Prediction of Inappropriate Myometrial Function

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Medicine. It has been composed by myself and has not been submitted in any previous application for any degree.

I, Lauren Lacey declare that:

1. My research has been conducted ethically and all of the work presented in this thesis, except where specifically stated, was original research performed by myself under the supervision of Professor Siobhan Quenby and Dr Andrew Blanks.
2. Participants were recruited by myself and Professor Quenby. The sample and data collection during the study were performed by myself with the support and help of my colleagues at the University of Warwick and University Hospitals Coventry and Warwickshire NHS Trust.
3. The laboratory work was carried out by myself with the support of my colleagues under the supervision of Dr Paul Brighton.
4. The data and the results presented are genuine and obtained during my research.
5. I have appropriately acknowledged and referenced within my thesis, where I have drawn on the work, ideas and help of others.
6. The thesis submitted is within the required word limit as specified by the University of Warwick.

Abstract

Preterm birth is a major clinical problem, worldwide 15000000 babies are born prematurely each year. Inappropriate myometrial function is a major cause of preterm labour. Preterm labour is the result of multiple pathological processes involving several underlying mechanisms. In all cases, a quiescent uterus in pregnancy changes to one that can produce coordinated, forceful contractions, following an increase in uterine conductivity and contractility, and cervical remodelling to facilitate cervical dilatation.

Currently there are several biochemical and clinical tests available to assist in the prediction of preterm birth. Many of these have a very high negative predictive value but their positive predictive value remains low. One in five women in the UK requires induction of labour. The outcomes of this process are again difficult to predict. Both of these areas of obstetrics would benefit from improvements in prediction of clinical outcomes.

Previously, phospholipase C like 1 (PLCL1) was identified as a novel intracellular protein found to be significantly downregulated in both the myometrium with the onset of spontaneous labour using sequencing techniques (Chan *et al.*, 2014). It acts as an IP_3 chelator, uncoupling phospholipase C from myometrial contractions, maintaining myometrial quiescence and therefore regulating a common pathway to inflammatory, oxytocin or prostaglandin mediated labour. We aimed to develop a clinical test utilising PLCL1 as a quiescence or susceptibility marker to other stimuli to premature labour and to determine if this marker could determine sensitivity to prostaglandins and syntocinon during the induction of labour process.

During a prospective observational cohort study, patients were recruited from a preterm prevention clinic throughout mid-pregnancy, and from the antenatal ward when attending for induction of labour at term. Cervical cytobrush samples were taken to obtain cervical epithelial cells. A novel assay was developed to quantify PLCL1 from these samples. There have been various challenges in the process, including the small and varying number of cells obtained, problems with interference from cervical mucus with protein quantification and difficulty adequately lysing our cells to release the protein.

We have demonstrated the presence of PLCL1 in cervical cytobrush samples using immunocytochemistry, SDS-PAGE, and western blotting and ELISAs. We have developed a method to isolate our cervical cells from the cervical mucus, lyse these cells and quantify PLCL1 using an ELISA.

Our findings demonstrate that PLCL1 is a promising novel protein which could be utilised in the prediction of preterm birth and outcomes of induction of labour. As a susceptibility marker, PLCL1 could be used in conjunction with other markers.

Abbreviations

AA	Arachidonic acid
ARM	Artificial rupture of membranes
ATCH	Adrenocorticotrophic hormone
ATP	Adenosine triphosphate
BMI	Body mass index
BRU	Biomedical Research Unit
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
CAM	Calmodulin
cAMP	cyclic adenosine monophosphate
CAP	Contractile associated proteins
cDNA	copy deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
COX	Cyclooxygenase
CRF	Case report form
CRH	Corticotrophin releasing hormone
C_t	Cycle threshold
CTG	Cardiotocograph
CV	Coefficient of variation
DAG	Diacylglycerol
DDT	DL-Dithyothreitol
DHEAS	Dehydroepiandrosterone sulfate
DNA	Deoxyribonucleic acid
DT	Diffusion tensor
ELISA	Enzyme linked immunosorbent assay
FASP	Fetal anomaly screening programme
fFN	Fetal fibronectin
GBS	Group B Streptococcus
GCP	Good Clinical Practice
GDP	Guanosine diphosphate
GP	General practitioner
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate

HPA	Hypothalamic pituitary axis
IARC	International Agency for Research on Cancer
ICC	Immunocytochemistry
IL	Interleukin
IP_2	Inositol bisphosphate
IP_3	Inositol triphosphate
IP_4	Inositol tetraphosphate
IV	Intravenous
KDa	Kilodaltons
LBC	Liquid based cytology
LLETZ	Large loop excision of transformation zone
mA	Milliamps
ml	Millilitres
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mm	Millimetres
MMP	Matrix metalloproteinases
MPA	Medroxyprogesterone
MRI	Magnetic resonance imaging
mRNA	messenger ribonucleic acid
mV	Millivolts
NaOH	Sodium hydroxide
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NO	Nitric Oxide
OE	Over expression
OXTR	Oxytocin receptor
PAM-G1	Placental alpha macroglobulin 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	Picograms
PG	Prostaglandin
PGDH	Prostaglandin dehydrogenase

PH	Pleckstrin homology
phIGFBP-1	phosphorylated insulin-like growth factor binding protein 1
PIMF	Prediction of Inappropriate Myometrial Function
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKG	Protein kinase G
PLC	Phospholipase C
PLCL	Phospholipase C like
PP1	Protein phosphatase 1
PPP	Phosphoprotein phosphatases
PPROM	Prelabour preterm rupture of membranes
PR	Progesterone receptor
PRIP	Phospholipase C related but catalytically protein
PV	Per vagina
RCOG	Royal College of Obstetricians and Gynaecologists
REC	Research Ethics Committee
RNA	Ribonucleic acid
RNA-seq	Ribonucleic acid sequencing
ROK	rhoA associated kinase
RT-PCR	Reverse transcription polymerase chain reaction
SCJ	Squamocolumnar junction
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SR	Sarcoplasmic reticulum
TBS	Tris buffered saline
TBS-T	TBS - Tween 20
TPM	Transcripts per million
UHCW	University Hospitals Coventry and Warwickshire
UK	United Kingdom
VOC	Volatile organic compounds
µl	microlitres

Chapter 1

1. Introduction

Preterm birth is a major worldwide problem in the field of obstetrics resulting in huge rates of neonatal mortality and morbidity. Due to the complex multifactorial nature of preterm labour, its prediction remains a challenge. Approximately one in five women in the United Kingdom (UK) requires induction of labour for a variety of clinical indications. Again, the outcomes following induction of labour are difficult to predict, with patients often requiring admission to hospital for several days prior to delivery. Both of these areas of obstetrics would benefit greatly from improvements in prediction of clinical outcomes.

This project aimed to ascertain if a new marker identified as important for the onset of labour, whose absence makes the uterus permissive to the onset of labour by uncoupling phospholipase C (PLC) from myometrial contractility via chelation of inositol triphosphate (IP_3), can be utilised as a susceptibility marker for the onset of labour.

1.1 The Uterus:

The female internal genital organs include the vagina, the uterus (including the cervix), the fallopian tubes and the ovaries. The uterus is a thick walled muscular organ with a central cavity. It is approximately 8 x 5 x 3cm in its non-pregnant state. Its walls are 1-2cm thick and surround the endometrial cavity (Bennett & Williamson, 2010). The body of the uterus forms its upper two thirds and includes the fundus, isthmus (internal os) and uterine horns. The body of the uterus lies between the layers of the broad ligament and it is freely movable. The cervix of the uterus forms its inferior lower one third. The wall of the body of the uterus is made up of three layers, the perimetrium (formed from the peritoneum), the

myometrium (consisting of smooth muscle fibres, held together by connective tissue with blood vessels throughout) and the endometrium (Moore & Agur, 2006).

The cervix is largely fibrous and was thought to have smooth muscle encircling the cervix with an outer longitudinal layer (Bennett & Williamson, 2010). More recent magnetic resonance imaging (MRI) studies utilising diffusion tensor (DT) MRI detected the existence of an inner longitudinal layer, extending along the length of the cervix parallel to the cervical canal and outer circumferential fibres which were more prominent in the upper cervix (Nott *et al.*, 2016a).

The uterus is supported by the muscles of the pelvic floor and three sets of ligaments, the pubocervical ligaments, the cardinal ligaments and the uterosacral ligaments. Its blood supply is mainly derived from the uterine artery, a branch of the anterior division of the internal iliac artery. A small component of its blood supply also comes from the ovarian artery via the ovarian ligament. The round ligament extends from the uterus laterally and attached to the pelvic sidewall (Edmonds, 2012).

The function of the uterus throughout the female reproductive life is to provide a site for implantation of an embryo. This is a dynamic process which involves complex interactions between the implanting embryo and the endometrium (Quenby & Brosens, 2013). Once successful implantation occurs the primary function of the uterus during pregnancy is to provide a safe location to protect the growing fetus in a largely quiescent environment.

During pregnancy the uterus increases hugely in both weight and volume (from 10ml to 5 litres at term). The increasing mass of the fetus, placenta, amniotic fluid and uterus pose remarkable haemodynamic, metabolic and mechanical demands on the mother as

pregnancy progresses (Edmonds, 2012). Importantly, as the uterus expands to enable the growth of the fetus it must also remain relaxed with a closed cervix (Edmonds, 2012). During pregnancy the fetus reaches a gestation at which it can survive the extrauterine environment. The uterus must remodel itself during gestation to be able to generate sufficiently forceful contractions to expel the fetus from the uterus during labour (Blanks *et al.*, 2007). This is facilitated by a complex set of structural, biochemical and electrophysical changes that allow the establishment of myometrial contractions which occur in a synchronised fashion and simultaneous cervical ripening and dilatation (Edmonds, 2012). During labour the fundal part of the myometrium contracts, whilst the lower segment of the uterus relaxes towards the cervix and thins out markedly (Terzidou, 2007).

1.2 The Myometrium:

The myometrium consists of smooth muscle bundles and connective tissue. During embryonic development, it is derived from the mesoderm of the urogenital ridge (Blanks *et al.*, 2007). The myometrium can be structured into three layers, the stratum subvasculare, the stratum vasculare and the stratum supravasculare (from innermost to outermost), the boundaries between the three layers are not clearly defined (Weiss *et al.*, 2006). In most organs, the organisation of smooth muscle is directly related the function of the organ. Uterine smooth muscle organisation has been extensively investigated and appears to be disordered although some controversy about this still exists. It has been described that there is an inner circular layer and an outer longitudinally orientated layer (Wray *et al.*, 2015). Weiss *et al*, 2006 utilised MRI DT imaging to investigate the architecture of muscle and connective tissue fibres in the non-pregnant human uterus. This showed that a circular layer occurs around the uterine activity and, at the level of the cervix there is an outer

circular layer and inner longitudinal layer. No further global structure could be identified (Weiss *et al.*, 2006).

Smooth muscle is comprised of cells, 1-5µm in diameter and 200-500µm in length. In the uterus these smooth muscle cells are aggregated into bundles 300µm +/-100µm in diameter (Blanks *et al.*, 2007; Hall & Guyton, 2011). These bundles are delineated by a small amount of connective tissue which is interspaced with microvessels (Blanks *et al.*, 2007). The smooth muscle cells are densely packed with myofilaments and associated dense bodies that occupy 80-90% of the cell volume and constitute the cells contractile machinery (Aguilar & Mitchell, 2010). The uterine contractile machinery is shown in Figure 1.1. The cell membranes are adherent to one another at multiple points so the force generated in one cell can be transmitted to the next. They are also connected by gap junctions that allow the easy passage of ions and action potentials from one cell to the next. This allows the fibres to contract together (Hall & Guyton, 2011). The bundles are then further organised into fasciculi, each is 1-2mm in diameter. The fasciculi are surrounded by a dense collagen matrix and the vasculature of the myometrium (Blanks *et al.*, 2007).

The myometrial smooth muscle is myogenic, the action potentials are produced by the muscle cells without the need for an extrinsic stimulus. The details of the mechanisms that underlie this process are not fully understood, however, the control of the myometrium is intimately connected to ion channel expression. The upregulation and downregulation of a huge variety of ion channels with different properties therefore can dampen or drive the firing of action potentials, in turn, leading to changes in intracellular calcium ion concentrations within the myocytes (Wray *et al.*, 2015).

1.3 Contraction of Uterine Smooth Muscle:

The predominant structural proteins expressed in myometrial smooth muscle cells are actin and myosin. In uterine smooth muscle there is approximately six fold more actin than myosin (Aguilar & Mitchell, 2010). Smooth muscle contraction is dependent upon the interaction of myosin with actin filaments. Actin is a soluble globular protein, at rest 80% is polymerised into filaments. These thin actin filaments form a part of the contractile machinery, they are predominantly composed of α and γ actin (two of 6 isoforms of actin which are expressed in the cell). These actin thin filaments slide along the myosin thick filaments to shorten the cell and cause contraction when the cell is stimulated (Aguilar & Mitchell, 2010). The pivotal event for triggering contraction in uterine smooth muscle is the phosphorylation of myosin light chains (MLC), catalysed by myosin light chain kinase (MLCK) (Mitchell *et al.*, 2013).

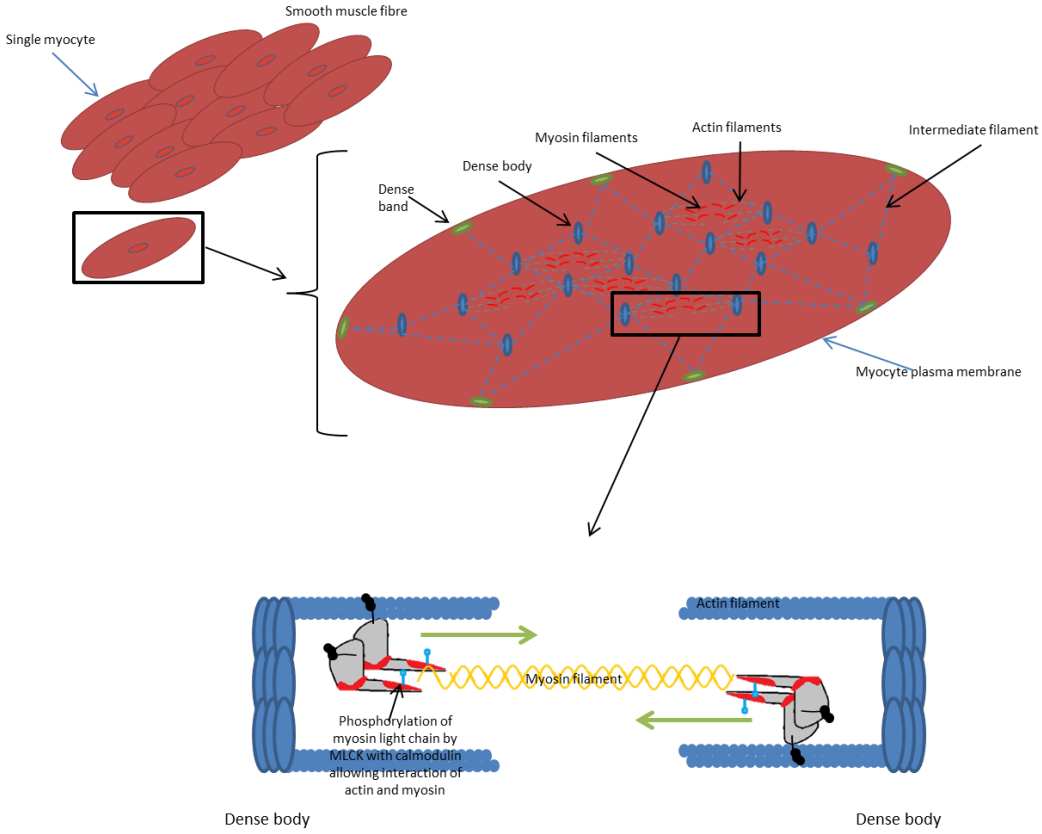

Figure 1.1: Uterine smooth muscle contractile machinery. This demonstrates a single myocyte taken from a smooth muscle fibre. Within it actin filaments can be seen with myosin filaments. These actin filaments are attached to dense bodies which are in turn attached to dense bands at the periphery of the cell. This arrangement is key for myocyte contraction. It demonstrates the interaction of the myosin filaments with the actin filaments following phosphorylation of the serine 19 residue of the myosin light chain by MLCK (itself active due to binding with the Ca^{2+}calmodulin complex).The myosin light chains are shown in red, with the myosin heavy chains in grey and yellow. Following phosphorylation of MLCK, at the expense of an ATP molecule, the head of the myosin protein can move, shortening the distance between the two dense bodies. Adapted from (Aguilar & Mitchell, 2010).

Also demonstrated in Figure 1.1 are the dense bodies and dense bands which form a critical part of the cells contractile machinery. Within each cell they serve to help the contractile

forces to be transmitted along the actin filaments from the poles of the cell to the centre and firmly attach the actin filaments to the cell cytoskeleton (Aguilar & Mitchell, 2010).

Myosin proteins are molecular motors who are defined by their ability to bind with actin and hydrolyse adenosine triphosphate (ATP) (Eddinger & Meer, 2007). Figure 1.1 shows the myosin proteins binding to the actin filaments and their ATPase enzyme activity. Both of these roles are performed by the myosin head.

Phosphorylation of serine 19 of MLC by MLCK is key to both the amplitude and duration of smooth muscle contraction. MLCK itself is activated by calcium ion signalling (Allen & Walsh, 1994; Butler *et al.*, 2013). The reverse, leading to uterine smooth muscle relaxation is mediated by myosin light chain phosphatase (MLCP) (Mitchell *et al.*, 2013). The balance between active MLCK and MLCP determines the phosphorylation of MLC and consequently myometrial contraction or relaxation as described (Blanks *et al.*, 2007). Furthermore, MLCK can be phosphorylated both at serine 19 and threonine 18. Threonine 18 phosphorylation may sustain contractile force by reducing the rate of dephosphorylation (Butler *et al.*, 2013).

