in risk of spontaneous preterm birth before 37 weeks (RR 0.65, 95% CI 0.54-0.79), and infant birth weight under 2500g (RR 0.63, 95% CI 0.49-0.81)(204).

The optimal dosage, mode of administration and duration of use remains unanswered. The two main forms of progesterone currently being used in the UK are intramuscular injections of 17-alpha hydroxyprogesterone caproate (17P) and vaginal progesterone pessaries. Table 1-6 summarises the data available.

Table 1-6 Characteristics of RCTs of progesterone for the prevention of spontaneous preterm birth

Study	Population screened	Population (n)	Intervention	Primary outcome	Results of primary outcome
Papiernik-Berkhauer (1970)(205)	'High preterm risk score'	99	Intramuscular 17P (250mg every 3 days) vs placebo from 28-32 weeks of gestation	PTB<37 weeks	Progesterone (2/50) vs placebo (2/49); OR 0.24 (95% CI 0.07-0.82)
Johnson *et al.* (1975) (206)	Prior PTB	50	Intramuscular 17P (250mg weekly) vs placebo from 'booking' until 24 weeks of gestation	PTB<37 weeks	Progesterone (2/18) vs placebo (12/25): OR 0.19 (95% CI 0.05-0.70)
Hartikainen-Sorri *et al.* (1975) (207)	Multiple pregnancy	77	Intramuscular 17P (250mg weekly) vs placebo from 28 until 37 weeks gestation	Perinatal death	Progesterone (4/78) vs placebo (2/76): OR 1.94 (95% CI 0.38-9.87)
Hauth *et al.* (1983) (208)	Women on active military duty (Lackland Airforce Base)	168	Intramuscular 17P (1000mg weekly) vs placebo from 16 to 20 weeks until 36 weeks of gestation	Preterm labour	Progesterone (5/80) vs (placebo (5/88): OR 1.11 (95% CI 0.31-3.96)

Study	Population screened	Population (n)	Intervention	Primary outcome	Results of primary outcome
Da Fonseca *et al.* (2003) (140)	Prior PTB, presence of cervical suture, uterine malformation	157	Vaginal progesterone (100mg daily) vs placebo from 24 to 28 weeks of gestation	PTB<37 weeks and <34 weeks	Progesterone 13.8% vs placebo 28.5% ($p<0.05$) for PTB <37 weeks and 2.7% vs 18.5% ($p<0.05$) for PTB <34 weeks
Meis *et al.* (2003) (141)	Prior spontaneous PTB	463	Intramuscular 17P (250mg) vs placebo (castor oil) from 16-20 weeks until 36 weeks	PTB<37 weeks	Progesterone 36.3% vs placebo (54.9%): RR 0.66 (95% CI 0.54-0.81)
Facchinetti *et al.* (2007) (209)	Presented in TPTL between 25 and 33^{+6} weeks gestation, acute symptoms arrested following use of tocolytics	60	Intramuscular 17P (341mg every 4 days) vs placebo until 36 weeks gestation	Cervical length by ultrasound	17P: reduction of risk of PTB (OR 0.15; 95% CI 0.04-0.58)
Fonseca *et al.* (2007) (142)	Short cervical length (≤15mm) on transvaginal ultrasound	250	Vaginal progesterone (200mg daily) vs placebo from 24 weeks to 33^{+6} weeks	Spontaneous PTB <34 weeks	Progesterone 19.2% vs placebo 34.4%; RR 0.56 (95% CI 0.36-0.86)
O'Brien *et al.* (2007) (210)	Prior spontaneous PTB	659	Vaginal progesterone (90mg daily) vs placebo	Spontaneous PTB <34 weeks	Progesterone 10% vs placebo 11.3%; OR 0.9 (95% CI 0.52-1.56)

Study	Population screened	Population (n)	Intervention	Primary outcome	Results of primary outcome
Rouse *et al.* (2007) (211)	Multiple pregnancy	661	Intramuscular 17P (250mg weekly) vs placebo (castor oil) from 16-20^{+3} weeks gestation until 34 weeks gestation	Composite of death or delivery <35 weeks	Progesterone 41.5% vs placebo 37.3%; RR 1.1 (95% CI 0.9-1.3)
Borna and Sahabi (2008) (212)	Women presenting in TPTL between 24 and 34 weeks gestation, in whom acute symptoms arrested following use of tocolytics	70	Vaginal progesterone (400mg daily) vs no therapy	Latency until delivery	Progesterone group: longer mean latency until delivery (36.1 ± 17.9 vs 24.5 ± 27.2) (mean ± SD) days (p=0.03)
Norman *et al.* (2009) (213)	Twin pregnancy	500	Vaginal progesterone gel (90mg daily) vs placebo gel from 24 until 34 weeks gestation	PTB or IUD <34 weeks gestation	24.7% (61/247) in the progesterone group and 19.4% in the placebo group; OR 1.36 (95% CI 0.89-2.09) (p=0.16)
Caritis *et al.* (2009) (214)	Triplet pregnancy	134	Intramuscular 17P (250mg weekly) vs placebo starting at 16-20 weeks and continued until 35 weeks gestation	Composite of delivery or fetal loss <35 weeks of gestation	Similar in both groups: 83% 17P vs 84% placebo; RR 1.0 (95% CI 0.9-1.1)

Study	Population screened	Population (n)	Intervention	Primary outcome	Results of primary outcome
Combs *et al.* (2010) (215)	Trichorionic-triamniotic triplets	81	Intramuscular 17P (250mg weekly) vs placebo starting at 16-22 weeks and continued until 34 weeks gestation	Composite neonatal morbidity	Similar in 17P and placebo groups (38% vs 41%; p=0.71)
Berghella *et al.* (2010) (203)	Prior SPTB (17-33^{+6} weeks gestation) and a short cervix (≤25mm) on transvaginal ultrasound (16-22^{+6} weeks gestation)	300	Intramuscular 17P (250mg weekly) starting from 16-22^{+6} after randomisation to cerclage or no cerclage for a short cervix on ultrasound	PTB<35 weeks	17P had no effect in either cerclage (p=0.64) or no cerclage (p=0.51) groups; OR 0.80 (95% CI 0.40-1.58)
Hassan *et al.* (2011) (216)	Singleton pregnancy with short cervix (10-20mm) at 19 to 23^{+6} weeks gestation	458	Vaginal progesterone gel (90mg daily) vs placebo gel from 20-23^{+6} weeks until 36^{+6} weeks gestation	PTB<33 weeks	8.9% (21/235) in the progesterone group and 16.1% (36/223) in the placebo group; RR 0.55 (95% CI 0.33-0.92)(p=0.02)
Grobman *et al.* (2012) (217)	Para 0 with singleton pregnancy with short cervix (<30mm) between 16 and 22^{+3}	657	Intramuscular 17P (250mg weekly) or placebo from 16-22^{+3} until 36 weeks	PTB <37 weeks	25.1% in the progesterone group vs 24.2% in placebo group; RR 1.03 (95% CI 0.79-1.35)

PTB, preterm birth; 17P, 17-alpha hydroxyprogesterone caproate; OR, odds ratio; RR relative risk; CI confidence interval; IUD, intrauterine death; TPTL, threatened preterm labour;

1.1.11.4 Antibiotics

The use of antibiotics in asymptomatic high-risk women is controversial. A Cochrane review (218) showed there was little evidence that screening and treating all pregnant women with asymptomatic bacterial vaginosis prevented spontaneous preterm birth. However, in women with a previous preterm birth, treatment of bacterial vaginosis may reduce the risk of PPROM and low birthweight. A meta-analysis by Simcox et al. (38) failed to show benefit and suggested that treating women at risk of spontaneous preterm birth with antibiotics did not reduce their risk of subsequent preterm birth. Conversely, the use of metronidazole in asymptomatic high-risk women appears to *increase* the risk of spontaneous preterm labour (219, 220), possibly due to changes in vaginal flora (221). As a result it is suggested that clindamycin, either oral (222) or vaginal (223), may be the better treatment choice for symptomatic bacterial vaginosis.

In view of the infection/inflammation link to preterm birth, many studies have looked at the use of antibiotics for the treatment of women with symptoms suggestive of preterm labour (224). ORACLE I showed that when erythromycin was given to women with PPROM, there was an associated increase in the number of women remaining undelivered after 7 days along with a range of health benefits for the neonate. Co-amoxiclav, however, was associated with an increased risk of necrotising enterocolitis in the neonate and therefore erythromycin has become the preferred choice for women with PPROM (225). The ORACLE II randomised control trial, however, found an increased risk of cerebral palsy at 7 years of age (OR 1.93; 95% CI 1.21-3.09 for erythromycin and OR 1.69; 95% CI 1.07-2.67 for co-amoxiclav) in children of women with intact membranes who received antibiotics for spontaneous preterm labour (226). This is likely to be because treating women with a sub-clinical infection could mask the infection and leave the fetus exposed to a hostile *in utero* environment, leaving the need for delivery going unnoticed.

Until a better understanding of the causes of preterm birth exists, we will continue to fail in the pursuit to prevent or stop preterm birth. Preterm birth is a persisting problem with some effective interventions available. Unfortunately, due to the heterogeneous nature of the condition, it is still difficult to tailor the correct treatment for each woman. If we were able to better phenotype the individual, then we could direct the correct intervention to the correct patient. To

be able to do this we need to search for better predictive strategies to help identify these high-risk women. This will likely need novel strategies (-omic technology), multiple biomarker approaches or larger prospectively acquired databases.

Using the SCOPE biobank, derived from a prospectively-acquired global cohort, this MD aimed to investigate three areas for their predictive ability:

- clinical risk factors
- biomarker discovery using proteomic technology
- directed candidate cytokine analysis.

2 Clinical Risk Factors for the Prediction of Spontaneous Preterm Birth

2.1 Introduction

The outcome of preterm birth arises from a number of different aetiologies, so it is probable that a variety of clinical factors (11-14) and biochemical changes (85, 86) may each confer increased risk, making accurate and consistent prediction and prevention difficult to achieve.

Despite these challenges, elucidating reliable prediction strategies for preterm birth is an important target for several reasons. Firstly, accurate identification of at-risk women will allow initiation of risk-specific strategies such as the use of progesterone (140, 141, 203). Second, the risk factors themselves might define populations useful for further assessments and interventions such as cervical screening (72, 167) or cerclage (195). Finally, identification of risk factors might provide important insights into mechanisms leading to preterm birth.

From a clinical point of view, women at risk of preterm birth are most commonly identified from their previous obstetric history, having had a preterm birth. As previously discussed, this excludes the primiparous population. More recently, there has been the appreciation that women with previous cervical surgery may also be at risk, so this group may be included, despite the lack of evidence to support subsequent monitoring.

In addition to previous preterm birth, previously researched screening strategies have considered a range of other historical and demographic risk factors such as maternal age, race and smoking status. Several risk scoring systems have been developed. The most recognised risk assessment tool is the Creasy score (144), which was developed by ascribing a numerical value to individual risk factors and by weighting risk factors differently depending on their importance. It was devised by looking at all women (966 patients) who registered for delivery at the National Women's Hospital in a 6 month period in 1978 in Auckland, New Zealand. They were screened at their initial booking visit and then again at 26-28 weeks. The scoring system was based on socioeconomic status, past

obstetric history, daily habits and problems in their current pregnancy. Women were then placed in a low-, medium- or high-risk category. The incidence of preterm labour in the final groups was 2%, 5% and 30% in the low-, medium-, and high-risk groups respectively. Sixty four per cent of the preterm births came from the high-risk group. This predictive method, however, lacks sufficient sensitivity and specificity for routine clinical use. Many women who deliver preterm are not identified by the risk-scoring system and the majority of women identified as high risk will not deliver preterm. Furthermore, because a traditional risk factor score is based largely on previous obstetric history, as expected its accuracy is particularly poor among women expecting their first child, who comprise half of those affected by PTB.

Another method of identifying high risk women is through the use of biomarkers, such as cervicovaginal fetal fibronectin (fFN). This is usually only used after 22 weeks gestation and may only be positive just a few weeks prior to the preterm birth which may be too late for any preventive strategy. Cervical length measurement has also been shown to have potential in highlighting women in whom intervention, such as progesterone or cerclage, may be beneficial (142).

The SCOPE (Screening for Pregnancy Endpoints) study, a prospective, multicentre cohort study of healthy nulliparous women had the primary aim of developing screening tests to predict pre-eclampsia, infants small for gestational age, and spontaneous preterm birth. A previous study (227), an analysis of the Southern hemisphere cohort, highlighted that different risk factors were present in women destined for spontaneous preterm birth following prelabour rupture of membranes or intact membranes.

In this part of my thesis I set out to:

- assess data from the global SCOPE cohort
- define clinical risk factor algorithms associated with spontaneous preterm birth before and after 34 completed weeks
- determine their predictive performance within a validation dataset
- examine how additional scan-derived data (fetal anthropometry, uterine artery Doppler and cervical length) might improve their predictive ability.

2.2 Methods

Six centres (Auckland, New Zealand; Adelaide, Australia; London, Manchester and Leeds UK; and Cork, Ireland) recruited nulliparous women with singleton pregnancies to the SCOPE study between November 2004 and August 2008. Women (n=8351) attending hospital antenatal clinics, obstetricians, general practitioners, or community midwives before 15 weeks' gestation were invited to participate. Exclusion criteria included women defined as high risk of pre-eclampsia, small for gestational age baby or spontaneous preterm birth because of underlying medical conditions (chronic hypertension requiring antihypertensive drugs, diabetes, renal disease, systemic lupus erythematosus, antiphospholipid syndrome, sickle cell disease, HIV), previous cervical knife cone biopsy, three or more terminations or three or more miscarriages, current ruptured membranes; known major fetal anomaly or abnormal karyotype; or intervention that could modify the outcome of pregnancy (such as aspirin, cervical suture).

Research midwives interviewed and examined women at 14-16 and 19-21 weeks' gestation. Women underwent an ultrasound scan at 19-21 weeks. At the time of interview, data were entered on an internet-accessed central database with a complete audit trail (MedSciNet). At 14-16 weeks' gestation the following data were collected: demographic information including age, ethnicity, immigration details, education, work, socioeconomic index, income level, living situation; the woman's birth weight and gestation at delivery and whether it was a singleton or multiple pregnancy; previous miscarriages, terminations, or ectopic pregnancies and whether these pregnancies were with the same partner as the current pregnancy or not; history of infertility, use of assisted reproductive technologies, duration of sexual relationship, and exposure to partner's sperm; gynaecological (including polycystic ovarian syndrome) and medical history, including hypertension while taking combined oral contraception, asthma, urinary tract infection, inflammatory bowel disease, thyroid disease, and thromboembolism; and family history (in mother and sisters) of obstetric complications (miscarriage, pre-eclampsia, eclampsia, gestational hypertension, spontaneous preterm birth, any preterm birth, gestational diabetes, stillbirth, and neonatal death) and family history (mother, father, sibling) of medical conditions (hypertension, coronary artery heart disease, cerebrovascular accident, type 1 and 2 diabetes, and venous thromboembolism). Information was collected on vaginal bleeding early in

pregnancy (gestation, severity and duration of bleeding, and recurrent bleeds), hyperemesis, and infections during pregnancy. Vegetarian status was recorded, and other dietary information before conception and during pregnancy was obtained from food frequency questions for fruit, green leafy vegetables, oily and other fish, and fast foods. Use of folate and multivitamins, cigarettes, alcohol (including binge drinking), and recreational drugs including marijuana, amphetamine, cocaine, heroin, ecstasy and LSD (lysergide) was recorded for before conception, first trimester, and at 15 weeks.

A lifestyle questionnaire was completed assessing work, exercise and sedentary activities, snoring, domestic violence, and social support. Psychological scales were completed to measure perceived stress (228), depression (229), anxiety (230), and behavioural responses to pregnancy (adapted from the Behavioural Responses to Illness Questionnaire (231)). Two consecutive manual blood pressure measurements (mercury or aneroid sphygmomanometer, with a large cuff if the arm circumference ≥33 cm and Korotkoff V for diastolic blood pressure) were recorded. Other maternal measurements included maternal height and weight and waist, hip, arm, and head circumference. Proteinuria in a midstream urine specimen was measured by dipstick or by protein:creatinine ratio. Random whole blood glucose and serum lipid concentrations (triglycerides, total cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol, total cholesterol: high density lipoprotein cholesterol ratio) were also measured.

Ultrasound examination at 19-21 weeks' gestation included measurements of the fetus (biparietal diameter, head circumference, abdominal circumference, and femur length), and Doppler studies of the umbilical and uterine arteries (232). All fetal measurements were adjusted for gestational age by calculating the multiple of the median for each gestational week. Mean uterine resistance index (RI) was calculated from the left and right uterine resistance index. If only a left or right uterine resistance index was available, this was used as "mean resistance index" (n=20). The presence or absence of notching in each uterine artery was recorded. An abnormal uterine artery Doppler result was defined as a mean resistance index >90th centile (>0.695).

Participants were followed prospectively, and research midwives collected data on pregnancy outcome and measurements of the baby. Data monitoring included individual checks of all data for each participant, including checks for any transcription errors of the lifestyle questionnaire, and detection of illogical or inconsistent data and outliers with customised software.

2.2.1 Primary outcome

The primary outcome was spontaneous preterm birth less than 37 weeks. Two subgroups were then formed: spontaneous preterm birth <34 completed weeks and spontaneous preterm birth 34-36 weeks. The referent group comprised women who delivered at ≥37 weeks.

2.2.2 Other definitions

The estimated date of delivery was calculated as follows: if the woman was certain of the date of her last menstrual period (LMP), the estimated date of delivery was adjusted only if a scan at <16 weeks' gestation found a difference of seven or more days between the scan gestation and that calculated by the LMP or a scan at 20±1 weeks found a difference of 10 or more days. If her date was uncertain, scan dates were used to calculate the estimated date of delivery.

2.2.3 Statistical analysis

All analyses were performed using the statistical software R. Continuous clinical variables in cases and controls were compared using either ANOVA, Student's t-test or the Wilcoxon Rank Sum, depending on data distribution. Categorical data were compared using either Chi-squared or the Fisher's exact test. For creating and testing predictive models, training and validation datasets (ratio 2:1) were created by randomly splitting the total dataset, stratified by geographical area.

2.2.4 Clinical variable selection

Figure 2.1 demonstrates how the variables were selected for the algorithm. Of the original 1140 variables, 286 variables remained after the following exclusions: paternal variables (n=85), missing data >10% (variables added after recruitment commenced, work related variables, clinical labs n=149), not easily applied when screening future populations (n=70) or p>0.1 in univariate comparisons in the training dataset (n=550). Nineteen variables were selected from the remaining 286 variables using the following criteria for removal: no extreme outliers or small numbers (n<5) in subgroups (n=109), addressed by other variable (n=8), ease of collection in the clinical setting (n=26) and no biological plausibility as risk factors for preterm birth (n=124).

Of the final 19 variables used for model building, 12 had no missing data, 3 had <1% missing data, 3 had between 1 and 5% missing data and 1 (cervical length scan) had 46% missing data. Missing clinical data was imputed with expectation maximisation (233) and used the median for three variables unrelated to the observed data. The expectation maximisation algorithm was implemented in the “mix” package in R, version 2.9.1 (234). For fetal biometry and uterine artery Doppler data, individuals with missing data were deleted.

Backwards logistic regression models were constructed for each endpoint in the training dataset. The model construction for each endpoint was performed with three sets of variables:

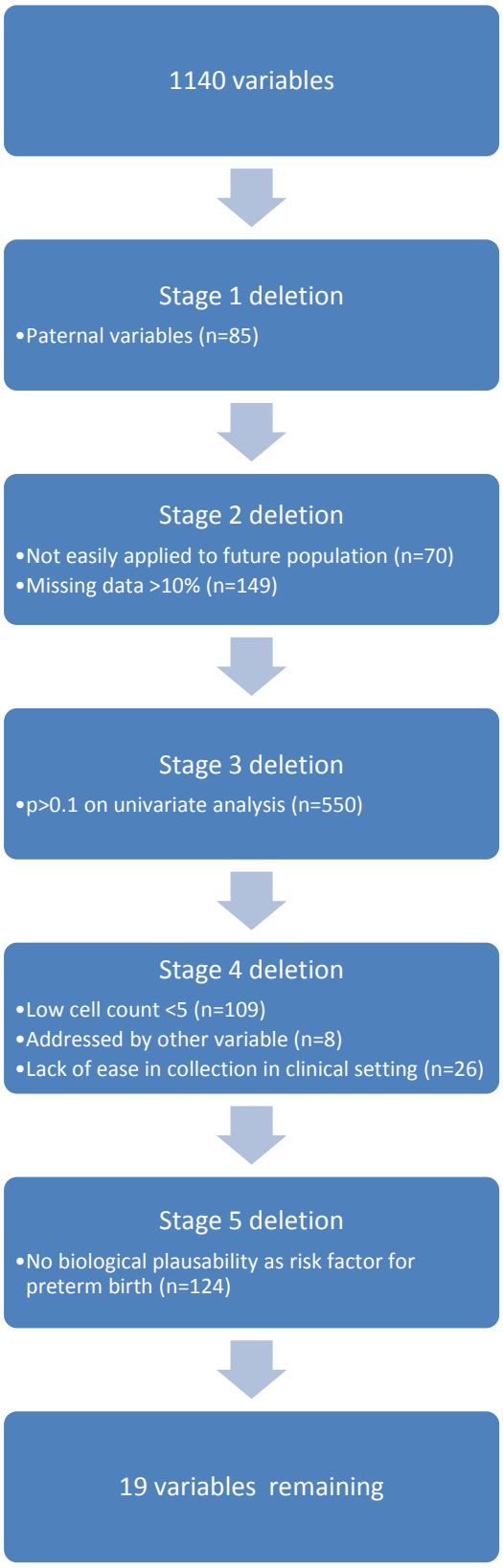
(1) clinical risk factors obtained at 14-16 weeks’ gestation

(2) clinical risk factors plus ultrasound scan measurements of fetal growth and uterine artery resistance index at 19-21 weeks

(3) clinical risk factors plus ultrasound scan measurements of fetal growth, uterine artery resistance index and cervical length at 19-21 weeks.

The model performance was then evaluated in the validation dataset.

Figure 2.1 Flow chart to demonstrate how variables were selected for clinical algorithm



2.3 Results

2.3.1 Recruitment and characteristics of participants

Seventy one percent (n=5989) of the 8351 women approached initially agreed to participate (Figure 2.2). Of the 5690 women recruited, pregnancy outcome was available in 5628 (99%). Two hundred and thirty six women (4.2%) had a spontaneous preterm birth, of whom 71 (30%) were delivered before 34 weeks and 165 (70%) delivered between 34 and 36 weeks. Among PTB cases delivered before 34 weeks, 34 (48%) had preterm prelabour rupture of membranes (PTB-PPROM) and 37 (52%) had intact membranes at the time of labour (PTB-IM). Similarly, PTB cases who delivered between 34-36 weeks comprised 72 (44%) PTB-PPROM and 93 (56%) PTB-IM. The median gestation at delivery was similar for PTB-PPROM [35.0 (interquartile range 33.2-36.3) weeks, n=106] and PTB-IM [35.6 (IQR 33.7-36.4) weeks, n=130, p=0.23].

Of the 851 women with an elevated mean uterine artery or bilateral notches, only 43 (5.1%) resulted in spontaneous PTB cases, 18.2% of the total number of spontaneous PTB cases (236). Of the 3009 women with cervical length measured, only 48 (1.6%) and 6 (0.2%), had a transvaginal cervical length less than 25 mm and less than 15 mm, respectively. Of the 48 with a cervix <25 mm, only two had spontaneous PTB.

After randomly splitting the dataset, there were 3752 women in the training and 1876 women in the validation datasets (Figure 2.2). After exclusion of participants who had a miscarriage or termination before 22 weeks or for whom there were missing fetal or uterine artery Doppler scan parameters, 96% of participants remained in the training (n=3583) and validation (n=1796) datasets. Transvaginal cervical length assessment was an optional component of consent, and these data were available in 56% of the women in both the training and validation datasets. The distribution of centres and preterm birth phenotypes in the training and validation datasets were very similar (Table 2-1).

Maternal characteristics and pregnancy outcomes in control pregnancies, and those spontaneous preterm births delivered before 34 weeks and at 34-36 weeks are shown in Table 2-2.

Figure 2.2 Flow diagram showing recruitment

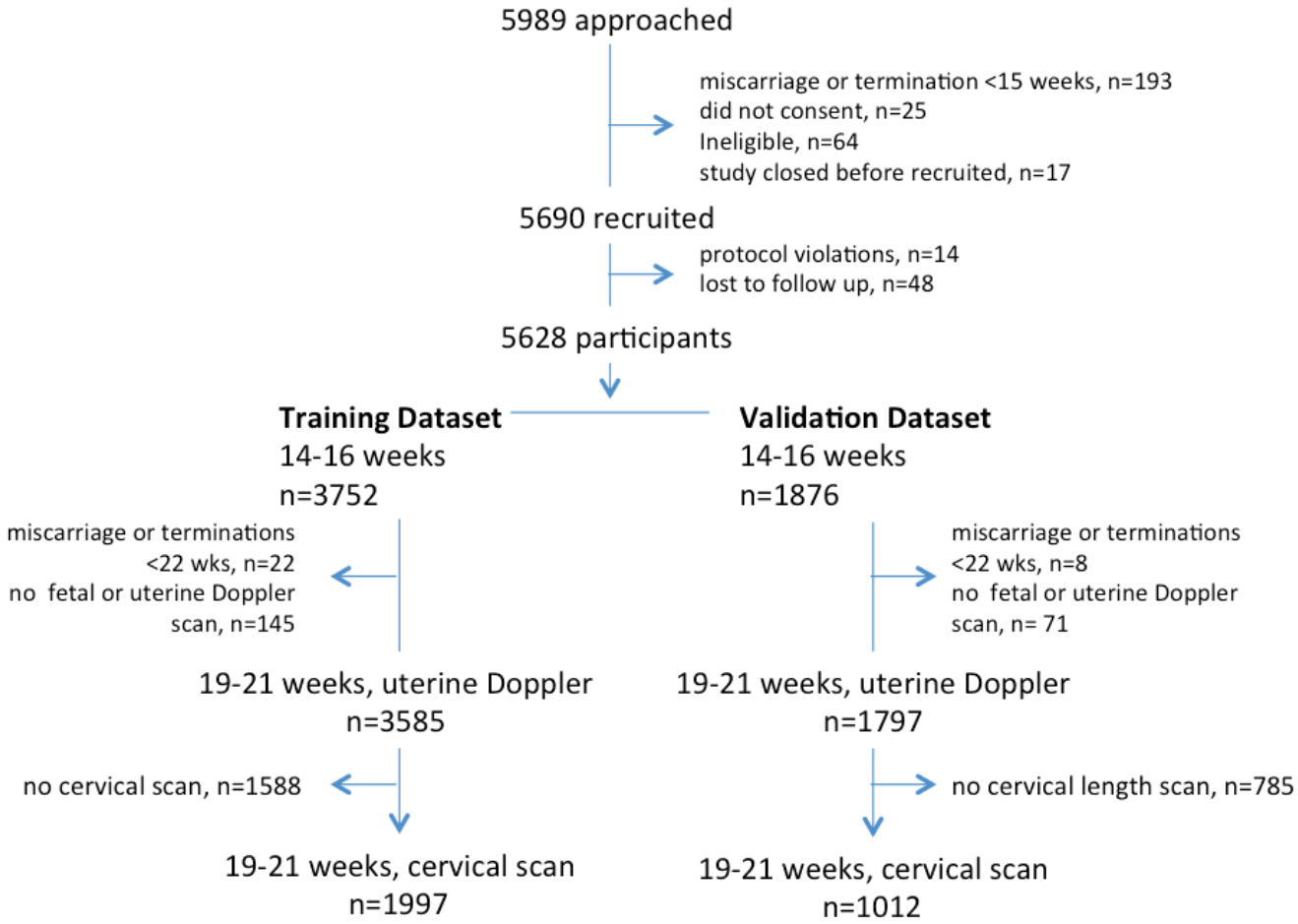


Table 2-1 Distribution of centres and preterm birth phenotypes in the training and validation sets. Number (%)

	Training n=3752	Validation n=1876
SCOPE Centres		
Auckland	342 (36)	690 (37)
Adelaide	789 (21)	375 (20)
London	125 (3)	60 (3)
Manchester	210 (6)	119 (6)
Leeds	105 (3)	39 (2)
Cork	1181 (31)	593 (32)
Spontaneous PTB	160 (4)	76 (4)
sPTB <34 weeks	48 (1)	23 (1)
sPTB 34-36 weeks	112 (3)	53 (3)

Table 2-2. Maternal Characteristics and Pregnancy Outcome

	Spontaneous PTB <34w n=71	Spontaneous PTB 34-36w n=165	No Spontaneous PTB n=5392	P-value
Maternal characteristics and outcome				
Maternal age (y)	28.6 (6)	28.1 (6)	28.7 (6)	0.41
Caucasian	61 (86)	152 (92)	4848 (90)	0.34
Married/defacto	57 (80)	155 (94)	4876 (90)	0.005
Socioeconomic Index	37.5 (15)	41.7 (17)	41.9 (16)	0.07
Education <12 years schooling	34 (48)	82 (50)	2008 (37)	0.001
Tertiary education	50 (70)	124 (75)	4498 (83)	0.0004
Full/part time work	53 (75)	140 (85)	4606 (85)	0.04
Unemployed/beneficiary	14 (20)	19 (12)	427 (8)	0.0004
Primigravida	46 (65)	114 (69)	4171 (77)	0.002
Number of previous miscarriages				
0	53 (75)	138 (84)	4662 (86)	< 0.0001
1	10 (14)	20 (12)	634 (12)	
2	8 (11)	7 (4)	96 (2)	
Smoking status				
non smoker	49 (69.0)	121 (73.3)	4093 (75.9)	0.005
ceased smoking in pregnancy	10 (14.1)	14 (8.5)	734 (13.6)	
current smokers	12 (16.9)	30 (18.2)	565 (10.5)	
Family history PTB				
single preterm birth	18 (25)	24 (15)	665 (12)	0.003
recurrent preterm birth	4 (6)	3 (2)	126 (2)	0.17
Vaginal bleeding before 15w				
0	39 (55)	121 (73)	4261 (79)	< 0.0001
1	20 (28)	34 (21)	836 (16)	
≥2	12 (17)	10 (6)	295 (5)	
Body mass index (kg/m^2) at 15w				
<18.5	2 (3)	4 (2)	78 (1)	0.61
18.5-24.9	33 (46)	93 (57)	2997 (56)	
25.0-29.9	22 (31)	46 (28)	1508 (28)	
≥30	14 (20)	22 (13)	809 (15)	
Systolic blood pressure (mmHg) at 15w	108 (11)	107 (11)	107 (11)	0.44
Diastolic blood pressure (mmHg)at 15w	67 (8)	65 (8)	65 (8)	0.31
Preeclampsia	2 (3)	5 (3)	271 (5)	0.69

	Spontaneous PTB <34w n=71	Spontaneous PTB 34-36w n=165	No Spontaneous PTB n=5392	P-value
Placental Abruption	9 (13)	3 (2)	31 (1)	< 0.0001
Fetal Outcome				
Delivery gestation (weeks)	29.2 (4.4)	35.8 (0.9)	39.8 (2.1)	< 0.0001
Birth weight (g)	1451 (704)	2711 (426)	3436 (572)	< 0.0001
SGA (<10th customised centile)	10 (14)	16 (10)	607 (11)	< 0.0001
Customised Birth weight centile	40 (32)	53 (31)	47 (29)	0.75
Admission to neonatal unit	57 (80)	100 (61)	490 (9)	< 0.0001
Perinatal death	10 (14.1)	1 (0.6)	20 (0.4)	< 0.0001

Values are “mean (sd)” for continuous variables, and “number (percentage)” for discrete variables. P-values generated by one-way ANOVA for continuous variables, and Chi-square test for discrete variables.