It has been suggested that phosphatases have a role in maintaining uterine quiescence for the duration of pregnancy and that mechanisms controlling phosphatase activity could be involved in the switch in phenotype that occurs in the labouring myometrium (Butler *et al.*, 2013). There are three known families of serine/threonine phosphatases, one of which is the phosphoprotein phosphatases (PPP). Only this family have a role in smooth muscle contraction. There are seven known phosphatases in the PPP family including PP1, PP2A, PP2B, PP4, PP5, PP6 and PP7. Currently only the first three have characterised roles in smooth muscle contraction (Butler *et al.*, 2013).

Protein phosphatase 1 (PP1) consists of the 35-38KDa catalytic subunit known as PP1C. There are several isoforms of PP1C. This subunit can bind to more than 100 regulatory subunits which modulate PP1C's activity and locate it within the cell. All myosin phosphatase enzymes contain a PP1 catalytic subunit and a regulatory subunit. MP-MYPT1 is the major smooth muscle phosphatase, it is composed of a PP1 catalytic subunit, an MYPT1 regulatory subunit and an M20 subunit. It controls contractile amplitude and duration through MLC dephosphorylation (Butler *et al.*, 2013).

Myocyte intracellular Ca^{2+} levels are regulated by a variety of ion channels. In uterine smooth muscle, a rise in intracellular Ca^{2+} concentration is important in the signalling pathway leading to contraction. Ca^{2+} concentration at rest is maintained at 10^4 times greater in the extracellular compartment when compared to the intracellular compartment (Aguilar & Mitchell, 2010). Uterine agonists including oxytocin, prostaglandin F2α, prostaglandin E2 and endothelin-1 bind to G-protein coupled receptors (GPCRs) in the myocyte plasma membrane. These GPCRs contain $G_{\alpha q}$ or G_{11} subunits. In the myocyte, bindings of these agonist to their receptors enables $G_{\alpha q}$ to stimulate phospholipase Cβ to act on its substrate phosphatidylinositol 4,5-bisphosphate (PIP_2), hydrolyse this into free IP_3 and membrane bound diacylglycerol (DAG), two second messengers. IP_3 can then bind with a receptor on the sarcoplasmic reticulum (SR) (IP_3 gated Ca^{2+} channels) causing the release of intracellular Ca^{2+} ions. SR release of Ca^{2+} stimulates calcium sensitive plasma membrane conductance, which in turn then activates voltage gated Ca^{2+} entry.

Calmodulin (CAM) is a 16 KDa EF hand protein which is the activated by the binding of four Ca^{2+} ions (Blanks *et al.*, 2007), this complex can then activate MLCK (in addition to other proteins) by binding to the activation site and the subsequent phosphorylation of serine 19

on the MLC. This activated MLC can then activate the pathway previously described leading to smooth muscle contraction (Aguilar & Mitchell, 2010).

Although an increase in intracellular Ca^{2+} is a major contributor to the control of smooth muscle contraction, hormones can also enhance contractile activity without directly increasing intracellular Ca^{2+} levels (Berridge, 1993; McKillen *et al.*, 1999; Word, 1995).

In addition, there are Ca^{2+} independent pathways which can regulate smooth muscle contraction. One pathway involves the GPCR $G_{\alpha 12\text{-}14}$ subunit which when it becomes activated in turn activates the small monomeric G protein rhoA-GDP. Activation of rhoA-GDP leads to the exchange of GTP for GDP and results in an activated rhoA-GTP which can then in turn activate rhoA associated kinase (ROK). ROK can then targets the substrate binding subunit of the MLCP which then inhibits the dephosphorylation of myosin light chain promoting smooth muscle contraction (Mitchell *et al.*, 2013). This is the likely means of calcium sensitisation of the contractile machinery of the uterine myocyte (McKillen *et al.*, 1999).

1.4 Relaxation of Uterine Smooth Muscle:

G-protein coupled receptors are key for uterine relaxation, the most important subunit being $G\alpha_s$. Activation of $G\alpha_s$ by β-adrenergic agonists (e.g terbutaline) leads to activation of adenylate cyclase which converts ATP to cyclic adenosine monophosphate (cAMP). Cyclic AMP then acts as a second messenger, activating protein kinase A (PKA). PKA phosphorylates MLCK at the Ca^{2+}CAM binding site hampering activation of MLCK (Word, 1995). PKA has other roles, it increases the activity of the Ca^{2+}ATPase in the plasma membrane and SR leading to a reduction in intracellular calcium concentrations and it can

also phosphorylate MLCP at a site adjacent to the ROK binding site so that it is no longer a target for ROK (Mitchell *et al.*, 2013).

The nitric oxide-cyclic guanosine monophosphate (cGMP) relaxation pathway has been demonstrated to be present in the human uterus (Buhimschi *et al.*, 1995). Nitric oxide (NO) can directly enter the myocyte by diffusion across the plasma membrane and activate guanylate cyclase to produce cGMP. Cyclic GMP can then activate protein kinase G (PKG). PKG also increases the activity of Ca^{2+}ATPase and can phosphorylate MLCP rendering it insensitive to ROK (Mitchell *et al.*, 2013).

1.5 The Cervix:

The cervix is a firm cylindrical structure which is composed predominately of connective tissue. The majority of the connective tissue is composed of collagen fibres embedded in proteoglycans. The firmness of the cervix is the result of the presence of collagen, both type I and type III. It is thought that the directionality of the collagen fibres may help to determines its ability to withstand the huge forces which are placed upon it during pregnancy (Nott *et al.*, 2016b). A small proportion of the cervix is composed of smooth muscle and fibroblast cells (10-15%) (Norman, 2007; Nott *et al.*, 2016b). Elastin is also found in the cervical stroma, however the majority of this is found in the vessel walls.

The cervix is formed from the lower third of the uterus with the tissues of the cervix being continuous with those of the uterus. It protrudes through the anterior wall of the vagina into its superior portion. It has a supravaginal (known as supravaginalis) and a vaginal portion (known as portio vaginalis). The vaginal section appears as a convex disc-shape, with the external os located in the centre of this. This is the area through which the vagina

communicates with the endocervical canal. The cervix is located in the true pelvis, posterior to the base of the bladder and anterior to the rectum. It is held in place by the uterosacral and cardinal ligaments. The cervix receives its principal blood supply from the branches of the uterine artery (Nott *et al.*, 2016b).

The cervix can be divided into the endocervix and the ectocervix. The endocervix is the luminal cavity within the cervix which forms the passageway between the external os and the internal os. The upper limit of the endocervix marks the transition to the endometrium. The endocervix is covered with simple single layer of mucus secreting columnar epithelium as shown in Figure 1.2.

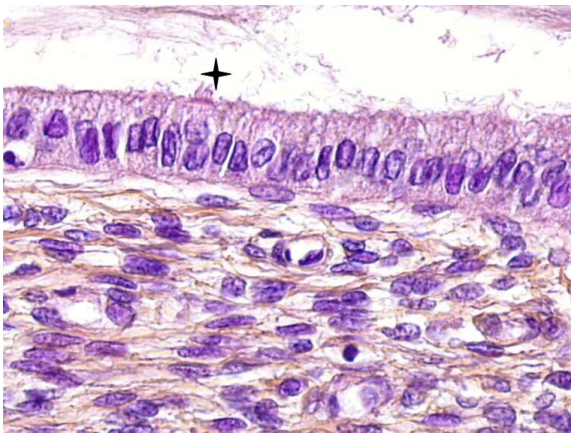

Figure 1.2: Histology of the normal endocervix. There is a single layer of simple columnar epithelium covering the endoocervix (International Agency for Research on Cancer). Reproduced with permission from IARC, available at http://screening.iarc.fr/atlashisto.php

The ectocervix is the portion of the cervix extending into the vagina. This is covered by squamous epithelium and is composed of multiple layers as shown in Figure 1.3

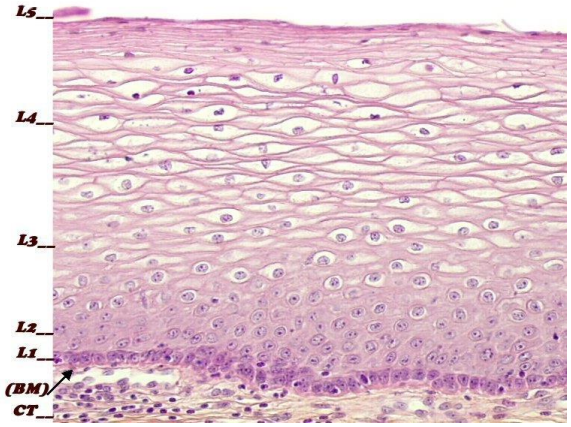

Figure 1.3: Histology of the normal ectocervix. Multiple layers including connective tissue (CT), the basement membrane (BM) and several layers (L) of squamous epithelium. L1 represents the basal cells, L2 the para basal cells, L3 the approximately 8 layers of intermediate cells, L4 the layers of superficial cells and L5 the exfoliating cells(International Agency for Research on Cancer). Reproduced with permission from IARC, available at http://screening.iarc.fr/atlashisto.php

The junction between the squamous and columnar epithelium is known as the squamocolumnar junction (SCJ) and is the location of most epithelial diseases of the cervix (Stevens & Lowe, 2000). The location of the SCJ varies dependent upon age and hormonal status. At birth, the SCJ is located close to the external os, during the reproductive years, it moves out to the ectocervix and then postmenopausally it retracts backwards into the endocervix. Eversion of the SCJ onto the ectocervix is called an ectropion. After puberty, the pH of the vagina becomes acidic and the exposure of these areas of the columnar epithelium to this acidic environment leads to metaplasia within the ectropion. These cells become squamous epithelium and a new SCJ is formed. The transformation zone is the area between the original SCJ and the new SCJ when the columnar epithelium has been changed. The transformation zone is the site of > 90% if precancerous cells.

The cervix is vital in the maintenance of pregnancy and the timely delivery of the fetus, throughout pregnancy it must remain closed despite multiple forces which are acting upon it (Nott *et al.*, 2016b).

1.6 Labour:

In humans, parturition is the result of complex interactions between many maternal, placental and fetal factors. These interactions lead to a change in the uterus and cervix, from the quiescent uterus of pregnancy, to a uterus which contracts in a coordinated fashion, leading to cervical dilatation and expulsion of the fetus into the world (Challis, 2000). Some of these interactions are shown in Figure 1.4. It is likely that labour is initiated by a variety of mechanisms, each contributing to the onset of labour (Smith, 2007). The regulation of uterine activity during pregnancy and labour has traditionally been divided into four physiological phases. Phase 0 during which the uterus is in a state of quiescence, phase 1 during which the uterus undergoes activation. Phase 2 during which the uterus is stimulated by uterotonics and finally phase 3 in which the uterus involutes following delivery (Norwitz *et al.*, 1999).

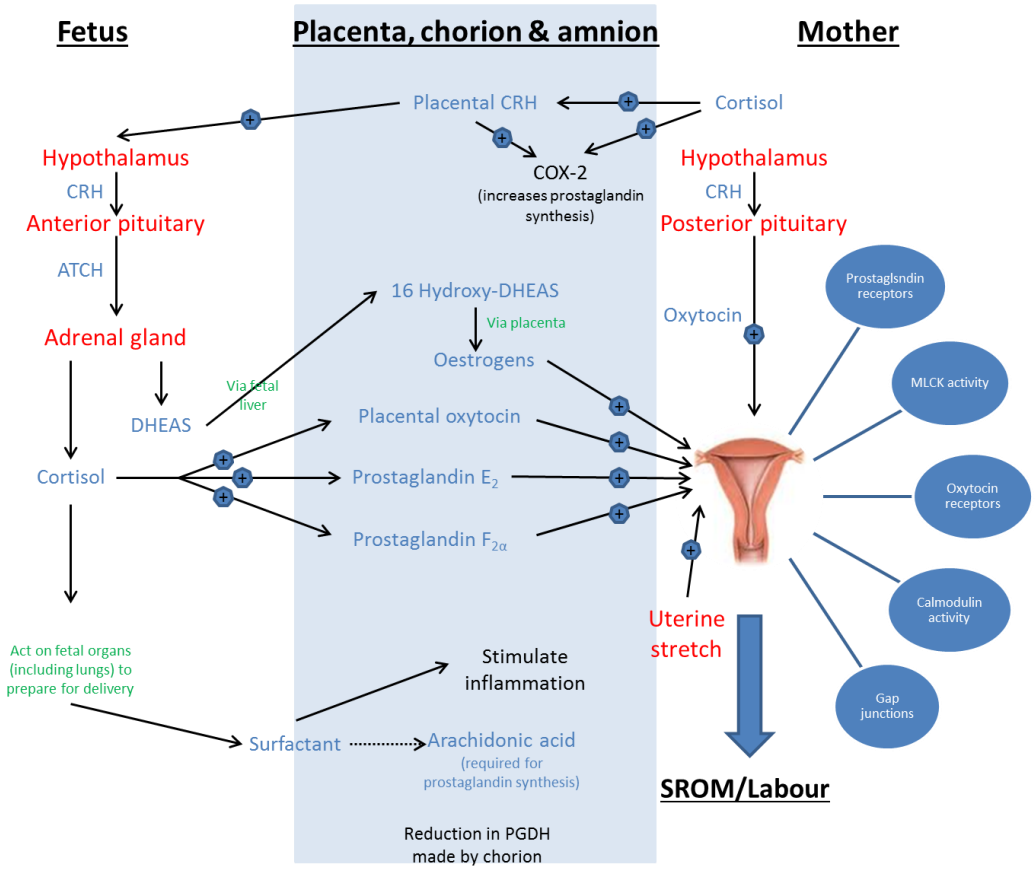

Figure 1.4: Some of the proposed mechanism of activation of labour: The three components (maternal, fetal membranes and placenta, and fetus) are thought to interact and play roles in the initiation of labour via the release of hormones and paracrine and autocrine factors. The order in which the events occur is likely to vary from patients to patient. Activation of the fetal HPA axis as the fetus matures leads to the release of CRH and ACTH from the fetal hypothalamus and anterior pituitary gland respectively. The resulting action on the fetal adrenal glands causes the release of fetal cortisol and DHEAS. Cortisol acts on the fetal tissues including the fetal lungs promoting maturation. DHEAS is metabolised by the placenta, producing oestrogen. These oestrogens can they act on the myometrium promoting the changes required for the uterus to change form the quiescent uterus of pregnancy to the contracting and excitable uterus of labour. Cortisol also acts of the placenta, leading to increased synthesis and release of oxytocin, prostaglandins and placental CRH. The maternal posterior pituitary gland also begins to synthesise more oxytocin Adapted from (Norwitz et al., 1999).

1.6.1 Initiation of labour:

Uterine contractions occur throughout gestation but four important parameters of these contractions change with the onset of labour; frequency, amplitude, duration and the direction of propagation (Aguilar & Mitchell, 2010). Overall, the process of initiation of labour is not well understood. There has been slow progress in the understanding of the biochemical mechanisms involved in the process of parturition in humans, in part due to difficulty in extrapolating from the endocrine-control mechanisms in various animals to the paracrine and autocrine mechanisms of labour in humans (Norwitz *et al.*, 1999). However, there are multiple factors which have been studied and are understood to individually promote the process of labour. It is likely that many of these factors are required to interact prior to the onset of labour. Some factors are illustrated in Figure 1.4. Independent of the trigger(s) of labour, the final pathway results in the development of coordinated uterine contractions and dilatation of the cervix in response to these.

For the baby to survive in the external environment, it would be logical for the fetus to be in control of initiating the process of labour, so that when it is ready to be born, it signals to the mother to go into labour. In normal labour there appears to be a time dependent relationship between uterine contractions, changes in the cervix and uterus (particularly myometrium) and cervical dilatation. Biochemical changes in the cervix and activation of the uterus precede uterine contractions, which in turn precede cervical dilatation (Snegovskikh *et al.*, 2006). For labour to occur, the factors which are maintaining uterine quiescence need to be removed and factors promoting uterine contractility recruited.

As gestation progresses, the uterus is placed under increasing tension, this may be one factor which contributes to the onset of parturition. Evidence which supports this includes,

the average onset of labour at earlier gestations in multiple pregnancies and in pregnancies with polyhydramnios (Smith, 2007).

The fetal hypothalamic-pituitary-adrenal (HPA) axis has been studied and has been demonstrated to be an important factor which contributes to the initiation of labour. As illustrated in Figure 1.4, the activation of the fetal HPA axis results in an increase in fetal adrenocorticotrophic hormone (ACTH). This ACTH stimulates the fetal adrenal gland to release dehydroepiandrostenedione sulfate (DHEAS), the fetal DHEAS is converted in the fetal liver to 16-hydroxy-DHEAS which then travels to the placenta and is metabolised into oestrogens. These oestrogens promote myometrial changes including increasing the number of prostaglandin receptors, oxytocin receptors, gap junctions, and increases in MLCK and calmodulin activity (Snegovskikh *et al.*, 2006). The oestrogens have also been suggested to augment uterine contractility by antagonising the progesterone receptors (PR) functions (Mendelson, 2009).

Activation of the fetal HPA axis also leads to an increase in cortisol production from the fetal adrenal glands. The rising concentration of fetal cortisol induces maturation of the fetal tissues including the fetal lungs. This in turn leads to the production of surfactant by the fetal lungs which can enter the amniotic fluid. It is thought that surfactant protein may stimulate inflammation in the fetal membranes, cervix and myometrium, contributing to the onset of labour (Smith, 2007). This concept that fetal surfactant provides a signal for the initiation of labour provides a link between fetal lung maturation which is essential for extra-uterine life and the onset of labour. Surfactant is also a source of arachidonic acid (AA) which is required for prostaglandin synthesis (Edmonds, 2012). Furthermore, cortisol can promote the expression of oxytocin and prostaglandins (PG), particularly prostaglandin E_2 (Snegovskikh *et al.*, 2006).

Placental corticotrophin releasing hormone (CRH) has been studied extensively and is understood to be a further factor which can contribute to the onset of labour. As term approaches there is an increase in the synthesis of CRH by the placenta. Cortisol causes this increase in the release of placental CRH which positively feeds back on the fetal HPA axis, stimulating further ACTH release from the fetal pituitary gland (Snegovskikh *et al.*, 2006). Increasing cortisol and CRH stimulate cyclooxygenase 2 (COX-2), leading to an increase in prostaglandins. Additionally CRH has been shown to induce the secretion of matrix metalloproteinases (MMPs) which play a role in the onset of labour via degradation of the extracellular matrix in the placenta and fetal membranes (Li & Challis, 2005)

Throughout pregnancy the chorion produces the enzyme prostaglandin dehydrogenase (PGDH) which inactivates prostaglandins. Late in pregnancy the production of this enzyme by the chorion falls. Therefore the underlying decidua, cervix and myometrium can be exposed to the increasing levels of prostaglandins, in particular PGE_2 (Johnson *et al.*, 2004). PGE_2 can also release metalloproteinases which can weaken the fetal membranes (Smith, 2007).

In most animals, withdrawal of progesterone from the maternal circulation is key to the onset of labour. However, in humans there is evidence against this mechanism of onset of labour as there is not a decline in maternal serum progesterone at the time of parturition, progesterone levels only decrease in the maternal circulation after delivery of the placenta (Edmonds, 2012; Mitchell *et al.*, 2013). Nevertheless, mifepristone (a progesterone receptor antagonist) is used to initiate labour in women undergoing second trimester termination of pregnancy and proves successful. Progesterone antagonists also appear to universally increase myometrial responsiveness to uterotonics, therefore supporting the

view that progesterone withdrawal must play a role in the onset of labour (Blanks & Brosens, 2012).

There are many known isoforms of the progesterone receptor expressed in the human. When progesterone binds to its receptors in the cytoplasm of the myocyte, it can influence cellular function by both modulating gene expression and by affecting intracellular signalling cascades. Progesterone promotes uterine relaxation throughout gestation. Mechanisms by which this is achieved include modulation of the expression of the contractile associated proteins, reduction in myometrial responsiveness to prostaglandins (as demonstrated by the administration of progesterone synthesis inhibitors increasing the effectiveness of exogenous prostaglandins as observed in second trimester termination of pregnancy), and inhibition of connexin-43 expression (Mesiano *et al.*, 2011).

There are several models suggested for how this relaxatory effect of progesterone is inhibited during the onset of labour without a decline of circulating progesterone levels being detectable. The leading hypothesis is that there is a functional withdrawal of progesterone, whereby myometrial cells become refractory to the effects of progesterone-progesterone receptor mediated relaxation. This has been proposed to occur by changes the expression of different isoforms of the progesterone receptor (in particular an increase in PR-A, when compared to PR-B), or by changes in progesterone coregulatory protein levels (Mesiano *et al.*, 2011).

1.6.2 Changes in the Uterus Prior to the Onset of Labour:

It is generally accepted that prior to the onset of labour in humans the myometrium undergoes a process of “activation” (Challis, 2000), whereby the muscle becomes both

more excitable and more susceptible to stimulation by procontractile hormones. To enable coordinated contraction of the uterus and good propagation of action potentials, upregulation of a variety of contractile associated proteins (CAP) are required to initiate powerful uterine contractions (Smith, 2007). These CAPs enhance the interaction between myosin and actin. For myosin and actin to interact, actin must be converted from its globular form to a filamentous form, it also must be attached to the myocytes cytoskeleton.

These CAPs result in an increase in the excitability of myometrial cells. With the onset of labour the distribution and function of several ion channels are altered to lower the intensity of the stimulus required to depolarise each myocyte, therefore lowering the threshold required to lead to calcium ion influx and ultimately generate myocyte contraction (Arrowsmith *et al.*, 2014; Smith, 2007). Prior to the onset of labour the resting membrane potential becomes less hyperpolarised (from -70mV in pregnancy to -45mV at parturition) (Parkington *et al.*, 1999) and inhibitory pathways that stimulate cAMP-PKA begin to decrease (Arrowsmith *et al.*, 2014; Challis, 2000). A number of potassium channels have been demonstrated to play a role in the myocyte resting membrane potential (Brainard *et al.*, 2007). McCloskey *et al* demonstrated the role of Kir7.1 as a key channel which contributes to maintaining a hyperpolarised membrane potential in the mid-trimester, the expression of this ion channel is then downregulated towards the end of pregnancy (McCloskey *et al.*, 2014).

Thirdly, prior to the onset of labour there is an increase in intercellular connectivity to aid in the development of synchronous contractions (Smith, 2007). This is important as strong coordinated contractions are required during labour, but also periods of rest in between contractions are needed during labour to allow the fetus to receive an adequate blood

supply and so fetal oxygenation is not impaired. It is essential that each myometrial cell is in close communication with its neighbour, with individual cells electrically coupled to allow currents to flow between them. In the myometrium, a group of proteins called connexins play an important role in the onset of labour. These proteins allow ionic coupling and the movement of certain metabolites between adjacent cells. The density of these proteins is a factor which governs the excitability of the uterus with an increased density prior to the onset of labour (Garfield *et al.*, 1977). In particular, connexins are upregulated before the onset of both term and preterm labour (Tabb *et al.*, 1992) and of particular importance in the myometrium are connexin-43, connexin-26, connexin-40 and connexin-45 (Blanks *et al.*, 2007). It has been demonstrated that a mutation in connexion-43 leads to a reduction in the force of myometrial contractions and impairment of myometrial responses to oxytocin (Tong *et al.*, 2009).

Simultaneous to these changes in CAPs, many cell surface receptors are also upregulated and downregulated. Many of these proteins are GPCRs which can then activate effector enzymes.

Both term and preterm labour are associated with an inflammatory response. This is evidenced by an increase in infiltration of macrophages and neutrophils into the myometrium, cervix and fetal membranes and an increase in proinflammatory cytokines in the amniotic fluid with the onset of labour. This proinflammatory invasion leads to the activation of NF-κB, which itself then leads to the upregulation of a variety of genes important for myometrial contractility. This includes the prostaglandin $F_{2\alpha}$ receptor (PTGFR), connexin-43, the oxytocin receptor and COX-2 (Mendelson, 2009).

1.6.3 Uterine Stimulants and Relaxants:

There are several uterine stimulants which play important roles in the onset and maintenance of labour, in particular oxytocin and prostaglandins. There are also several uterine relaxants which are often utilised in clinical practice although their efficacy is low.

Oxytocin is a nine amino acid neuropeptide produced by the magnocellular neurones of the hypothalamus and is stored in the neurohypophysis of both the mother and the fetus. It is produced as a propeptide and then undergoes a variety of modifications (Arrowsmith & Wray, 2014). It is also produced by the maternal decidua, the amnion and the chorion in the human (Chibbar *et al.*, 1993). The action of oxytocin on myometrial smooth muscle cells is mediated thorough the activation of the oxytocin receptor (OXTR). This is a G-protein coupled receptor is coupled to both $G_{\alpha q/11}$ and $G_{\beta\gamma}$ and activates several isoforms of phospholipase C-β (PLC-β). This signalling cascade then progresses as described leading to myometrial contractions (Blanks *et al.*, 2007). There are also understood to be many other processes activated as a result of oxytocin binding to its receptor. The myometrium's increased sensitivity to oxytocin with the onset of labour is mediated by an increase in the density of the OTR particularly in the fundal region of the uterus. This increased density occurs in both preterm and term labour. The mechanism by which it occurs is thought to be multifactorial (Fuchs *et al.*, 1984; Kimura *et al.*, 1996).

Eicosanoids are signalling lipid molecules which act locally and are derived from the cell membrane. They are synthesised from the oxidation of 20-carbon unsaturated fatty acid chains, mainly arachidonic acid. There are four major groups of eicosanoids; prostaglandins, prostacyclins, thromboxane and leucotrienes. Eicosanoid metabolism is complex. Simply, glycerophospholipids are released from the plasma membrane by the

action of the enzymes phospholipase A_2, or phospholipase C or D and diacylglycerol lipase (Rang & Dale, 2012), this ultimately leads to the release of arachidonic acid. A variety of different enzymes can then act on arachidonic acid, producing different eicosanoids. The prostaglandin H2 synthase (PGH_2 synthase) complex has both cyclooxygenase (COX) and peroxidase activity. There are two forms of COX activity in humans, COX-1 (PGHS-1) and COX-2 (PGHS-2). PGH_2 synthase can act on arachidonic acid producing prostaglandin H_2 (PGH_2) which can then be converted into a variety of prostaglandins including $PGF_{2\alpha}$ and PGE_2 (Bennett & Williamson, 2010). Different prostaglandins are named using a letter which relates to their specific ring structure. A variety of prostaglandins have actions on the uterus and cervix during pregnancy and labour. The endometrium and myometrium produce a variety of prostaglandins both in the non-pregnant and pregnant state. The sensitivity of the uterus to prostaglandins increases throughout gestation (Rang *et al.*, 2003). In obstetric practice, several synthetic prostaglandins are used to induce labour and promote uterine contractions, these include dinoprostone (PGE_2), carboprost (15-methyl $PGF_{2\alpha}$) and gemeprost or misoprostol (PGE_1 analogues).

There are four principal prostaglandins produced in the human body, PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 (prostacyclin). They are produced throughout the body, with each cell type usually producing one or two dominant products (Ricciotti & FitzGerald, 2011). Prostaglandin synthesis and the different receptors each product of the pathway can bind to are shown in Figure 1.5.

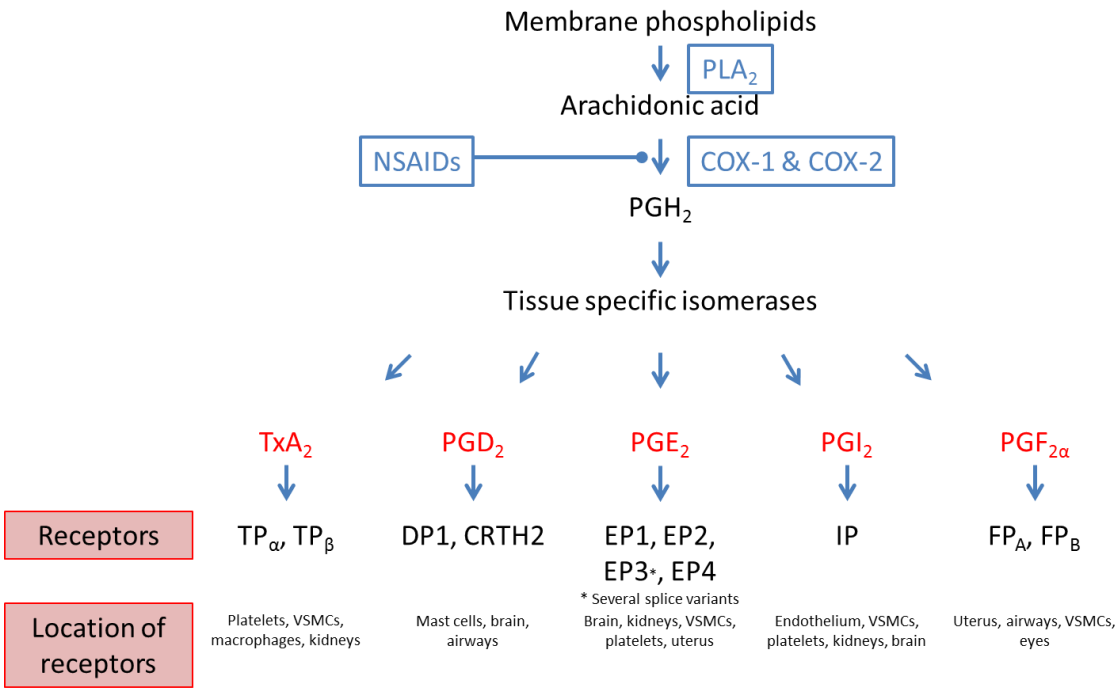

Figure 1.5: The biosynthetic pathway of the prostaglandins and the different receptors each of the prostaglandins can bind to. Adapted from (Ricciotti & Fitzgerald, 2011).

There are known to be seven prostaglandin receptors in the human body. There are four PGE_2 receptors known as EP1, EP2, EP3 and EP4, there is one PGF receptor, FP (also known as PTGFR), one PGD receptor, PD1, and one PGI receptor, IP. All of these receptors have distinct biochemical properties, locations in the body and differential affinities for their ligands (Blesson & Sahlin, 2014).

The actions of prostaglandin E_2 (PGE_2) on the myometrium are complex, principally due to the variety of PGE_2 receptors expressed. Additionally alternate splicing of the mRNA encoding these proteins, particularly of the c-terminus, results in the activation of different signal transduction pathways with differently spliced transcripts. Generally, EP1 is coupled to $G_{\alpha q}$ and EP3 to $G_{\alpha i}$ and are stimulatory leading to Ca^{2+} release via IP_3 signalling and adenylate cyclase inhibition respectively. EP2 and EP4 are generally relaxatory. Studies

have looked at the differential expression of these receptors throughout the uterus and have suggested that EP2 is the predominant PGE_2 receptor expressed in the lower segment of the uterus and hence the action of PGE_2 on the lower segment of the uterus would be predominately relaxatory, as is required for labour to progress (Blanks *et al.*, 2007).

Activation of PTGFR by binding of $PGF_{2\alpha}$ is associated with labour at term. The binding of $PGF_{2\alpha}$ increases the intracellular calcium ion levels by stimulating the release of stored calcium ions and it also increases the expression of matrix metalloproteinases. Recent studies have also demonstrated that the binding of $PGF_{2\alpha}$ to its receptors also promotes the expression of many of the proteins required for activation of the uterus prior to the onset of labour and include connexion-43, the oxytocin receptor and PTGS-2 (Xu *et al.*, 2013).

Clinically there is huge interest in uterine relaxants known as tocolytics, principally for the prevention of preterm birth but also for the management of intrapartum fetal distress, impaired fetal growth and to help with external cephalic version at term. Those in current use include beta-agonists, calcium channel blockers, oxytocin receptor antagonists, prostaglandin synthase inhibitors, nitric oxide donors and magnesium sulphate (Royal College of Obstetricians and Gynaecologists, 2011a).

Selective β2 agonists including salbutamol and terbutaline mediate their action on the uterus through the β2 adrenergic receptors. Activation of these receptors leads to release of $G_{\alpha s}$. This in turn can then increase GTP dependent adenylate cyclase activity. Adenylate cyclase converts ATP to cAMP which can then activate PKA to act on many downstream targets and cause relaxation (Dokhac *et al.*, 1986).

Calcium channel blockers such as nifidipine relax the uterus by inhibiting L-type calcium channels and therefore voltage gated Ca^{2+} entry. Oxytocin receptor antagonists such as atosiban, competitively bind to the oxytocin receptors but do not activate it therefore inhibiting the action of oxytocin. Prostaglandin synthase inhibitors (cyclooxygenase inhibitors) such as indomethacin are mainly used following cervical cerclage. Their use is limited to the second trimester due to their effect on the fetal heart and kidneys. They inhibit prostaglandin synthesis and therefore inhibit uterine contractile activity. Nitric oxide can diffuse across the myocyte cell membrane and activate guanyl cyclase, leading to the activation of PKG as discussed. Finally, magnesium sulphate can act via extracellular and intracellular pathways to decrease intracellular calcium ion concentration (Mattison, 2013).

Despite the wide variety of tocolytics available, there are currently no high quality evidence that tocolytics improve outcomes in patients who present in preterm labour, and, therefore current guidance is that it is reasonable not to give tocolytics drugs to patients who present in preterm labour except in the circumstances to prolong gestation to allow administration of antenatal corticosteroids to promote fetal lung development, or allow transfer of a patient to a healthcare unit which has the facilities to care for preterm babies (Royal College of Obstetricians and Gynaecologists, 2011a).

1.7 Cervix in Pregnancy:

In addition to the importance of contraction of the upper uterine segment, relaxation of the lower uterine segment with dilatation of the cervix is key to a successful vaginal delivery. As discussed, the cervix is principally composed of connective tissue and a small amount of smooth muscle (Granström *et al.*, 1989). Biochemical changes in the cervix need to occur prior to the onset of uterine contractions. In preparation for the onset of labour

the cervix is remodelled throughout pregnancy in processes called cervical softening and prior to parturition the cervix undergoes ripening. Cervical softening refers to the changes in the biomechanical properties of the cervix occurring throughout gestation. Ripening refers to the changes in the cervix as a load bearing structure, this is clinically identified using the Bishop Score (this is discussed in further detail in Chapter 4 Section 4.3 and 4.4) (House *et al.*, 2009). Overall, prior to the onset of labour the cervix changes from a tightly closed structure which is maintaining an intrauterine pregnancy to a soft organ capable of dilating to accommodate the delivery of a baby. Ekman *et al*, 1986 demonstrated that disturbances in the processes of cervical softening and ripening cause problems with dysfunctional and protracted labour highlighting the importance of these changes prior to the onset of labour (Ekman *et al.*, 1986).