2.3.2 Variables of the algorithm at 15 weeks gestation

Of the 19 variables used for the model building, some were better performing than others for each sub-phenotype. The clinical risk factors recorded at 15 weeks' gestation that performed best for each of the phenotypes following multivariate analysis are included in the models. They are shown along with their odds ratios for the different sub-phenotypes in Table 2-3, Table 2-4 and Table 2-5. The cervical length scan variable is not included in these models as this made no difference to the performance of the algorithm (see Table 2-9).

Table 2-3 Clinical risk factors at 15 weeks gestation for spontaneous preterm birth less than 34 weeks

Clinical Risk Factor	**Odds ratio**
One vaginal bleed before 15w	1.94 (0.91,3.86)
≥ 2 vaginal bleeds before 15w	5.11 (2.21,10.82)
>12 months to conceive with current partner	2.07 (0.99,4)
One previous miscarriage	1.32 (0.53,2.82)
≥2 miscarriages	3.62 (1.01,9.94)
Consumed/inhaled/injected other recreational drugs at gestation at 15w	4.7 (1.43,12.8)
Unemployed or sickness beneficiary at 15 w	2.08 (0.85,4.6)
Any folate intake in 1st trimester	0.44 (0.17,1.3)
Did not consume oily fish in pregnancy prior to 15w	1.57 (0.87,2.82)

Table 2-4 Clinical risk factors at 15 weeks gestation for spontaneous preterm birth at 34-36 weeks gestation

Clinical Risk Factor	Odds ratio
One vaginal bleed before 15w	1.86 (1.18,2.86)
≥ 2 vaginal bleeds before 15w	1.13 (0.43,2.42)
Participant's birthweight	1.19 (1.01,1.39)
Family history of pre-eclampsia (first degree relative)	1.81 (1.04,2.99)
Any sister had a history of LBW baby	2.6 (1.22,5.01)
Quit smoking in pregnancy	0.55 (0.24,1.08)
Smoking at 15w pregnancy	1.85 (1.11,2.95)

Table 2-5 Clinical risk factors at 15 weeks gestation for all spontaneous preterm birth

Clinical Risk Factor	Odds ratio
One vaginal bleed before 15w	1.94 (1.31,2.81)
≥ 2 vaginal bleeds before 15w	2.13 (1.16,3.66)
>12 months to conceive with current partner	1.66 (1.08,2.49)
Participant's birthweight	1.14 (0.99,1.31)
Family history of pre-eclampsia (first degree relative)	1.57 (0.96,2.45)
One previous miscarriage	1.04 (0.62,1.65)
≥2 miscarriages	2.82 (1.26,5.63)
Any sister had a history of LBW baby	1.85 (0.9,3.46)
Quit smoking in pregnancy	0.7 (0.39,1.18)
Smoking at 15w pregnancy	1.45 (0.89,2.28)
Consumed/inhaled/injected other recreational drugs at gestation at 15w	2.61 (1.11,5.59)
Any education post school institution or university	0.66 (0.45,1)

2.3.3 Variables of the algorithm at 20 weeks gestation

The odds ratios of the included clinical risk factors from 15 weeks gestation and the Dopplers and fetal biometry at 20 weeks are shown in Table 2-6, Table 2-7 and Table 2-8.

In comparison to the 15 weeks models, for sPTB less than 34 weeks, history of previous miscarriages, folate and oily fish intake were no longer included in the algorithm whereas alcohol intake was now included. Uterine artery Doppler and fetal biometry were not identified as being important in this algorithm. The sPTB 34-36 weeks algorithm required the uterine artery Doppler and fetal biometry variables to be included along with level of education. The all sPTB algorithm required the addition of uterine artery Doppler and fetal biometry variables but no longer included previous history of miscarriage. Cervical length data again did not improve the prediction and is therefore not included (see Table 2-9).

Table 2-6 Clinical risk factors and scan data at 20 weeks for the prediction of spontaneous preterm birth less than 34 weeks

Clinical Risk + Doppler + Biometry	Odds ratio
One vaginal bleed before 15w	1.97 (0.89,4.05)
≥ 2 vaginal bleeds before 15w	3.94 (1.43,9.31)
>12 months to conceive with current partner	2.22 (1.02,4.48)
Consumed/inhaled/injected other recreational drugs at gestation at 15w	6.88 (2.12,18.72)
Units of alcohol per week in 1st trimester	0.61 (0.32,1.15)
Unemployed or sickness beneficiary at 15 w	2.58 (1.06,5.64)

Table 2-7 Clinical risk factors and scan data at 20 weeks for the prediction of spontaneous preterm birth at 34-36 weeks gestation

Clinical Risk + Doppler + Biometry	Odds ratio
One vaginal bleed before 15w	1.8 (1.12,2.81)
≥ 2 vaginal bleeds before 15w	1.22 (0.47,2.64)
Participant's birthweight	1.18 (1,1.39)
Family history of pre-eclampsia (first degree relative)	1.8 (1.03,2.99)
Any sister had a history of LBW baby	2.57 (1.2,5)
Quit smoking in pregnancy	0.49 (0.2,1)
Smoking at 15w pregnancy	1.71 (1,2.8)
Mean uterine artery RI at 19-21w	0.83 (0.69,1)
Head circumference: abdominal circumference ratio at 19-21w	0.64 (0.45,0.91)
Any education post school institution or university	0.7 (0.44,1.14)

Table 2-8 Clinical risk factors and scan data at 20 for the prediction of all spontaneous preterm birth

Clinical Risk + Doppler + Biometry	Odds ratio
One vaginal bleed before 15w	1.9 (1.27,2.8)
≥ 2 vaginal bleeds before 15w	1.92 (0.98,3.45)
>12 months to conceive with current partner	1.61 (1.02,2.46)
Participant's birthweight	1.16 (1.01,1.34)
Family history of pre-eclampsia (first degree relative)	1.62 (0.98,2.56)
Any sister had a history of LBW baby	1.98 (0.96,3.73)
Quit smoking in pregnancy	0.6 (0.31,1.06)
Smoking at 15w pregnancy	1.52 (0.93,2.4)
Consumed/inhaled/injected other recreational drugs at gestation at 15w	2.72 (1.14,5.9)
Mean uterine artery RI at 19-21w	0.85 (0.72,1)
Head circumference: abdominal circumference ratio at 19-21w	0.68 (0.5,0.92)
Any education post school institution or university	0.66 (0.45,1.01)

2.3.4 Performance of the clinical risk factor algorithms

The model performances in the training and validation datasets for the different endpoints, using the three different sets of variables, are shown in Table 2-9. The addition of fetal biometry, Doppler and cervical length data did not improve the performance of any of the models.

The model which performed the best was the one to predict preterm birth <34 weeks gestation with an AUC of 0.74, which achieved a similar performance (AUC 0.75) in the validation dataset.

Table 2-9 Model performance in training and validation sets for different end points

	AUC (95% CI) in training dataset	AUC (95% CI) in validation dataset
PTB <34 weeks		
Clinical	0.74 (0.67,0.81)	0.75 (0.65,0.85)
Clinical + Doppler + Biometry	0.72 (0.64,0.80)	0.69 (0.56,0.82)
Clinical + Doppler + Biometry + Cervical length	0.65 (0.53,0.77)	0.55 (0.41,0.68)
PTB 34-36 weeks		
Clinical	0.65 (0.60,0.70)	0.50 (0.42,0.58)
Clinical + Doppler	0.69 (0.64,0.74)	0.51 (0.43,0.60)
Clinical + Doppler + Biometry + Cervical length	0.70 (0.64,0.76)	0.53 (0.40,0.65)
PTB all		
Clinical	0.66 (0.62,0.70)	0.60 (0.53,0.67)
Clinical + Doppler + Biometry	0.68 (0.64,0.73)	0.57 (0.49,0.64)
Clinical + Doppler + Biometry + Cervical length	0.71 (0.66,0.77)	0.58 (0.48,0.67)

2.3.5 Clinical risk factor algorithm for spontaneous preterm birth <34 weeks using multivariate analysis

The spontaneous preterm birth less than 34 weeks gestation clinical risk factor algorithm performed the best and was equally as good when tested in the validation dataset. Table 2-10 shows the clinical risk factors identified as being most important for identifying nulliparous women at risk of preterm birth less than 34 weeks and the frequencies in each group of the SCOPE cohort.

Table 2-10 Clinical risk factors at 15 weeks included in clinical risk factor algorithm for preterm birth <34 weeks

	Clinical risk factors at 15 weeks		
Risk Factors	***No PTB n=5557 (%)***	***PTB n=71 (%)***	***Adjusted OR***
One vaginal bleed before 15w	870 (16)	20 (28)	1.94 (0.91,3.86)
≥ 2 vaginal bleeds before 15w	305 (5)	12 (17)	5.11 (2.21,10.82)
>12 months to conceive with current partner	680 (12)	18 (25)	2.07 (0.99,4)
Consumed/inhaled/injected other recreational drugs at gestation at 15w	77 (1)	7 (10)	4.7 (1.43,12.8)
Unemployed or sickness beneficiary at 15 w	446 (8)	14 (20)	2.08 (0.85,4.6)
One previous miscarriage	654 (12)	10 (14)	1.32 (0.53,2.82)
≥2 miscarriages	103 (2)	8 (11)	3.62 (1.01,9.94)
Any folate intake in 1st trimester	5340 (96)	61 (86)	0.44 (0.17,1.3)
Did not consume oily fish in pregnancy prior to 15w	2221 (40)	34 (48)	1.57 (0.87,2.82)

Model formulae for PTB <34 weeks:

15 week model: -4.48 + 0.73*unemployed or sickness beneficiary at 15 weeks+0.27*one previous miscarriage+1.29*two or more miscarriages+0.73*more than 12 months to conceive with current partner+0.66*one vaginal bleed before 15w+1.63*two or more vaginal bleeds before 15w+0.45*did not consume oily fish in pregnancy prior to 15w-0.82*any folate intake in 1st trimester.

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2.3.6 Prediction of spontaneous preterm birth <34 weeks in the training and validation sets

The algorithm was used to predict spontaneous preterm birth <34 weeks in both the training and validation sets for comparison.

Table 2-11 shows the performance of the algorithm for the prediction of spontaneous preterm birth <34 weeks based on clinical risk factors at 15 weeks' gestation in training and validation sets.

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Table 2-11 Prediction of spontaneous preterm birth <34 weeks based on clinical risk factors at 15 weeks gestation in training and validation sets

		Sensitivity *(95% CI)*	Specificity *(95% CI)*	Positive Predictive Value *(95% CI)*	Negative Predictive Value *(95% CI)*
90% specificity probability threshold: 0.0255	Training	33 *(20 to 48)*	91 *(91 to 92)*	5 *(3 to 8)*	99 *(98 to 99)*
	Validation	35 *(16 to 57)*	92 *(91 to 94)*	5 *(2 to 10)*	99 *(99 to 100)*
95% specificity probability threshold: 0.0360	Training	21 *(11 to 35)*	95 *(95 to 96)*	5 *(3 to 10)*	99 *(99 to 99)*
	Validation	30 *(13 to 53)*	96 *(95 to 97)*	8 *(3 to 16)*	99 *(99 to 100)*

2.4 Discussion

Traditional methods of screening for preterm birth have been based on obstetric history, current symptoms or epidemiological risk factors such as maternal age, race and smoking status. The most commonly known scoring system is Creasy's score (144), which is a modified Papiernik-Berkhauer system (235). However, these methods fail to identify over 50% of pregnancies that deliver preterm (i.e. they have a low sensitivity) and the majority of women who screen positive will ultimately deliver at term (i.e. they also have low positive predictive values).

The single greatest risk factor for preterm birth is a history of a prior unexplained spontaneous preterm birth, with a history of two prior preterm births yielding a risk of over 50% (90, 236). The model yielded through this large prospective international cohort of healthy nulliparous women by necessity does not include this risk factor, which therefore potentially makes it useful for other nulliparous women, who contribute around half of all preterm births (237). Using information which can be acquired in early pregnancy a clinical risk factor algorithm with a modest predictive performance for spontaneous preterm birth in both discovery and validation datasets has been developed. This study has highlighted other less well known risks which appear important, such as vaginal bleeding in early pregnancy, prior early miscarriage, months to conceive with current partner, unemployment, use of recreational drugs, folate intake and lack of consumption of oily fish.

The algorithm and collection of risk factors for the most clinically relevant group of preterm birth, those delivering less than 34 weeks, exhibited the best predictive performance overall – the area under the receiver operating characteristics curve was 0.74 – with an excellent negative predictive value of 99%. However, only 33% of preterm births in this group would have been predicted using the algorithm. The performance was similar (with a sensitivity of 35% and NPV of 99%) when we tested the algorithm on the validation cohort. The addition of ultrasonographic details, (biometry, Doppler and cervical length) did not improve performance, in part due to the very small numbers of women who had a preterm birth when this was measured. This identified risk assessment tool was more significant for the clinically important early (<34

weeks) preterm cohort as opposed to the later group (34-36 weeks). Although this may identify smaller numbers of preterm babies, they account for the greatest proportion of perinatal morbidity and mortality and long-term disability. There are clear patterns in the risk factors highlighted in this algorithm which demonstrates the multifactorial nature of preterm birth (18, 23).

Socioeconomic status

Low socio-economic status (SES) has previously been strongly linked to prematurity (96, 103, 105, 128-130, 238-242) and the algorithm has identified unemployment or sickness beneficiary and drug use as being important risk factors for spontaneous preterm birth. All these factors are more prominent in the most deprived areas. Socio-economic disadvantage is unlikely to be a direct, independent determinant of preterm birth; instead, it probably leads to less healthy behaviour, exposure to psychosocial stress and social evaluative threat, and psychological reactions to stress that shorten gestation (105). The contributions of genital tract colonisation, infection and inflammation have been identified as significant predictors of preterm birth, particularly for early preterm birth (<32 weeks) and bacterial vaginosis is more common among the socially disadvantaged (26, 34, 106-110). Several case-control studies have demonstrated an increased risk of spontaneous preterm birth in women who are unemployed (OR 1.5)(243). This can be found to be even higher in women receiving unemployment or sickness benefit (very preterm birth OR 1.88-2.64) (244, 245).

It has been shown that pregnant women living in low SES areas experience more stressful life events during their pregnancy and chronic stressors often result from the environment they live in (101, 111-113). These include financial insecurities, poor and crowded housing conditions, living without a partner, unsatisfying marital relationships, domestic violence, and stressful working conditions (101, 114, 115). They also often have a more restricted social support network (116) which is often less available during their pregnancy which can increase the impact of those stressors.

Toxins

Recreational drug use was another important risk factor. Cocaine use, although generally low during pregnancy, has previously been associated with preterm

birth (105, 246-248). However, in poor, inner-city areas, its use during pregnancy may be extremely prevalent (249), and in these settings, its mediating role may be substantial. Marijuana (250, 251) and narcotic (252) use, and high alcohol (253-255) and caffeine (>300mg/day) (256, 257) consumption are also more common among the socially disadvantaged, but these factors have not been convincingly shown to have an impact on gestational duration (242, 246-248, 257-263).

Nutrition

The nutritional status of the mother has previously demonstrated a clear association with preterm birth (264). The algorithm highlighted the lack of oily fish consumption (265-267) and use of folate in the first trimester as being important, which was in keeping with previous studies. Fish oil supplementation has been shown to prolong gestation in pregnant women with the most marked differences in those with previous pregnancy complications and in low and middle, but not in high, fish consumers (267-269). Recent reports suggest that low folic acid intake increases the risk of preterm delivery and that supplementation may be associated with a 50-70% reduction in early spontaneous preterm birth (270-272).

Fertility history

Taking more than 12 months to conceive with one's current partner was another variable identified in this analysis and this has previously been demonstrated in a study in Denmark (273). Women who have suffered a threatened miscarriage have been independently shown to have an increased incidence of preterm delivery (50, 274).

Implantation/placentation

Vaginal bleeding and number of miscarriages may be reflective of disruption at the uteroplacental interface (275). Bleeding in early pregnancy most often originates from the placenta and it is thought that bleeding between the chorionic membrane and the uterine wall may result in a spectrum of effects on pregnancy development and outcome. At the end of pregnancy this is likely to be due to a chronic inflammatory reaction within the decidua and placental

membranes, with weakening and eventual rupture of the membranes or resulting in myometrial activity (275-281).

The derived algorithms are likely to be indicative of the best performance achievable using clinical and ultrasonographic data to predict preterm birth in a healthy nulliparous population, because of the study's prospective design, cohort size, comprehensive range of candidate predictors, high quality data, and completeness to follow-up. Clinical risk estimates for disease are already established in other areas of medicine and biomarkers are often added to improve performance. Unfortunately there have been no clinically useful biomarkers identified for spontaneous preterm birth yet (282), although these remain the subject of intense interest.

The cohort size was sufficient to permit division into a training and validation cohort. Other strengths of the study included prospective acquisition of high quality data from questionnaires administered at face to face interviews, informed by detailed standard operating procedures and the use of a real-time database, with automatic checking procedures, ensuring reduced data entry and transcription errors. For a dataset of this size, the rate of missing data was extremely low. The intensive two-stage data monitoring added confidence in data integrity.

Despite these strengths, there will always be limitations in such far-ranging data collection. It is likely that measurement errors, such as in self-reported family history (283) could have occurred. The outcome data for each case were reviewed by the local principal investigators which ensured accurate and consistent diagnosis of spontaneous preterm birth. One of the challenges when predicting rare events in prospective cohorts such as SCOPE is the relatively low number of cases compared with those studies derived from larger but less detailed epidemiological databases. While the latter might have substantially greater number of events, their interpretation is constrained by less accurate diagnosis.

There is no consensus as to the best method for selection of clinical variables (284). Given the rich dataset of potential predictors for preterm birth, an initial reduction step based on significance of univariate testing was used and then

selection of a subset of candidate variables based on *a priori* knowledge. Although this could have introduced variable selection bias, it was reassuring that the clinical risk factors and their strength of association with preterm birth were consistent with the literature.

The ideal use of a predictive tool would be to identify healthy nulliparous women at low or high risk for spontaneous preterm birth. Those at low risk could continue with standard antenatal care, whereas high risk women would follow a more intensive pathway, such as ultrasound assessment of cervical length, or fetal fibronectin testing, to allow early initiation of targeted interventions, such as supplemental progesterone or cerclage.

The large multicentre prospective design with excellent follow-up is a major strength of this study. The ultimate aim of the SCOPE study is to develop tests to predict pregnancy outcome with potential to translate into clinical care. The population in which the algorithm is developed needs to be identifiable if a screening test is to be used in clinical care (285) and recruitment has been a clearly defined population of nulliparous women to enable the detection of similar populations for validation and clinical use. Important clinical risk factors in healthy nulliparous women for spontaneous preterm birth at less than 34 weeks gestation have been identified. The algorithm provides a modest improvement in predictive performance compared with current practice. It has been validated in a separate sample set, however will need further validation in a separate cohort.

Disease biomarkers may improve the prediction further but as yet no useful biomarkers have been identified to improve the prediction of spontaneous preterm birth. It may be that a clinical risk factor-biomarker combination is likely to yield the best clinical performance, such as that used in Down's syndrome screening (286-288).The next part of my thesis will be to investigate the plasma proteome in search of preterm birth predictive biomarkers.

3 Proteomic Identification of Biomarkers for the Prediction of Spontaneous Preterm Birth

3.1 Introduction

Proteomics can be defined as the systematic analysis of proteins for their identity, quantity and function, in a non-targeted and unbiased manner. The proteome is dynamic, varying in response to external and internal influences during development, ageing, health and disease (289).

The proteome is the total set of expressed proteins in a cell, tissue or organism (290). There are estimated to be over 100,000 proteins compared to just 30,000 genes (290). As the function of proteins is so closely tied to their cellular, tissue and physiological context, the ultimate goal is to characterise the information flow through protein pathways and networks (291), with an eventual aim to understand the functional relevance of proteins under disease conditions (292). Proteomic methods are hypothesis generating and allow the simultaneous analysis of many molecules including known and novel candidates, and are therefore ideally placed to search for biomarkers (290). As a result of the heterogeneous nature of humans and the complexity of disease, especially preterm birth, a panel of biomarkers rather than a single marker may be required to achieve the high sensitivity and specificity required for clinical applications (293).

Proteomics is a particularly rich source of biological information because proteins are involved in almost all biological activities and changes in protein structure and expression levels signal, in most cases, the presence of disease (291). This means that proteins may be excellent targets in disease diagnostics, prognostics and therapeutics.

Human plasma is an extreme challenge to study due to the large dynamic range of the proteins of interest. The proteins in human plasma range from common “high-abundance” proteins, comprising 2%-50% of the total protein mass (typically the µg/mL-mg/mL range) to those considered to be the more specific tissue biomarkers present in the pg/mL-ng/mL range (294). Approximately 22

high abundance proteins account for 99% of the plasma proteome by mass, the transport protein albumin for 50% alone (295). The remaining 1% are low abundance proteins: cytokines, chemokines and peptide hormones, which are of primary interest for biomarker discovery (295). Removal of the most abundant components by immunodepletion or fractionation before analysis has markedly expanded the number of lower abundance components that can be detected (294).

Once samples have been depleted of the most abundant proteins, fractionation is required to simplify the samples further. This allows the plasma samples to be separated out into fractions containing different low abundance proteins.

3.1.1 Immunodepletion

The presence of higher abundance proteins interferes with the identification and quantification of lower abundance proteins. While biomarkers could be found at any abundance level, the chances of finding a biomarker will increase with the number of proteins profiled (296). In addition, the more promising biomarkers are more likely to be the low abundant proteins that either leak into the plasma from different tissues as a result of disease or play a role as signal molecules, rather than the more common generic plasma proteins (297).

Albumin, in very high abundance, is an ideal candidate for complete selective removal prior to performing a proteomic analysis of lower abundance proteins. However, depletion also results in a risk of losing proteins of interest other than those specifically depleted. It is well known that albumin interacts with a diverse number of compounds, such as low abundance cytokines, peptide hormones and lipoproteins, compounds that might be of interest in biomarker discovery. Therefore, depletion of the transport and carrier protein albumin under non-denaturing conditions may lead to the depletion of many other potentially interesting compounds. However, no single proteomic technique is capable of visualizing every component of a proteome and compromises are always required. Instead each technique displays a different analytical window of proteins and depletion strategies should simply be seen as one way of extending the analytical window so that more potentially interesting proteins may be identified.

Depletion has become a routine and accepted technique and a number of methods are available. Traditionally this issue is tackled using immunodepletion columns to specifically remove the most abundant proteins. These columns are expensive and have limited loading capacity. It involves the application of plasma to immobilised purified polyclonal antibodies in order to remove these proteins (298).

3.1.1.1 Multiple Affinity Removal Column (MARS)

Various commercial products are available for depletion. When compared to other methods the Agilent Multiple Affinity Removal Column (MARS, Agilent Technologies Inc, CA) offers good depletion efficiency, specificity and reproducibility (296). A MARS column has been developed which can deplete 14 high-abundant proteins, removing approximately 94% of the total protein mass. The 14 high-abundant proteins are human serum albumin, transferrin, haptoglobin, IgG, IgA, α1-antitrypsin, fibrinogen, α2-macroglobulin, α1-acid glycoprotein, complement C3, IgM, apolipoprotein AI, apolipoprotein AII and transthyretin. MARS columns are highly reproducible from sample to sample without introducing additional variation (296). Reproducibility is a prerequisite for accurate quantitative protein profiling for biomarker discovery.

3.1.1.2 ProteoMiner™ Beads

ProteoMiner™ technology (Bio-Rad Laboratories Inc, CA) is a novel sample preparation tool used for the compression of the dynamic range of the protein concentration in complex biological samples. This is accomplished through the use of a large, highly diverse bead-based library of combinatorial peptide ligands. Each bead features a different hexapeptide ligand with affinity for specific proteins in a sample. Samples are applied to the beads, allowing proteins to bind ligands to which they have the highest affinity. Proteins in excess are washed away, and those proteins bound to the beads are eventually eluted, allowing further downstream analysis. In contrast, the medium- and low-abundance proteins are concentrated on their specific affinity ligands. This reduces the dynamic range of protein concentrations whilst maintaining representatives of all proteins within the original sample.

3.1.2 High Performance Liquid Chromatography

Once the samples are depleted of the most abundant proteins, the next step is to simplify the sample further using fractionation. This is commonly applied in multiple dimensions by high performance liquid chromatography (HPLC) producing a simplified fraction separated by different properties (299).

The Agilent mRP-C18 column provides optimised separation by hydrophobicity for human serum after depletion of the high-abundance proteins using the MARS column. This combined workflow enables high recovery, excellent resolution and reproducibility in reducing sample complexity for biomarker research. The ability to recover >98% of immunodepleted plasma also provides the opportunity to validate biomarkers that are differentially expressed in control versus disease/treated plasma. The ability to reproducibly fractionate at the protein level with high resolution enables efficient separation and reduced overlap of proteins in collected RPLC fractions.

3.1.3 Identification of proteins using Mass Spectrometry

Once samples have been depleted and fractionated they can be analysed by a mass spectrometer (MS). The MS has low sensitivity for intact proteins and so samples are usually cleaved into peptides by a protease (usually trypsin) to allow identification and analysis. Mass spectrometry is capable of producing, separating and detecting molecular ions (297).

3.1.4 Quantification methods in Mass Spectrometry

Many potential diagnostic applications of proteomics depend on quantification of proteins in addition to their identification. The size of the peaks generated within the mass spectrum, indicate the intensity, i.e. the number of ions detected, at each m/z; however this information can only be used for relative quantification as it does not correlate with the amount of protein in the original sample. Quantitative MS methods can be divided into those using stable isotope labels, and those which are ‘label free’. Stable isotope labelling methods involve chemical modification of peptides to incorporate stable isotopes, labelled peptides behave almost identically to non labelled peptides but the differences in mass can be detected by the MS. Label free quantification can be achieved by comparing signal intensity against a given peptide (300).

3.1.4.1 Isobaric Tags for Relative and Absolute Quantification (iTRAQ)

This strategy is a ‘shotgun’ proteomics approach using iTRAQ labelling to achieve relative quantification (301-304). The iTRAQ kit allows the differential labelling of peptides from distinct proteomes. The labels consist of three elements, a peptide reactive group that enables the N-terminal labelling of peptides, a balance group that ensures labels are indistinguishable by mass, and a reporter group of variable mass which enables them to be differentiated by the release of this reporter ion. The iTRAQ kit can be used to label up to eight samples which can then be treated as one in subsequent fractionation steps, allowing for deep plasma penetration at the expense of high sample throughput. During tandem-MS, the peptide fragments into a product ion series which is used for peptide sequencing by database searching, and an iTRAQ reporter ion which is used for relative quantification (305). All individual plasma samples are referenced against the same ‘master pool’ allowing inter-experiment comparisons to be made.