The structural integrity of the cervix is an important requirement for a normal pregnancy and delivery of a fetus at term. The cervix maintains its structure throughout pregnancy despite the vast changes and growth which occur in the uterus (House *et al.*, 2009). During pregnancy the cervix starts as rigid tubular structure and then throughout gestation is remodelled into a structure which has the ability to soften, thin out and has the capability to dilate to allow parturition. The process of cervical remodelling involves a change in the turnover of the extracellular matrix and reorganisation of the collagen network (Sennström *et al.*, 2000). There are concomitant increases in cervical stromal hydration, changes in concentration of hyaluronic acid and dermatan sulphate, extravasation of leucocytes and activation of cervical fibroblasts (Ghulé *et al.*, 2012). The most common collagens found in the cervix are fibrillary collagen type 1 and type 3. These changes in the collagen network that occur prior to the onset of labour are associated with both a reduction in collagen concentration (by up to 50%) and an increase in collagen solubility (80-90% solubility at term). Proteoglycans decrease by 50% in pregnancy. Their role is in stabilising the collagen

fibres, therefore a reduction in proteoglycans further aids in the disorganisation of the collagen fibres. Hyaluronan (a polysaccharide component of the cervix) increases in concentration in the third trimester, this increases hydration of the cervix and also increases the production of proinflammatory cytokines (House *et al.*, 2009). The proinflammatory cytokines can facilitate an increase in metalloproteinases which can then degrade collagen (Smith, 2007).

Synthetic prostaglandins are widely used to ripen the cervix in the process of induction of labour (this is discussed in more detail in Chapter 4). Studies on first trimester cervical biopsies have suggested that prostaglandins lead to cervical remodelling predominately by causing disorganisation of the collagen network and vasodilation. Ghulé *et al*, 2012, studied the effect of synthetic PGE_1 (misoprostol) on the cervix. Cervical biopsies were taken from patients undergoing first trimester medical termination of pregnancy prior to administration of misoprostol. A second sample was taken approximately 90 minutes following misoprostol administration (Ghulé *et al.*, 2012). This study demonstrated that PGE_1 leads to increased expression of tight junction proteins in the cervix. They hypothesised this increase could mediate cervical remodelling by altering the paracellular permeability (Ghulé *et al.*, 2012).

1.8 From Basic Science to Clinical Practice:

The normal physiological processes involved with the onset of human parturition have been discussed. Deviation from these normal physiological processes can lead to several clinical problems including the onset of labour prior to fetal maturity or continuation of pregnancy past 42 weeks.

1.8.1 Preterm Labour and Mid-trimester Pregnancy Loss:

Preterm birth and mid-trimester pregnancy loss are complex multifactorial syndromes that have multiple clinical subtypes (Eidem *et al.*, 2015). In both preterm labour and mid-trimester pregnancy loss there is an asynchrony between the labour process and fetal maturation (Challis, 2000). Inappropriate myometrial function is a major contributor to preterm labour and mid-trimester pregnancy loss. It is caused by several disease processes which activate one or more of the common pathways described previously, required for the onset of labour in patients who deliver preterm (Romero *et al.*, 2014).

Miscarriage can be defined as spontaneous loss of pregnancy before the fetus reaches viability. 1-2 % of second trimester pregnancies (12-23 weeks) miscarry before viability (Royal College of Obstetricians and Gynaecologists, 2011b). There are several risk factors for miscarriage including maternal age, number of previous miscarriages, acquired thrombophilia (antiphospholipid syndrome), congenital uterine anatomical abnormalities, fetal and parental chromosomal abnormalities, cervical weakness, infections such as bacterial vaginosis, endocrine disorders (thyroid disease and diabetes mellitus) and many inheritable thrombophilias including deficiencies in antithrombin, protein C and protein S, and abnormal procoagulant factors including factor V Leiden and prothrombin (Robertson *et al.*, 2006; Royal College of Obstetricians and Gynaecologists, 2011b).

In the UK, over 52000 babies (7.3%) were born prematurely in 2012 (The National Institute for Health and Care Excellence, 2015) and this accounts for up to 70% of infant deaths, posing a serious clinical problem. Preterm babies suffer from a high rate of mortality and morbidity, and those babies who manage to survive have an increased incidence of severe handicap. The major long term consequence is neurodevelopmental disability (The

National Institute for Health and Care Excellence, 2015). Additionally, preterm labour contributes to massive emotional, social and economic costs. Improving therapies for preterm labour is especially important as the neonatal survival rate and quality of health of babies born prematurely improves dramatically for each week spent *in utero* beyond 23 weeks gestation. Previously survival rates at 23 weeks were quoted at approximately 8%, whereas at 28 weeks this survival rate increased to approximately 74%, an improvement in survival of 3% per day (Challis, 2000). Survival rates are improving further with improvement in care with time. There has been no decline in the preterm birth rate in the UK over the last 10 years, however, there has been an increased rate of survival for extreme preterm births (the rates of disability remain unchanged)(The National Institute for Health and Care Excellence, 2015).

Current tocolytic therapies designed to prevent preterm labour by targeting the contractile mechanisms of the myometrial smooth muscle cells exhibit relatively poor efficacy. One problem with the current clinical trial data is that there is a low rate of preterm labour in women randomised. This leads to difficulty showing any benefit from tocolysis. This problem is not helped by the lack of clinical tests which can accurately predict preterm labour. These tocolytic therapies could be considered to be trying to assess the symptoms and not the underlying cause(s) that activate the labour process (Romero *et al.*, 2014).

This inability to predict preterm labour and delivery itself is also a huge clinical problem for many reasons and leads to numerous unnecessary hospital admissions.

Approximately 75% of women delivering preterm do so following the onset of preterm labour (with or without preceding preterm prelabour rupture of membranes) with the remaining 25% of women delivering preterm iatrogenically for fetal or maternal indications

(The National Institute for Health and Care Excellence, 2015). Women who have experienced either preterm labour, delivery of a low-birth weight infant, second-trimester pregnancy loss or first-trimester pregnancy termination are at increased risk of subsequent preterm labour. The recurrence risk increases with the number of prior preterm labours. The recurrence risk of preterm delivery ranges from 15-50%, dependent upon both the number of previous preterm deliveries and the gestational age at which the previous deliveries occurred (Goldenberg *et al.*, 2008; McManemy *et al.*, 2007). Women with an early prior spontaneous preterm labour (less than 28 weeks of gestation), had a three-fold increased risk of recurrent spontaneous preterm labour and were more likely to be associated with subsequent early spontaneous preterm labour at less than 28 weeks of gestation (Challis, 2000).

Previously, women who delivered preterm twins were thought to not be at an increased risk of recurrence of preterm labour in a subsequent singleton pregnancy. This was explained by factors such as uterine over distension and polyhydramnios that occur in twin pregnancies and are unlikely to recur in subsequent singleton pregnancies. However, some recent studies have suggested an increased risk of preterm delivery in subsequent pregnancies (Facco *et al.*, 2007; Schaaf *et al.*, 2012).

The risk for spontaneous early preterm labour increases with maternal age and decreases with height; it is higher in women of African and South Asian origin than in the Caucasian population, in cigarette smokers and in those conceiving after the use of ovulation induction drugs (Goldenberg *et al.*, 2008). However, screening for preterm labour on the basis of previous obstetric history and therapeutic intervention in the high-risk group is likely to have a small impact on the overall rate of prematurity. Only approximately 10% of spontaneous early preterm births occur in women with this history.

There are multiple causes of preterm labour which include infection, ischaemia, uterine over distension, cervical disease and haemorrhage. Conditions of maternal or fetal distress may activate the fetal HPA axis early as the fetus needs to escape the hostile uterine intrauterine environment. Infection activates inflammatory pathways leading to the production of chemokines, cytokines, prostaglandins and proteases which stimulate the common pathways of labour causing preterm labour. Haemorrhage leads to thrombin release, this then stimulates myometrial contractility (Elovitz *et al.*, 2000).

However, many preterm deliveries are idiopathic. So in addition to the role of the fetus, much of the literature suggests that the mother must also play a complex role in this process.

Multiple causes of preterm labour and mid-trimester loss have been discussed. Due to the huge variety in these causes, multiple pathological processes must underlie the onset of labour and the preceding changes required in the uterus and cervix. As a consequence of this complexity, prediction of the onset of labour and therefore those who are at risk of preterm labour and mid-trimester miscarriage remains a challenge.

1.8.2 Prediction of Preterm Labour:

Currently there is not a "Gold Standard" test available for the prediction of preterm labour. There are several tests available for use in the clinical setting to help with the decision making process and assessing risk in women who are a high risk of preterm labour. These tests initially had focused on those women who presented with symptoms of preterm labour. Increasingly these tests are also being investigated to determine their roles in prediction of preterm delivery in the asymptomatic high and low risk population. A

sensitive early pregnancy screening test would be ideal. This could then facilitate the timely administration of individualised prophylactic treatments to those who were then deemed to be at the highest risk (Cecatti *et al.*, 2016).

Even with improvements in prediction of preterm labour, one problem remains and is an area of constant research, there is limited and conflicting evidence about preventative treatments for spontaneous preterm delivery.

Currently there are three main treatments utilised worldwide for the prevention of preterm birth, progesterone (vaginal and intramuscular), pessaries and cervical cerclage. There are multiple studies examining the benefits and risks of each of these options. Currently there isn't a consensus about which if any are beneficial, both in terms of prevention of preterm delivery and neonatal and childhood outcomes, and in which groups of patients if any, they are beneficial.

Recently, establishing a core set of clinical outcomes to evaluate these interventions is hoped to ensure that data from trials can be compared and combined to improve the quality of the evidence available for the prevention of preterm birth (van 't Hooft *et al.*, 2016). At present, significant heterogeneity exists from study to study. As a consequence of this, it is difficult to obtain answers to these important questions from systematic reviews and meta-analyses. It also raises questions about the validity of combining women who are deemed to be at a risk of preterm labour due to a variety of factors into one group. With multiple pathological mechanisms underlying preterm labour, it may be beneficial to categorise high risk women into subgroups, dependent upon their risk factor for preterm labour, for example, previous preterm labour, history of cervical surgery, known congenital uterine anomaly and then to assess these preventative measures.

For example, the results of the OPTTIMUM trial published in May 2016 demonstrated that vaginal progesterone was not associated with a reduction in risk of preterm birth or composite neonatal outcome, and also had no long term harm or benefit on outcomes in children ages 2 years (Norman *et al.*, 2016). However, following on from this a systematic review published by Romero and colleagues concluded that vaginal progesterone reduces the risk of preterm birth and neonatal morbidity and mortality in women with a singleton pregnancy and a mid-trimester transvaginal cervical length of <25mm (Romero *et al.*, 2016). Reduction in rates of preterm labour are important, but more important, is the effect on outcomes of children ages 2 years or more, many studies do not have this outcome data. Having a core set of outcomes to be measured may help to reduce inconsistencies in the findings of clinical trials and will allow the production of more powerful meta-analyses to answer these important clinical questions.

1.8.2.1 Biomarkers:

Many biomarkers have been associated with preterm delivery. There are currently a range of biochemical tests utilising these biomarkers available to help to determine which patients may be at the highest risk of preterm labour. These include cervical fetal fibronectin (fFN) (most commonly used in the UK), cervical phosphorylated insulin-like growth factor-binding protein (phlGFBP-1), placental α macroglobulin 1 (PAM-G1) or IL-6 test (Hezelgrave *et al.*, 2015; Min *et al.*, 2016). However these tests have a low positive predictive value. An improvement in the positive predictive value of these biomarker tests would be very useful. The maximal benefit of antenatal corticosteroids occurs within 48 hours of administration with some benefit seen in the reduction of respiratory distress of the baby seen up to 7 days following their administration. An improvement in the prediction of the preterm delivery would allow more appropriate use of antenatal

corticosteroids to achieve their maximum benefit, especially as the data about administration of further steroid doses and its affects upon outcomes is not clear (Hezelgrave *et al.*, 2015).

1.8.2.1.1 Fetal Fibronectin:

Fetal fibronectin (fFN) is a glycoprotein which is found between the chorion and the decidua, it is also found in the amniotic fluid and in placental tissue. Prior to fusion of the chorion with the decidua fFN is detectable in the cervicovaginal fluid. The level then decreases until it is no longer detectable by 22 weeks gestation. The presence of fFN after this process is completed at subsequent gestations is thought to be due to either mechanical disruption or inflammatory changes at the choriodecidual junction and is therefore associated with an increased risk of preterm birth (Hezelgrave *et al.*, 2015). Currently most obstetric units in the UK which use fFN testing use it as a qualitative test, with a positive or negative result. A positive result conventionally corresponds to a fFN level of ≥50ng/mL.

Owing to its high negative predictive value, fFN testing has been demonstrated to reduce unnecessary interventions, change management and reduce healthcare costs, allowing resources to be concentrated on those who are at a higher risk (Foster & Shennan, 2014).

Outside of the research setting, fFN testing is utilised to aid clinical decision making in women symptomatic for preterm labour with intact membranes. Symptomatic patients with a positive test result have a 10-40% risk of delivery within 7-14 days of the test, this increases to 60% risk of delivery at <37 weeks gestation (Hezelgrave *et al.*, 2015). In asymptomatic high risk women, fFN has a positive predictive value of 13.6% for

spontaneous preterm birth before 30 weeks (Min *et al.*, 2016). Again, the main benefit of this test is in its negative predictive value. A negative fFN test can help to reassure clinicians that a patient has a low risk for spontaneous preterm birth. In asymptomatic high risk women, fFN has a negative predictive value of 98.6% for spontaneous preterm birth before 30 weeks.

Current ongoing research is also aiming to determine the role of quantitative fFN results in clinical practice and how this tool may help to improve prediction of preterm labour. The current literature suggests that women symptomatic for preterm labour with a fFN concentration >200ng/mL are at a higher risk of preterm delivery, with 37% delivering within 14 days (Hezelgrave *et al.*, 2015). Abbott *et al*, 2013 performed an observational study that involved 300 symptomatic women for preterm labour. The relative risk of spontaneous preterm delivery within 14 days of the test increased as the fFN concentration increased when compared to the group of patients with results of the fFN test ranging from 0-9ng/mL. Patients with a fFN concentration of 50-199ng/mL had a relative risk (RR) of 4.3, patients with a concentration of 200-499ng/mL had an RR of 16.1 and those with a concentration of ≥500ng/mL had an RR of 26 (Abbott *et al.*, 2013).

Furthermore, a prospective observational cohort study of 1448 asymptomatic high risk women who had quantitative fFN levels taken between 22 and 28 weeks gestation also demonstrated an increasing risk of preterm labour with increasing fFN levels. Risks of spontaneous preterm birth at less than 34 weeks gestation were 2.7% (fFN <10ng/ml), 11.0% (fFN 10-49ng/ml), 14.9% (fFN 50-199ng/ml), 33.9% (200-499ng/ml) and 47.6% (fFN >500ng/ml) (Abbott *et al.*, 2015).

1.8.2.1.2 Phosphorylated Insulin-Like Growth Factor Binding Protein 1:

Phosphorylated insulin-like growth factor binding protein 1 (phIGFBP1) is also a protein biomarker which can be detected using a test on cervicovaginal fluid. It is produced by decidual cells and is released into the cervicovaginal fluid following presumed damage to the choriodecidual junction as occurs with the onset of labour (Hezelgrave *et al.*, 2015). Similar to the fFN test, the presence of phIGFBP1 has a high negative predictive value in symptomatic women and is therefore useful in helping to exclude preterm labour, but its positive predictive value is low (Hezelgrave *et al.*, 2015). A recent systematic review concluded that phIGFBP1 test has a low predictive accuracy for preterm birth at <34 weeks and <37 weeks gestation and for delivery within 7 and 14 days of testing in symptomatic women (Conde-Agudelo & Romero, 2016). The phIGFBP1 test is less expensive than the fFN test and has a higher negative predictive value. This therefore makes it again a more useful test to rule out preterm labour than a test to predict patients who are going to deliver prematurely.

1.8.2.1.3 Placental Alpha Microglobulin 1:

Placental α macroglobulin 1 (PAM-G1) is made by the decidua, it is not present in the cervicovaginal fluid in the absence of labour, a few small studies have demonstrated a high negative predictive value and moderate predictive value for preterm delivery, however further data are needed (Hezelgrave *et al.*, 2015).

1.8.2.2 Transvaginal Cervical Length Scanning:

The current National Institute for Health and Care Excellence (NICE) guideline for Preterm Labour and Birth published in 2015 advises about the role for transvaginal cervical length

scanning in the diagnosis of preterm labour. They state that transvaginal cervical length scanning can be used in women who present with symptoms of preterm labour with intact membranes who are 30+0 weeks or more. If the transvaginal cervical length is >15mm, this suggests delivery is unlikely and the patient is not in preterm labour. If the transvaginal cervical length is <15mm, view the patient as being diagnosed as in preterm labour and offer treatment in line with this (The National Institute for Health and Care Excellence, 2015). This role of transvaginal cervical length scanning is in the diagnosis of preterm labour, there is currently limited guidance available for the role of transvaginal cervical length scanning in the assessment of risk of preterm labour.

One problem which exists when researching this topic is the significant heterogeneity that exists between the different studies performed. The populations studied vary considerably, from women who are asymptomatic low risk patients for preterm labour, to asymptomatic high risks patients to symptomatic patients, and often includes singleton and multiple pregnancies. Multiple different pathologies are likely to underlie the mechanisms of preterm labour in the different groups and therefore performing an analysis grouping all patients into may be questionable.

There are several studies in the literature focusing of each of these groups. Additionally the cervical length cut-off used to define short cervical length also varies and often different gestational outcomes are the primary outcome measure.

Asymptomatic low risk women are a particularly important group to investigate. A large percentage of women who have spontaneous preterm birth are women who are in their first pregnancy and do not have any specific risk factors for preterm birth. Therefore

without targeting this group and developing a test which is applicable to this group of women, any strategy aiming to reduce preterm birth rates would only have a small impact.

This does imply that a screening test is required which can be offered to all nulliparous women. A systematic review and meta-analysis in 2014 by Barros-Silva and colleagues evaluated transvaginal cervical length measurement as a screening test for preterm birth taken in the mid-trimester. They concluded that transvaginal cervical length as a screening test had a high specificity and positive likelihood ratio with a low false positive rate and therefore a short cervix is highly predictive for preterm birth in both singleton and multiple pregnancies and in both high and low risk women (Barros-Silva *et al.*, 2014). Unfortunately the sensitivity of this test is not as promising with a high false negative rate and therefore a normal cervical length in the second trimester cannot be taken as reassuring when considering risk of preterm birth. This would therefore make this test very difficult to justify its use widely in the National Health Service (NHS). In this review, each study included used a different cervical length cut off to define a short cervix and therefore the authors had difficulty in defining a short cervix.