Most labelling based quantification methods have limitations; which include increased time and complexity of sample preparation, requirement for higher sample concentration, high cost of the reagents, incomplete labelling, and the requirement for specific quantification software. Moreover, iTRAQ experiments are limited to the comparison of eight samples in a single experiment and are therefore often used for pooled samples.

3.1.4.2 Label Free Method

All label-free techniques include the following fundamental steps: (i) sample preparation including protein extraction, reduction, alkylation, and digestion; (ii) sample separation by liquid chromatography (LC or LC/LC) and analysis by MS/MS; (iii) data analysis including peptide/protein identification, quantification, and statistical analysis. This allows high sample throughput at the expense of reduced proteome penetration. In contrast to peptide-labelling methods, label free quantification allows you to analyse a larger number of individual samples rather than analysing pooled samples.

Peptide quantification is generally based on two categories of measurements. The first is the measurements of ion intensity changes such as peptide peak heights in chromatography. The second is based on the spectral counting of

identified peptides after MS/MS analysis. Peptide peak intensity or spectral count is measured for individual LC-MS/MS runs and changes in peptide abundance are calculated via a direct comparison between different analyses. Compared with isotope labelling methods, label-free quantification needs to be more carefully controlled, due to the possible error which may be introduced by run-to- run variations in the performance of the LC and MS(306).

3.1.5 MS Limitations

The application of mass spectrometry methods to proteomic biomarker identification is ideal due to the ability to analyse complex mixtures without prior knowledge of the components. The greatest limitations of mass spectrometry analyses are the sensitivity of the instruments and their ability to handle complex mixtures, although this technique continues to show promise in the search for biomarkers (307).

3.2 Aim of this study

Spontaneous preterm birth is well suited to a proteomic approach given the likely serologic changes that precede its clinical manifestation. The hypothesis is that proteomic differences exist in maternal plasma several weeks prior to the onset of clinical symptoms in women destined to develop spontaneous preterm birth. The aim is to use plasma proteomics to differentiate women having a subsequent spontaneous preterm birth from those having term deliveries.

3.3 Methods

3.3.1 Patient Plasma Samples

EDTA-plasma from women at 20±1 weeks gestation who had uncomplicated pregnancies (controls) or who subsequently delivered prematurely were obtained from the SCOPE biobank (Auckland, New Zealand). Blood was collected by venepuncture into BD EDTA-Vacutainers®, placed on ice and centrifuged at 2400 x g for 10 minutes at 4°C. Plasma was stored at -80°C within four hours of collection. Sample collection and storage conditions were closely regulated for all samples. Plasma samples were defrosted on ice and always maintained below 4ºC during experiments.

3.3.1.1 Ethical Approval and Participants

The participants were healthy nulliparous women with singleton pregnancies recruited to the Screening for Pregnancy Endpoints (SCOPE) study between November 2004 to July 2007 in Auckland, New Zealand and Adelaide, Australia (Australian and New Zealand Clinical Trials Registry ACTRN12607000551493) (308). SCOPE was a prospective, multicentre cohort study with the main aim of developing screening tests to predict pre-eclampsia, small for gestational age infants, and spontaneous preterm birth.

The women were recruited to the SCOPE study (see 2.2) between 14 and 16 weeks through hospital antenatal clinics, obstetricians, general practitioners, community midwives, and self-referral in response to advertisements or recommendations of friends. Women were excluded if they were judged to be at high risk of pre-eclampsia, small for gestational age babies, or spontaneous preterm birth because of underlying medical conditions (chronic hypertension requiring antihypertensive drugs, diabetes, renal disease, systemic lupus erythematosus, antiphospholipid syndrome, sickle cell disease, HIV), previous cervical cone biopsy, three or more previous miscarriages/terminations of pregnancy, current ruptured membranes, known major fetal anomaly or abnormal karyotype, or who had received interventions that might modify pregnancy outcome (such as aspirin or cervical suture). Full ethical approval was obtained and all patients gave written informed consent.

A research midwife interviewed and examined women who agreed to participate at 14-16 weeks' and again at 19-21 weeks' gestation. At the time of the interview, data were entered into an internet-accessed central database with a complete audit trail (MedSciNet). Participants were followed up prospectively, with pregnancy outcome data and baby measurements collected by midwives, usually within 72 hours of birth. Stringent data monitoring included individual checking of all data for each participant, including checks for any transcription errors of the lifestyle questionnaire, and using a customised software package to detect any systematic data entry errors.

3.3.2 Study Population

Spontaneous preterm birth was defined as spontaneous preterm labour or preterm rupture of membranes at less than 37 weeks' gestation. Uncomplicated pregnancy was defined as a pregnancy with no antenatal obstetric or medical complications and resulting in delivery of an appropriately grown, health baby at 37 or greater weeks' gestation.

3.3.3 Experimental Samples from SCOPE

Plasma samples were obtained for women at 20 weeks gestation who subsequently had a spontaneous preterm birth (random samples with and without PPROM) (n=12), preterm birth with PPROM (n=12), and preterm birth without PPROM (n=12). Three distinct control groups (n=12) were obtained, matched for centre only.

3.3.4 Samples for Workflow Development

Venous blood samples were obtained from healthy women with singleton pregnancies booked for antenatal care and delivery at the Ninewells Hospital, Dundee as part of a previous study (309). Approval was given by the Dundee and Manchester Local Research Ethics committees and written informed consent obtained for all samples. Plasma samples were prepared by laboratory staff at the University of Dundee and transported on dry ice. Non-pregnant samples were obtained with informed consent from healthy volunteers.

3.3.5 Comparison of Plasma Sample Preparation Workflows for Proteomics

Replicates of plasma from pregnant women at 20 weeks were either immunodepleted of the 14 most abundant plasma proteins using MARS 14 HPLC column (Agilent, UK) or treated using ProteoMiner™ (Bio-Rad) beads. The depleted plasma samples were then further fractionated using a reverse phase HPLC column. Following these treatments the plasma samples were compared by 1D SDS gels and Bradford protein assay.

3.3.5.1 ProteoMiner™ Beads

Two plasma replicates of 200µL were mixed with ProteoMiner™ Beads (Bio-Rad) according to manufacturer's instructions. Briefly, the beads were prepared by rinsing with PBS buffer then mixed with crude plasma on a rotating wheel for 2 hours at room temperature. Unbound proteins were then removed with wash steps. After the appropriate binding and washing steps, proteins were eluted using 8M Urea, 1% acetic acid, vortexed and the eluate collected.

3.3.5.2 MARS 14 Immunodepletion

Plasma (40µL x 2) was depleted of high abundance plasma proteins using the MARS 14 Hu column (4.6x100mm) with a 1200 HPLC system (Agilent) with thermostat-controlled autosampler and fraction collector. The manufacturer's recommended protocol was followed using proprietary MARS A and B buffers. Briefly, 40µL of EDTA plasma was thawed on ice, centrifuged at 10,000g for 10 minutes at 4°C, then diluted with 120µL Buffer A and injected onto the column at 0.125mL/min. UV absorbance was detected at 280nm. A single fraction of the flow-through was collected per sample.

All the samples prepared using ProteoMiner™ and MARS 14 were desalted and concentrated further using Amicon 10kD molecular weight cut off (Millipore, UK) spin columns. Using the spin columns they were buffer-exchanged into 50mM AmBic 5% acetonitrile.

Both the MARS 14 and the ProteoMiner™ treated samples were then reduced and alkylated using dithiothreitol added to 5mM final concentration and heated

at 60°C for 15 minutes. Iodoacetamide was added to 20mM final concentration and the samples placed in the dark for one hour.

3.3.5.3 Bradford Protein Assay

The concentration of protein in each sample was determined using Bradford Protein Assay Reagent (Bio-Rad). Standards (0-1mg/mL) were prepared using Bovine Serum Albumin dissolved in ammonium bicarbonate. To 200µL of Bradford Protein Assay Reagent, 10µL of sample or standard were added, mixed and incubated for 10 minutes at room temperature. Measurements were made in triplicate at 595nm using a Spectramax M5 plate reader (Molecular Devices, UK). Concentration of samples was calculated by plotting a standard curve of concentration against blank-adjusted optical density measurement of the standards.

3.3.5.4 Fractionation of samples

To simplify the complex samples, proteins were fractionated using a Macroporous Reversed-Phase C18 Column (mRP-C18, 4.6mm x 50mm, Agilent, UK). Mobile phases were A: water + 0.1% Trifluoroacetic acid (TFA); B: Acetonitrile + 0.1% TFA. At a flow rate of 0.75mL/min with a gradient of 5-45% B over 3.5 minutes, and UV absorbtion at 280nm, fractions were collected every 0.60 minutes.

3.3.5.5 Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis

Equal volumes of the mRP protein fractions were reduced and denatured by dilution 1:10 in distilled water, addition of an equal volume of Lamelli buffer (0.125M Tris (pH 6.8), 5% SDS, 50% Glycerol, 25% β- mercaptoethanol, 0.05% w/v bromophenol blue) and heating at 90 ºC for 5 minutes. Samples were loaded onto a 1.5mm stacking gel and 10% resolving gel with Amersham Full-Range Rainbow Molecular Weight Markers (12-225 kDa, GE Healthcare, UK). Electrophoresis was carried out in Running buffer (25mM Tris-base, 191mM Glycine, 3.4mM SDS, pH8.0) using a Mini Protean III Electrophoresis System (BioRad, Hemel Hempstead, UK).

3.3.5.6 Comassie Staining

To visualise protein bands, gels were stained in Comassie stain (brilliant blue R-250 10% acetic acid 45% methanol) and rinsed in destain (10% acetic acid 45% methanol) with each step carried out with agitation.

3.3.6 MS of samples

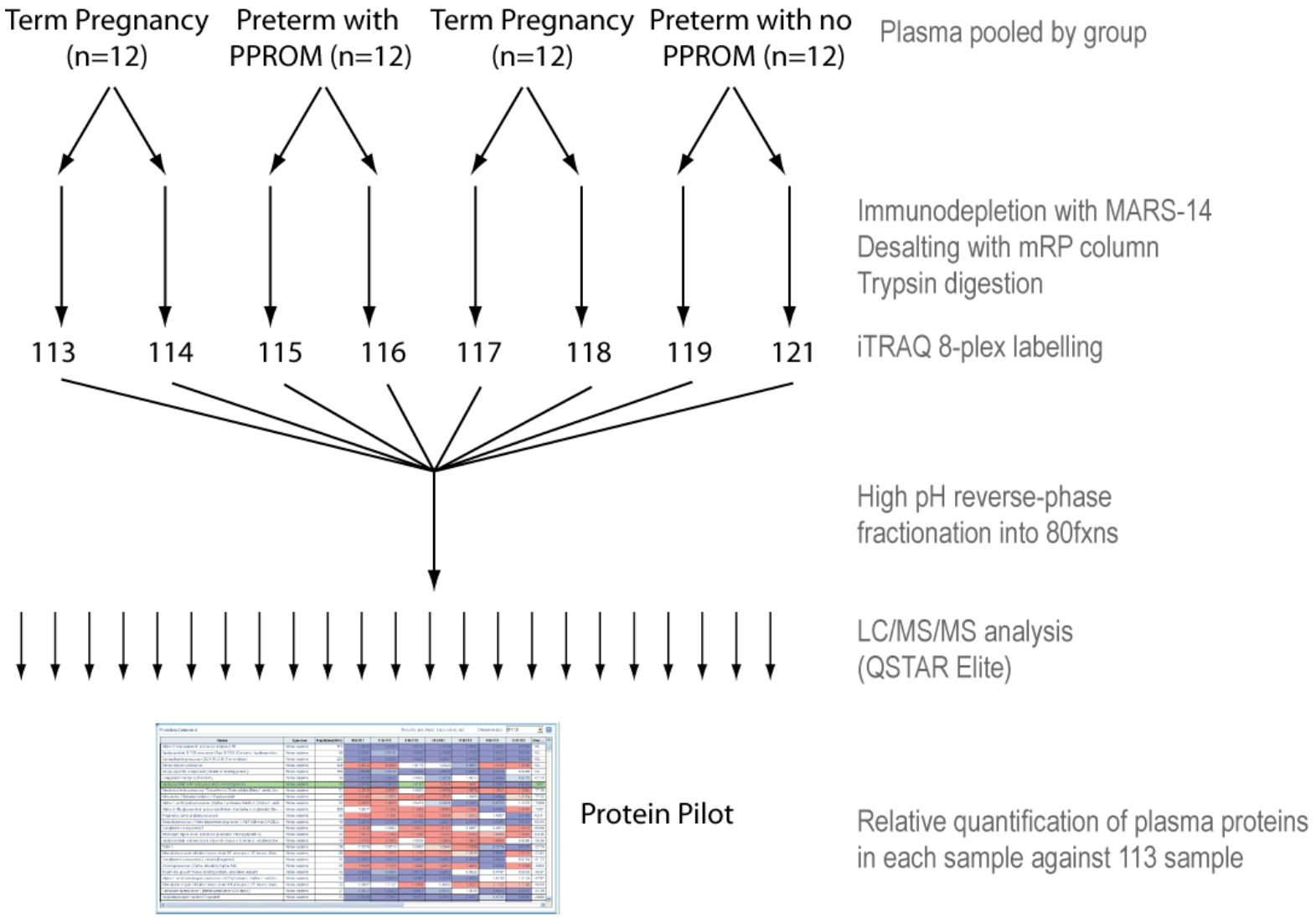
Fractions collected from mRP column (3.3.5.4) were dried down in a rotary evaporator (Eppendorf, UK). Each fraction was resuspended in 50µl AmBic + 5% Acetonitrile and Trypsin (Proteomics grade, Sigma) was added to an approximate protein: enzyme ratio of 50:1. Tryptic digestion was carried out at 37°C overnight.

The samples were analysed with HPLC chip-6350 QTOF nano LC-MS platform (Agilent) with 60 minute analysis times. Database searching was done with Mascot 2.2 (Matrix Science, UK) against the SwisProt Human protein database.

3.3.7 Identification of candidate biomarkers using iTRAQ-based relative quantification

SCOPE plasma samples from preterm labour with ruptured membranes (n=12), preterm labour without ruptured membranes (n=12), and corresponding control groups (n=12 each) were pooled based on equal volume. Two aliquots of each pool were then immunodepleted by the MARS 14 column and subsequently desalted on the mRP column (See Figure 3.1). One fraction containing the entire elution was collected using the manufacturer's recommended desalting method. The fraction was reduced and alkylated using dithiothreitol (5mM final concentration) and iodoacetamide (20mM final concentration) then trypsin digested at 1:20 enzyme: protein ratio. Digests were dried down (approx. 80µg protein) and resuspended in 30µL 0.5M TEAB.

Figure 3.1 iTRAQ workflow for samples obtained from the SCOPE Study



3.3.7.1 iTRAQ labelling

The manufacturer's protocol was followed to label peptides using 8-plex iTRAQ Reagents (Isobaric Tags for Relative and Absolute Quantification, Applied Biosystems, Warrington, UK). The eight iTRAQ reagents were resuspended in 50µL isopropanol and transferred to the appropriate sample digests and incubated at 23°C for 2 hours. On completion labelled samples were combined into one mixture, and then divided into two halves before being dried in the rotary evaporator.

3.3.7.2 High pH reverse phase fractionation of iTRAQ labelled peptides

High pH RP fractionation was performed using a 4.6x150mm Extend SB-300 HPLC column (Agilent) using the same 1200HPLC system as the mRP column. Fresh mobile phases were prepared; A: water + 0.1% ammonium hydroxide; B: Acetonitrile + 0.1% ammonium hydroxide (NH_4OH). (1mL min, gradient from 5-40% with 0.35 minute fraction collection). One iTRAQ sample (50% of total) was resuspended in 900µL of 5% ACN, 0.1% NH_4OH, vortexed hard and centrifuged at 16 000g for 5 minutes. The supernatant was removed to a glass vial for injection. 80 fractions were collected in total which were dried down completely in a rotary evaporator.

3.3.7.3 Analysis of iTRAQ Labelled Samples

The standard iTRAQ method was followed on the QSTAR Elite (Applied Biosystems, UK) with 120min analysis time using the method named iTRAQ_120mins.

3.3.7.4 Data analysis

Data were interrogated using ProteinPilot version 3.0 (Applied Biosystems, Warrington, UK) with trypsin as the digest agent and default settings for iTRAQ 8-plex labelling. Peptide identifications were made using the Paragon algorithm (310) searching against the Uniprot Human database. A 95% confidence interval cut off was used for significant peptides. False discovery rate was calculated by searching all peptide data against a concatenated database containing both forward and reversed protein sequences.

Relative quantification was carried out using ProteinPilot v3.0 software. An average relative quantification ratio is calculated from the unique iTRAQ reporter ions associated with the peptides which identify each protein. A normalisation factor is applied to each iTRAQ reporter ion ratio to compensate for any differences in the amount of starting protein material prior to labelling. Relative ratios were calculated against a user defined denominator, in this study 113 (Term Pregnancy) was used.

3.3.8 Identification of candidate biomarkers using label-free methods

Plasma samples in the SCOPE cohort taken at 20 weeks gestation were obtained from 12 women with preterm birth (delivery <37 weeks), 12 controls (delivery >37 weeks) and six replicates to assess technical variability. The 40µL SCOPE plasma samples were diluted with 160µL MARS buffer. The 24 sample cohort was randomly divided with either 1µg/ml or 1.5µg/ml horse myoglobin spiked into the samples. These known concentrations were spiked into the samples prior to depletion to allow for technical reproducibility to be assessed. 145µL of each sample was injected onto the MARS 14 column and subsequently desalted on the mRP column. Six replicate SCOPE plasma samples (R1-6), with no proteins spiked in were also injected randomly as another measure of reproducibility (see Table 3-1), and to determine the ability of the software to identify quantitative differences in myoglobin levels. The samples were then fractionated into six fractions using reverse-phase chromatography (mRP-C18, Agilent). Each fraction was trypsin digested and run on a LC-QTOF (Agilent) platform to acquire label-free MS data. Analysis was performed using LC-MS Progenesis (Non-Linear Dynamics).

Table 3-1 Sample run on MARS 14 column to demonstrate random placement of technical variates

Sample No.	Plate position	Outcome	Myoglobin Spike
R1	**P1A**	**REPLICATE**	**NONE**
R2	**P2A**	**REPLICATE**	**NONE**
1	P1C	PT	1µg/mL
2	P1D	PT	1.5µg/mL
3	P1E	T	1µg/mL
4	P1F	T	1.5µg/mL
5	P1G	PT	1µg/mL
6	P1H	T	1µg/mL
7	P2A	T	1.5µg/mL
8	P2B	PT	1.5µg/mL
9	P2C	PT	1µg/mL
10	P2D	PT	1µg/mL
R3	**P2E**	**REPLICATE**	**NONE**
11	P2F	T	1µg/mL
12	P2G	PT	1.5µg/mL
13	P2H	PT	1.5µg/mL
14	P3A	T	1.5µg/mL
15	P3B	PT	1.5µg/mL
R4	**P3C**	**REPLICATE**	**NONE**
16	P3D	T	1.5µg/mL
17	P3E	T	1.5µg/mL
18	P3F	PT	1µg/mL
19	P3G	T	1.5µg/mL
20	P3H	T	1µg/mL
R5	**P4A**	**REPLICATE**	**NONE**
21	P4B	T	1µg/mL
22	P4C	PT	1.5µg/mL
23	P4D	PT	1µg/mL
24	P4E	T	1µg/mL
R6	**P4F**	**REPLICATE**	**NONE**

3.3.8.1 High pH reverse phase fractionation

High pH RP fractionation was performed using a 4.6x150mm Extend SB-300 HPLC column (Agilent) using the same 1200HPLC system as the mRP column. Fresh mobile phases were prepared; A: water + 0.1% ammonium hydroxide; B: acetonitrile + 0.1% ammonium hydroxide (NH_4OH). (1mL min, gradient from 5-40% with 0.35 minute fraction collection dried down completely in a rotary evaporator. 180 fractions (6 from each sample) were collected in total.

3.3.8.2 Data analysis

The spectra were analysed using LC-MS Progenesis (Non-Linear Dynamics) to detect features that best discriminated sample groups. These features were filtered then submitted as targeted MS/MS lists to identify them via database searching (Mascot). The data generated from fraction 4 was also analysed to interrogate the quantification of the spiked proteins.

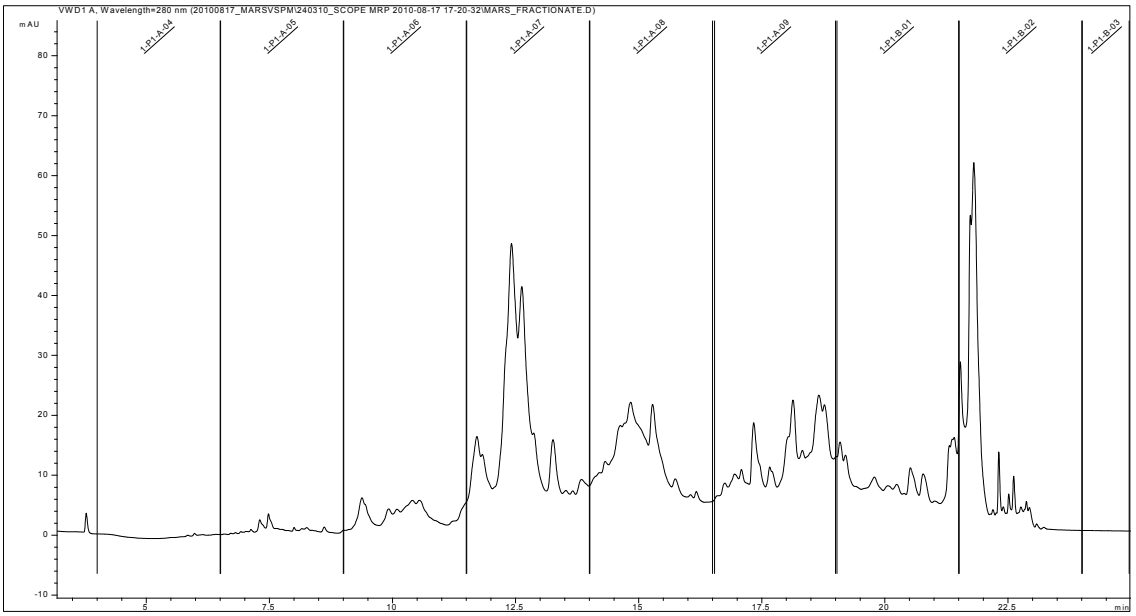
3.4 Results

3.4.1 Comparison of Plasma Sample Preparation Workflows for Proteomics

3.4.1.1 Protein fractionation using Reverse Phase HPLC

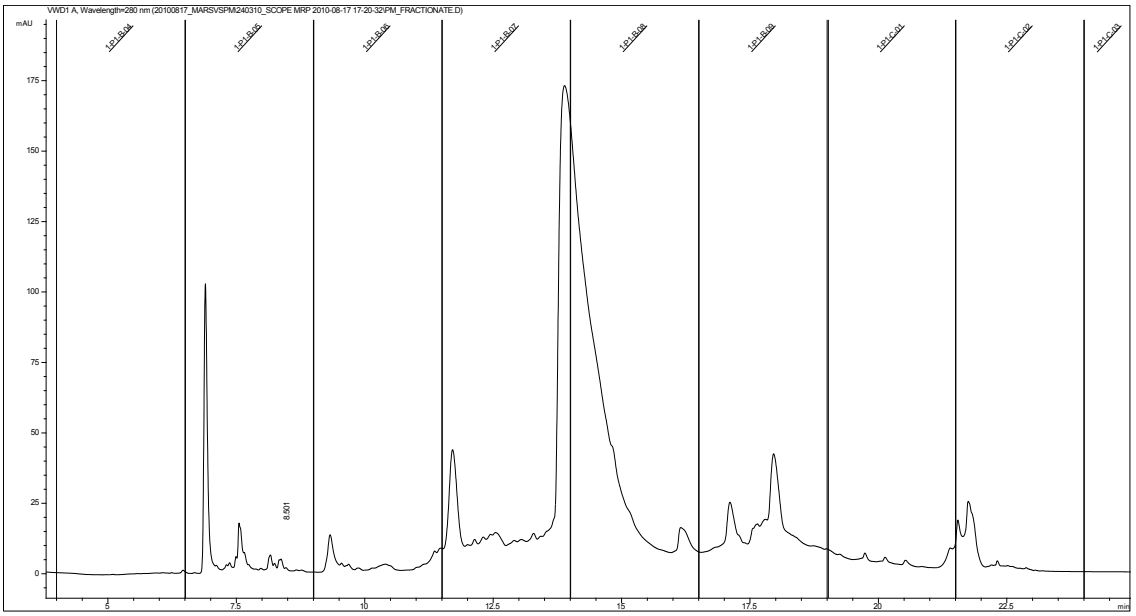
To visualise the protein profiles of plasma following MARS 14 depletion or ProteoMiner^{TM} beads depletion, samples were fractionated by reverse phase chromatography then analysed by SDS-PAGE. The UV chromatogram produced by the MARS 14 demonstrated good separation with resolution of several peaks (Figure 3.2). The ProteoMiner^{TM} beads chromatogram was dominated by a large peak with several other poorly resolved peaks (Figure 3.3).

Figure 3.2 shows mRP chromatograms of plasma prepared with MARS 14 at UV 280nm



UV chromatogram produced by the MARS 14 demonstrated good separation with resolution of several peaks. A flow rate of 0.75mL/min with a gradient of 5-45% B over 3.5 minutes, and UV absorption at 280nm, fractions were collected every 0.60 minutes.

Figure 3.3 shows mRP chromatograms of plasma prepared with ProteoMiner^{TM} beads at UV 280nm



UV chromatogram produced by the ProteoMiner^{TM} beads dominated by a large peak with several others poorly resolved. A flow rate of 0.75mL/min with a gradient of 5-45% B over 3.5 minutes, and UV absorption at 280nm, fractions were collected every 0.60 minutes.

3.4.1.2 Protein Assay Results

Bradford Assays were performed as described on the individual fractions to assess how much protein was present in each sample following depletion. Table 3-2 shows the result to compare MARS 14 to ProteoMiner™ beads. Although the capacity of the ProteoMiner™ beads was five times greater than the MARS columns, the yields were very similar.

Table 3-2 Comparison of amount of protein (*µ*g) in each fraction using MARS depletion and ProteoMiner™ beads depletion

mRP Fraction	Amount of Protein (µg) MARS 14	Amount of Protein (µg) ProteoMiner™ Beads
1	0	0
2	0	0
3	6	6
4	25	50
5	50	100
6	50	50
7	12	12
8	25	12
Total	168	230

3.4.1.3 SDS Gels to compare MARS 14 and ProteoMiner™ Beads

When visualised on protein gels the two sets of samples showed completely different band patterns (Figure 3.4 and Figure 3.5) highlighting the fact they had enriched different populations of proteins.

Figure 3.4 SDS gel of proteins present following depletion using MARS 14. Desalt is the protein elution collected in the total sample prior to fractionation

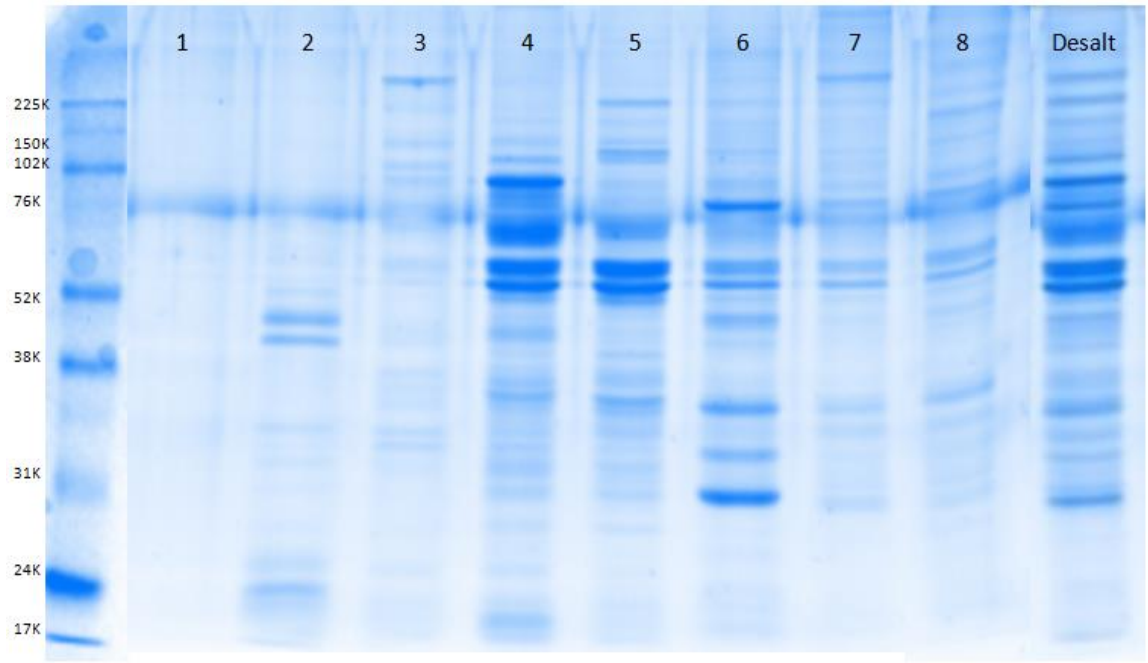
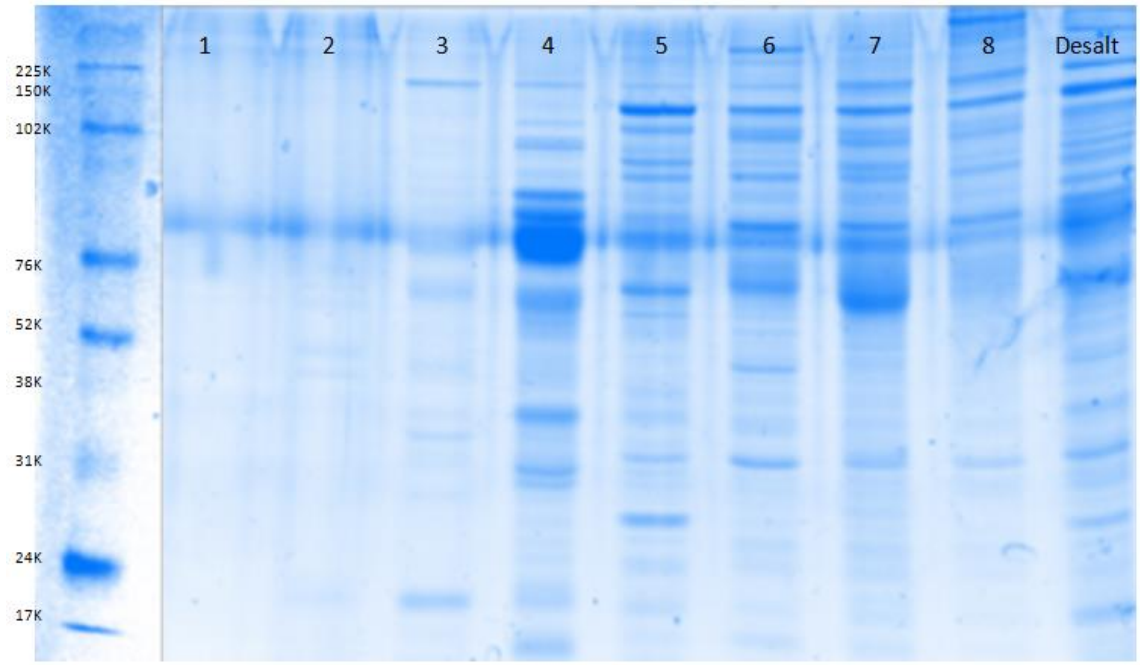


Figure 3.5 SDS gel of proteins present following depletion using ProteoMiner™ beads. Desalt is the protein elution collected in the total sample prior to fractionation



3.4.1.4 MS/MS of fractions

94 proteins were identified on MS/MS following depletion with MARS 14 and 85 proteins following depletion with ProteoMiner™ beads. Appendix A lists the proteins identified following depletion with MARS 14 and ProteoMiner™ beads.

Of the 14 proteins which should have been depleted by the MARS column, eight were still present in the ProteoMiner™ treated plasma. These were some of the most abundant proteins and therefore visualisation of lower abundance proteins was more challenging. It was noted that most of the proteins identified are found at 100µg/mL or higher in plasma. C-reactive protein was an example of a protein which circulates at a lower level (<10µg/mL in human plasma) and was observed in both methods. However, the concentration of proteins observed was remote from the concentration associated with likely biomarkers (pg/mL – ng/mL).

Overall, the MARS treated plasma showed more bands, a better reversed phase fractionation, and more protein identifications when analysed by mass spectrometry. Table 3-3 summarises the differences between MARS 14 depletion and ProteoMiner™ bead treatment.

Table 3-3 Summary of properties of MARS depletion and ProteoMiner™ beads treatment

	MARS 14	**ProteoMiner™**
Technology	Immobilised Polyclonal	Hexameric Peptide Library
Format	HPLC column	Microcentrifuge tubes
Plasma capacity	30µL to 40µL	200µL to 1mL
Workflow	Automated procedure, sequential runs on HPLC	Manual processing of many samples simultaneously
Reproducibility	Very good	Untested
Approx. cost per sample	£10	£3
Approx. yield	30µL plasma gives 150µg protein	200µL plasma gives 200µg protein
Compatibility with downstream applications	Desalting and concentration required	Ideal for protease digestion
Notes	Depletion of albumin-bound proteins	No quantification on proteins which saturate binding sites

3.4.2 iTRAQ quantitative analysis of term versus preterm samples

3.4.2.1 iTRAQ sample preparation

When the eight samples were MARS depleted and desalted on mRP column the UV chromatograms overlay almost perfectly (see Figure 3.6 and Figure 3.7). This demonstrated that there was little technical variation introduced at this stage of the workflow. The high pH reverse phase HPLC chromatogram showed an even separation of the labelled peptides over the 80 fraction collection window (see Figure 3.8).

Figure 3.6 UV chromatogram of MARS 14 flowthrough

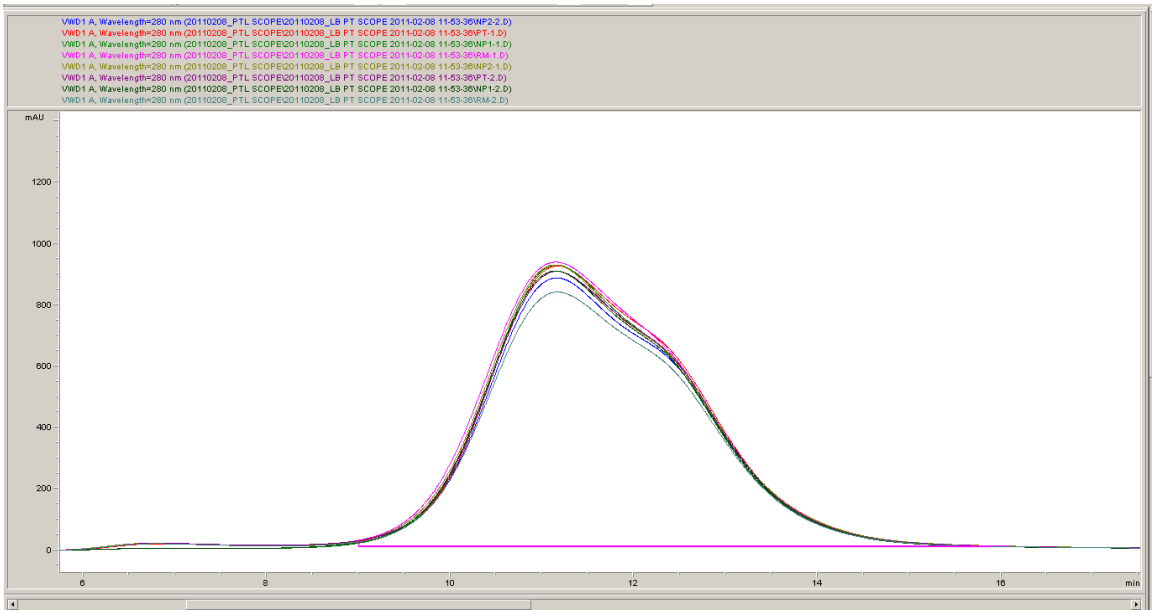


Figure 3.7 UV chromatogram of mRP eluate

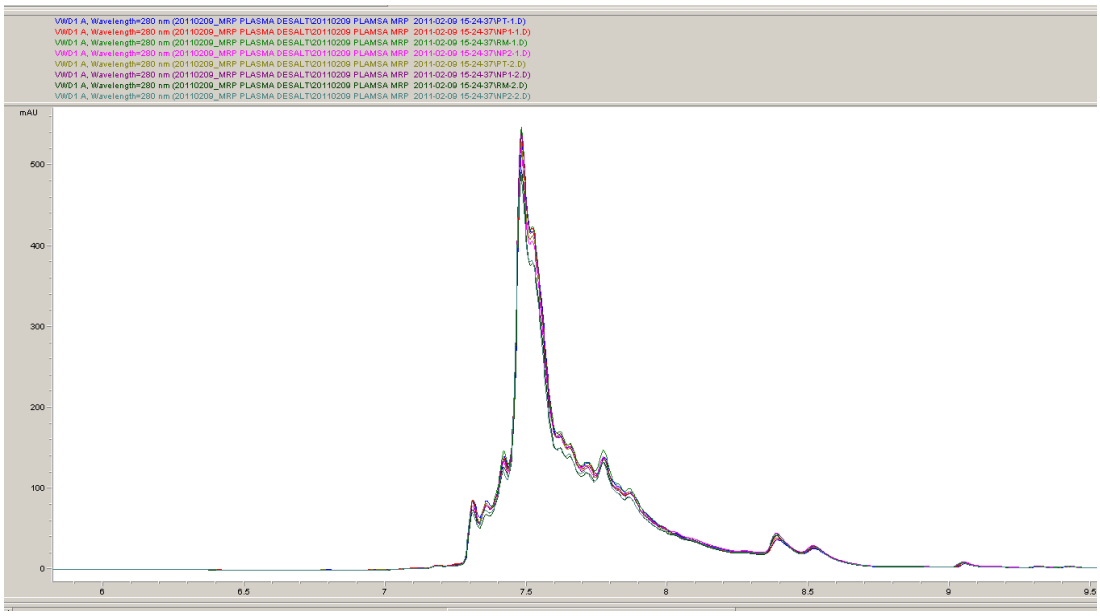
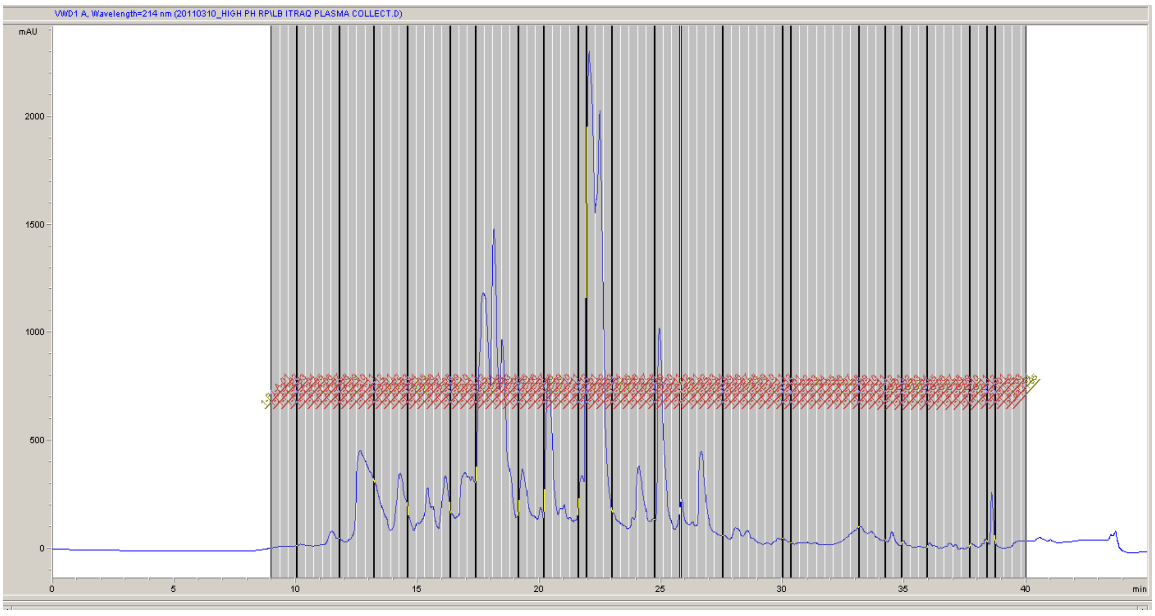


Figure 3.8 UV chromatogram of high pH Reversed Phase HPLC fractionation



3.4.2.2 Identification of proteins

Following MS/MS of the iTRAQ labeled plasma, 243 high confidence protein identifications were identified; with relative quantification data available for 117 of these (see Appendix B).

3.4.2.3 Normalisation factor

ProteinPilot data were filtered to look for proteins with good technical reproducibility and good reproducibility between two controls. Only when consistent quantification was observed was the peptide quantification data included within the analysis.

Table 3-4 shows the normalisation factors applied by ProteinPilot to the iTRAQ reporter ions. The majority of proteins in a sample would be present at an equal abundance and therefore it should equal 1. It can be seen that labels 115 and 119 had unacceptable normalization factors so were therefore removed from subsequent analysis. This still allowed for technical reproducibility to be assessed but limited the number of cases which could be analysed.

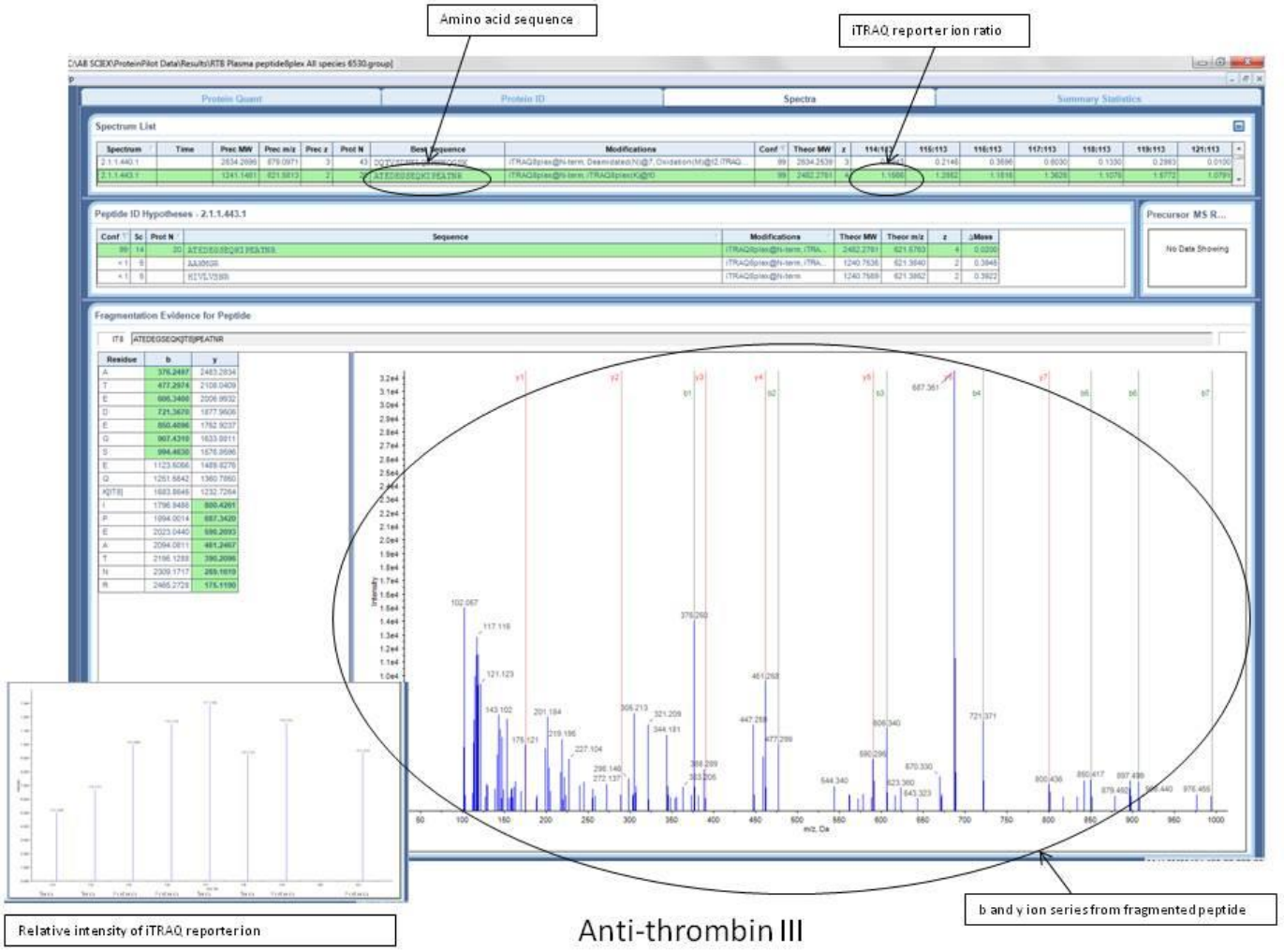
Table 3-4 ProteinPilot normalisation factors for iTRAQ reporter ions

Ratio	Comparative groups	Normalisation factor
114:113	Term:Term	1.2379
115:113	Preterm with PRROM:Term	**0.3322**
116:113	Preterm with PPROM:Term	1.1123
117:113	Term:Term	1.4383
118:113	Term:Term	1.1251
119:113	Preterm no PPROM:Term	**0.1790**
121:113	Preterm no PPROM:Term	1.0432

3.4.2.4 Quantitative data

An example of a peptide assigned to anti-thrombin III and its iTRAQ quantification data is shown in Figure 3.9. The relative intensities of the reporter ions can be used to demonstrate the relative quantification of each peptide identified in relation to each sample pool. The statistical p value calculated by ProteinPilot tested the null hypothesis that the protein was unchanged between the sample and the reference group (113). The data were interrogated to identify fold changes <0.8 and >1.2 in proteins between control and case groups, to identify biological difference.

Figure 3.9 Example of a peptide assigned to anti-thrombin III and its iTRAQ quantification data



3.4.2.5 Technical replicates

An example of a good technical replicate was seen when comparing two pools from the same group. For example, 113 and 114 were proteins both from the same term pregnancy control group. It can be seen in Table 3-5 that there was no significant difference in the ratio of the protein apolipoprotein C-II in the two samples labeled with 113 and 114. Another example is seen with complement C4-B and 117 and 118 as the ratios to 113 were very similar.

Table 3-5 Example of good technical replicate

Comparison	**Term:Term**	**Preterm with PPROM: Term**	**Term:Term**	**Term:Term**	**Preterm no PPROM:Term**
Name	**114:113** ***(p value)***	**116:113** ***(p value)***	**117:113** ***(p value)***	**118:113** ***(p value)***	**121:113** ***(p value)***
Apolipoprotein C-II	1.00 *(1.00)*	1.02 *(0.78)*	1.00 *(1.00)*	1.03 *(0.75)*	1.27 *(0.12)*
Complement C4-B	0.93 *(0.27)*	1.04 *(0.37)*	0.94 *(0.26)*	0.95 *(0.13)*	1.04 *(0.72)*

3.4.2.6 Proteins of interest

3.4.2.6.1 Term pregnancy versus Preterm birth with PPROM

When comparing the iTRAQ data from women who delivered at term compared to women who experienced preterm birth with PPROM, there were five proteins with significant differences between the groups (Table 3-6).

Table 3-6 Proteins of interest for term pregnancy versus preterm birth with PPROM

Comparison	**Term: Term**	**Preterm with PPROM: Term**	**Term: Term**	**Term: Term**	**Preterm no PPROM: Term**
iTRAQ labels	**114:113** ***(p value)***	**116:113** ***(p value)***	**117:113** ***(p value)***	**118:113** ***(p value)***	**121:113** ***(p value)***
Hemopexin	0.98 *(0.77)*	0.92 ***(0.05)***	0.98 *(0.81)*	0.95 *(0.18)*	0.99 *(0.79)*
Beta-2-glycoprotein 1	0.90 *(0.16)*	0.91 ***(0.03)***	1.01 *(0.89)*	1.02 *(0.66)*	1.12 *(0.15)*
Coagulation factor XII	0.99 *(0.91)*	0.87 ***(<0.01)***	0.94 *(0.37)*	0.94 *(0.17)*	1.07 *(0.80)*
Alpha-1-antichymotrypsin	0.80 *(0.13)*	0.81 ***(0.03)***	0.93 *(0.37)*	0.89 *(0.21)*	0.87 *(0.58)*
Complement C2	0.86 *(0.20)*	0.88 ***(0.04)***	0.91 *(0.26)*	0.91 *(0.14)*	0.93 *(0.44)*

3.4.2.6.2 Term pregnancy versus preterm birth without PPROM

The comparison between iTRAQ data from term pregnancy and preterm birth without PPROM identified nine candidate proteins (Table 3-7).

Table 3-7 Proteins of interest for term pregnancy versus preterm birth without PPROM

Comparison	**Term: Term**	**Preterm with PPROM: Term**	**Term:Term**	**Term:Term**	**Preterm no PPROM:Term**
Name	**114:113** ***(p value)***	**116:113** ***(p value)***	**117:113** ***(p value)***	**118:113** ***(p value)***	**121:113** ***(p value)***
Complement factor B	0.90 (*0.10)*	1.01 *(0.89)*	1.00 *(0.96)*	1.00 *(0.94)*	1.17 ***(0.02)***
Alpha-1B-glycoprotein	1.06 *(0.55)*	0.95 *(0.33)*	1.06 *(0.52)*	0.97 *(0.49)*	1.16 ***(0.03)***
Fibrinogen beta chain	0.91 *(0.19)*	0.94 *(0.33)*	0.97 *(0.75)*	0.99 *(0.84)*	1.26 ***(<0.01)***
Complement component C6	0.98 *(0.77)*	1.00 *(0.96)*	1.11 *(0.13)*	1.00 *(0.92)*	1.15 ***(0.04)***
Protein AMBP	0.99 *(0.93)*	0.99 *(0.85)*	1.01 *(0.93)*	0.98 *(0.65)*	1.12 ***(0.05)***
C4b-binding protein alpha chain	1.05 *(0.62)*	1.04 *(0.54)*	1.09 *(0.35)*	1.03 *(0.60)*	1.19 ***(0.04)***
Apolipoprotein C-III	0.82 *(0.31)*	0.92 *(0.22)*	0.87 *(0.55)*	1.04 *(0.57)*	1.27 ***(0.01)***
Fibrinogen gamma chain	0.93 *(0.55)*	0.93 *(0.24)*	1.07 *(0.56)*	1.00 *(0.95)*	1.17 ***(0.03)***
Disintegrin and metalloproteinase domain-containing protein 12	0.87 *(0.48)*	1.13 *(0.25)*	1.02 *(0.91)*	1.11 *(0.48)*	1.43 ***(0.05)***

3.4.2.6.3 Term pregnancy versus spontaneous preterm birth

Comparison of iTRAQ data for term pregnancy vs all preterm, (with and without PPROM) identified, one protein, Fetuin-B, as being different between groups (Table 3-8).

Table 3-8 Protein of interest for term pregnancy versus spontaneous preterm birth

Comparison	**Term:Term**	**Preterm with PPROM:Term**	**Term:Term**	**Term:Term**	**Preterm no PPROM:Term**
Name	**114:113** ***(p value)***	**116:113** ***(p value)***	**117:113** ***(p value)***	**118:113** ***(p value)***	**121:113** ***(p value)***
Fetuin-B	0.88 *(0.31)*	1.18 ***(0.05)***	1.05 *(0.70)*	1.12 *(0.07)*	1.12 ***(<0.01)***

3.4.3 Results of label-free methods

3.4.3.1 Label-free sample preparation

When the six replicate samples (see 3.3.8) were MARS depleted and desalted on the mRP column, the UV chromatograms overlayed almost perfectly. This demonstrated that minimal variation was introduced at this stage of the workflow (see Figure 3.10 and Figure 3.11). However, the large biological variation of the samples can be seen by the difference in the poor overlay of the MARS and mRP chromatograms of the term and preterm plasma samples (see Figure 3.12 and Figure 3.13). Figure 3.14 demonstrates the mRP fractionation of a preterm sample into 6 equal fractions.

Figure 3.10 MARS UV chromatograms of the replicate samples (n=6)

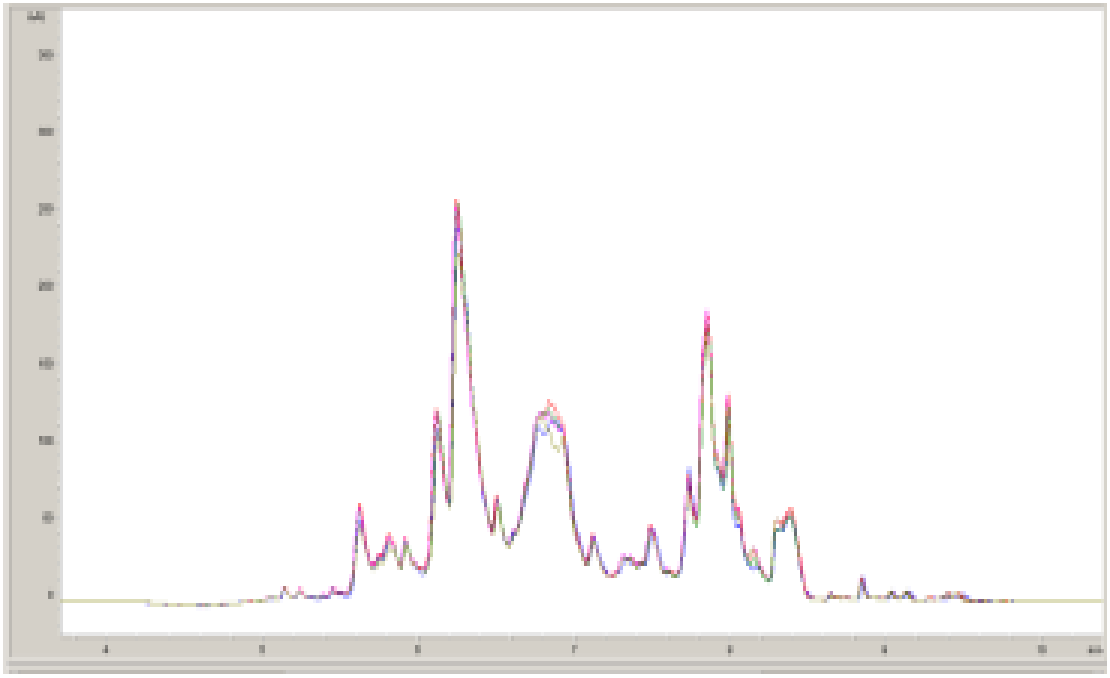


Figure 3.11 mRP UV chromatograms of the replicate samples (n=6)

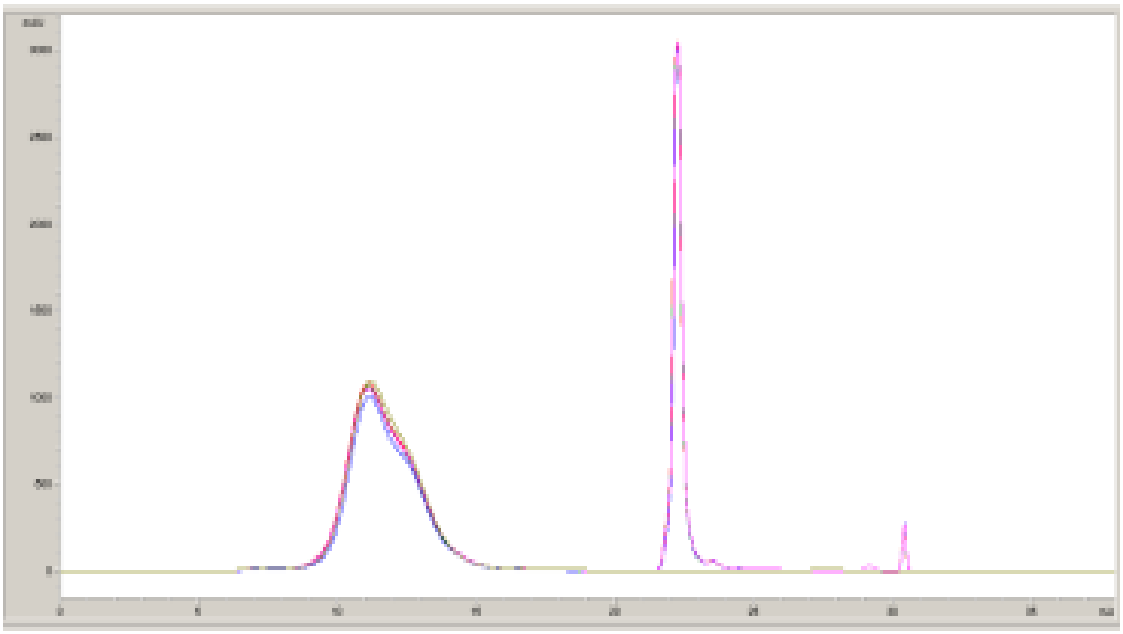


Figure 3.12 MARS UV chromatograms of 20 weeks plasma samples (n=24)