Prior to this review Honest and colleagues had published a similar systematic review in 2003. They concluded that a cervical length of <25mm taken at 20 weeks gestation is useful in predicting preterm birth before 34 weeks gestation (Honest *et al.*, 2003).

Crane & Hutchens focused on high risk women (history of preterm labour, cervical surgery or known congenital uterine anomaly) in their systematic review in 2008. Their analysis led to the conclusion that at 20 weeks gestation a cervical length of <25mm has a positive likelihood ratio of 4.31 for preterm birth, at 20-24 week a positive likelihood ratio of 2.18 and at >24 weeks a positive likelihood ratio of 4.01 (Crane & Hutchens, 2008).

Transvaginal cervical length measurement has also been studied as a predictor of preterm birth in women who present to hospital with symptoms of preterm labour. Boots *et al* (2014) published a systematic review examining this group. Their analysis suggested that a cut off of <15mm was the most accurate length to predict preterm delivery within the subsequent 7 days. They concluded that cervical length scanning in symptomatic patients can be an aid when making management decisions about these patients, particularly as a long cervix is accurate at excluding preterm labour (Boots *et al.*, 2014). This has subsequently been included in the recent NICE guidance on Preterm Labour & Birth.

The use of transvaginal cervical length scanning to assess for cervical shortening is a very useful test as patient with a short cervix are at an increased risk of preterm birth. As the cervix shortens to ≤15mm there is almost a 50% chance of delivery at <32 weeks gestation in both women assessed to be high and low risk (Bolt *et al.*, 2011). However it remains difficult to implement this test into routine clinical practice as cervical length cut offs and who these are applicable too are still not clear from the studies performed. Additionally the management options following identification of a shortened cervical length have conflicting evidence, as discussed above.

Salomon *et al* undertook a large prospective study over three years of 6614 cervical length measurements of low risk women. This measurement was taken as part of routine antenatal care from 16-36 weeks gestation. If women had more than one measurement taken in their pregnancy only the first measurement was utilised in analysis. Patients were excluded if they had abnormal fetal growth, abnormal fetal karyotype, had a known congenital uterine anomaly, delivered preterm or had a multiple pregnancy (Salomon *et al.*, 2009). Cervical length changes throughout pregnancy and therefore gestational age is an

important parameter to take into consideration when utilising cervical length measurements as a tool to aid in the prediction of preterm birth. Several studies prior to Salomon *et al* had demonstrated that cervical length decreases with increasing gestational age (Brieger *et al.*, 1997; Iams *et al.*, 1996). This study resulted in the generation of reference values for cervical length, based upon a large sample of low risk women and utilised a statistical approach which was not based upon the assumption of cervical length being normally distributed (Salomon *et al.*, 2009). This will be used further in Chapter 5.

Currently NICE guidance for the diagnosis of preterm labour does not suggest the use of transvaginal cervical length measurement and fFN testing in combination to enhance the prediction of preterm birth (The National Institute for Health and Care Excellence, 2015). Currently research is looking at this to identify which women should be targeted at further intervention. The EQUIPP study involved over 1000 asymptomatic high risk women. These women had serial cervical length scans and quantitative fFN testing every 2-4 weeks from 22-30 weeks gestation. This data has been utilised to develop an application QUiPP which gives a risk of spontaneous preterm birth at <30 weeks, <34 weeks and <37 weeks and the risk of delivery within 1 week, 2 weeks and 4 weeks. This is based upon inputting data about symptoms, history of cervical surgery, previous preterm prelabour rupture of membranes/preterm birth/mid-trimester miscarriage, current gestational age, cervical length and fFN result (Kuhrt *et al.*, 2016).

Following on from this work data has been published utilising clinical history, fFN test results and transvaginal cervical length in a preterm surveillance clinic to triage women into groups. Those determined to be low risk utilising this method were discharged from the clinic and those determined to be high risk were admitted and hospital polices followed. The results of this demonstrated a low incidence of spontaneous preterm birth and lack of

neonatal complications in those patients who were discharged from the surveillance clinic, suggesting that these high risk patients were appropriately triaged by the clinic (Min *et al.*, 2016). Of the women who were admitted, one third delivered before 30 weeks gestation.

In Chapter 5, I will further review the specific literature regarding preterm birth prediction related to my project.

1.8.3 Post-Term Pregnancy:

Post-term pregnancy can be defined as pregnancy which progresses to 42 weeks or further. As discussed, gestational age at delivery is a key determinant of fetal outcome. In addition to preterm delivery being associated with an increase in perinatal mortality and morbidity, post term births are also associated with increased perinatal mortality and morbidity (Edmonds, 2012). There is still a lack of understanding regarding the reasons about why some women do not go into spontaneous labour. The direct measurement of contractility showing that postdates myometrium contracts poorly is consistent with clinical findings suggestive of poor uterine contractility including, longer labours, increased caesarean section rates and increasing risk of postpartum haemorrhage (Arrowsmith *et al.*, 2014; Arrowsmith *et al.*, 2011).

Due to the increasing risks of post-term pregnancy, current NICE guidelines published in 2008 advise that women with uncomplicated pregnancies should usually be offered induction of labour between 41+0 and 42+0 weeks. Currently vaginal PGE_2 is the preferred method of induction of labour unless there are clinical contraindications (The National Institute for Health and Care Excellence, 2008).

In addition to induction of labour for post-term pregnancy, many other women have their labour induced for a variety of maternal medical and fetal indications. Induction of labour is a common obstetric intervention with the 2013-2014 maternity statistics for NHS England reporting a 25% rate of induction of labour (Vallikkannu *et al.*, 2016).

In Chapter 4, I will further review the literature specifically relating to my project.

1.9 Identification of New Biomarkers:

Over the past 10 years with the advent of new molecular technologies and these becoming more readily available, it has become possible to look at differential gene expression, and for this to be used to screen for new diagnostic/therapeutic targets for a variety of diseases and clinical problems including preterm birth. For example, comparison of gene expression in the myometrium of those patients not in labour compared to those in labour could provide a very useful insight into the process of labour. A variety of different techniques can be used to do this including cDNA microarrays and high throughput RNA sequencing (RNA-seq) with subsequent reverse transcription polymerase chain reaction (RT-PCR) and northern blot/western blot experiments. RNA-seq is a technology which can look at a snapshot at a moment in time of RNA presence and quantity of RNA from a genome. In addition to sequencing the RNA transcripts of a cell, it also allows differentially spliced gene transcripts, post-translational gene modifications, mutations and small nucleotide polymorphisms to be examined.

As discussed, both induction of labour and preterm labour are clinical problems with different consequences both for the patients themselves and the NHS and society as a whole. These are areas of obstetrics which could benefit greatly from better prediction and

subsequent management. Many recent studies have aimed to identify new targets for this, particularly as the details of the mechanism by which the uterus changes from the quiescent uterus of pregnancy to the excitable uterus of labour leading to birth are not well understood.

Chan *et al* recently published data on this subject. They used RNA-seq to compare the transcriptome of myometrial samples taken at caesarean section on women not in labour (elective caesarean sections) with those in labour (defined as regular contraction less than 3 minutes apart with a cervical dilatation of more than 2cm) at term (defined in this study as 38-40 weeks gestation). They studied for the first time the full complement of mRNA in human uterine samples in these two different groups. Using three statistical methods to analyse the gene expression results, they demonstrated that of the 19879 expressed genes, 764 genes were differentially expressed in the labouring compared to the non-labouring samples. Many of the upregulated genes related to those previously known to change in labour including those in the prostaglandin E_2 synthesis pathway, oxytocin receptor and β-adrenergic receptors and proinflammatory cytokines (including chemokine ligand 20 (CCL20), C-X-C motif chemokines 3, 5 &6 and C-C motif chemokine 11(CCL11)) (Chan *et al.*, 2014).

One gene which was noted to be in the top ten differentially expressed genes was a relatively newly identified protein called phospholipase C-like 1 (PLCL1). This was significantly down regulated in the labouring samples with 94.30 transcripts per millions (SD+/- 30.50) in the on-labouring samples compared to 10.93 transcripts per million (SD+/- 3.78) in the labouring samples.

In 2015 Eidem and colleagues performed a systematic review and meta-analysis reviewing preterm birth transcriptomics research with the aim of further understanding the genes and pathways involved in the preterm birth subtype. They included all studies of gestational tissues including placenta, decidua, myometrium, cervix, umbilical cord and fetal blood. This identified 134 studies, 17 of which located at myometrium and 9/17 which focused on labour. They looked for overlap between the studies in differentially expressed genes. This identified 15 genes present in 4 or more of the studies focusing on transcriptomics on labouring myometrium. These genes included some which may be expected and have been discussed above in the sections about initiation of labour such as PTGS2, but also the novel protein PLCL1 (Eidem *et al.*, 2015).

Leading on from this we wish to investigate if this novel finding of downregulation of PLCL1 with the onset of labour can be translated into a clinically useful test (Chan *et al.*, 2014).

1.9.1 Phospholipase C Like-1 (PLCL1) Protein:

1.9.1.1 Background:

Phosphatidylinositol 4,5-bisphosphonate (PIP_2) can be hydrolysed to inositol (1,4,5) triphosphate (IP_3) and diacylglycerol (DAG) by the enzyme phospholipase C (PLC). This is an important process in intracellular cell signalling as both IP_3 and DAG are second messengers and go on to perform a variety of further tasks. Following this reaction, IP_3 can then either be dephosphorylated to form inositol (1,4) bisphosphate (IP_2) or phosphorylated to produce inositol (1,3,4,5) tetraphosphate (IP_4). IP_3 has the empirical structure $C_6H_{15}O_{15}P_3$. It is composed of an inositol ring with three bound phosphate groups. There are many types of PLC, on the basis of their structure they have been divided into three classes, β, γ and δ.

All PLC proteins have conserved X and Y domains which are responsible for their catalytic activity (Otsuki *et al.*, 1999).

Prior to work published by Kanematsu and his colleagues in 1992, it was understood that there were only three types of proteins which interacted with IP_3 including IP_3 receptors on the endoplasmic reticulum and those two enzymes related to IP_3 metabolism discussed above(Kanematsu *et al.*, 1992).

To examine IP_3 protein interaction further Kanematsu *et al* prepared an IP_3 affinity media to purify IP_3 interacting proteins. From rat brain cytosol, using an affinity column, they identified two cytosolic IP_3 binding proteins. The molecular mass of these proteins was estimated using SDS-polyacrylamide gel electrophoresis. One had a molecular mass of 85KDa which was later identified as phospholipase Cδ and a second protein with a molecular mass of approximately 130KDa. They sequenced these proteins to ensure that they were not the proteolytic products of the IP_3 receptor from the cell membrane. These proteins were found to show no similarities with the receptor and therefore the 130KDa protein was deemed as an unidentified novel protein. In their paper in 1992, Kanematsu et al postulated a role for the 130KDa protein in compartmentalisation of IP_3 (Kanematsu *et al.*, 1992).

In 1996, Kanematsu and his colleagues published further details about this novel protein from rat brain cytosol. The full length cDNA was isolated and the protein was expressed in COS-1 cells. The protein was found to be composed of 1096 amino acids corresponding to a molecular mass of 122.8 KDa (Similar to that expected from the previous work). Analysis of the gene and amino acids sequences revealed many similarities with PLC-δ. In particular,

the two catalytic domains X and Y showed approximately 50% homology to PLC-δ (Kanematsu *et al.*, 1996). This is shown in Figure 1.6.

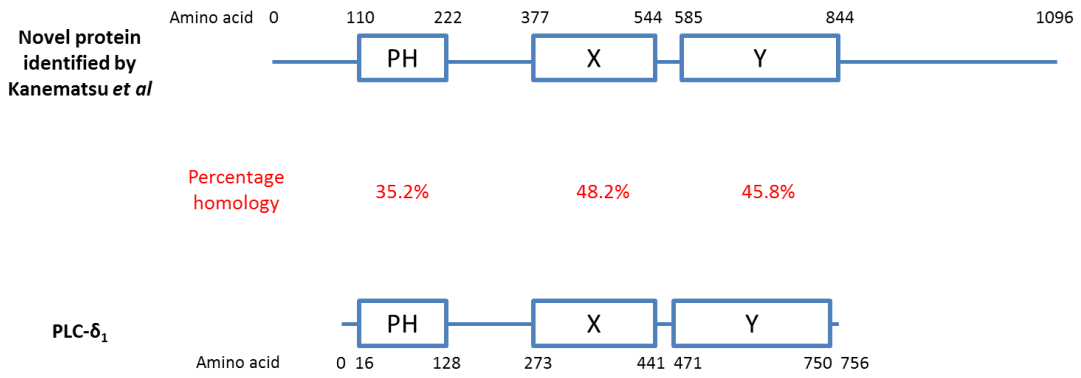

Figure 1.6: A comparison of the protein domains of newly isolated 1096 amino acid protein with PLC-δ (new protein above, PLC-δ below).As can be seen the catalytic domains X and Y show significant homology to the X and Y domains of PLC-δ_1. Adapted from (Kanematsu et al., 1996).

A Pleckstrin homology (PH) domain is a protein domain of approximately 120 amino acids. It was first described in the Pleckstrin protein in platelets (Alberts, 2002). It is defined by six weakly conserved sequence blocks and within the sixth block, a single tryptophan residue is conserved always in all proteins carrying the PH domain (Kanematsu *et al.*, 1996). It can bind to lipid head groups such as that of PIP_2 and IP_3 (Berg *et al.*, 2002). It is via this mechanism, the PH domains enable a protein that they are part of to dock on to the cell membrane and interact with other proteins (Alberts, 2002). The PH domain contains a hydrophobic core which helps to stabilise the overall structure of the domain. The newly identified 130KDa protein also contained a PH domain at its N-terminus with 35% homology to that of the PLC-δ, as shown in Figure 1.6. This protein became known as phospholipase C like (PLCL) protein due to its similarity with PLC.

Subsequent to the identification of PLCL, in 1999, Otsuki *et al* identified another type of PLCL in rats which they then called PLCL2 with the previous protein then being called PLCL1 (Otsuki *et al.*, 1999). The tissue distribution of PLCL1 and PLCL2 in rats is different with PLCL1 mainly being expressed in the central nervous system and PLCL2 being expressed more ubiquitously (Kanematsu *et al.*, 2000).

The protein PLCL1 has also become known by several other names dependent upon the author writing about the protein. The most frequently used being phospholipase C-related but catalytically inactive protein (PRIP) but also PLCE, PLDL and PPRIR127.

Proteolytic experiments with trypsin and the construction of chimeric molecules between PLC-δ1 and PLCL1 have demonstrated that the PLCL1 contains the following domains, PH, EF-hand, X and Y catalytic regions and a C2 domain, this is very similar to the PLC-δ1 family The results of these experiments demonstrated the structural domains of PLCL1 as shown in Figure 1.7 (Kanematsu *et al.*, 2005).

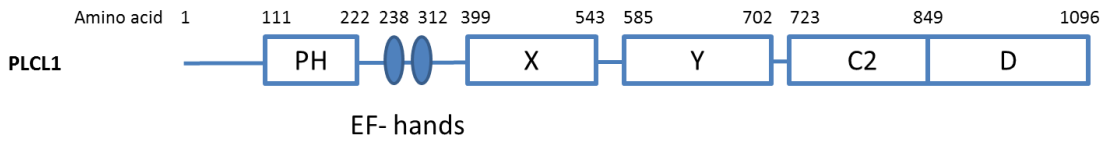

Figure 1.7: The structural domains of PLCL1. Adapted from (Kanematsu et al., 2005)

Importantly, as mentioned, the X and Y domains do not have catalytic activity. This has been postulated to be due to the lack of conservation of critical amino acid residues within these domains (Kanematsu *et al.*, 2005).

The human PLCL molecule was identified by Kohno and his colleagues in 1995. It shows sequence homology to the rat PLCL1 protein. This protein was found to be deleted in human small cell lung carcinoma and hence was initially designated as phospholipase C-deleted in lung carcinoma. It is located on chromosome 2 position 2q33 (Kohno *et al.*, 1995).

1.9.1.2 Role of PLCL1:

As mentioned previously, the PH domain of a protein can bind to IP_3 and leading on from this a hypothesis was developed suggesting that PLCL1 could have a role in regulation of IP_3 signalling via binding to soluble IP_3 in the cytosol. Kanematsu *et al* tested this hypothesis. They analysed Ca^{2+} ion release in response to the addition of IP_3 using permeablised COS-1 cells. When increasing amounts of recombinant PH domain of the PLC-L1 protein were added, the experimental results suggested that this PH domain was able to compete with the IP_3 receptor in the endoplasmic reticulum membrane for IP_3 resulting in the decreased release of Ca^{2+} from the endoplasmic reticulum. Within increasing amount of the protein domain added, the measured intracellular Ca^{2+} level became less (Kanematsu *et al.*, 2005). The bradykinin receptor is a G-protein coupled receptor which is couple to both G_q and G_i. G_q stimulated PLC. To further test these findings COS-1 cells and COS-1 cells stably expressing PLCL1 were stimulated with bradykinin and changes in free intracellular Ca^{2+} concentration were monitored in the absence of extracellular Ca^{2+}. As expected, the response of the COS-1 cells expressing PLCL1 to bradykinin stimulation in relation to measured intracellular Ca^{2+} was less than the response seen in the COS-1 cells. IP_3 can also be metabolised by IP_3-5 phosphatase and 3-kinase enzymes. Further experiments also showed that at these enzymes are also inhibited by high concentrations of the PLCL1/PH

domain, although the concentrations were much higher than physiological ones (Kanematsu *et al.*, 2005).