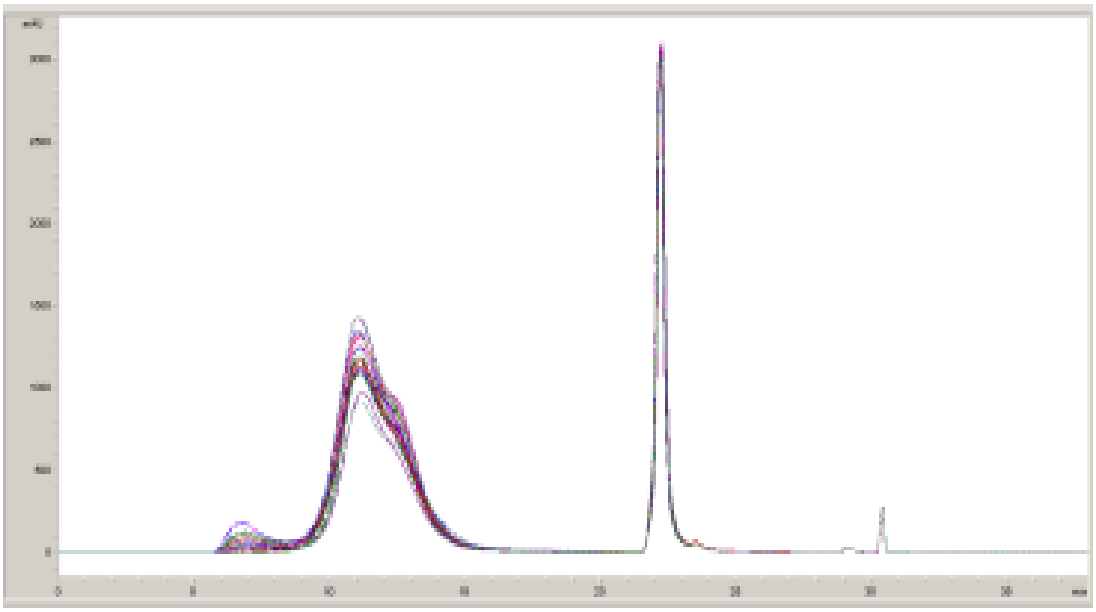


Figure 3.13 mRP UV chromatograms of 20 week plasma samples (n=24)

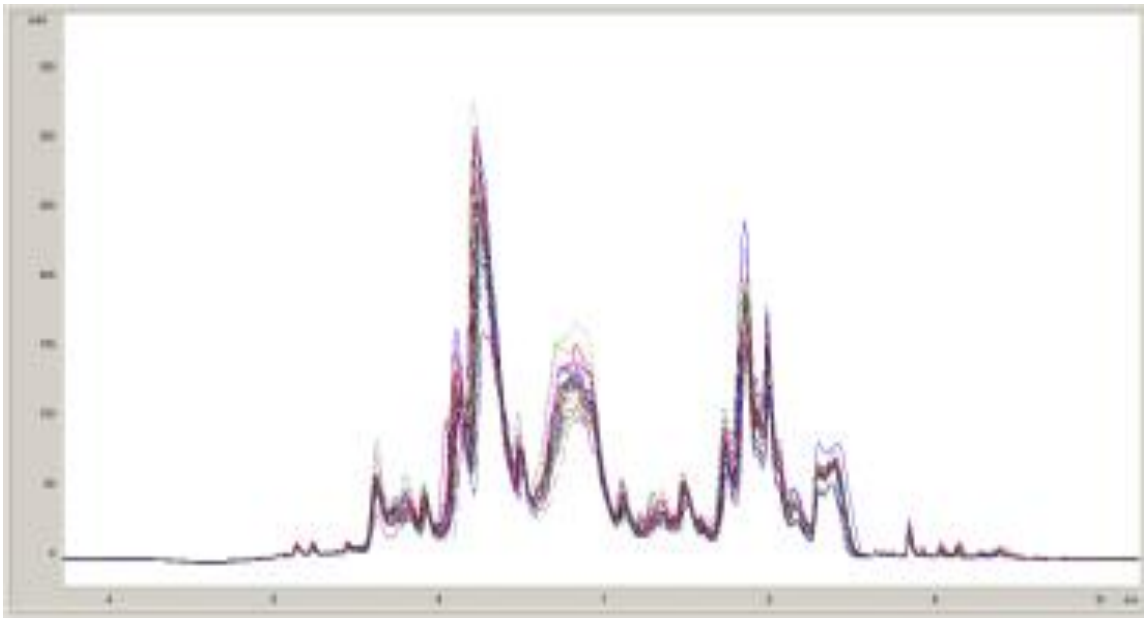
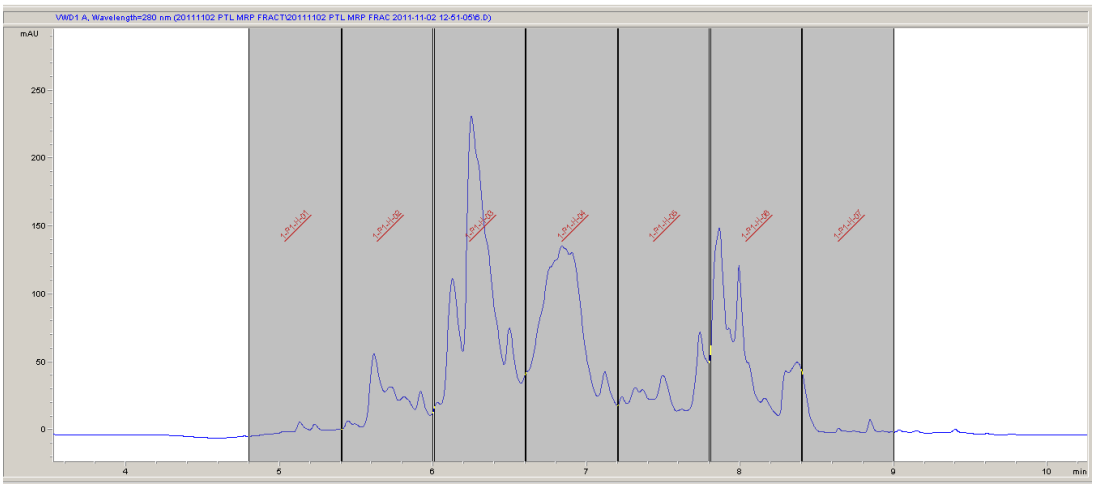


Figure 3.14 Example of mRP fractionation to demonstrate 6 even fractions



3.4.3.2 Identification of proteins

LC-MS Progenesis was used to detect features that best discriminated between the sample groups. These features were filtered then submitted as targeted MS/MS lists to identify them via database searching (Mascot). If a protein was different between groups then all of the peptides from that protein should behave the same. In the myoglobin control experiment, 100% of the peptides identified from myoglobin discriminated between the sample groups (see Table 3-9). The preterm birth versus term birth comparison demonstrates that only a small number of the peptides identified from each protein were changed and therefore these are more likely to represent background noise than actual changes in protein abundance (Table 3-10).

Table 3-9 Discriminating peptides demonstrating myoglobin detected in both samples

	Discriminating peptides	**Total peptides**
Myoglobin (horse)	3	3
Complement component C8B	1	17

Table 3-10 Identifying peptides between preterm and term birth

	Identifying peptides	**Total peptides**
Beta-2-glycoprotein 1	2	18
Complement component C6	1	27
Pregnancy-specific glycoprotein-7	1	3
Ceruloplasmin	1	59
Complement component C4	3	86
Inter-alpha-trypsin inhibitor heavy chain 2	1	34
Angiotensinogen	1	16
Complement component C5	1	58

3.5 Discussion

The ultimate goal of this plasma biomarker discovery was to accurately quantify thousands of proteins within a small number of well-characterised clinical samples. Given the high dynamic range and complexity of the plasma proteome, the current proteomics technology forces compromises between proteome penetration and sample number. In the proteomics community, there is no consensus on how to achieve this balance. Immunodepletion of highly abundant plasma proteins is part of most plasma biomarker workflows. Whilst the performance of immunodepletion columns has been shown to be reproducible (311), their limited loading capacity and the complexity of the depleted plasma leaves significant scope for the development of alternative methods to enrich for low abundance proteins.

ProteoMiner™ (PM) beads have, to date, mostly been used for 2D gel applications. Sihlbom *et al* (312) demonstrated that the beads are an efficient and simple prefractionation method to increase the possibility of penetrating low abundance proteins not accessible before depletion. The results from the analyses presented here, however, indicate that the ProteoMiner™ beads do not represent an improved sample preparation workflow compared to other immunodepletion methods. Advantages of the beads include their high loading capacity, single use (which avoids the potential for contamination between samples), efficient parallel processing of samples, and compatibility with all major downstream protein analysis techniques.

Disadvantages of the PM beads include the fact that they have uncharacterised reproducibility, and their mode of action makes it impossible to predict what proteins will bind to the beads and with what affinity. Their mechanism of action means that any proteins that saturate the beads (high abundance or high affinity ones) will elute at equal levels across all samples so any biological differences could be missed (false negatives). As some proteins will not bind to the beads, these proteins will also be lost potentially leading to additional false negative findings. The current study has demonstrated that plasma treated with the PM beads still contained many of the most abundant proteins that the MARS-14 column had been able to remove, especially albumin. This makes it less likely that lower abundance proteins of interest will be identified in subsequent MS analyses.

MARS 14 is widely used and known to be reproducible (296). There are however two drawbacks which need to be acknowledged. The first is the removal of albumin which will lead to the co-depletion of proteins and bioactive factors bound to this carrier. The second is the limited capacity of the column which means that only small volumes of plasma can be processed. The lower cost and higher capacity of the PM beads are however outweighed by their inferior ability to remove high abundance proteins and therefore make lower abundance plasma proteins available for MS analysis.

The iTRAQ data has demonstrated that a modest number of proteins can be identified and quantified using this workflow. From the normalisation factors, it can be ascertained that the iTRAQ labelling was not successful in all pooled samples. This was demonstrated by the MARS and mRP chromatograms which showed almost identical amounts of protein present in the samples prior to labelling. iTRAQ labelling failure appears to be a sporadic issue noted by many users. Unfortunately, by removing two samples there were no valuable technical duplicates for the two preterm groups, which limit the significance of any observed differences.

Using pooled samples is a common feature of proteomic experiments and has also been reported in microarray/genomics experiments (313). It would be difficult to perform a 12 vs 12 individual sample analysis using the iTRAQ technique due to high instrument time, expense and issue with quantifying across separate experiments. So given the choice, we are more likely to demonstrate change with pooled samples of 12, as opposed to 2 or 3 individual samples from each group. It is also likely that a pool of 12 will be big enough for one outlier sample to not skew the pool.

As with all biomarker studies, the false positive rate can be very high, therefore conclusions cannot be drawn too quickly. The limitations of this study are that it is a single experiment and so would need to be repeated two or three more times to combine and compare the data. It can be seen however, that although there are 15 statistically significant protein differences, the small fold changes are such that demonstrating a clinical significance would be unlikely. Table 3-11 shows the names of the proteins identified as being different between the three comparison groups. The proteins found are generally related to systemic

inflammation and it is likely that these will be less helpful due to being be non-specific.

Table 3-11 Proteins identified in each of the three comparison groups

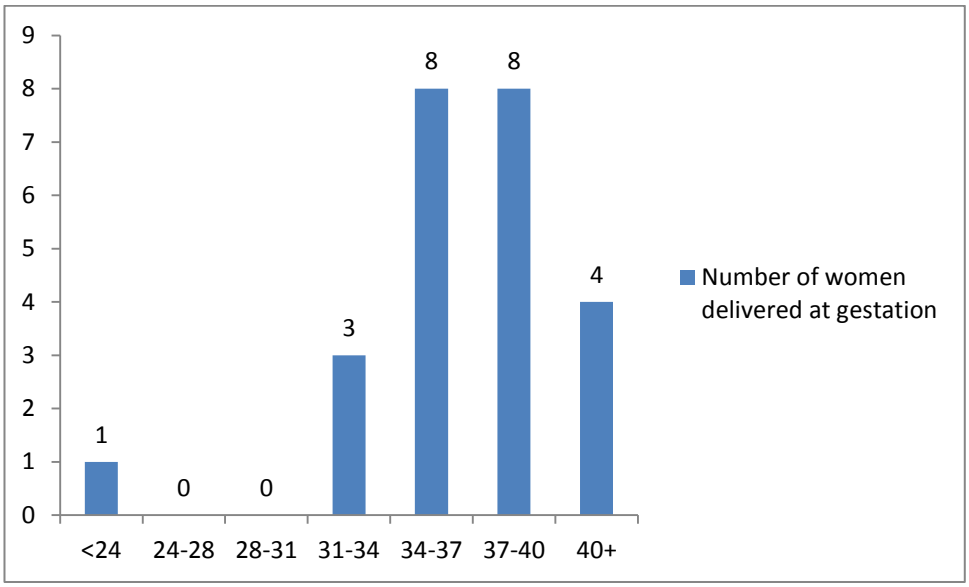
PTB-PPROM vs Term	PTB-IM vs Term	Preterm vs Term
Hemopexin	Complement factor B	Fetuin-B
Beta-2-glycoprotein 1	Alpha-1B-glycoprotein	
Coagulation factor XII	Fibrinogen beta chain	
Alpha-1-antichymotrypsin	Complement component C6	
Complement C2	Protein AMBP	
	C4b-binding protein alpha chain	
	Apolipoprotein C-III	
	Fibrinogen gamma chain	
	Disintegrin and metalloproteinase domain containing protein 12	

Although technically a good study, the label-free methods failed to detect any proteins which could show a difference between the preterm birth and term birth women. The ability to detect a 1.5x fold change in an exogenous protein at 1µg/ml was however, convincingly demonstrated. It is therefore unlikely that there are any endogenous proteins that discriminate term and preterm birth at a concentration above ~1µg/ml in 20 week plasma samples. This fits with previous studies which fail to identify any significant biochemical biomarkers for prediction of preterm birth and is in contrast to pre-eclampsia where dozens of candidate markers have been identified e.g. PlGF, PP13 (282, 301).

One of the potential issues that this study highlights is that the preterm birth and term birth samples used were actually quite similar. When the gestation of the samples is plotted (Figure 3.15) it can be seen that there may be only a two-week difference in gestation between cases and controls. Therefore a large biochemical difference would be very difficult/impossible to identify at 20 weeks.

This difference may be clinically very relevant however, and may be the difference between needing neonatal care or not.

Figure 3.15 Delivery gestation of women from Proteomics studies



Given access to a larger number of samples of the early preterm birth cohort I may have been able to biochemically discriminate them from the controls. The fact that over half of the samples were late preterm birth may have made the sample groups too similar. It is not worth doing any further proteomic analysis with this cohort as there is probably too much overlap.

With pre-eclampsia, adding biochemical markers adds strongly to the prediction algorithm e.g. PlGF; however no biochemical marker to add to a preterm birth clinical algorithm has been identified. Another way of assessing biochemical differences is to look at a known number of low abundance proteins, such as, cytokines, rather that looking at the global proteome, and the next chapter of my thesis aims to do this.

4 Maternal Plasma Cytokines as Predictors of Spontaneous Preterm Birth

4.1 Introduction

In order to predict and prevent preterm birth, and minimise the adverse neonatal outcomes associated with prematurity, it is essential to understand the causes of preterm labour. The biomolecular mechanisms leading to labour, both preterm and term, are not well understood. Although PTL and labour at term share a common terminal pathway, comprising uterine contractions, cervical dilation ± rupture of membranes, research shows that PTL is induced by significantly different factors than labour at term (23).

Labour in humans results from a complex association of fetal and maternal factors, although the exact mechanism still remains uncertain (23). The human placenta plays a major role in pregnancy and the cascade of labour processes are activated via placental mediators exerting endocrine, paracrine, and autocrine actions (314). Whilst some of the mediators are involved in myometrial activation and preparation, others are involved in myometrial stimulation leading to delivery (315).

In the early stages of pregnancy, myometrial substances, like progesterone, nitric oxide, and relaxin, contribute to the retention of the pregnancy (314). At late stages of gestation, fetal hypothalamus maturation signals act on the placenta causing the production of hormones, including CRH, in an endocrine manner; the signals then enhance the production of more hormones, such as oestrogens and neuropeptides, that contribute to cervical ripening and uterine contractility (316). These molecules act directly on the myometrium through specific receptors, while cytokines and multiple growth factors are also produced, inducing prostaglandin production which additionally contributes to labour (314).

In situations leading to preterm labour, as in maternal stress, overdistension, haemorrhage and fetal infection, it is thought that cytokines trigger placental signalling sooner, thus leading to preterm birth (314). Infection/inflammation

predominates as a cause of early preterm birth, whereas maternal or fetal HPA axis activation is more commonly associated with late preterm birth after 32 weeks gestation (18). Goldenberg *et al*. reported that up to 80% of women who deliver before 30 weeks of gestation have evidence of bacterial infection of the amniotic fluid and/or membranes, compared with only 30% of those who deliver after 37 weeks gestation (26).

The mechanisms by which intrauterine infections lead to preterm labour are related to activation of the innate immune system (23). The cervix, fetal membranes and mucus plug serve to provide a mechanical and chemical barrier to prevent ascending infection from accessing the genital tract. However, when these barriers fail, microorganisms are recognised by pattern-recognition receptors, for example toll-like receptors, which in turn elicit the release of proinflammatory chemokines and cytokines—such as interleukin-8, interleukin-1β, and tumour necrosis factor-α. Microbial endotoxins and proinflamatory cytokines stimulate the production of prostaglandins, other inflammatory mediators, and matrix-degrading enzymes. Prostaglandins stimulate uterine contractility, whereas degradation of extracellular matrix in the fetal membranes leads to PPROM (23, 26, 317). An understanding of the innate immune system may enable the cascade of events in preterm labour to be arrested early on, which may improve our ability to target specific subgroups for the prevention of preterm birth.

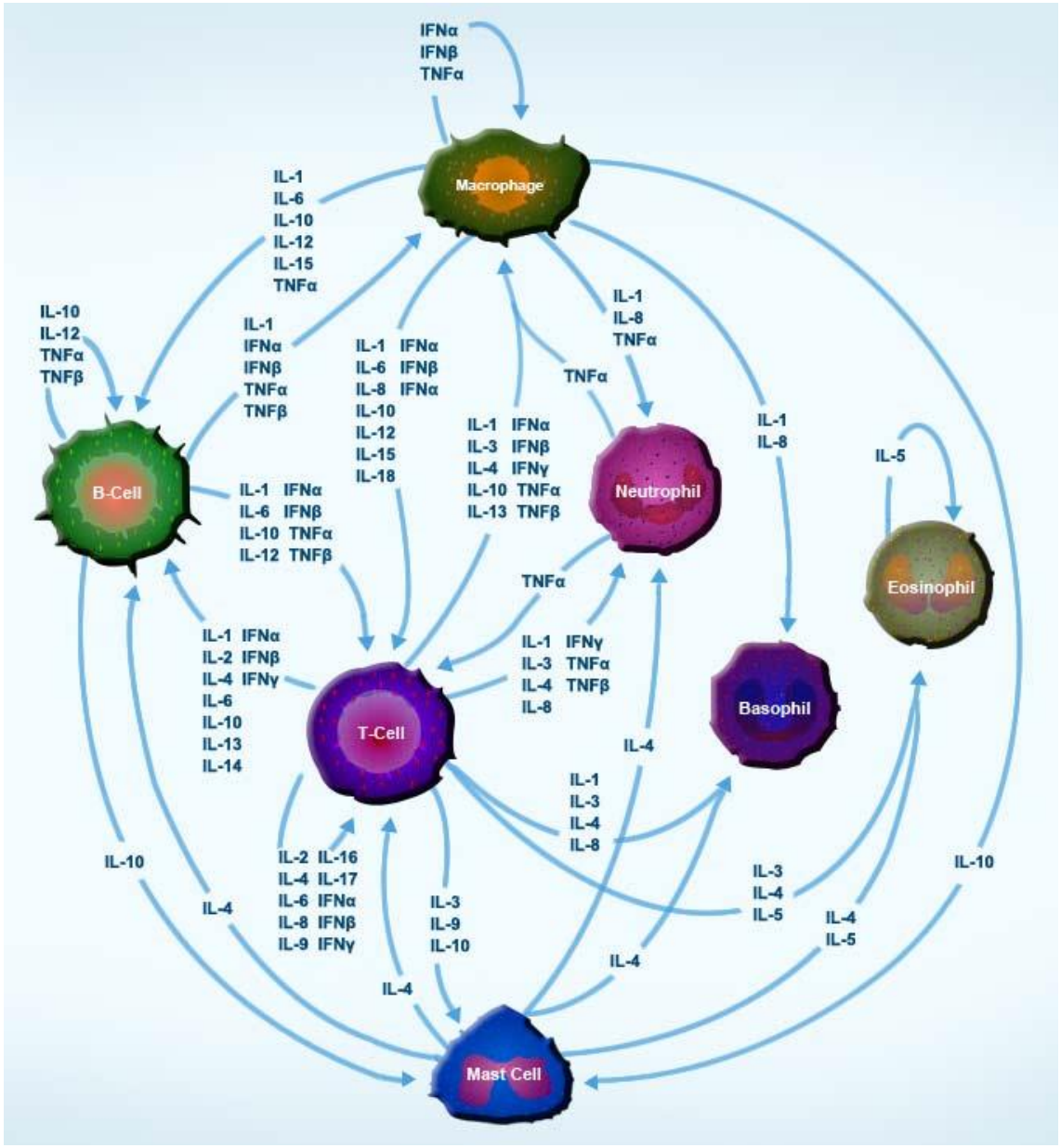
Inflammation is the mechanism by which the body responds to the presence of infective and non-infective insults. It allows for the destruction of the invading pathogen whilst preserving normal tissue integrity and function. Inflammation is mediated through the interaction of a complex network of pro-inflammatory (Th1) and anti-inflammatory (Th2) cytokines and other immunomodulators (151). Pregnancy is thought to be predominantly a Th2 dominant state allowing maternal regulation, but preventing rejection of the semi-allogenic fetus. Nearer term there is a gradual shift towards a Th1 state which culminates in labour and delivery. An early shift to Th1 activity is thought to be involved in preterm delivery and preterm prelabour rupture of membranes (318).

Cytokines are pleiotropic glycoproteins which help to regulate all biological processes, and in particular are involved in immune regulation. They generally have a molecular mass of less than 30 kDa and are structurally characterised

as belonging to one of four groups: the haematopoietin family, the interferon family, the chemokine family, and the tumour necrosis family. Cytokines can mediate their biological effects at picomolar concentrations because of their high affinities. Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action). Their pathways have been extensively investigated within maternal and fetal tissues in order to ascertain a possible link between inflammation and spontaneous preterm labour (319-327). Although most studies have examined these biomarkers after the onset of labour or rupture of membranes, several have examined their role as systemic inflammatory biomarkers, studying plasma or serum cytokines (86, 328-334) in the prediction of spontaneous preterm labour.

Figure 4.1 illustrates the complicated network of cytokine and chemokine signalling. This interdependent relationship makes isolating the roles of individual cytokines and chemokines hard, and their effects complicated to understand and predict.

Figure 4.1 Cytokine network depicting the interaction of various cell types. (Taken from (335))



4.1.1 Proinflammatory cytokines and preterm labour

There is strong evidence to support a role for cytokines in the initiation of inflammation/infection-induced preterm labour (32, 106, 336-344). The most commonly studied have been the proinflammatory cytokines IL-1β, TNF-α and IL-8. It is thought that during the course of ascending intrauterine colonisation, microorganisms may reach the decidua, where they can stimulate a local inflammatory reaction and the production of proinflammatory cytokines and inflammatory mediators (platelet-activating factor, prostaglandins, leucotrienes, reactive oxygen species, NO, amongst others) (23). If labour is not started from this inflammatory process, then microorganisms may also cross intact membranes into the amniotic cavity, where they can stimulate the production of other inflammatory mediators by resident macrophages and other host cells. Those microorganisms that have gained access to the fetus may elicit a systemic inflammatory response syndrome, characterised by increased concentrations of IL-6 (345, 346) and other cytokines (347), as well as cellular evidence of neutrophil and monocyte activation (348).

IL-1 was the first cytokine to be implicated in the onset of spontaneous preterm labour associated with infection (349). Evidence in support of the participation of IL-1 is shown in Table 4-1.

Table 4-1 Evidence to demonstrate IL-1 is associated with preterm labour

Study	Evidence
Romero *et al* (1989)(350)	IL-1 is produced by human decidua in response to bacterial products
Romero *et al* (1989)(351)	IL-1 stimulates prostaglandin production by human amnion and decidua
Romero *et al* (1989)(352)	Increased IL-1 concentration and bioactivity in the amniotic fluid of women with preterm labour and infection
Sadowsky *et al* (2003)(353)	IL-1 stimulates myometrial contractions
Romero *et al* (1991)(354) & Romero *et al* (1992)(355)	Administration of IL-1 to pregnant animals induced preterm labour and preterm birth which could be blocked by the administration of its natural antagonist: IL-1 receptor-antagonist (IL-1ra)

Similarly, the evidence supporting the role of tumour necrosis factor-alpha (TNF-α) in the mechanisms of preterm parturition is shown in Table 4-2.

Table 4-2 Evidence to demonstrate TNF-α is associated with preterm labour

Study	Evidence
Romero *et al* (1998) (28)	TNF-α stimulates prostaglandin production by the amnion, decidua, and myometrium
Casey *et al* (1989) (356) & Romero *et al* (1991) (357)	Human decidua can produce TNF-α in response to bacterial products
Romero *et al* (1989) (358)	AF TNF-α bioactivity and immunoreactive concentrations are elevated in women in preterm labour and with intra-amniotic infection
Romero *et al* (1989) (358)	In women with PPROM and intra-amniotic infection, TNF-α concentrations are higher in the presence of labour
Chwalisz *et al* (1994) (359)	TNF-α application on the cervix induces changes that resemble cervical ripening
Hirsch *et al* (2006) (360)	TNF-α is involved in the mechanisms of bacterial-induced preterm parturition in animal models

4.1.2 Anti-inflammatory cytokines and preterm labour

A key cytokine involved in the maintenance of pregnancy is IL-10. Compared to first and second trimester tissues, the production of IL-10 in the placenta is significantly reduced at term prior to labour. This suggests that the reduction of IL-10 is a physiological event that favours an inflammatory state around the time of the onset of labour (361). IL-10 has been shown to be reduced in the placental tissues of pregnancies complicated by preterm labour and chorioamnionitis compared to normal controls (362) and in animal models, the administration of IL-10 has been associated with improved pregnancy outcome (363).

4.1.3 Multiplex Technology

Conventionally, enzyme-linked immunosorbent assays (ELISAs) have been the technique used to measure protein concentrations in biological samples. However, this method is time consuming and only allows for the analysis of a single cytokine using a minimum of 200µL of sample. Over the last decade, multiplex cytokine technologies have become readily available. These allow multiple analytes to be measured in a smaller volume of biological sample.

Bio-Plex Pro™ Assays are magnetic bead-based multiplex assays designed to measure multiple members of the diverse group of proteins in a minimal volume of matrix such as serum, plasma, tissue culture supernatant and other biological fluids.

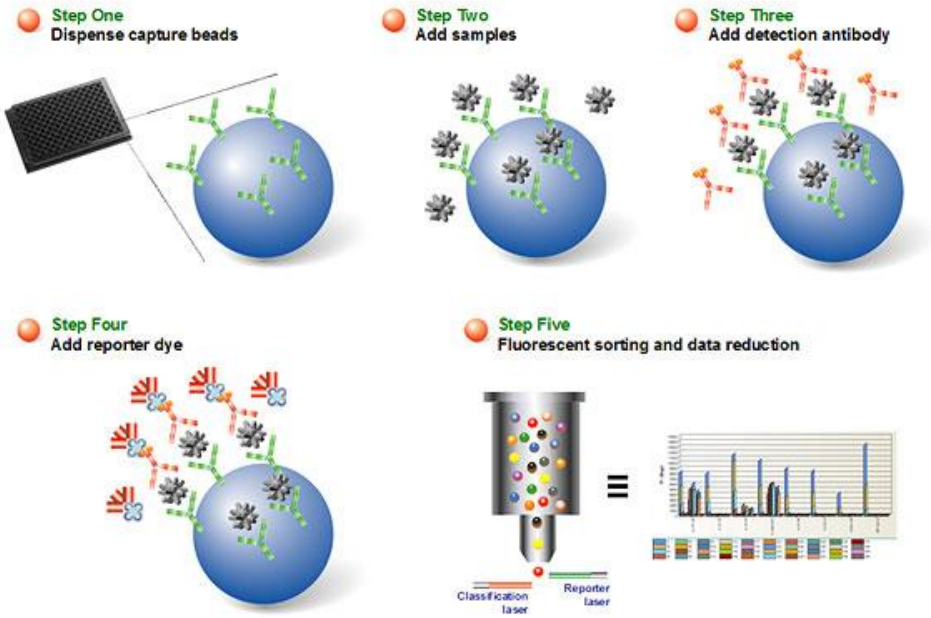
The Bio-Plex Pro™ suspension is built around three principles:

1. Fluorescently dyed microspheres (beads) each with a distinct colour code to permit discrimination of individual tests within a multiplex suspension.
2. A flow cytometer with two lasers and optics to measure the different molecules bound to the surface of the beads
3. A high-speed digital signal processor that efficiently manages the fluorescence data.

The assay principle (Figure 4.2) is to have antibodies directed against the intended cytokine coupled to the beads. The coupled beads react with the

sample containing the biomarker of interest. After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE). Phycoerythrin serves as a fluorescent indicator, or reporter.

Figure 4.2 Schematic representation of an immunoassay sandwich-based assay workflow (Taken from BioRad: Bio-Plex Assays. http://www.bio-rad.com/en-uk/applications-technologies/bio-plex-multiplex-immunoassays)



The contents of each well are then drawn up into the array flow system. A red (635nm) laser illuminates the fluorescent dyes within each bead to provide identification. At the same time, a green (532nm) laser excites PE to generate a reporter signal. A data processor manages the output and presents data as Median Fluorescence Intensity (MFI) and concentration (pg/mL).