Other proteins which interact with PLCL1 have also been identified and include protein phosphatase 1α, $GABA_A$ receptor associated protein, the β subunit of $GABA_A$ receptors and protein phosphatase 2A. This is shown in Figure 1.8.

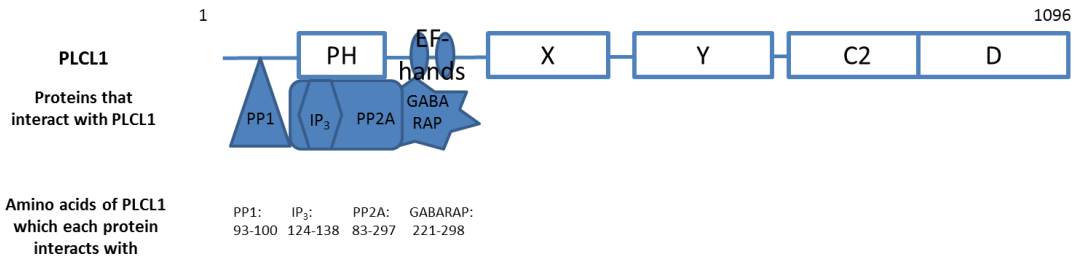

Figure 1.8: The proteins which interact with PLCL1 and the residues which are responsible for the interaction. Adapted from (Kanematsu et al., 2005).

In particular studies have looked at the interaction of PLCL1 with protein phosphatase 1α (PP1α). It has been suggested that when PLCL1 itself is not phosphorylated, PP1α can bind to its N-terminus and PP1α remains inactive. However if PLCL1 is phosphorylated by protein kinase A (PKA), this releases PP1α to be active (Kanematsu *et al.*, 2005).

All of the work described so far was performed in rats with the exception of Kohno *et al* from 1995. Following on from this, more work was needed to be performed in the human. Murakami and his colleagues performed Western blotting and RT PCR analysis on human samples. They looked at human brain, kidney, heart, skeletal muscle, liver spleen and lung. This work demonstrated that PLCL1 was much less widely expressed at both the RNA and protein level than PLCL2. Using Western blotting, PLCL1 was demonstrated only in human

brain, whereas PLCL2 was present in human brain, heart, skeletal muscle, liver, spleen and lung. RT PCR experiments showed similar results with PLCL1 being present only in human brain and kidney (Murakami *et al.*, 2006).

1.9.2 Role of PLCL1 in Pregnancy and Labour:

Chan *et al* 2014, collected myometrial samples taken at caesarean section from both patients in spontaneous labour at emergency caesarean section (patients were having regular contractions and their cervix was more than 2cm dilated at delivery), and those not in labour, at elective caesarean section. Samples were subject to high throughput RNA sequencing to ascertain of the presence of RNAs and quantify these at the time of sampling. This process resulted in over 700 hundred differentially expressed genes. As discussed, one of the top ten downregulated genes in the labouring samples was a new protein which had not previously been studied in reproductive tissues, PLCL1. The downregulation of PLCL1 in labouring myometrium is illustrated in Figure 1.9 (Chan *et al.*, 2014).

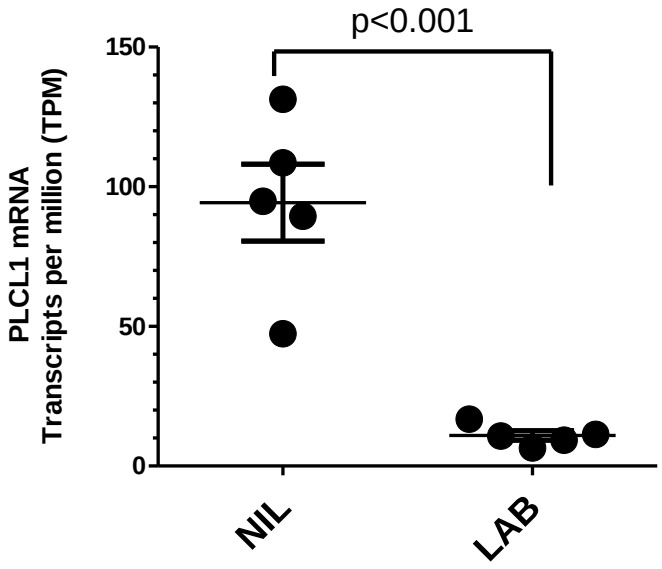

Figure 1.9: PLCL1 is significantly downregulated in labouring myometrium when compared to non-labouring myometrium, 94±14 vs 11±2 transcripts per million (LAB vs NIL) (Chan et al., 2014).

Further myometrial biopsies were utilised to support these findings. PLCL1 expression in human myometrium was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting and RT PCR experiments in both labouring (LAB) and non-labouring samples (NIL). This work has supplemented the previous findings and shown that PLCL1 is significantly downregulated in labouring compared to non-labouring myometrium both at the RNA level using RT PCR and at the protein level using SDS-PAGE and Western blotting as can be seen in Figure 1.10 (Brighton *et al.*, 2014).

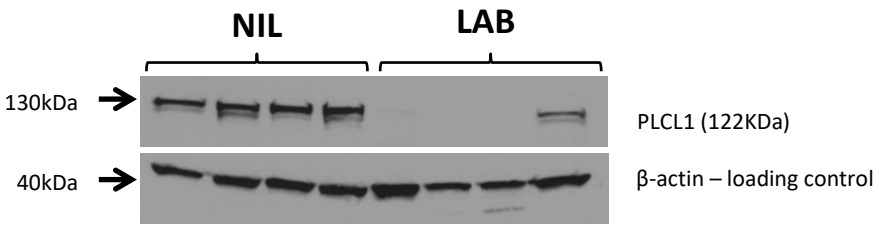

Figure 1.10: PLCL1 is down regulated in human labouring myometrial samples demonstrated by SDS-PAGE and Western blotting. LAB – labouring myometrium, NIL – non-labouring myometrium (Brighton et al., 2014).

Kanematsu *et al*, 2005 looked at the involvement of PLCL1 in Ca^{2+} signalling. They added recombinant PH domain of the PLCL1 protein to COS-1 cells and observed that the amount of calcium released into the cells decreased with increasing amount of recombinant protein added, concluding that PLCL1 via its PH domain could compete with the receptors on endoplasmic reticulum for IP_3, inhibiting receptor activation and calcium release (Kanematsu *et al.*, 2005).

Based upon the previously demonstrated inhibition of IP_3 signalling by PLCL1, and its gestation dependent regulation in myometrial smooth muscle, it was hypothesised that PLCL1 serves to silence PLC-dependent Ca^{2+} signalling via IP_3 chelation in myometrial cells, therefore in cells expressing high levels of PLCL1, we would expect minimal increases in intracellular calcium levels in response to addition of ligands including oxytocin, PGE_2 and $PGF_{2\alpha}$.

Further experimental work using a primary myometrial cell line looked at the effects of over and under expression of PLCL1 on oxytocin medicated calcium signalling. siRNA-mediated knockdown experiments were performed to examine the function of PLCL1 in

myometrial cell lines by examining the effects on oxytocin mediated calcium signalling. Additionally, PLCL1 was overexpressed in the primary myometrial cells to determine the effect of an increase in PLCL1 in these cells. The results of these experiments are illustrated in Figure 1.11 support the conjecture that PLCL1 acts to sequester phosphoinositides and therefore can attenuate IP_3 dependent calcium release (Brighton *et al.*, 2014).

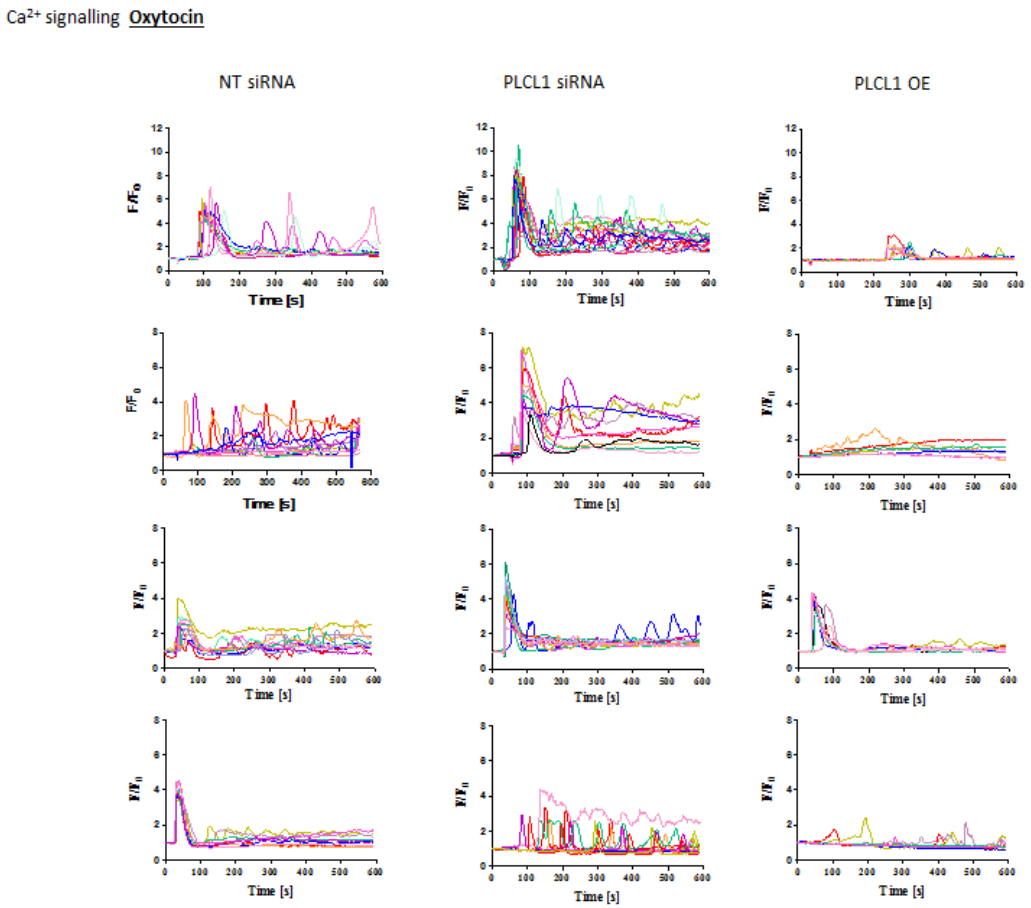

Figure 1.11: Demonstrating the effects of over and under expression of PLCL1 on oxytocin medicated calcium signalling in primary myometrial cells line. Primary myometrial cells were transfected with non-targeting (NT) (left panels) or PLCL1 (central panels) siRNA. Oxytocin was administered at time 0. PLCL1 is under-expressed in the central samples and is overexpressed (OE) in the samples shown on the right panel. Florescence probes were utilised to detect calcium release into the cytosol. Primary myometrial cells were then challenged with oxytocin and confocal imaging of intracellular

calcium oscillation recorded over 10 minutes. Florescence intensities were measured in region of interest and are expressed as a fold increase from time 0 (F/F_0) The cells transfected with PLCL1 siRNA (central panels) displayed a robust and sustained Ca^{2+} release over the time recorded. Myometrial cells in which PLCL1 was overexpressed (right panels) show minimal Ca^{2+} oscillations in response to PLC activation by oxytocin administration(Brighton et al., 2014).

Similar results have been demonstrated using primary myometrial cell lines when prostaglandin E_2 and prostaglandin $F_{2\alpha}$ were used as the ligand to activate the pathway (Brighton *et al.*, 2014).

Following on from this work, the division of Translational and Systems Medicine at the University of Warwick have demonstrated that PLCL1 expression in myometrial cells is progesterone dependent. Primary myometrial cell lines were cultured with progesterone (cAMP + medroxyprogesterone (MPA)). This resulted in an increase in PLCL1, both at the RNA and protein level, as shown in Figure 1.12. Withdrawal of progesterone from primary myometrial cells leads to a reduction in expression of PLCL1 (Brighton & Blanks, 2015).

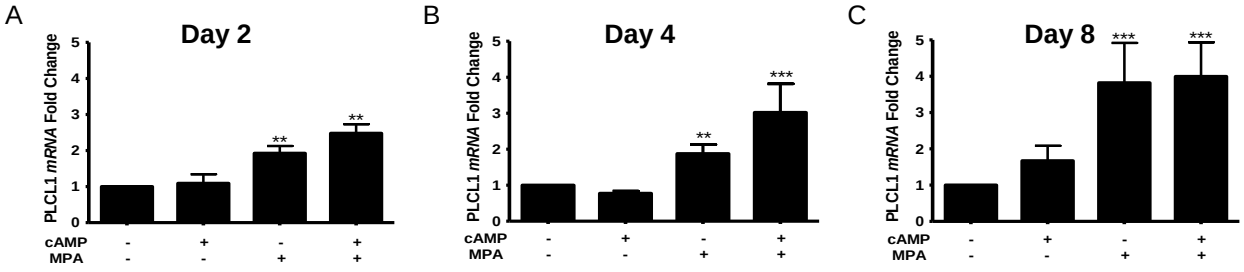

Figure 1.12: Progesterone regulates PLCL1 expression in primary myometrial cells. PLCL1 transcripts were measured in cells treated with cAMP or MPA or both for A 2 days, B 4 days or C 8 days. The panes show the relative increase in PLCL1 mRNA levels. The presence of MPA and cAMP leads to a significant increase in expression of PLCL1(Brighton & Blanks, 2015).

PLCL1 is a novel intracellular protein which is significantly down regulated with the onset of labour in the myometrium, it is progesterone dependent and it is involved in calcium signalling. It is proposed that PLCL1 is a potent suppressor of agonist mediated IP_3 signalling in human myometrium.

In the non-labouring myometrium or in patients not susceptible to the onset of labour, when a ligand (e.g. oxytocin, PGE_2 or $PGF_{2\alpha}$) binds to its receptor, $G_{\alpha q}$ then stimulates phospholipase Cβ to act on its substrate PIP_2 and hydrolyse this into the products of free IP_3 and membrane bound DAG. There are high levels of PLCL1 which can then acts as an IP_3 sink inhibiting IP_3 mediated signalling, maintaining myometrial quiescence and therefore regulating a common pathway to inflammatory, oxytocin or prostaglandin mediated labour.

We hypothesise that when PLCL1 levels are low as in labour or possibly in patients susceptible to labour at any gestation, the inhibition of IP_3 mediated calcium release from the sarcoplasmic reticulum is absent. These patients therefore following initiation of labour by the variety of pathways discussed will go into labour.

PLCL1 is an exciting biomarker as its mechanism of action is involved in the final common pathway of labour. It therefore could be a very useful biomarker as could be used as a predictor of inset of labour independent of the cause of labour and independent of gestation.

In pregnancy it is not possible to access the myometrium of women unless they are undergoing a caesarean section, however the cervix is accessible. The Human Protein Atlas demonstrates that PLCL1 is expressed epithelial cells and the basement membrane in the

cervix as illustrated in Figure 1.13 (The Human Protein Atlas)

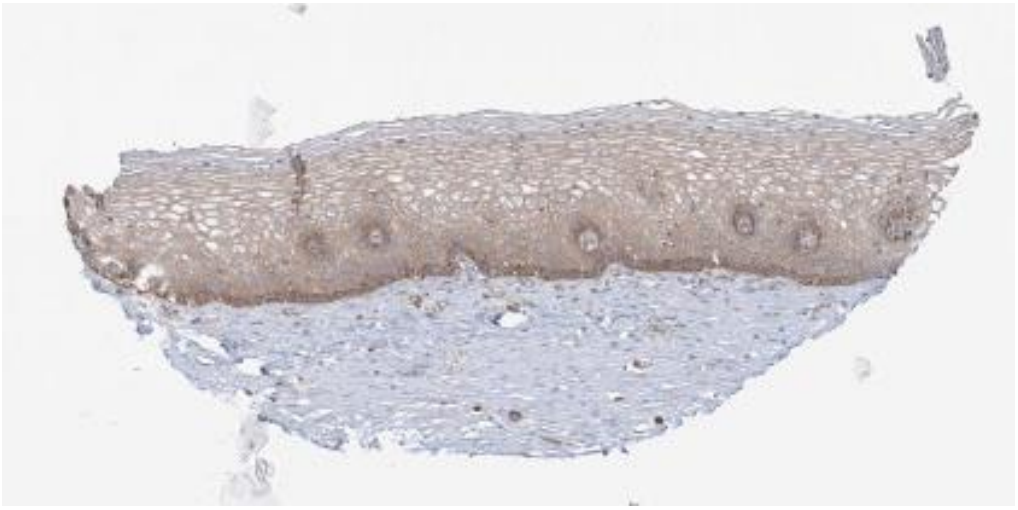

Figure 1.13: Expression of PLCL1 in the human cervix (The Human Protein Atlas).

Human cervical samples were obtained from the University of Edinburgh by the division of Translation and Systems Medicine at the University of Warwick from patients who were >37 weeks gestation at caesarean section. These slides were stained for the presence of PLCL1 using immunohistochemistry. They confirmed that PLCL1 is widely expressed in the cervix (Brighton & Blanks, 2014).

A previous study demonstrated that cervical epithelial cells could be obtained antenatally from women at risk of preterm labour. Whitworth *et al* 2007 used an endocervical cytobrush to sample epithelial cells of the cervix of pregnant women with a history of preterm labour. The purpose of the study was to describe the leucocyte population in pregnancy and how it changed over increasing gestation. The results were analysed in women who developed marked cervical shortening and in whom preterm labour reoccurred. The cytobrush technique was found to obtain and substantial number of cells (4-6 x 10^6 cells per sample) and was safe in pregnancy (not associated with any adverse events). A reduced number of cervical macrophages were associated with recurrent

preterm labour suggesting that reduced cervical immune defence to ascending infection contributed to recurrent preterm labour (Whitworth *et al.*, 2007).

1.10 Aims of the Project:

This project aimed to answer the following questions;

1. Can we detect PLCL1 in cervical epithelial cells obtained from cervical cytobrush samples taken from pregnant women?
2. Can we quantify PLCL1 in these samples?
3. Can we use PLCL1, an intracellular protein in cervical epithelial cells to develop a novel test to help to predict the onset of labour?
    a. Could be utilised in the prediction of preterm birth in high risk women?
    b. Could this be used as a susceptibility marker in conjunction with other markers?
    c. Can PLCL1 levels help to predict the duration and efficacy of the induction of labour process with prostaglandin and oxytocin?

Chapter 2

2. Study Protocol: Prediction of Inappropriate Myometrial Function. A prospective Observational Cohort Study

2.1 Background:

Inappropriate myometrial function is a major contributor to preterm labour and birth and can be caused by a variety of mechanisms. As discussed in Chapter 1, the onset of labour is the result of complex interactions between many maternal, fetal and placental factors which lead to a change in the uterus from a quiescent state throughout pregnancy to a contractile state. In synchrony with the changes in the uterus, the cervix undergoes a process of ripening and remodelling, allowing for cervical dilatation in response to coordinated uterine contractions facilitating birth of the fetus.

There are many triggers factors that contribute to labour. However which ever factor(s) triggers the onset of labour there are changes which need to occur in the uterus and cervix as part of a final common pathway to labour. These changes allow contraction of the uterus by myometrial smooth muscle, a process dependent upon the activation of signalling cascades ultimately permitting the interaction of myosin and actin filaments.

Phospholipase C like 1 (PLCL1) was identified as a novel intracellular protein which is significantly downregulated with the onset of labour in the uterus and cervix. Its presence uncouples PLC from myometrial contractions via chelation of IP_3 and therefore it maintains uterine quiescence. It is hypothesised that PLCL1 can act as a susceptibility marker to other stimuli for the initiation of labour. PLCL1 is expressed in both the myometrium and cervical epithelial cells. Cervical epithelial cells can be safely collected from pregnant women using a cervical cytobrush.

A new test which could help to predict the onset of labour would be very useful in two groups. Firstly, for the population who is at an increased risk of preterm labour but is asymptomatic, or the symptomatic population in threatened preterm labour with clinically recognisable contractions, bleeding or rupture of membranes. An improvement in prediction of preterm delivery would allow more timely administration of antenatal corticosteroids and, where appropriate administration of magnesium sulphate for neuroprotection, increasing survival rates and reducing disability. Women could then be admitted to appropriate hospitals with the necessary neonatal care availability as resource allocation would improve.

Secondly, it would be beneficial for women requiring induction of labour. Currently this group can spend up to five or more days in hospital with painful contractions whilst the currently available chemical agents (prostaglandins) or mechanical methods ripen the cervix. A test informing patients and their clinician which women will go into labour following induction and which will not, would enable better utilisation of NHS resources.

We aimed to be able to assess both myometrial quiescence with PLCL1 and trigger factors/stimuli for the onset of labour. In the protocol we included existing and new tests for infection in the high risk asymptomatic patients group. With induction of labour patients, the trigger for labour is known, prostaglandin E_2. This chapter describes the entire protocol that we developed. The thesis results chapters will describe the work on PLCL1 alone.

2.2 Objectives of the Study:

To investigate whether we could develop a test to help to predict which patients will go into preterm labour or have a successful induction of labour. Our novel approach to this problem was to investigate whether:

- Does a lack of PLCL1 in cervical samples or serum predict the onset of labour?
    - Can we detect PLCL1 in cervical samples?
    - Can we quantify PLCL1?
    - Does the PLCL1 level correlate with clinical outcome?

In addition to a cervical cytobrush sample at speculum examination, further swabs were taken from women recruited to the study to;

- Examine the microbiome of the vaginal and cervical flora in high risk asymptomatic women. A comparison will be made between the microbiomes of those patients who go on to deliver preterm with the microbiomes of the patients who deliver at term.
- Assess whether the electronic nose can detect volatile organic compounds (VOC) in vaginal fluid to develop a bedside test for the detection of group B Streptococcus (GBS), bacterial vaginosis and following on from this whether it can assist in the prediction of preterm labour.

2.3 Methods:

2.3.1 Ethical Approval and Research & Development Approval:

"Prediction of Inappropriate Myometrial Function" (PIMF) was approved by the NRES Committee West Midlands South Birmingham on 14th January 2014 (REC reference 13/WM/0486) following submission of the study protocol, patient information sheet, consent form, general practitioner (GP) letter and invitation letter to patients (Appendices 1-5). The PIMF study was approved by University Hospitals Coventry & Warwickshire Research & Development Department (after Ethical Committee approval) on 17th January 2014 (Appendix 6).

2.3.2 Patient Recruitment:

All patients who booked to have their antenatal care through our University Teaching Hospital received a newsletter with their antenatal notes informing them about the research projects in the Department of Obstetrics & Gynaecology (a sample newsletter can be seen in Appendix 7). The aim of this newsletter was to inform patient about projects they may be approached to take part in during their pregnancy. Following this, all participants who were recruited to the study were given a Patient Information Leaflet (Appendix 2) to read and understand, and the study was discussed with each potential participant by a researcher involved in the study after the patient had been given an appropriate amount of time to consider taking part.

2.3.3 Clinical Subjects & Methodology:

Participants were recruited at UHCW NHS Trust from February 2014 onwards from the preterm prevention clinic, the labour ward triage area and the antenatal ward. Written consent was obtained from all study participants prior to examination and taking endocervical and high vaginal swabs(Appendix 3). With permission, each participant's general practitioner was informed by letter regarding their taking part in the study (Appendix 4).

2.3.4 Materials for Sample Collection:

Cotton swab (Charcoal Wood Cotton Tip)

Catch-All™ Sample Collection swabs

10% potassium hydroxide

0.9% sodium chloride

ThinPrep® components

- Vials filled with 20ml of Preservcyt® solution stored at room temperature until use
- Cytobrush sample

BD SurePath™ components

- Vials filled with 10ml SurePath® Preservative Fluid stored at room temperature until use
- Cytobrush sampler

Dulbecco's phosphate buffered saline (DPBS)

- Potassium chloride – 200.0mg/L
- Potassium phosphate monobasic – 200.0mg/L
- Sodium chloride – 8000.0mg/L
- Sodium phosphate dibasic – 2160.0mg/L

2.3.5 Sections of the Project:

2.3.5.1 Phospholipase C like 1 (PLCL1):

Discussed in Chapter 1, Section 1.9.2.

2.3.5.2 Microbiome:

Previously it has been demonstrated that some alterations in the normal bacterial flora of the vagina are associated with preterm labour (Witkin, 2015). We aimed to examine this relationship further by taking sterile swabs from the vagina and cervix and extracting DNA and RNA. Changes in the endocervical/vaginal microbiota will be profiled using Illumina MiSeq sequencing amplified 16S rRNA genes followed by bioinformatics analysis. Targeted RT-PCR will also be used to examine bacterial species such as Enterobacteria and Bifdobacteria relative to the universal bacterial primer set. Pyrosequencing and RT-PCR will be performed in the cohort of patients considering the final systemic findings. Samples will be retained for subsequent studies. This is not discussed further in this thesis.

2.3.5.3 Enose:

The normal bacterial flora in the vagina produces an odour. Samples were collected as described. These samples will be subsequently heated to room temperature to release volatile organic compounds (VOCs) and gases that emanate from biological samples as a consequence of dysbiosis due to perturbation of vaginal microflora. This will be evaluated using electronic nose technology.

An electronic nose is the colloquial name for an instrument made of an array of chemical sensors combined with pattern recognition software. Developed at the University of Warwick it is an instrument designed to replicate the human olfactory system. It is made of an array of chemical sensors combined with pattern recognition software. In this proposal such an instrument will assess defined chemical components within the headspace (vapours in the gas above and in equilibrium with a solid or liquid which are sampled), but identify odours through their multi-component nature. This is an alternative approach to more traditional VOC analysis techniques, such as mass spectrometry (MS) and gas chromatography (GC). Though both of these techniques are still important in sample analysis, they only provide information on the individual chemical components within a sample (instead of the whole, as with an e-nose).

Traditionally e-nose instruments employ an array of chemical sensors with overlapping sensitivity. As each sensor is different in some way (usually broadly tuned to a chemical group), the interaction between the sample and each sensor is unique. From these responses we are able to generate a 'fingerprint' of an aroma created by the profile of sensor responses. These instruments are very powerful at analysing the overall chemical

composition of samples and identifying which samples are different, but not the individual chemical components within them.

Analysis will be undertaken with Dr James Covington through a continuing collaboration with Warwick Medical Bio-Engineering Unit. The initial aim is to improve the diagnosis of bacterial vaginosis and GBS infections in order to appropriately initiate maternal antibiotic therapy. Additionally the microbiome results of patients who went on to deliver preterm will be compared to those patients who deliver at term. These results will not be discussed further in this thesis.

2.3.6 Study Centre:

The study was conducted at University of Warwick. Patients were seen in the Obstetrics & Gynaecology Department of University Hospitals Coventry and Warwickshire NHS Trust, UK.

The Site File was kept under lock at Biomedical Research Unit (BRU), in the PRI suite at University Hospitals Coventry and Warwickshire NHS Trust. The Key was with the BRU administrator. Notes and all other relevant forms are also kept at the BRU.

2.3.7 Study Design:

This was a prospective observational cohort study.

We collected:

1. Clinical data including age, body mass index (BMI), obstetric history, medical history, pregnancy complications, progress in labour, pregnancy outcomes

including gestation at delivery, fetal weight, mode of delivery, complications at delivery and cervical length if measured by transvaginal ultrasonography in the preterm prevention clinic.

2. Swabs/cytobrush samples of cervical cells, see Section 2.3.11.
3. Blood Sample Collection**:** we obtained approval to collect blood but have not yet started this. Blood samples (up to 20 ml) will be collected with patient permission using sterile BD vacutainer blood collection apparatus. Blood components will be separated by centrifugation. Samples will be aliquoted and stored in -80^{o}C freezer facilities in preparation for subsequent analysis.

All samples collected were given a code number specific to each patient and to each patient visit. Samples were stored in Arden Tissue Bank until they were transferred to the Clinical Science Laboratory at University Hospital of Coventry and Warwickshire for further processing (See Chapter 3). Patient details and data were stored on password secure database systems in an anonymised fashion.

2.3.8 Patient Groups:

Three different groups of patients were invited to take part in the study.

2.3.9 Inclusion Criteria:

2.3.9.1 Group 1:

Participants from this group were recruited from the preterm prevention clinic at UHCW NHS Trust. These patients attended this clinic due to a history of:

- preterm labour (<34 weeks),
- second trimester miscarriage,
- cervical surgery (previous large loop excision of the transformation zone (LLETZ) or previous cone biopsy).

  Or,
- a presentation to the Early Pregnancy Assessment Unit earlier in their pregnancy with a dilated cervix and exposed intact fetal membranes and therefore had a rescue cervical cerclage.

  Or,
- If they were known to have a congenital uterine anomaly.

Prior to attending the clinic, patients were invited to participate in the study via a patient invitation letter (Appendix 5) and a patient information sheet (Appendix 2). Patients attended the preterm prevention clinic every two to four weeks from the second trimester onwards, during which time they had a speculum examination to take a high vaginal swab which was sent to the routine pathology laboratory for culture and sensitivity, and a transvaginal scan to determine cervical length and to check for funnelling of the cervix.

Written informed consent was taken at each patient's first visit to the clinic. Samples were repeated each time the participant attended the clinic if they continued to consent to take part in the study. The patient information was collected at each visit on a specific Case Report Form (CRF) (sample shown in Appendix 8).

If patients were identified as being at an increased risk of preterm delivery in this pregnancy due to clinical history or ultrasound findings, interventions were offered in line with hospital protocols and guidance. These included the use of vaginal progesterone

(Cyclogest) pessaries, cervical cerclage, bed rest and 2% clindamycin cream, dependent upon the clinical scenario and patient choice.

2.3.9.2 Group 2:

Participants from this group were recruited from the Labour Ward Triage area of UHCW NHS Trust. All patients who presented with symptoms of preterm labour (including lower abdominal pain, spontaneous rupture of membranes or per vagina bleeding) from February 2014 onwards when a researcher was present were seen. A clinical history was taken and risk factors reviewed. Prior to examination the patient was given a patient information leaflet (Appendix 2) and given some time to read this. Participants were consented to take part in the study, swabs taken including a routine high vaginal swab which was sent to the pathology laboratory for culture and sensitivity. The clinical history and examination findings were then presented to a clinician not involved in the study to determine the management plan for all patients in the study. Data was collected about the study participant on a specific case report form (sample shown in Appendix 8).

2.3.9.3 Group 3:

Participants were recruited from the antenatal ward at UHCW NHS Trust. Patients who attended the ward for induction of labour at term were recruited from April 2014 onwards. When each patient attended, the researcher explained the process of induction of labour. During this time the study was discussed with each potential participant and a patient information sheet given. Participants had the time to consider participation this whilst their routine observations were taken and cardiotocography was performed by the ward midwife. Patients were then consented to take part in the study and swabs taken. Data

were collected about the study participant on a specific case report form (sample shown in Appendix 8).

2.3.10 Exclusion Criteria:

- Multiple pregnancy
- History of preterm labour (Group 1) in a multiple pregnancy
- Recent abnormal smear test.

2.3.11 Sample Collection:

2.3.11.1 Group 1 & Group 2

Groups 1 & 2 had swabs taken for all three arms of the project. The protocol for obtaining the swabs was as follows.

1. A sterile speculum examination was performed using a water based lubrication gel and the cervix visualised. A routine high vaginal cotton swab (Charcoal Wood Cotton Tip) was placed in the posterior fornix of the vagina for 10 seconds and then sent to the UHCW NHS Trust pathology laboratory for culture and sensitivity testing including examination under the microscope for the presence of Clue cells.
2. Microbiome swabs: Two Catch-All™ Sample Collection swabs were utilised, one placed in the posterior fornix of the vagina and one in the endocervical canal, each for 15 seconds.
3. Enose swabs: Two further Catch-All™ Sample Collection swabs were then placed in the posterior fornix for 15 seconds, each was processed by adding 5ml of 10% KOH

to one and 5ml of 0.9% NaCl to the other. The swab was left in the solutions for 1 minute before removing it from the liquid and then sealing the sample.

4. PLCL1 cytobrush sample: A cytobrush was used to collect cervical cells by gentle rotation once in the endocervical canal. This aimed to obtain cervical cells from several layers of the squamous epithelium and basement membrane (See Chapter 1, Figure 1.3). Cells were immediately placed in a ThinPrep or SurePath vial and the sample snap frozen in liquid nitrogen and transferred to Arden Tissue Bank. Optimisation of cervical cell collection is discussed in Chapter 3.

2.3.11.2 Group 3:

Groups 3 participants had swabs taken for only the PLCL1 arm of the project. As above, a cytobrush was used to collect endocervical cells by gentle rotation once in the endocervical canal, placed in a SurePath vial and the sample snap frozen in liquid nitrogen and transferred to Arden Tissue Bank as described above.

Further details about the processing of the samples taken for the Microbiome and Enose arms of this project will not be included and are beyond the scope of this thesis. Data relating to these samples will be published at a later date.

2.4 Primary Outcome Measure:

Quantity of PLCL1 in each sample

2.5 Secondary Outcome Measures:

Analysis of microbiome and Enose data

- Not discussed further in this thesis

2.6 Study safety:

The technique of sampling the endocervix with a cytobrush was developed by Whitworth *et al* for their study looking at cervical leukocyte sub-populations in idiopathic preterm labour. This was not associated with an increased risk of preterm labour and was tolerated well by patients (Whitworth *et al.*, 2007).

2.7 Withdrawal Criteria:

Women could voluntarily withdraw from the study at any stage.