4.2 Aim of this study

Although multiple inflammatory mediators including cytokines have been evaluated before in women with a previous preterm birth, they have not been specifically studied in a nulliparous, low risk population. The aim of this study was therefore to examine a panel of cytokines in maternal plasma taken at 20 weeks gestation to discover a marker, or combination of markers, for the prediction of spontaneous preterm birth less than 35 weeks gestation. Maternal blood is a readily accessible source, which is far easier to collect than amniotic fluid for example, and so would make an excellent non-invasive screening test.

4.3 Methods

4.3.1 Patient plasma samples

EDTA-plasma from women at 20±1 week gestation who had uncomplicated pregnancies (controls) or who subsequently delivered prematurely were obtained from the SCOPE biobank (Auckland, New Zealand) (See 3.3.1.1). Blood was collected by venepuncture into BD EDTA-Vacutainer®, placed on ice and centrifuged at 2400 x g for 10 minutes at 4°C. Plasma was stored at -80°C within 4 hours of collection. Sample collection and storage conditions were closely regulated for all samples. Plasma samples were defrosted on ice and always maintained below 4ºC during experiments.

4.3.2 Study population

Spontaneous preterm birth was defined as spontaneous preterm labour or preterm rupture of membranes at less than or equal to 34+6 weeks' gestation. Uncomplicated pregnancy was defined as a pregnancy with no antenatal obstetric or medical complications and resulting in delivery of an appropriately grown, healthy baby at 37^{+0} or greater weeks' gestation.

4.3.3 Experimental samples from SCOPE

Plasma samples were obtained for women in the southern hemisphere cohort of the SCOPE biobank who subsequently had a spontaneous preterm birth (n=40), of which n=21 were preterm birth with PPROM (PTB-PPROM), and n=19 were preterm birth with intact membranes (PTB-IM). One control group (n=40), with term delivery ≥37 weeks, was obtained; matching for centre only.

4.3.4 Multiplex analysis

For this study, cytokine analysis was performed using Bio-Plex Pro™ Assays for cytokine, chemokine and growth factors (BioRad Laboratories, Hemel Hempstead, UK). The samples were run according to manufacturers instructions on a Luminex-100 cytometer (Luminex Corporation, Austin, Texas), equipped with STarStation Software (Version 2.0: Applied Cytometry Systems, Dinnington, UK). The kits were available as pre-determined panels including pre-mixed beads, detection antibodies and standards enabling a considerable time saving.

Twenty-seven cytokines, chemokines and growth factors were analysed during the study. Table 4-3 shows the different assays performed.

Table 4-3 Bio-Plex™ Pro Human Cytokine Standard 27-Plex Group 1

IL-1β	IL-10	IFN-γ
IL-1ra	IL-12p70	IP-10
IL-2	IL-13	MCP-1
IL-4	IL-15	MIP-1α
IL-5	IL-17A	MIP-1β
IL-6	Eotaxin	PDGF-BB
IL-7	Basic FGF	RANTES
IL-8	G-CSF	TNF-α
IL-9	GM-CSF	VEGF

The cytokine standards were reconstituted and serially diluted. The multiplex bead working solution was prepared. Sample dilution was undertaken at 1:2. The 96-well plate was pre-soaked with the assay buffer and then removed by vacuum filtration. This was repeated using the multiplex bead working solution and subsequently the assay buffer. The plasma sample (50μL) was transferred into each well, covered with foil and incubated at room temperature with shaking at 300 RPM for 30 minutes. At the end of the first incubation, the buffer was removed by vacuum filtration and each well underwent three washes with 100μL assay buffer ensuring vacuum filtration to remove buffer after each wash.

Detection antibody solution (25µL) was added to each well, covered and incubated for 30 minutes at room temperature, with shaking at 300 RPM. The plate was washed three times with 100µL assay buffer again with vacuum filtration after each wash. Streptavidin-PE (50µL) was added to each well, covered and incubated for 10 minutes at room temperature, with shaking at 300 RPM. Buffer was removed by vacuum filtration and the plate washed 3 times with assay buffer, with vacuum filtration each time. The beads were resuspended in 125µL assay buffer and incubated at 110 RPM for 30 seconds. The plate was then read on the Bio-Plex Pro ^{TM} system.

Concentrations in ng/mL were calculated from the standard curves using 4PL logistic regression with Bio-Plex Pro^{TM} software.

4.3.5 Data analysis

Data analysis was performed using GraphPad Prism Version 6.04, USA. Mann-Whitney U tests were performed as the Shapiro-Wilk test demonstrated a non-normal distribution of data. The differences were tested to a significance of $P<0.05$.

Another method of analysing the cytokine data is Bayesian network formalism. This involves a statistical procedure that can characterise a network of variables (in this case cytokines) and represent their interactions as a directed acyclic graph (DAG). The connections between the nodes (variables) are known as ‘edges', and imply causal directionality, i.e. the status (relative concentration, in this case) of a downstream cytokine is dictated by that of its immediate upstream ‘parent(s)' These models are very useful in exploring the complex nature of cytokine networks, given that they can also capture synergistic and antagonistic interactions at nodes having more than one parent. Computational machine-learning-based methods undertake the inference process. In order to establish a prior cytokine network in this target set, a seed network is learned from literature-mining algorithms. Such prior knowledge speeds up the analysis and yields more robust networks, while minimising the risk of introducing bias. In this study, all 27 cytokines were set as random variables. Their interactions in term and preterm birth were then analysed independently.

Support vector machines (SVM) are a way of classifying the data to know which cytokines are the most important when deciding whether a woman had a preterm birth or not. It is computer based and was done with a 5-fold cross validation in the search for the best possible model.

4.4 Results

Table 4-4 shows the 40 case samples with gestation of delivery and membrane status. The control samples were deliveries >37 weeks.

Table 4-4 Gestation of delivery and membrane status for samples in preterm birth group

Sample ID	Gestation of delivery (weeks)	Membranes	Sample ID	Gestation of delivery (weeks)	Membranes
1324	34+2	Intact	1175	34+1	PPROM
1222	25+5	Intact	1350	24+3	PPROM
1218	34+1	Intact	1336	34+5	PPROM
1347	32+6	Intact	3103	34+3	PPROM
2896	22+5	Intact	3308	25+3	PPROM
3357	31+3	Intact	4019	33+3	PPROM
2959	25+2	Intact	3834	26+6	PPROM
3038	24+3	Intact	4689	34+3	PPROM
3948	33+5	Intact	323	34	PPROM
11	32+1	Intact	885	34+4	PPROM
269	32+3	Intact	1177	32+1	PPROM
869	23	Intact	1196	33+5	PPROM
814	31+3	Intact	1455	26+5	PPROM
1373	34+5	Intact	1573	31+6	PPROM
3342	28+1	Intact	2067	34	PPROM
2923	32+1	Intact	2558	32+5	PPROM
1294	32+5	Intact	3234	33+2	PPROM
3567	28+4	Intact	3003	32+3	PPROM
1986	34+1	Intact	2630	33	PPROM
			2910	33+5	PPROM
			3473	33+3	PPROM

Table 4-5 compares the baseline characteristics of the 40 cases and 40 controls in this cohort.

Table 4-5 Baseline characteristics of cases and controls

	Control ≥37 weeks (n=40)	**Spontaneous PTB ≤34+6 (n=40)**	**PTB-IM ≤34+6 (n=19)**	**PTB-PPROM ≤34+6 (n=21)**
Maternal Age				
<20	10% (4)	10% (4)	15.8% (3)	4.8% (1)
20-34	65% (26)	75% (30)	73.7% (14)	76.2% (16)
≥35	25% (10)	15% (6)	10.5% (2)	19.0% (4)
Ethnicity				
Caucasian	87.5% (35)	92.5% (37)	89.5% (17)	95.2% (20)
Others	12.5% (5)	7.5% (3)	10.5% (2)	4.8% (1)
Marital Status				
Single	12.5% (5)	7.5% (3)	15.8% (3)	0
Married	47.5% (18)	57.5% (23)	42.1% (8)	71.4% (15)
Defacto	42.5% (17)	35% (14)	42.1% (8)	28.6% (6)
Years of schooling				
<12	30% (12)	25% (10)	26.3% (5)	23.8% (5)
12,13	65% (26)	70% (28)	73.7% (14)	66.7% (14)
>13	5% (2)	5% (2)	0	9.5% (2)
Number of previous miscarriages				
0	85% (34)	82.5% (33)	78.9% (15)	85.7% (18)
1	10% (4)	12.5% (5)	15.8% (3)	9.5% (2)
≥2	5% (2)	5% (2)	5.3% (1)	4.7% (1)
Any vaginal bleeding < 15wks				
No	85% (34)	70% (28)	63.2% (12)	76.2% (16)
Yes	15% (6)	30% (12)	36.8% (7)	23.4% (5)
Smoking status at 15 weeks				
Never smoked	75% (30)	67.5% (27)	57.9% (11)	76.2% (16)
Smoked pre-pregnancy, quit before pregnancy	7.5% (3)	5% (2)	5.3% (1)	4.7% (1)
Smoked in pregnancy, quit before 15 weeks	5% (2)	7.5% (3)	5.3% (1)	9.5% (2)
Smoker	12.5% (5)	20% (8)	20.7% (6)	9.5% (2)
BMI at booking(kg/m^2)				
<18.5	0	0	0	0
18.5 to<25	30% (12)	55% (22)	47.4% (9)	61.9% (13)

25 to <30	27.5% (11)	32.5% (13)	36.8% (7)	28.6% (6)
≥30	15% (6)	12.5% (5)	15.8% (3)	9.5% (2)
SGA <10th centile for customised birthweight				
No	100% (40)	85% (34)	84.2% (16)	85.7% (18)
Yes	0	15% (6)	15.8% (3)	14.3% (3)
Placental abruption				
No	100% (40)	95% (38)	94.7% (18)	95.2% (20)
Yes	0	5% (2)	5.3% (1)	4.8% (1)
Gestational age at delivery				
<24 weeks	0	5% (2)	10.5% (2)	0
24-29+6	0	22.5% (9)	26.3% (5)	19.1% (4)
30-34+6	0	72.5% (29)	63.2% (12)	80.9% (17)
35-36+6	0	0	0	0
>37 weeks	100% (40)	0	0	0

In total 27 cytokines were measured. Cytokines were compared between term or preterm birth, term or preterm birth with intact membranes (PTB-IM) and term or preterm birth with PPROM (PTB-PPROM). Not all samples recorded cytokine levels that were detectable therefore these were recorded as 0ng/mL. Table 4-6 compares the median and range of the 27 cytokines among the term controls, total cases and case subgroups.

Table 4-6 Associations between maternal plasma cytokine median (range) concentration (ng/mL) and spontaneous preterm birth

	Term controls (n=40)	**Spontaneous PTB ≤34+6 (n=40)**	**PTB-IM ≤34+6 (n=19)**	**PTB-PPROM ≤34+6 (n=21)**
IL-1b	1.57 (0.34-6.25)	1.57 (0.51-4.68)	1.61 (0.51-4.68)	1.41 (0.68-4.07)
IL-1ra	100.72 (37.45-932.64)	97.03 (13.91-11539.23)	97.03 (13.91-11539.23)	97.03 (32.65-494.71)
IL-2	1.62 (0-24.83)	1.16 (0-44.51)	1.16 (0-44.51)	1.16 (0-16.24)
IL-4	2.54 (0.63-6.91)	2.22 (1.08-7.69)	**2.05*** (1.10-7.69)	2.29 (1.08-4.53)
IL-5	3.16 (0.86-13.68)	2.91 (1.12-10.21)	2.98 (1.12-10.21)	2.79 (1.25-7.13)
IL-6	7.68 (1.89-30.57)	7.63 (2.94-31.97)	7.63 (2.94-31.97)	7.63 (3.84-21.34)
IL-7	6.83 (2.35-26.51)	6.12 (2.53-17.6)	**6.01*** (2.53-17.6)	6.33 (2.89-14.85)
IL-8	7.19 (1.62-24.18)	6.63 (2.25-29.07)	6.63 (2.25-29.07)	6.63 (2.62-18.28)
IL-9	5.32 (0.03-27.01)	5.35 (0.92-30.79)	4.59 (1.85-16.99)	6.00 (0.92-30.79)
IL-10	6.23 (1.72-37.85)	5.42 (2.29-34.11)	5.23 (2.29-34.11)	5.61 (2.47-17.12)
IL-12	17.9 (4.6-94.05)	16.42 (7.05-172.75)	14.45 (7.05-172.75)	20.89 (7.43-37.58)
IL-13	4.61 (1.46-33.97)	4.29 (1.74-31.99)	**4.10*** (1.74-31.99)	4.95 (2.45-12.76)
IL-15	0 (0-11.96)	0 (0-49.63)	0 (0-49.63)	0 (0-5.42)
IL-17A	17.73 (0-135.28)	16.28 (0-114.91)	16.06 (0-114.91)	21.88 (0-84.74)
Eotaxin	16.98 (5.61-60.8)	18.44 (0.14-373.24)	14.1 (0.14-373.24)	20.52 (6.3-68.13)
Basic FGF	35.92 (2.77-16.9)	33.96 (8.74-91.63)	33.03 (10.15-91.63)	36.54 (8.74-69.91)

G-CSF	50.77 (16.94-116.79)	44.63 (16.14-87.57)	**39.61*** (16.14-76.25)	54.21 (16.14-87.57)
GM-CSF	0 (0-24.56)	0 (0-18.02)	0 (0-18.02)	0.01 (0-15.66)
IFN-g	126.54 (15.08-424.31)	117.82 (36.93-504.87)	121.49 (36.93-504.87)	115.98 (48.53-301.92)
IP-10	157.64 (71.84-787.23)	168.51 (79.6-4.2.71)	162.81 (101.11-329.94)	182.32 (79.60-402.71)
MCP-1	6.99 (4.42-18.08)	7.09 (3.05-21.00)	6.63 (3.05-21.00)	7.57 (4.16-14.49)
MIP-1a	4.02 (1.42-10.34)	4.03 (1.96-8.00)	3.93 (2.11-7.62)	4.19 (1.96-8.00)
MIP-1b	11.8 (6.58-24.63)	12.36 (5.96-32.31)	13.89 (7.86-32.31)	11.78 (5.96-23.10)
PDGF-BB	170.45 (2.32-998.17)	182.20 (13.89-1387.54)	150.86 (33.73-1387.54)	187.93 (13.89-733.13)
RANTES	1841.7 (128.74-4539.5)	1986.25 (356.9-5195.62)	2164.44 (523.58-5057.17)	1771.41 (356.9-5195.62)
TNF-a	33.9 (5.62-128.38)	33.03 (11.53-371.16)	33.40 (11.53-371.16)	32.65 (11.53-105.75)
VEGF	9.01 (2.74-25.83)	6.42 (3.00-22.89)	8.16 (3.00-22.89)	9.39 (3.92-22.14)

* P <0.05 vs controls

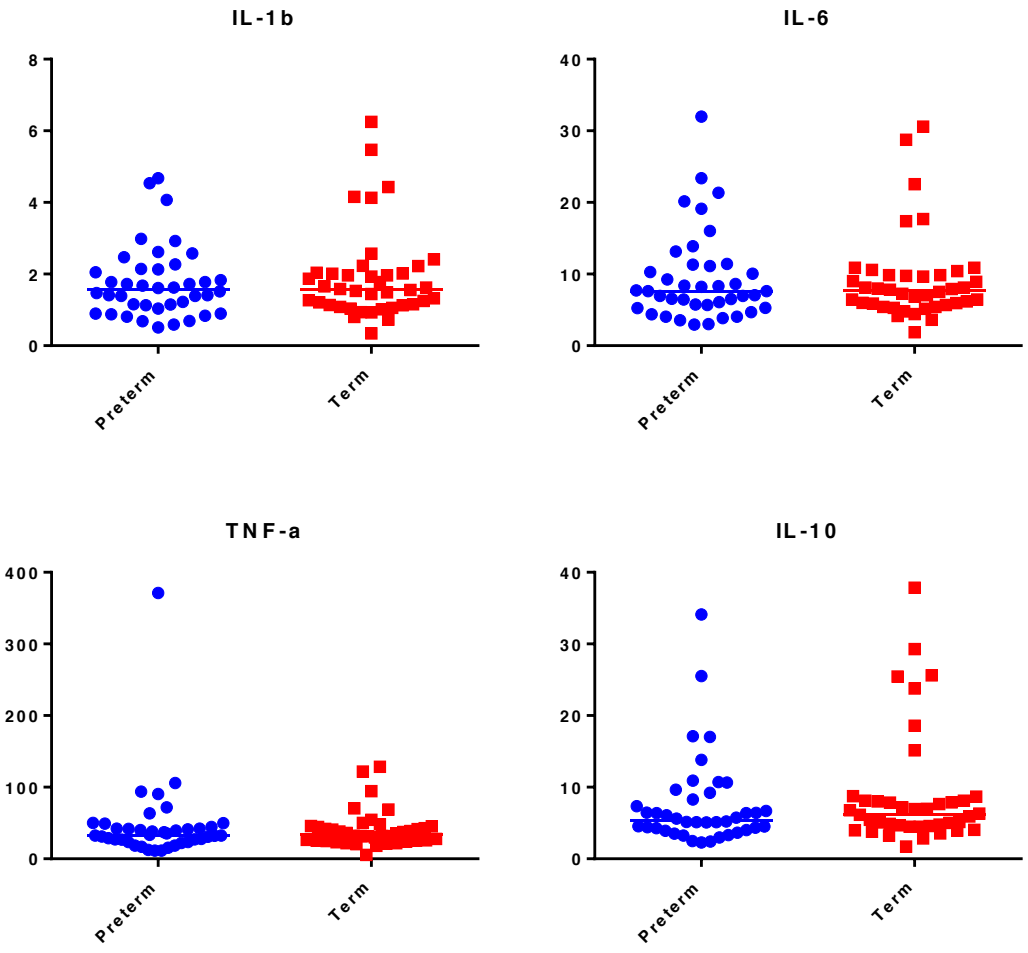
4.4.1 Term versus preterm labour

No cytokines showed a statistically significant difference between the term controls and the spontaneous preterm birth group. However, with the majority of the cytokines a downward trend was noted in the spontaneous preterm birth group as compared to the term group.

Cytokine	***P* value**	**Cytokine**	***P* value**
IL-1b	0.7250	Eotaxin	0.7430
IL-1ra	0.8764	Basic FGF	0.4507
IL-2	0.8909	G-CSF	0.2567
IL-4	0.1544	GM-CSF	0.5943
IL-5	0.2276	IFN-g	0.6237
IL-6	0.9447	IP-10	0.3819
IL-7	0.1734	MCP-1	0.7143
IL-8	0.3613	MIP-1a	0.5448
IL-9	0.5134	MIP-1b	0.3588
IL-10	0.4032	PDGF-BB	0.5512
IL-12	0.3463	RANTES	0.2629
IL-13	0.3293	TNF-a	0.8764
IL-15	0.7561	VEGF	0.4251
IL-17A	0.7904		

An example of the major pro- (IL-1b, IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines are shown below (Figure 4.3).

Figure 4.3 Charts demonstrate concentration (ng/mL) of cytokine (a) IL-1b (b) IL-6 (c) TNF-α and (d) IL-10 in each preterm and term sample



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4.4.2 Term versus preterm birth with preterm prelabour rupture of membranes

As no cytokines were significant between the term and preterm groups, a further comparison was done between those with preterm prelabour rupture of membranes versus women who delivered >37 weeks. No statistically significant difference was found between the two groups.

Cytokine	P value	Cytokine	P value
IL-1b	0.6495	Eotaxin	0.3655
IL-1ra	0.5792	Basic FGF	0.4140
IL-2	0.5852	G-CSF	0.7699
IL-4	0.8470	GM-CSF	0.1582
IL-5	0.6316	IFN-g	0.6316
IL-6	0.3998	IP-10	0.2797
IL-7	0.5877	MCP-1	0.2274
IL-8	0.5049	MIP-1a	0.5708
IL-9	0.1996	MIP-1b	0.5708
IL-10	0.6493	PDGF-BB	0.7782
IL-12	0.4658	RANTES	0.2380
IL-13	0.4969	TNF-a	0.4582
IL-15	>0.9999	VEGF	0.7041
IL-17A	0.4808		

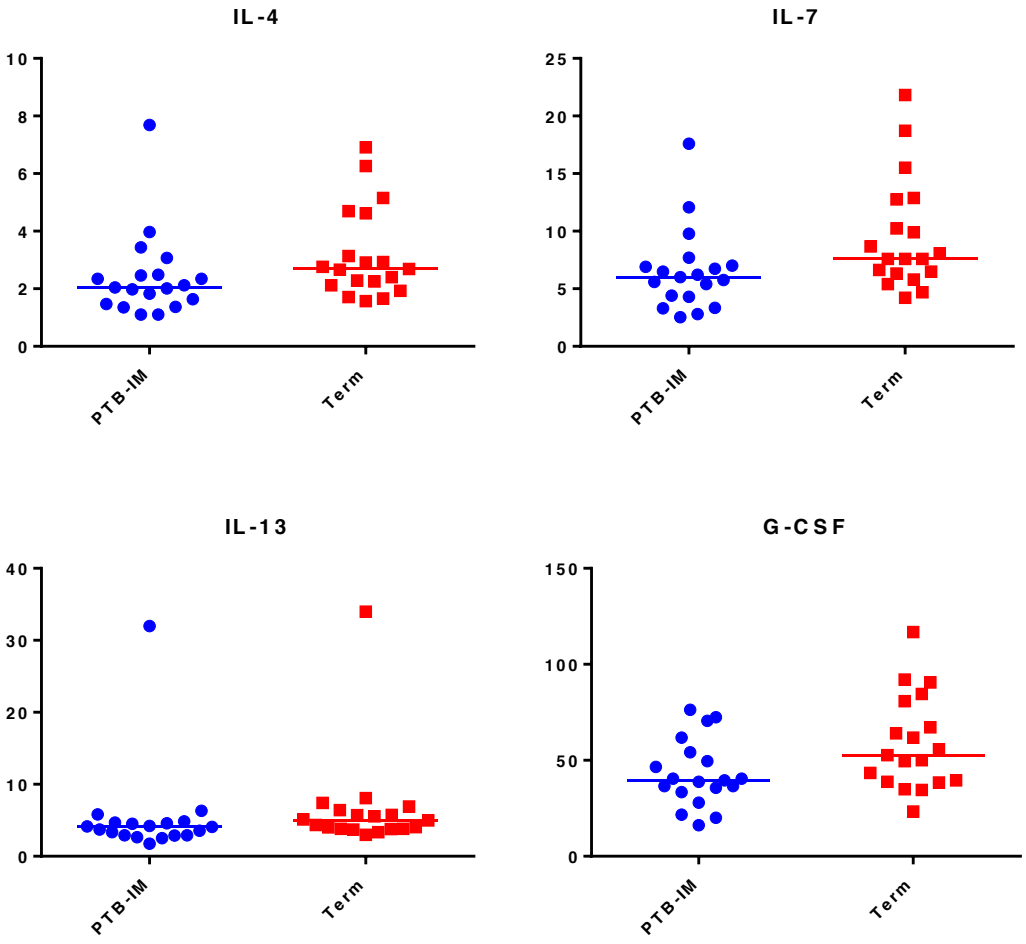
4.4.3 Term versus preterm birth with intact membranes

A third subgroup comparison was done between those with preterm birth with intact membranes and women who delivered >37 weeks. Four cytokines showed a statistically significant difference ($p<0.05$) between the term group and the preterm birth with intact membranes group IL-4, IL-7, IL-13 and G-CSF (Figure 4.4).

Cytokine	P value	Cytokine	P value
IL-1b	0.4224	Eotaxin	0.6082
IL-1ra	0.4307	Basic FGF	0.1009
IL-2	0.7224	G-CSF	**0.0422***
IL-4	**0.0407***	GM-CSF	0.5841
IL-5	0.0600	IFN-g	0.2935
IL-6	0.5782	IP-10	0.8011
IL-7	**0.0150***	MCP-1	0.5586
IL-8	0.0601	MIP-1a	0.2146
IL-9	0.8343	MIP-1b	0.5111
IL-10	0.1790	PDGF-BB	0.2093
IL-12	0.0522	RANTES	0.7012
IL-13	**0.0454***	TNF-a	0.4307
IL-15	0.8980	VEGF	0.1563
IL-17A	0.2930		

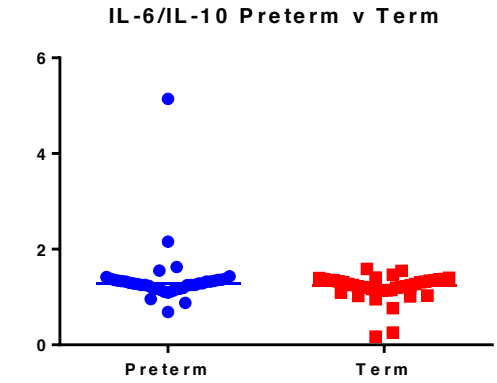
* *P* <0.05 vs controls

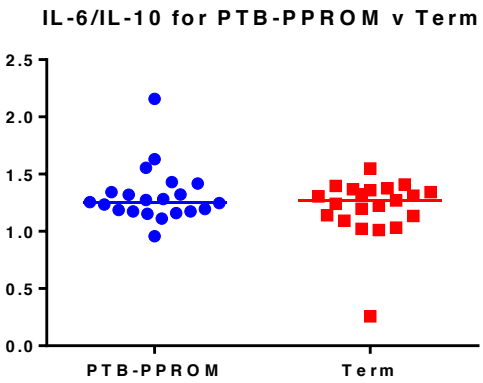
Figure 4.4 Charts demonstrate concentration (ng/mL) of cytokine (a) IL-4 (b) IL-7 (c) IL-13 and (d) G-CSF in each preterm with intact membranes and term sample

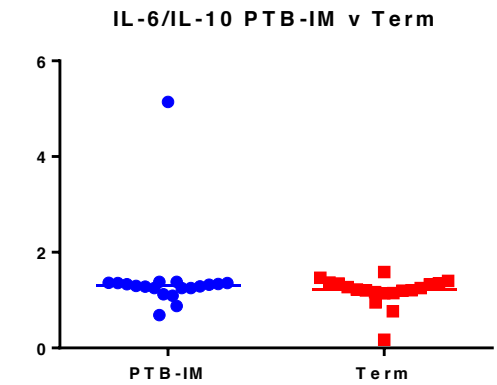


4.4.4 Ratios between pro- and anti-inflammatory cytokines

To interrogate the data further I have also looked at the ratios between the major pro- (IL-6) and anti-inflammatory (IL-10) cytokines in the three groups of spontaneous preterm birth, preterm birth with preterm prelabour ruptured membranes (PTB-PPROM) and preterm birth with intact membranes (PTB-IM) when compared with term controls. Again no statistical significance was seen between the groups.







4.4.5 Bayesian Network Formalism

Figure 4.5 shows the cytokine network for term birth and Figure 4.6 the cytokine network for spontaneous preterm birth. Interestingly, despite the analysis being done independently it can be seen that the overall pathway for the two different populations is the same. The main drivers for both of the networks are IL-1β and IL-15 with multiple interactions downstream from them. The major 'hubs' in the networks are also the same and include IL-1ra, IP-10, MCP-1, IL-6, IL-8, IFN-γ and TNF-α. The striking difference between the two networks is the difference in concentration of IL-2, which is downstream from IL-1β. In preterm labour it is noted that IL-1β causes a low concentration of IL-2 to be present which in turn causes a low concentration of IFN-γ and TNF-α.

Figure 4.5 Bayesian cytokine network for term labour

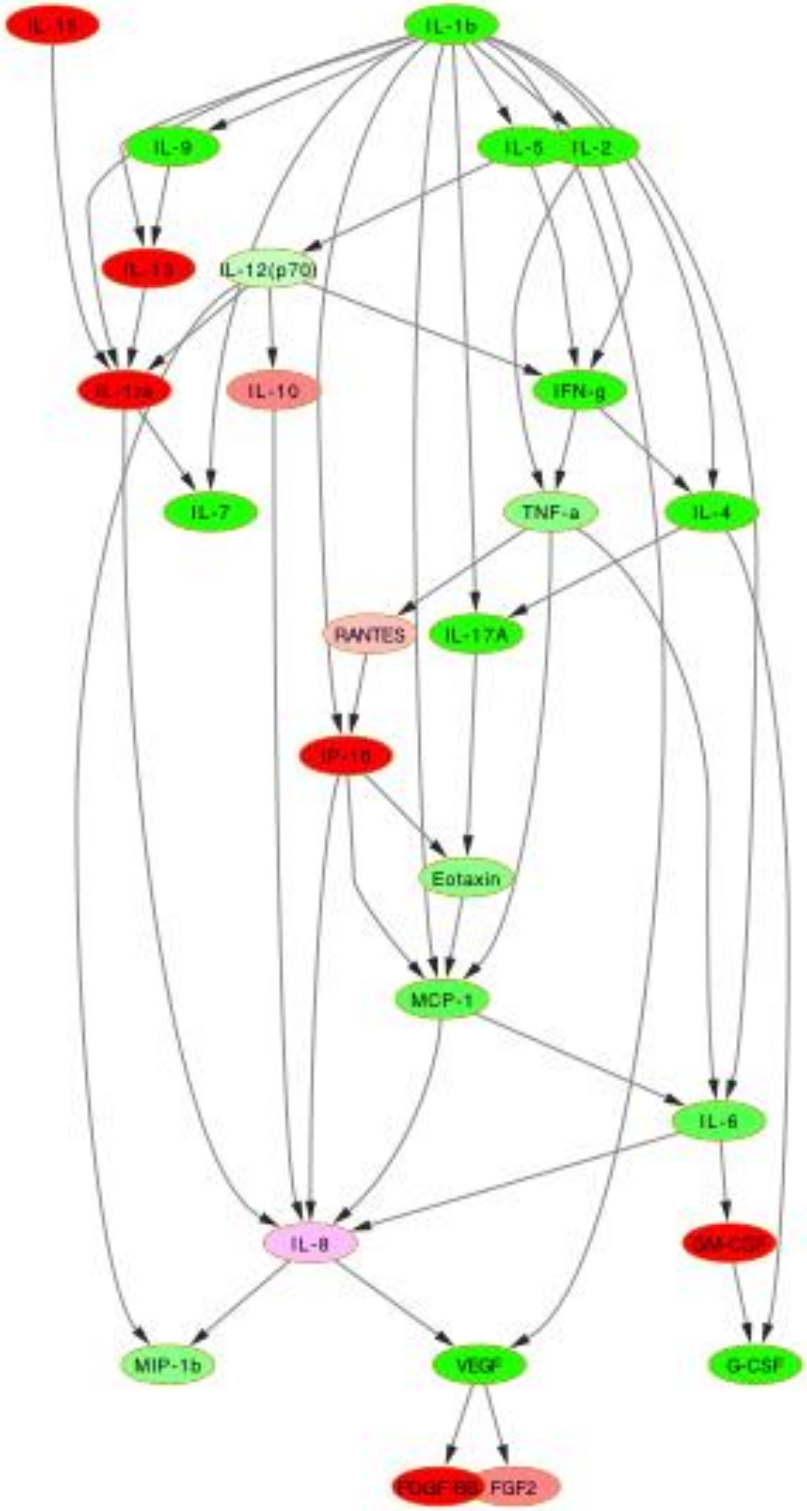
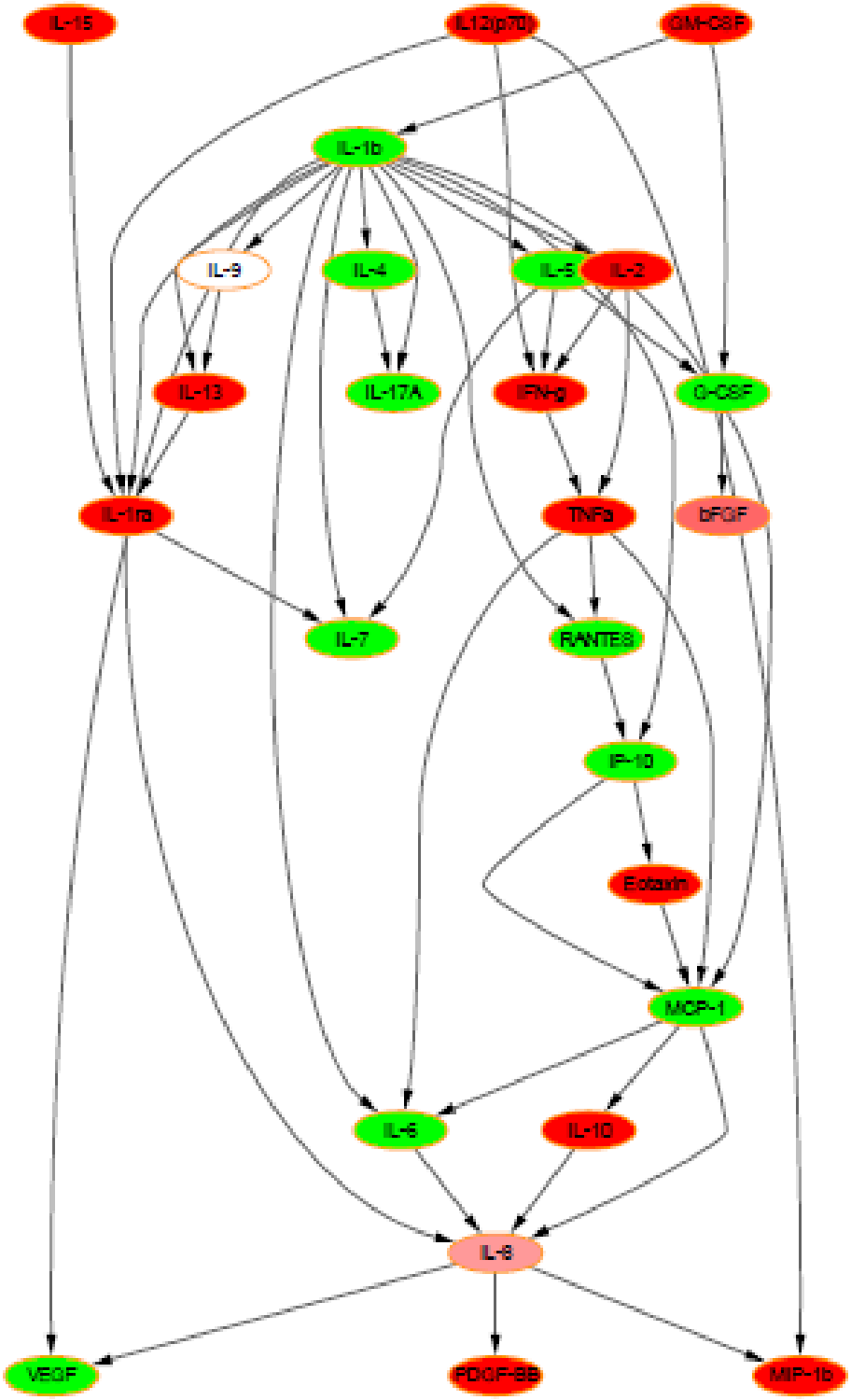


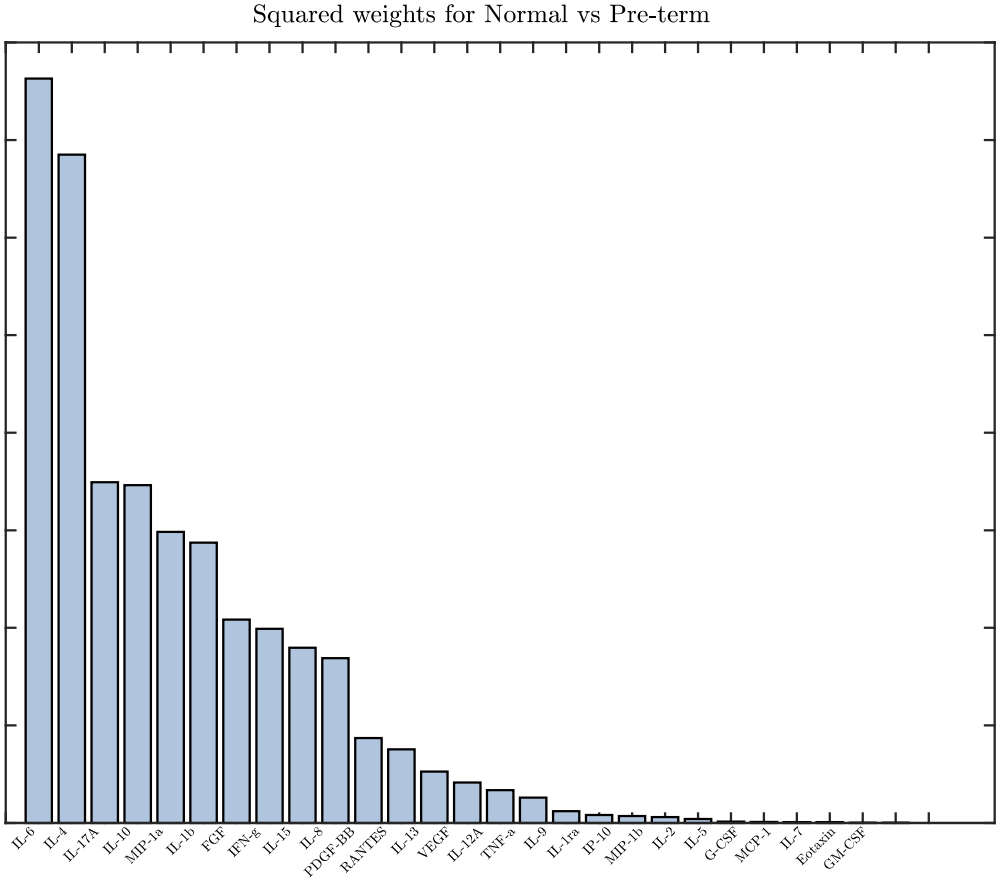
Figure 4.6 Bayesian cytokine network for preterm labour



4.4.6 Support Vector Mechanisms

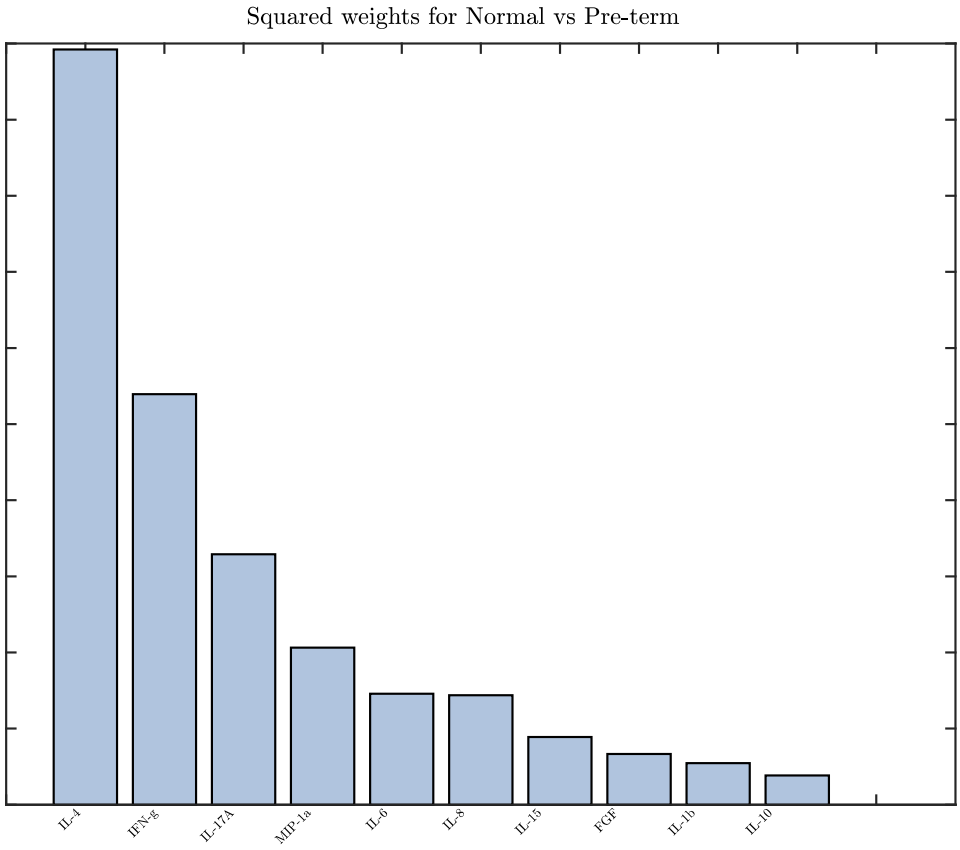
The first analysis performed was on all 27 cytokines and gave a 5-fold cross validation accuracy of 63.75%. This means that the panel of cytokines decided whether a woman delivered preterm correctly 63.75% of the time. It also ranked the cytokines as to their importance in making the decision. Figure 4.7 shows the ranking of all 27 cytokines revealing IL-6 and IL-4 to be very important in the decision.

Figure 4.7 Ranking of 27 cytokines



These results may help to form a panel of biomarkers that may be used to predict if a woman is at risk of preterm birth. Clearly, 27 markers is a large number and so the next step was to reduce the number of cytokines to see if it improved or worsened the accuracy. A 10 cytokine classifier was done and gave a 5-fold cross validation accuracy of 65%. Interestingly, the most important cytokine was IL-4 but this time IL-6 was not as relevant (Figure 4.8)

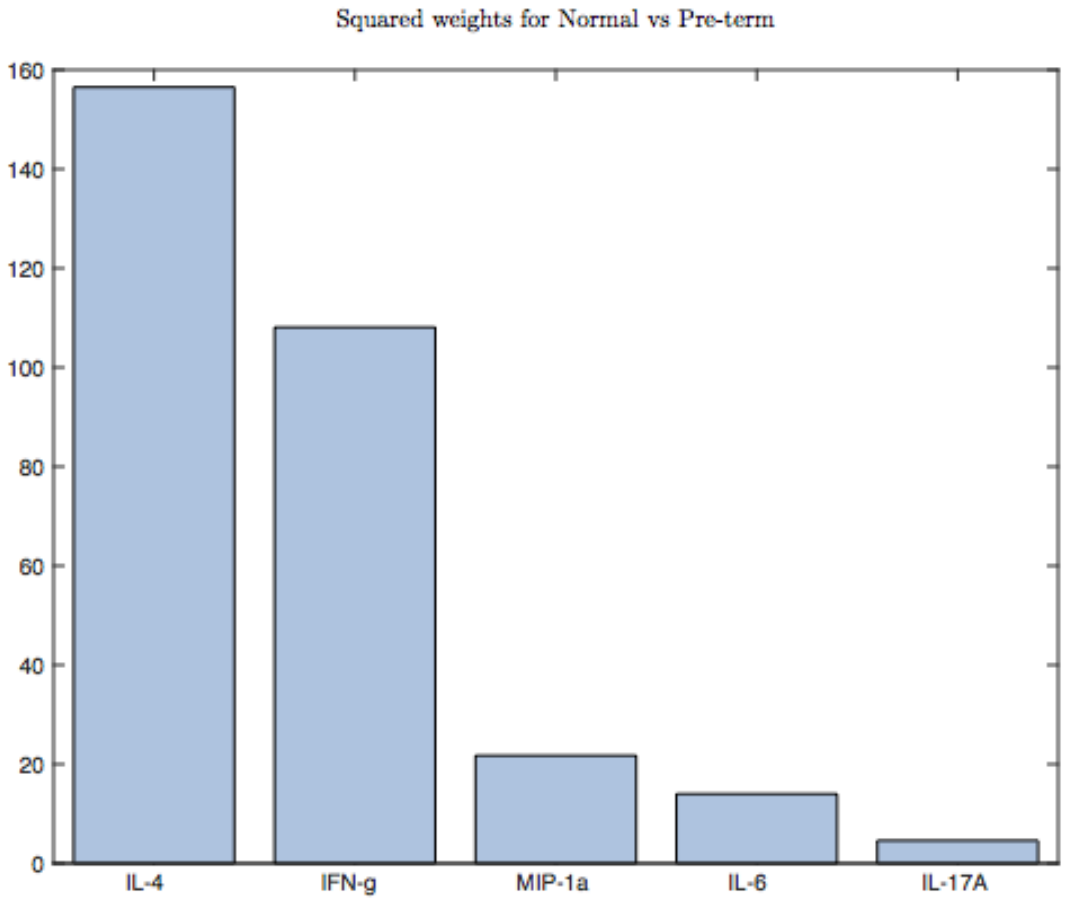
Figure 4.8 Ranking of 10 cytokines



To try and improve the accuracy further, the least relevant cytokines were removed and the process was repeated with only the two and five most relevant cytokines. With just two cytokine markers (IL-4 and IFN-γ) a 5-fold cross validation accuracy of 68.75% was gained.

With five cytokines, adding in IL-6, IL-17α, MIP-1α; a slightly improved accuracy of 71.25% was noted (Figure 4.9). Another observation was that IL-4 is slightly more important than IFN-γ but you do need both. In other words these two markers can do the job reasonably well on their own but it is more accurate with all five. This could lead further study into the five most relevant cytokines to allow the analysis of their role in preterm birth. They would obviously also require validation as a panel of biomarkers for predicting women at risk of preterm birth.

Figure 4.9 Ranking of 5 cytokines



4.5 Discussion

The ultimate goal in predictor algorithm biomarker discovery is to identify disease-associated biomarkers that can be used in clinical practice. Currently, the most robust biomarker in the prediction of preterm birth is cervicovaginal fetal fibronectin (152). However, the most reliable results are on those women who already have symptoms of preterm labour. Unfortunately, this is often too late in the disease process to try to prevent preterm birth from happening. Using biological samples taken at twenty weeks gestation, prior to the onset of clinical symptoms and signs, would enable women who would be most at risk to be highlighted at an early stage of pregnancy. This would allow identification of those women who would benefit from enhanced antenatal care, in the form of specialist consultant review and possibly cervical scanning. The women most at risk could then be offered potential management strategies in the form of infection screening, progesterone treatment or cervical cerclage.

By studying a panel of twenty-seven cytokines I was able to ascertain any differences in plasma cytokine levels between women who had a preterm birth less than 35 weeks gestation and those who went on to have a term delivery. This study demonstrated that none of the cytokines tested showed any significant differences between those women who delivered preterm and those delivered at term. These findings agree with those of the systematic review by Conde-Agudelo *et al.* (173). They reported that of the 72 studies reviewed, which evaluated a total of 18 novel plasma and/or serum biomarkers, none had good predictive accuracy.

A similar study to this was reported in 2010 (364) which demonstrated a significant association of matrix metalloproteinase-9 with spontaneous preterm birth. The authors highlighted that this positive finding could have occurred by chance due to the number of cytokines studied. We did not analyse MMP-9, therefore are unable to confirm or refute this finding.

The only comparison groups that had significant findings were spontaneous preterm birth with intact membranes when compared with women who delivered at term. There was an overall tendency for IL-4 to be lower in women who had a preterm birth and this is similar to other studies where IL-4 was significantly lower in amniotic fluid of women with clinical signs of intrauterine infection and

inflammation (365, 366) who went on to deliver preterm. The interleukin 4 (367) is a cytokine that induces differentiation of naive helper T cells (Th0 cells) to Th2 cells. It is closely related and has functions similar to Interleukin 13 and is a key regulator in humoral and adaptive immunity which could play a role in the inflammatory process of preterm birth.

There was also a statistically significant level of granulocyte-colony stimulating factor (G-CSF) in women who delivered preterm. The results demonstrated a trend to lower levels in women who delivered preterm birth than those women who delivered at term. This is in contrast to Goldenberg *et al* (368) who found that G-CSF levels were elevated in asymptomatic pregnant women at 24-28 weeks who went on to deliver <32 weeks. This was thought to provide evidence that early spontaneous preterm birth is associated with an inflammatory process that is identifiable by the presence of a cytokine in maternal plasma several weeks before an early spontaneous preterm birth. G-CSF (367) is a glycoprotein that stimulates the bone marrow to produce granulocytes and stem cells and release them into the bloodstream. G-CSF also stimulates the survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils which may be important in preterm birth caused by infective processes.

IL-7 and IL-13 were also lower in women who delivered preterm than in women who went on to have term delivery and this appears to be a novel finding as this has not been reported in published literature before. IL-7 (367) a hematopoietic growth factor secreted by stromal cells in the bone marrow and thymus. It is important for B and T cell development. IL-13 (367) is cytokine secreted by many cell types, but especially Th2 cells and is a mediator of allergic inflammation and disease.

The most widely studied cytokines (IL-1β, IL-6 and IL-10) did not appear to play a major role in this cohort, which may be by chance or reflect the different populations studied. One of the reasons for this could be these and other cytokines do not exert their influence as individuals.

When the 27 cytokines were studied using Bayesian network analysis it was identified that the key players were IL-1ra, IP-10, MCP-1, IL-6, IL-8, IFN-γ and

TNF-α. The majority of these were pro-inflammatory (Th1) which would be as expected. The interesting difference between the term and preterm birth networks was the difference in concentration of IL-2, which is downstream from IL-1β. In preterm labour it was noted that IL-1β caused a low concentration of IL-2 to be present which in turn caused a low concentration of IFN-γ and TNF-α. IL-2 has key functions in immune response and therefore may be of importance in the inflammatory process of preterm labour. Whilst this approach has clarified the nature of these interrelationships, it has also enabled identification of key cytokines and relationships that govern preterm labour. These interrelationships with surrounding nodes may therefore warrant further investigation *in silico* or as targets for interventive treatment in the future.

This study has both strengths and limitations. The main strengths are the focus on the systemic cytokine response and prospective collection of maternal plasma before the onset of labour or rupture of membranes. Other strengths include a diverse study population, rigorous assessment of gestational age, restriction to spontaneous preterm birth and the separate characterisation of PPROM and preterm labour, Despite the large size of our biobank, the small number of spontaneous preterm birth (n=40) that was analysed provides low power to detect the differences.

These results clearly need confirmation with other studies. Further research is necessary to understand the pathogenesis of preterm birth and to improve the prevention strategies we use. It may be that cytokine mediation is not present at this range in the systemic circulation and that studying local levels in amniotic fluid or cervical mucus may have a clearer result. However, cytokines that are important in the preterm birth pathway have been identified and to progress and validate these results in future studies will be exciting.

5 General Discussion and Future Work

Preterm birth is a global issue affecting approximately 12.9 million births worldwide each year (1). Preventative strategies need good prediction tools in order to highlight the women most in need of care. One of the main difficulties when researching spontaneous preterm birth is the lack of uniform definition, with some reports defining it as less than 37 weeks, others less than 34 weeks or even less than 32 weeks. Some studies also report the differences of whether membranes have ruptured or not. Therefore given the heterogeneous pathophysiology of the condition, the limited clinical utility of any individual biomarker for predicting preterm birth is understandable. Also, even with the same patterns of preterm birth, it appears that biomarkers will perform differently across different ethnic populations and/or geographical locations. It may be that a multiple-marker test based on a combination of clinical and biological markers may be a more reliable predictor. An example of one that has been previously proposed is the five marker test (cervicovaginal fetal fibronectin, cervical length and serum alpha fetoprotein, G-CSF, and defensins or alkaline phosphatase) evaluated by Goldenberg *et al.* in the Preterm Prediction Study (86). The combined markers showed better predictive accuracy than when used alone. In addition, the overlap of the markers was small, supporting the notion that there are several different heterogeneous pathways that lead to spontaneous preterm birth.

The overall aim of this study was to develop a predictive tool using both clinical data and plasma markers to try and detect those women at greatest risk of preterm birth. The cohort used was a nulliparous, low risk population, meaning the most pertinent risk factor of previous preterm birth was not included. These are women who in routine clinical practice we would have no other means of identifying those that will later deliver preterm. A test which would allow a proportion of these women to be identified as high risk in mid gestation would allow a targeted programme of cervical surveillance and the potential for prophylactic treatments such as progesterone or cerclage.

The clinical risk factor algorithm provided a modest predictive performance for spontaneous preterm birth in this nulliparous, low risk population. The most clinically relevant group, those delivering less than 34 weeks, exhibited the best

predictive performance, with an area under the ROC curve of 0.74. Like most predictive tools for spontaneous preterm birth, this showed an excellent negative predictive value of 99%, due to the fact that most women delivered at term. However, its positive prediction would only have highlighted 33% of the births less than 34 weeks meaning we would still miss a significant proportion. Due to the study's prospective design, cohort size, comprehensive range of candidate predictors, high quality data, and completeness to follow-up, the derived algorithms are likely to be indicative of the best performance achievable using clinical data to predict preterm birth in a healthy nulliparous population. The clinical risk tool performs to a similar standard when compared to more traditional scoring systems such as the Creasy score (144), however, as this tool does not require the history of a previous preterm birth, it has the added benefit of also being suitable for nulliparous women. As only 5% of women from this cohort delivered preterm, this still provided a better prediction than current clinical prediction from patient history.

Clinical risk estimates for disease are already established in other areas of medicine and biomarkers are often added to improve performance. By using plasma proteomics I sought a biomarker which might improve the sensitivity of the clinical risk factor algorithm. However, proteomics being relatively new and emerging technology, I was unable to enter the depth of the proteome to discover low abundance proteins of interest. The proteins that were discovered appeared to be mainly plasma proteins related to systemic inflammation and therefore would not be specific enough for a marker for spontaneous preterm birth.

Following on from this, I used a more targeted approach, using Luminex technology, to try to reach these lower abundance proteins. Cytokines are inflammatory mediators and have been frequently measured in cervicovaginal secretions and amniotic fluid of women who deliver preterm. It has been proposed that ascending infection leads to activation of inflammatory pathways which precede cervical shortening (369). This study was unable to detect a single cytokine in maternal plasma sensitive enough to be a marker for women who develop preterm labour. It did however highlight a group of cytokines that may be most relevant. A panel of five cytokines IL-4, IFN-γ, IL-6, IL-17α and MIP-1α appeared to be the most sensitive with a predictive accuracy of 71.25%. These cytokines should be analysed further to validate these findings. Another interesting result was the analysis of cytokines as a network to compare the

differences between term and preterm birth. This will allow a better understanding of the pathways and also provide further areas to explore in the investigation of predicting preterm birth.

The use of cervical scanning and progesterone to reduce preterm births do appear to have a positive effect in reducing preterm birth and so is seems the importance of prematurity clinics to provide this surveillance remains crucial. It still remains difficult to highlight those women with no previous history who may benefit form this treatment.

The biobank showed that almost 5% of the low risk women in the SCOPE population will deliver preterm. It therefore means that further research is needed into this important group of women to prevent further the problems of preterm birth. Sample collection was deliberately done at 20 weeks gestation as this is a common time when patients are reviewed in hospital for their fetal anomaly scan and so access for the blood sample was easy. Also, if a predictive tool was developed it would still allow time for a cervical scan to be preformed at a good time to allow chance of progesterone or cerclage to still have effect. It is also late on in pregnancy to be able to try to pick up changes already happening which could predispose women to deliver preterm. If a sample was taken too early, the inflammatory changes we expect to be happening may not yet be detectable in the blood.

Leading on from my study there are a range of further research questions to be addressed. These can only be answered by the use of high quality, international data which will only be attained with the use of collaboration internationally to set up biobanks. One of the weaknesses of the SCOPE cohort is the very small numbers of very early preterm births. The only way to move forward in the quest to reduce preterm birth is to understand the different aetiologies better and to work together globally in the search for predictive and preventative strategies. One of the common pit falls of researching predicting spontaneous preterm birth is the assumption that prematurity is all the same. A large international biobank would allows us to increase the number of cases along with separating out the different phenotypes of preterm birth as it is very likely that different biomarkers would be predictive of the different phenotypes.

Along with improving the numbers and types of cases we need to be more sophisticated with the analysis of data. New omic technologies have enabled us to examine a number of different biological samples in very sophisticated techniques; however, this has to be supported by the accurate analysis of data. Bioinformatics is having a promising renaissance and so it is important to be part of this future.

I believe that with improvements in discovery techniques along with intelligent analysis of data we may be able to identify a panel of biomarkers for each preterm birth phenotype. This will allow us to highlight women at risk of preterm birth in the mid trimester enabling them to access a screening programme and intervention in order to reduce the numbers of babies being born too soon.

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7 List of Abbreviations

17P	17-alpha hydroxyprogesterone caproate
ACTH	Adrenocorticotropic hormone
AF	Amniotic fluid
AmBic	Ammonium bicarbonate
AMBP	Alpha-1-microglobulin/bikunin precursor
ANOVA	Analysis of variance
AUC	Area under the curve
BMI	Body mass index
BV	Bacterial vaginosis
CI	Confidence interval
CL	Cervical length
CRH	Corticotrophin-releasing hormone
DES	Diethylstilbestrol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
fFN	Fetal fibronectin
FGF	Fibroblast growth factor
GA	Gestational age
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIV	Human immunodeficiency virus
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
IgA	Immunoglobulin A
IFN-γ	Interferon gamma
IGFBP-1	Insulin-like growth factor binding protein-1
IgG	Immunoglobulin G

IL-1	Interleukin-1
IL-1β	Interleukin-1 beta
IL-1ra	Interleukin-1 receptor antagonist
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-8	Interleukin-8
IL-9	Interleukin-9
IL-10	Interleukin-10
IL-12p70	Interleukin-12 (p70)
IL-13	Interleukin-13
IL-15	Interleukin-15
IL-17A	Interleukin-17A
IP-10	Interferon gamma-induced protein 10
iTRAQ	Isobaric tags for relative and absolute quantification
IVH	Intraventricular haemorrhage
kDa	Kilodalton
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LEEP	Loop electrocautery excision procedure
LLETZ	Large loop excision of the transformation zone
LMP	Last menstrual period
LSD	Lysergic acid diethylamide
MARS	Multiple affinity removal column
MCP-1	Monocyte Chemoattractant Protein-1
MFI	Median fluorescence intensity
MIP-1α	Monocyte Chemoattractant Protein-1 alpha

MIP-1β	Monocyte Chemoattractant Protein-1 beta
MMP-9	Matrix metallopeptidase 9
mRP	Macroporous reverse phase
MS	Mass spectrometer
NEC	Necrotising enterocolitis
NO	Nitric oxide
NPV	Negative predictive value
OR	Odds ratio
PBS	Phosphate-buffered saline
PDA	Patent ductus arteriosus
PDGF	Platelet derived growth factor
PE	Phycoerythrin
pHIGFBP-1	Phosphorylated insulin-like growth factor binding protein-1
PlGF	Placental growth factor
PM	Proteominer beads
PP13	Placental protein 13
PPROM	Preterm prelabour rupture of membranes
PPV	Positive predictive value
PTB	Preterm birth
PTB-IM	Preterm birth with intact membranes
PTB-PPROM	Preterm birth with prelabour rupture of membranes
PTL	Preterm labour
PVL	Periventricular leukomalacia
QTOF	Quadrupole-time-of-flight
RDS	Respiratory Distress Syndrome
RI	Resistance index
ROC	Receiver operating characteristic
RP	Reverse phase
RR	Relative risk

SA-PE	Streptavidin-phycoerythrin
SCOPE	Screening for pregnancy endpoints
sd	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sens	Sensitivity
SES	Socioeconomic status
SGA	Small for gestational age
Spec	Specificity
sPTB	Spontaneous preterm birth
sPTD	Spontaneous preterm delivery
SVM	Support vector machines
RR	Relative risk
RPM	Revolutions per minute
TEAB	Triethylammonium bicarbonate
TFA	Trifluoroacetic acid
TNF	Tumour necrosis factor
TVS	Transvaginal scan
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

8 Appendix

Appendix A Proteins identified following depletion with MARS 14 and ProteoMiner™ Beads

MARS 14 Column	ProteoMiner™ Beads
Ceruloplasmin	Apolipoprotein A-I
Complement C4-B	Fibrinogen alpha chain
Inter-alpha-trypsin inhibitor heavy chain H4	Fibrinogen beta chain
Hemopexin	Apolipoprotein A-IV
Apolipoprotein A-IV	Clusterin
Antithrombin-III	Serum albumin
Inter-alpha-trypsin inhibitor heavy chain H1	Vitronectin
Inter-alpha-trypsin inhibitor heavy chain H2	Serum paraoxonase/arylesterase 1
Alpha-1B-glycoprotein	Fibrinogen gamma chain
Vitamin D-binding protein	Complement C4-A
Alpha-1-antichymotrypsin	Inter-alpha-trypsin inhibitor heavy chain H4
Vitronectin	Complement C1r subcomponent
Clusterin	Ig mu chain C region
Alpha-2-HS-glycoprotein	Ig gamma-1 chain C region
Keratin, type II cytoskeletal 2 epidermal	Prothrombin
Complement factor B	Apolipoprotein E
Prothrombin	Ig gamma-3 chain C region
Complement component C9	Hemopexin
Heparin cofactor 2	Ceruloplasmin
Complement factor H	Plasminogen
Inter-alpha-trypsin inhibitor heavy chain H3	Ig kappa chain C region
Keratin, type I cytoskeletal 10	Apolipoprotein C-III
Sex hormone-binding globulin	Complement C1q subcomponent subunit B
Serum amyloid P-component	Alpha-2-HS-glycoprotein
Plasminogen	Alpha-1-antichymotrypsin
Complement C1s subcomponent	Ig gamma-2 chain C region
Leucine-rich alpha-2-glycoprotein	Ig gamma-4 chain C region
Kininogen-1	C4b-binding protein alpha chain
Keratin, type II cytoskeletal 1	Complement C1s subcomponent
Complement C1r subcomponent	Complement C1q subcomponent subunit C
Gelsolin	Apolipoprotein D
Lumican	Transthyretin
Serum paraoxonase/arylesterase 1	Inter-alpha-trypsin inhibitor heavy chain H1
Fibrinogen alpha chain	Pregnancy-specific beta-1-glycoprotein 4
C4b-binding protein alpha chain	Apolipoprotein C-I

Complement C1q subcomponent subunit C	Vitamin K-dependent protein S
Complement component C8 alpha chain	Antithrombin-III
Fibrinogen beta chain	Ig lambda chain C regions
Carboxypeptidase N catalytic chain	Vitamin D-binding protein
Complement component C8 beta chain	Ig kappa chain V-III region SIE
Complement component C8 gamma chain	Pregnancy-specific beta-1-glycoprotein 3
Complement factor I	Keratin, type II cytoskeletal 1
Afamin	Inter-alpha-trypsin inhibitor heavy chain H2
Pregnancy-specific beta-1-glycoprotein 1	Ig alpha-1 chain C region
Protein AMBP	Apolipoprotein C-II
Pregnancy-specific beta-1-glycoprotein 6	Sex hormone-binding globulin
Complement C1q subcomponent subunit B	Complement C3
Coagulation factor XII	Heparin cofactor 2
Pregnancy-specific beta-1-glycoprotein 9	Alpha-1-antitrypsin
Fibrinogen gamma chain	Alpha-1B-glycoprotein
Apolipoprotein C-III	Haptoglobin
Fetuin-B	Serum amyloid A-4 protein
Complement C1r subcomponent-like protein	Fibrinogen-like protein 1
Keratin, type II cytoskeletal 5	Serotransferrin
Kallistatin	Apolipoprotein A-II
Complement component C7	Lipopolysaccharide-binding protein
N-acetylmuramoyl-L-alanine amidase	Sulfhydryl oxidase 1
Complement component C6	Kininogen-1
Glutathione peroxidase 3	Complement factor B
Vitamin K-dependent protein S	C4b-binding protein beta chain
Alpha-1-antitrypsin	Hemoglobin subunit beta
Complement C2	Ig kappa chain V-IV region Len
Keratin, type II cytoskeletal 1b	Complement factor H
Retinol-binding protein 4	Afamin
Keratin, type I cytoskeletal 9	Ig kappa chain V-II region RPMI 6410
Apolipoprotein C-I	Histidine-rich glycoprotein
Histidine-rich glycoprotein	Coagulation factor XII
Apolipoprotein C-II	E3 ubiquitin-protein ligase TRIM33
Carboxypeptidase B2	Tsukushin
Coagulation factor IX	Apolipoprotein F
Ficolin-3	Fibulin-1
Plasma kallikrein	Coagulation factor V
Attractin	Keratin, type I cytoskeletal 10
Keratin, type I cytoskeletal 27	C-reactive protein
Serum amyloid A-4 protein	Protein S100-A9
C-reactive protein	Gelsolin

Monocyte differentiation antigen CD14	Apolipoprotein C-IV
Alpha-2-antiplasmin	Collectin-11
Fibulin-1	Alpha-2-antiplasmin
Carbonic anhydrase 1	Protein FAM59B
Apolipoprotein E	Fetuin-B
Apolipoprotein C-IV	Complement factor I
Beta-2-glycoprotein 1	Hyaluronan-binding protein 2
Adiponectin	Complement component C9
Complement C5	Histone-lysine N-methyltransferase SUV39H1
Hyaluronan-binding protein 2	
Mannan-binding lectin serine protease 1	
Apolipoprotein M	
Fibronectin	
Adenylate cyclase type 10	
Mannose-binding protein C	
Dopamine beta-hydroxylase	
Thyroxine-binding globulin	
C4b-binding protein beta chain	

Appendix B Following MS/MS of the iTRAQ labeled plasma, 243 high confidence proteins were identified with relative quantification data available for 117 of these

N	Unused	Accession	Name	Peptides (95%)	114:113	PVal 114:113	116:113	PVal 116:113	117:113	PVal 117:113	118:113	PVal 118:113	121:113	PVal 121:113
1	329.97	sp|P02774|VTDB_HUMAN	Vitamin D-binding protein	546	0.98	0.56	1.12	**<0.01**	1.08	0.11	1.14	**<0.01**	1.23	**<0.01**
2	286.14	sp|P08603|CFAH_HUMAN	Complement factor H	352	0.99	0.88	0.91	**<0.01**	1.03	0.45	0.91	**<0.01**	1.15	**<0.01**
3	215.47	sp|P02765|FETUA_HUMAN	Alpha-2-HS-glycoprotein	405	0.88	0.06	0.97	0.32	0.91	0.14	0.95	0.08	1.02	0.73
4	212.66	sp|P0C0L5|CO4B_HUMAN	Complement C4-B	257	0.93	0.27	1.04	0.37	0.94	0.26	0.95	0.13	1.04	0.72
5	203.28	sp|P00747|PLMN_HUMAN	Plasminogen	264	1.04	0.35	0.97	0.28	1.05	0.22	0.94	**0.03**	1.13	**<0.01**
6	194.49	sp|P00450|CERU_HUMAN	Ceruloplasmin	307	0.81	**0.02**	0.86	**0.01**	0.84	**0.03**	0.94	0.22	0.95	0.49
7	191.69	sp|P02790|HEMO_HUMAN	Hemopexin	343	0.98	0.77	0.92	**0.05**	0.98	0.81	0.95	0.18	0.99	0.79
8	163.62	sp|P04114|APOB_HUMAN	Apolipoprotein B-100	120	0.79	**<0.01**	0.83	**<0.01**	0.83	**<0.01**	0.91	**0.01**	0.83	0.11
9	154.79	sp|P43652|AFAM_HUMAN	Afamin	165	0.98	0.64	1.12	**<0.01**	1.06	0.26	1.12	**<0.01**	1.20	**<0.01**
10	152.87	sp|P00751|CFAB_HUMAN	Complement factor B	160	0.90	0.10	1.01	0.89	1.00	0.96	1.00	0.94	1.17	**0.02**
11	140.39	sp|P01042|KNG1_HUMAN	Kininogen-1	242	1.13	0.14	1.01	0.80	1.15	0.14	1.09	0.14	1.26	**<0.01**
12	132.65	sp|P06727|APOA4_HUMAN	Apolipoprotein A-IV	134	0.79	**<0.01**	0.89	**<0.01**	0.92	**0.05**	1.07	**0.01**	0.83	**0.00**
13	131.18	sp|P02671|FIBA	Fibrinogen alpha chain	151	0.92	0.18	0.77	**<0.01**	0.74	**<0.01**	0.73	**<0.01**	0.95	0.61

		_HUMAN												
14	129.67	sp|P00734|THRB_HUMAN	Prothrombin	218	1.12	0.18	1.04	0.48	1.16	0.08	1.06	0.27	1.14	0.10
15	119.13	sp|P02749|APOH_HUMAN	Beta-2-glycoprotein 1	171	0.90	0.16	0.91	**0.03**	1.01	0.89	1.02	0.66	1.12	0.15
16	115.8	sp|Q14624|ITIH4_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H4	190	0.98	0.68	0.92	0.10	0.97	0.63	0.96	0.38	1.07	0.24
17	113.67	sp|P04217|A1BG_HUMAN	Alpha-1B-glycoprotein	254	1.06	0.55	0.95	0.33	1.06	0.52	0.97	0.49	1.16	**0.03**
18	108.39	sp|P25311|ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	195	0.91	0.18	1.04	0.60	0.93	0.48	1.00	0.99	1.14	0.29
19	100.43	sp|P10909|CLUS_HUMAN	Clusterin	153	1.01	0.86	0.99	0.80	0.97	0.69	0.99	0.89	1.14	0.06
20	97.51	sp|P01008|ANT3_HUMAN	Antithrombin-III	113	0.88	0.11	1.04	0.47	0.95	0.59	1.01	0.80	1.15	0.11
21	83.95	sp|P06396|GELS_HUMAN	Gelsolin	82	0.85	0.02	1.00	0.91	0.89	0.08	1.02	0.43	0.99	0.86
22	74.11	sp|P19823|ITIH2_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H2	90	1.00	0.99	1.01	0.85	1.00	0.99	1.01	0.93	1.09	0.25
23	71.01	sp|P02675|FIBB_HUMAN	Fibrinogen beta chain	65	0.91	0.19	0.94	0.33	0.97	0.75	0.99	0.84	1.26	**<0.01**
24	69.55	sp|P13671|CO6_HUMAN	Complement component C6	66	0.98	0.77	1.00	0.96	1.11	0.13	1.00	0.92	1.15	**0.04**
25	69.32	sp|P00748|FA12_HUMAN	Coagulation factor XII	83	0.99	0.91	0.87	**<0.01**	0.94	0.37	0.94	0.17	1.07	0.80
26	68.41	sp|P02751|FINC_HUMAN	Fibronectin	55	1.02	0.81	0.84	**<0.01**	1.19	**0.04**	0.99	0.83	0.99	0.89
27	68.07	sp|P04004|VTNC_HUMAN	Vitronectin	98	1.13	0.20	1.06	0.30	1.09	0.37	1.04	0.40	1.22	0.44
28	63.	sp|P051	Complement factor I	60	0.98	0.76	0.95	0.29	0.95	0.52	0.93	**0.03**	1.09	0.18

	82	56|CFAI_HUMAN												
29	58.4	sp|P02760|AMBP_HUMAN	Protein AMBP	63	0.99	0.93	0.99	0.85	1.01	0.93	0.98	0.65	1.12	**0.05**
30	56.82	sp|P23142|FBLN1_HUMAN	Fibulin-1	75	1.12	0.08	1.07	0.17	1.16	**0.02**	1.09	0.13	1.33	**<0.01**
31	56.03	sp|P19827|ITIH1_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H1	54	0.82	0.08	0.93	0.51	0.84	0.06	0.97	0.72	1.01	0.96
32	55.11	sp|P09871|C1S_HUMAN	Complement C1s subcomponent	56	0.91	0.21	0.96	0.35	0.96	0.52	0.96	0.26	1.04	0.55
33	49.29	sp|P10643|CO7_HUMAN	Complement component C7	39	0.96	0.76	1.03	0.75	1.09	0.44	1.05	0.47	1.24	0.46
34	48.99	sp|P04003|C4BPA_HUMAN	C4b-binding protein alpha chain	55	1.05	0.62	1.04	0.54	1.09	0.35	1.03	0.60	1.19	**0.04**
35	45.36	sp|P07357|CO8A_HUMAN	Complement component C8 alpha chain	47	1.01	0.94	0.93	0.49	1.01	0.94	0.97	0.78	1.06	0.63
36	44.79	sp|P00736|C1R_HUMAN	Complement C1r subcomponent	34	1.12	0.22	0.99	0.87	1.19	**0.04**	1.01	0.90	1.16	0.12
37	44.57	sp|Q06033|ITIH3_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H3	56	0.90	0.20	1.07	0.29	0.93	0.47	1.04	0.52	1.02	0.82
38	44.43	sp|P02748|CO9_HUMAN	Complement component C9	49	0.87	0.12	0.92	0.22	0.93	0.45	1.00	0.97	1.02	0.81
39	43	sp|P01011|AACT_HUMAN	Alpha-1-antichymotrypsin	40	0.80	0.13	0.81	**0.03**	0.93	0.37	0.89	0.21	0.87	0.58
40	40.56	sp|Q16610|ECM1_HUMAN	Extracellular matrix protein 1	35	1.20	0.09	1.22	**<0.01**	1.36	**<0.01**	1.21	**<0.01**	1.24	**<0.01**
41	38.09	sp|P02750|A2GL_HUMAN	Leucine-rich alpha-2-glycoprotein	59	1.12	0.27	0.98	0.76	1.09	0.36	1.13	**0.02**	1.16	**0.03**
42	37.3	sp|P08697|A2AP_HUMAN	Alpha-2-antiplasmin	45	0.89	0.22	0.98	0.74	0.97	0.76	0.98	0.69	1.02	0.91

43	34.5	sp|Q96PD5|PGRP2_HUMAN	N-acetylmuramoyl-L-alanine amidase	32	0.88	0.34	0.91	0.28	0.86	0.29	0.94	0.44	0.87	0.31
44	32.41	sp|P01019|ANGT_HUMAN	Angiotensinogen	35	0.82	0.10	1.00	0.98	0.91	0.39	1.07	0.44	1.16	0.25
45	31.21	sp|P03952|KLKB1_HUMAN	Plasma kallikrein	26	1.26	**0.04**	1.12	0.12	1.53	**<0.01**	1.20	0.04	1.51	**<0.01**
46	30.88	sp|P01031|CO5_HUMAN	Complement C5	22	0.94	0.59	0.91	0.12	0.98	0.89	0.90	0.05	0.93	0.30
47	30.03	sp|P05160|F13B_HUMAN	Coagulation factor XIII B chain	25	1.05	0.76	1.03	0.73	1.17	0.25	1.04	0.65	0.99	0.93
48	29.95	sp|P02655|APOC2_HUMAN	Apolipoprotein C-II	30	1.00	1.00	1.02	0.78	1.00	1.00	1.03	0.75	1.27	0.12
49	29.71	sp|Q9UGM5|FETUB_HUMAN	Fetuin-B	29	0.88	0.31	1.18	**0.05**	1.05	0.70	1.12	0.07	1.31	**<0.01**
50	27.97	sp|P36980|FHR2_HUMAN	Complement factor H-related protein 2	31	1.16	0.18	1.17	**0.02**	1.57	**<0.01**	1.35	**<0.01**	1.19	0.11
51	27.23	sp|P04196|HRG_HUMAN	Histidine-rich glycoprotein	24	1.25	**<0.01**	1.32	**<0.01**	1.73	**<0.01**	1.86	**<0.01**	0.95	0.91
52	27.06	sp|P36955|PEDF_HUMAN	Pigment epithelium-derived factor	24	0.89	0.18	1.06	0.41	1.02	0.85	1.15	**<0.01**	0.97	0.87
53	26.88	sp|P02753|RET4_HUMAN	Retinol-binding protein 4	30	1.11	0.56	0.92	0.49	1.23	0.41	0.98	0.86	1.20	0.21
54	26.58	sp|P51884|LUM_HUMAN	Lumican	21	1.17	0.17	1.03	0.49	1.09	0.35	1.11	**0.04**	1.04	0.60
55	26.32	sp|Q14520|HABP2_HUMAN	Hyaluronan-binding protein 2	21	1.10	0.38	1.04	0.60	1.31	**0.02**	1.18	**0.01**	1.28	0.06
56	25.9	sp|P26927|HGFL_HUMAN	Hepatocyte growth factor-like protein	24	1.05	0.69	0.97	0.65	0.96	0.77	0.90	0.11	0.97	0.76
57	24.15	sp|Q13219|PAPP1_H	Pappalysin-1	19	0.94	0.61	1.28	**<0.01**	1.23	0.09	1.44	**<0.01**	1.50	**<0.01**

58	24.12	UMAN sp|P05452|TETN_HUMAN	Tetranectin	23	1.14	0.38	1.06	0.42	1.20	0.21	1.20	0.24	1.04	0.71
59	23.94	sp|P02649|APOE_HUMAN	Apolipoprotein E	18	1.14	0.42	0.82	0.07	0.99	0.95	1.00	0.98	0.88	0.25
60	23.83	sp|P07358|CO8B_HUMAN	Complement component C8 beta chain	24	1.18	0.40	0.93	0.50	1.17	0.45	0.99	0.94	1.05	0.95
61	23.29	sp|P27918|PROP_HUMAN	Properdin	23	0.94	0.61	0.99	0.94	0.97	0.80	1.00	0.99	1.13	0.17
62	23.11	sp|P06681|CO2_HUMAN	Complement C2	21	0.86	0.20	0.88	**0.04**	0.91	0.26	0.91	0.14	0.93	0.44
63	22.81	sp|P02775|CXCL7_HUMAN	Platelet basic protein	21	0.99	0.93	1.01	0.90	1.73	**<0.01**	1.74	**<0.01**	1.48	**<0.01**
64	22.66	sp|P17936|IBP3_HUMAN	Insulin-like growth factor-binding protein 3	18	0.92	0.48	1.01	0.94	0.95	0.65	0.98	0.84	1.24	0.17
65	20.47	sp|P12259|FA5_HUMAN	Coagulation factor V	17	1.11	0.43	1.11	0.13	1.08	0.49	1.09	0.16	1.16	0.72
66	19.22	sp|P02656|APOC3_HUMAN	Apolipoprotein C-III	45	0.82	0.31	0.92	0.22	0.87	0.55	1.04	0.57	1.27	**0.01**
67	17.73	sp|P67936|TPM4_HUMAN	Tropomyosin alpha-4 chain	13	0.87	0.44	0.97	0.80	1.40	0.21	1.53	**<0.01**	1.00	0.99
68	16.68	sp|P02679|FIBG_HUMAN	Fibrinogen gamma chain	16	0.93	0.55	0.93	0.24	1.07	0.56	1.00	0.95	1.17	**0.03**
69	16.65	sp|P07225|PROS_HUMAN	Vitamin K-dependent protein S	15	0.92	0.49	0.96	0.51	0.96	0.74	0.97	0.64	1.07	0.66
70	16.59	sp|P02776|PLF4_HUMAN	Platelet factor 4	10	0.94	0.32	1.25	**0.05**	2.09	**<0.01**	2.15	**<0.01**	1.59	**<0.01**
71	16.48	sp|P02652|APOA2_HUMAN	Apolipoprotein A-II	13	0.74	**<0.01**	1.23	<0.01	0.99	0.93	1.21	**<0.01**	1.31	**0.03**
72	16.31	sp|P11464|PSG	Pregnancy-specific beta-1-glycoprotein 1	15	0.95	0.70	1.21	0.33	1.54	0.32	1.65	0.37	1.42	0.44

		1_HUMAN												
73	16.27	sp|Q12805|FBLN3_HUMAN	EGF-containing fibulin-like extracellular matrix protein 1	13	0.93	0.75	0.94	0.66	1.02	0.90	0.94	0.67	0.92	0.67
74	16.24	sp|P35858|ALS_HUMAN	Insulin-like growth factor-binding protein complex acid labile subunit	13	0.98	0.84	1.01	0.91	1.00	0.98	1.01	0.94	1.03	0.79
75	15.99	sp|P04278|SHBG_HUMAN	Sex hormone-binding globulin	12	0.85	0.43	1.13	0.58	0.93	0.72	1.06	0.78	1.12	0.75
76	15.35	sp|P00742|FA10_HUMAN	Coagulation factor X	16	1.16	0.60	0.98	0.91	1.14	0.61	0.95	0.53	1.07	0.55
77	15.01	sp|Q04756|HGFA_HUMAN	Hepatocyte growth factor activator	11	1.13	0.28	1.03	0.74	1.36	0.05	1.22	0.17	1.05	0.70
78	14.71	sp|P33151|CADH5_HUMAN	Cadherin-5	11	0.98	0.82	1.08	0.20	0.99	0.89	0.99	0.84	1.10	0.28
79	14.7	sp|P05543|THBG_HUMAN	Thyroxine-binding globulin	11	0.91	0.70	0.99	0.97	1.04	0.84	1.14	0.36	1.01	0.95
80	14.2	sp|O75636|FCN3_HUMAN	Ficolin-3	21	0.89	0.40	1.14	0.27	0.91	0.58	1.03	0.80	1.01	0.96
81	14.12	sp|P05546|HEP2_HUMAN	Heparin cofactor 2	10	1.03	0.87	0.92	0.26	1.03	0.80	0.97	0.53	0.96	0.63
82	13.17	sp|P02654|APOC1_HUMAN	Apolipoprotein C-I	15	1.13	0.47	1.11	0.23	1.13	0.60	1.19	0.08	1.11	0.67
83	13.02	sp|Q9NZP8|C1RL_HUMAN	Complement C1r subcomponent-like protein	16	1.02	0.89	1.00	0.97	0.96	0.78	1.00	0.95	1.08	0.54
84	12.86	sp|P05155|IC1_HUMAN	Plasma protease C1 inhibitor	8	1.15	0.48	1.12	0.41	1.41	0.04	1.35	0.07	1.19	0.23
85	12.73	sp|P08519|APOA_HUMAN	Apolipoprotein(a)	13	0.59	**0.04**	0.64	0.08	0.61	**0.01**	0.60	0.07	0.68	0.08
86	12.65	sp|P01344|IGF2_HUMAN	Insulin-like growth factor II	11	0.89	0.40	0.89	0.33	0.79	0.15	0.89	0.28	1.06	0.71
87	12	sp|P07360|CO8	Complement component C8 gamma chain	11	0.98	0.84	0.93	0.37	0.97	0.67	1.05	0.55	0.94	0.45

		G_HUMAN												
98	11.9	sp|P00746|CFAD_HUMAN	Complement factor D	7	1.25	0.14	0.90	0.18	1.12	0.44	1.01	0.92	1.00	0.97
99	11.59	sp|P05019|IGF1_HUMAN	Insulin-like growth factor I	9	0.85	0.11	0.95	0.55	0.84	0.16	0.93	0.39	1.22	0.08
00	11.56	sp|P22792|CPN2_HUMAN	Carboxypeptidase N subunit 2	7	1.43	0.44	0.91	0.44	1.09	0.56	0.96	0.65	1.01	0.89
01	11.52	sp|P49908|SEPP1_HUMAN	Selenoprotein P	11	1.17	0.49	1.06	0.49	1.19	0.41	1.04	0.57	1.07	0.63
02	11.24	sp|P19320|VCAM1_HUMAN	Vascular cell adhesion protein 1	8	1.34	0.38	1.10	0.60	1.07	0.74	1.03	0.80	1.23	0.27
03	10.65	sp|P16070|CD44_HUMAN	CD44 antigen	8	1.20	0.17	1.19	0.17	1.16	0.25	1.09	0.43	0.70	0.25
04	10.25	sp|P54108|CRIS3_HUMAN	Cysteine-rich secretory protein 3	9	0.99	0.91	1.14	0.17	1.09	0.65	1.11	0.29	1.13	0.49
05	10.04	sp|P60709|ACTB_HUMAN	Actin, cytoplasmic 1	5	0.65	0.39	0.85	0.60	1.12	0.37	1.40	0.16	0.88	0.58
06	9.97	sp|P15169|CBPN_HUMAN	Carboxypeptidase N catalytic chain	8	1.13	0.61	1.09	0.58	1.08	0.76	1.04	0.72	1.17	0.27
07	9.68	sp|P00740|FA9_HUMAN	Coagulation factor IX	7	1.06	0.60	1.04	0.68	1.00	0.99	0.98	0.83	1.24	0.15
08	9.66	sp|O75882|ATRN_HUMAN	Attractin	5	1.42	0.06	1.00	0.99	1.20	0.27	1.00	1.00	1.08	0.49
09	9.48	sp|Q00887|PSG9_HUMAN	Pregnancy-specific beta-1-glycoprotein 9	11	0.78	0.57	1.06	0.78	0.85	0.46	0.99	0.92	1.04	0.87
10)	9.39	sp|P27169|PON1_HUMAN	Serum paraoxonase/arylesterase 1	7	0.96	0.91	0.97	0.91	1.11	0.80	1.24	0.40	0.96	0.90
11	9.38	sp|P02743|SAMP_HUMAN	Serum amyloid P-component	9	0.86	0.66	0.87	0.52	0.85	0.60	0.97	0.91	0.97	0.90
10	9.3	sp|O43	Disintegrin and	6	0.87	0.48	1.13	0.25	1.02	0.91	1.11	0.48	1.43	**0.05**

2	6	184|ADA12_HUMAN	metalloproteinase domain-containing protein 12											
103	9.36	sp|P41222|PTGDS_HUMAN	Prostaglandin-H2 D-isomerase	7	1.23	0.55	1.10	0.39	1.31	0.46	1.21	0.19	1.06	0.75
104	9.2	sp|Q9BXR6|FHR5_HUMAN	Complement factor H-related protein 5	7	0.86	0.56	0.82	0.08	0.78	0.37	0.86	0.16	0.91	0.95
105	9.11	sp|P05362|ICAM1_HUMAN	Intercellular adhesion molecule 1	5	0.94	0.77	0.92	0.70	0.91	0.64	0.91	0.64	0.86	0.54
106	9.09	sp|P02746|C1QB_HUMAN	Complement C1q subcomponent subunit B	13	1.16	0.30	0.95	0.84	1.03	0.85	0.93	0.68	1.04	0.87
107	8.92	sp|P35542|SAA4_HUMAN	Serum amyloid A-4 protein	7	1.14	0.35	1.19	0.22	1.29	0.14	1.26	0.06	1.29	**0.05**
108	8.65	sp|P43121|MUC18_HUMAN	Cell surface glycoprotein MUC18	6	1.08	0.65	1.00	0.99	1.02	0.90	0.97	0.84	1.04	0.82
110	8.3	sp|Q6UXB8|PI16_HUMAN	Peptidase inhibitor 16	7	1.21	0.33	1.14	0.47	1.15	0.54	1.08	0.62	1.33	0.18
111	8.21	sp|P01034|CYTC_HUMAN	Cystatin-C	7	1.04	0.77	0.99	0.91	1.08	0.46	1.00	1.00	0.98	0.89
112	8.09	sp|Q6H9L7|ISM2_HUMAN	Isthmin-2	4	0.89	0.70	1.29	0.10	0.86	0.32	0.94	0.65	1.42	0.12
113	8.06	sp|P01024|CO3_HUMAN	Complement C3	7	0.88	0.78	1.04	0.84	0.95	0.82	0.86	0.39	0.99	0.95
114	8.03	sp|O00533|CHL1_HUMAN	Neural cell adhesion molecule L1-like protein	5	1.01	0.97	0.89	0.61	0.91	0.77	0.89	0.69	0.86	0.71
115	8	sp|P59666|DEF3_HUMAN	Neutrophil defensin 3	7	1.07	0.85	1.24	0.34	1.06	0.88	1.14	0.57	1.07	0.81
116	7.97	sp|P14151|LYAM1_HUMAN	L-selectin	5	1.21	0.45	1.21	0.23	1.20	0.47	1.09	0.56	1.23	0.39
117	7.82	sp|P02745|C1QA_HUMAN	Complement C1q subcomponent subunit A	12	0.75	0.45	1.05	0.77	0.77	0.28	0.92	0.62	0.52	0.19