2.8 Study Flow Diagram:

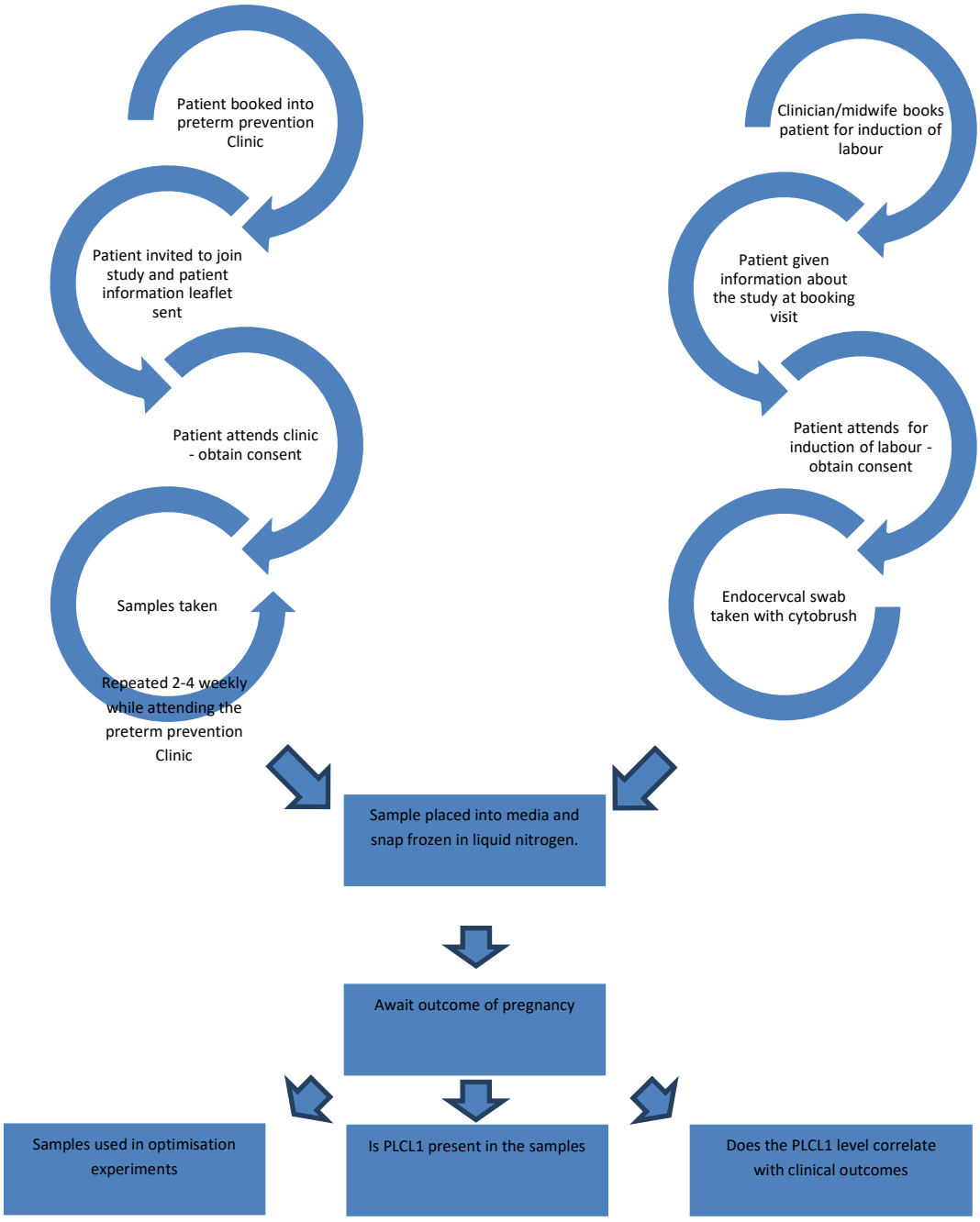

Figure 2.1: Study flow diagram for PIMF for both group 1 and group 3 patients

2.9 Confidentiality:

Clinical data, laboratory data and clinical outcomes were recorded and analysed on a structured proforma (sample shown in Appendix 8). Samples analysed at the University of Warwick were identified by a code which allowed reversible anonymity. Anonymised data were stored on NHS computers and University computers which had access controls, in order to facilitate analysis of the data and follow up of outcomes using spreadsheets. The main location for data storage was the Biomedical Research Unit (BRU) at UHCW NHS Trust. Manual data were filed and the filing cabinets, cupboards and/or rooms and were locked. Out of hours access is available to authorised personnel only. The principal investigator ensured that the confidentiality of participants taking part in the study was preserved. Participants' identification data were required for the registration process. We preserved the confidentiality of participants taking part in the study and were registered under the Data Protection Act.

2.10 Indemnity:

The indemnity for the study was provided by the study sponsor.

2.11 Sponsor:

University of Warwick acted as the main sponsor for this study (Appendix 9).

2.12 Funding:

Biomedical Research Unit, University Hospitals Coventry and Warwickshire NHS Trust

2.13 Audits and Inspection:

Monitoring and audit was conducted under the normal practices of University Hospitals Coventry and Warwickshire NHS Trust and the University of Warwick. This study was subject to inspection and audit by the sponsor and other regulatory bodies to ensure adherence to Good Clinical Practice (GCP).

2.14 Further Information, Group 2:

Following the initial two months of patient recruitment to PIMF a decision was made by the study team to no longer recruit patients from the labour ward triage area at present. The reason for this was despite numerous hours spent, only one patient who attended with symptoms of preterm labour while the study team were present went on to deliver preterm. A decision was therefore made to concentrate on the other groups.

Chapter 3

3. Development of an assay to detect and quantify PLCL1 in cervical epithelial cells obtained using the cytobrush technique

3.1 Materials:

3.1.1 BD SurePath™ components:

Prepstain® Density reagent

3.1.2 General buffers and solutions:

All buffers and solutions were made with distilled water and stored at room temperature unless stated otherwise.

Dulbecco's Phosphate buffered saline (DPBS)

- Potassium chloride – 200.0mg/L
- Potassium phosphate monobasic – 200.0mg/L
- Sodium chloride – 8000.0mg/L
- Sodium phosphate dibasic – 2160.0mg/L

Tris buffered saline (TBS) (10X)

TBS-Tween 20 (TBS-T)

- 0.05% Tween 20 (polyoxyethylensorbitan monolaureate) in TBS

Sputolysin® Reagent – Calbiochem (10x) (Stored at 5°C)

- ~6.5mM dithiothreitol in 100mM phosphate buffer pH 7.0

3.1.3 For Reagent Based Cell Lysis:

RIPA buffer (10X) - (Stored at 5°C)

- One tablet of Protease inhibitor per 10ml (Pierce™ Protease Inhibitor Tablet Mini)
- 100µl Phosphatase inhibitor per 10ml (Sigma phosphatase inhibitor cocktail 2)

3.1.4 For Mechanical Cell Lysis:

Polytron system PT 1200E Manual disperser

3.1.5 For Sonication for Cell Lysis:

Ultrasonic Water Bath - Grant - XUBA3

3.1.6 For Filtration:

40µm Falcon Cell Strainer

3.1.7 Protein Quantification Materials and Reagents

Bovine serum albumin (BSA) solution 4mg/ml in 0.1M NaOH

- 52mg BSA powder (Sigma)
- 13ml 0.1M NaOH (Fisher)

Bradford Reagent for 1-1400µg/ml protein (Sigma)

- Serial dilutions made using 0.1M NaOH

3.1.8 For Polyacrylamide Gel Electrophoresis (PAGE) & Western Blotting Technique:

Sample buffer

- NuPAGE® SDS Sample Buffer (4x) (ThermoFisher)
- DL-Dithiothreitol (DDT)

Running Buffer

- Tris glycine SDS (10x) (ThermoFisher)

Mini-PROTEAN® TGX™ precast gels (10%)

Tetracell Tank (BioRad)

Precision Plus Protein™ Prestained Standard (Bio-Rad)

Filter paper - extra thick

Blotting pads

Nitrocellulose (unsupported), 0.2µm Hybond-EC transfer membrane

Transfer Buffer

- Transfer buffer (20x) (ThermoFisher)
- Methanol (20%)

Blocking agent

5% Non-fat dried milk

- TBS-T
- Non-fat dried milk powder

3.1.8.1 Antibodies:

Primary

Dilutions were made using TBS-T

Antibody	Dilution	Manufacturer
PLCL1 Rabbit polyclonal	1:500	Sigma Aldrich
B-actin Mouse monoclonal	1:100000	Abcam

Secondary

Dilutions made with 5% non-fat dried milk

Antibody	Dilution	Manufacturer
Horseradish peroxidase (HRP) – conjugated goat anti-rabbit IgG	1:1000	Abcam
Horseradish peroxidase (HRP) – conjugated rabbit anti-mouse IgG	1:100000	Abcam

Substrate

Pierce® ECL Western Blotting Substrate (ThermoFisher)

- Detection reagent 1
- Detection reagent 2

3.1.9 Cytospin:

Shandon EZ single cytofunnell with white filter cards and caps (ThermoFisher)

Charged glass slides

3.1.10 Immunocytochemistry:

Novolink Polymer Detection System (Leica Biosystem)

- Peroxidase block,

  3% hydrogen peroxide

- Protein Block

  0.4% Casein in phosphate-buffered saline, with stabilisers, surfactant, and 0.2% Bronidox L as a preservative

- Post Primary Block

  Polymer penetrations enhancer containing 10% (v/v) animal serum in tris-buffered saline/0.09% ProClin™ 950

- NovoLink Polymer

  Anti-mouse/rabbit IgG-Poly-HRP (each at 8µg/ml) containing 10% (v/v) animal serum in tris-buffered saline/0.09% ProClin™ 950

- DAB Chromogen

  1.74% w/v 3,3' – diaminobenzidine, in a stabiliser solution

- NovoLink DAB Substrate Buffer (Polymer)

  Buffered solution containing 0.05% hydrogen peroxide and preservative.

- Hematoxylin

  0.02% hematoxylin

TBST

3.1.10.1 Antibodies:

Primary

Dilutions were made using TBS-T

Antibody	Dilution	Manufacturer
PLCL1 **Rabbit polyclonal**	1:250	Sigma Aldrich
CD117 **Mouse monoclonal**	1:300	LifeSpan Biosciences

*Xylene (*70% & 100%)

Alcohol

Mountant

Cover slips

3.1.11 Enzyme-Linked Immunosorbent Assay (ELISA):

Human inactive phospholipase C-like protein1 (PLCL1) ELISA kit- CUSABIO

Assay plate (96 coated wells)

Standard (freeze dried)

Biotin- antibody (100x concentrate)

HRP-avidin (100x concentrate)

Sample diluent

Wash Buffer (25x concentrate)

TMB substrate

Stop solution

Adhesive strips

3.1.12 Reverse Transcription Polymerase Chain Reaction (RT-PCR):

RNA extraction and sample preparation

RNA STAT-60 (AMS Biotechnology) – ice cold

Nuclease-free Eppendorf tubes

Chloroform - ice cold

75% ethanol/25% nuclease free water

TE buffer

NanoDrop® ND-1000 spectrophotometer

CDNA reverse transcription

Quantitect Reverse Transcription Kit (Quigen)

gDNA wipeout buffer

Quantiscript Reverse Transcriptase

Quantiscript RT buffer (5x)

RT primer mix

RNase-free water

SYBR Green Master Mix PCR reagents (Fisher Scientific)

3.1.13 Trypan Blue Exclusion:

Trypan blue solution 0.4% (ThermoFisher)

PBS

Haemocytometer

3.2 Methods:

3.2.1 Optimisation:

This novel project aimed to quantify an intracellular cervical protein (in the current literature, no other marker of preterm labour has been developed from an intracellular cervical protein). We therefore needed to develop a method to collect and then process our samples of cervical cells. This process involved overcoming several issues to answer our research questions including:

1. What solution to put our samples in prior to freezing?
    a. It needed to not interfere with the structure of the PLCL1 protein
    b. This needed to allow the subsequent development of a method of removal of cervical mucus from the sample prior to total protein and PLCL1 quantification
2. How to separate the cells from the cervical mucus?
3. How to lyse the cervical cells effectively without affecting the protein structure?
4. Is PLCL1 present in our cervical samples?
    a. Western blotting
    b. Immunocytochemistry (ICC)
    c. ELISA
    d. RT-PCR
        i. Cell viability
5. Can we quantify PLCL1 in our cervical samples?
    a. ELISA
6. Due to the variety in sample size and number of cells obtained per patient – how can we adjust results to account for these differences?

a. Quantification of PLCL1 as a proportion of the total protein concentration of the sample

3.2.2 Collection of Cervical Cells Samples and Separation from Cervical Mucus

From 2014-2015, 3.21 million cervical samples were taken as part of the NHS Cervical screening Programme (Screening and Immunisation Team Health and Social Care Information Centre, 2015). Since 2009 all cervical samples have been prepared using liquid based cytology (LBC). There are currently two principal systems available for liquid based cytology, ThinPrep® and SurePath®. These systems are used to prepare cells to screen for the presence of atypical cells, cervical cancer and both low-grade and high-grade squamous intraepithelial lesions. Our University Teaching Hospital utilises the ThinPrep® 2000 system in the preparation of cervical cells in the Cervical Screening Programme. This is a computerised process. The ThinPrep® 2000 processor controls dispersion, collection and transfer of cervical cells from the sample to a glass slide. The process allows the break-up of cervical mucus and blood in the samples. Fluid is drawn through a filter using negative pressures. This leads to a collection of an even layer of diagnostic cervical cells on a glass slide which can then be fixed, stained and examined under the microscope.

Initially we utilised this method for our sample collection but we could not use the ThinPrep® 2000 processor for several reasons. It leads to the production of only one glass slide with a thin layer of cells. This sample size was too small for us to quantify PLCL1 from. The process is also performed at room temperature and so we had concerns this may affect protein structure in our samples. We used the ThinPrep vials to collect our initial 8 patient samples. Samples were collected by the study team using the cytobrush and then immersed in vials filled with 20ml of PreservCyt® solution. They were capped, labelled

using the study number, flash frozen in liquid nitrogen and stored in the Arden Tissue Bank prior to further sample processing.

On the day of sample processing, each sample was defrosted and then kept on ice. Samples were vortexed for 15 seconds and transferred to labelled 50ml conical tubes and centrifuged at 1200rpm for 5 minutes. The supernatant was discarded and the pellet resuspended in 1ml of RIPA buffer. This method did not remove the cervical mucus which affected the subsequent experiments.

Following liaison with the cytology department at UHCW NHS Trust pathology laboratory, we then decided to use the BD SurePath™ system as this would allow the full processing following their protocol to eliminate cervical mucus.

Samples were collected by the study team using the cytobrush and immediately immersed in vials filled with 10ml SurePath® Preservative Fluid. This is an alcohol-based (21.7% ethanol, 1.2% methanol & 1.1% isopropanol), preservation solution that serves as a transport, preservative and antibacterial medium for gynaecological specimens. This was capped, labelled using the study number, flash frozen in liquid nitrogen and stored in the Arden Tissue Bank prior to further sample processing.

On the day of sample processing, each sample was defrosted and stored on ice. 15ml centrifuge tubes were labelled and filled with 4ml PrepStain® Density Reagent. Samples were vortexed in the vials for approximately 20 seconds prior to sample processing. Each sample was then gently loaded on to the density gradient medium using a 10ml sterile syringe ensuring the sample was on top of the density reagent. Samples were centrifuged for 2 minutes and 15 seconds at 200g at 4°C as per the SurePath Process WorkFlow. The

supernatant was aspirated using a tube vacuum. All visible mucus in the supernatant and including that adherent to the sides of the centrifugation tubes was removed at this point. Samples were then centrifuged again for 10 minutes at 800g at 4°C. Remaining fluid was aspirated from the tubes using a gel loading tip on a tube vacuum. The remaining pellet of cervical cells was kept on ice prior to being used in subsequent experiments.

Further optimisation experiments were carried out following immunocytochemistry, SDS-PAGE and Western blotting, PCR and ELISA experiments (Chapter 3, Sections 3.2.3 and 3.2.5).

3.2.3 Protein Extraction for ELISAs and SDS-PAGE/Western Blot Analysis:

3.2.3.1 Background:

Cell lysis is the first step required for protein extraction. Cells can be lysed using both mechanical methods and using detergents. Conventionally, cells were lysed using mechanical methods, these included use of a homogeniser, sonication, and freezer thaw cycling. A homogeniser mechanism of action involves shearing of the cell membranes by forcing the cell through a narrow space. There are several problems with this method. The process itself can cause localised heating of the sample leading to denaturing of the protein, therefore all samples should be kept on ice using this process. Additionally, the reproducibility using this method may not be good. Sonication works using high frequency sound waves, this indirectly causes agitation and shearing of the cell membranes also leading to cell lysis. As with homogenisation, this can also cause a heating effect and therefore needs to be carried out on ice. Freeze thaw cycling disrupts the cell membranes though ice crystal formation. This process can be lengthy as many cycles are required to

lead to adequate cell lysis. More commonly detergents are now used for cell lysis, they disrupt the cell membrane leading to cell lysis, however many detergents also can cause proteins to be denatured. RIPA buffer is a cell lysis buffer which is compatible with many downstream experiments and therefore can be used on our samples which were then used for protein assays including ELISA, SDS-PAGE and Western blotting.

3.2.3.2 Initial Cell Lysis

Cervical cell samples were prepared as described above. Cells were lysed by adding 500µl of protein lysis RIPA buffer.

3.2.3.3 Total Protein Quantification:

The protein concentration of each sample was determined by the Bradford Method. This method of protein determination is based on the binding of a dye, Coomasie Blue G, to the protein (particularly to arginine and hydrophobic amino acid residues). This binding shifts the absorption maximum of the dye from red to blue (Bradford, 1976).

A set of standards were made in 1.5ml eppendorfs using BSA, diluted with 0.1M NaOH to the concentration of; 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0 mg/ml. Patient cervical cell protein samples were diluted 1:5 using 10µl of sample and 40µl of 0.1M NaOH. 5µl of each standard/patient sample were added to the wells in triplicate in a 96-well clear bottomed plate.

The aim was to ensure that the patient samples fell in the linear portion of the standard curve. Occasionally for patient samples containing a large amount of total protein, this

process of protein quantification needed to be repeated with a 1:10 dilution (10µl of patient samples with 90µl of 0.1M NaOH).

200µl of Bradford reagent was added to each well and left to stand for 5-15 minutes. The absorbance was then read on the plate reader at 595nm.

All results were analysed using Microsoft Excel. The coefficient of variation (CV) was calculated for each of the triplicates. CV is a standard measure of dispersion and is defined as the ratio of the standard deviation to the mean, it is often expressed as a percentage

$$CV\ (\%) = \frac{Standard\ deviation\ (\sigma)}{Mean\ (\mu)}\ x\ 100$$

This was used to help to determine the repeatability of the assay. If the CV was greater than 5%, the value that was furthest away from the median was excluded from further analysis. A standard curve was plotted using the GraphPad software and patient sample results interpolated to determine the protein concentration of the unknown samples. The values obtained were then multiplied by their dilution factor to obtain the protein content of each sample in mg/ml.

3.2.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) & Western Blotting:

Following protein quantification per sample, each sample was divided and one portion of the sample was diluted using RIPA buffer. The amount of RIPA buffer added was calculated to ensure each sample contained a standard protein concentration prior to commencing the SDS-PAGE and Western Blotting.

Polyacrylamide gel electrophoresis is a technique during which charged molecules are separated dependent upon their mass or charge. Proteins are forced though a sieving gel matrix by an electrical current. The sizes of the pores in the gel are dependent upon the concentration of acrylamide and bisacrylamide. Gels with a lower percentage of acrylamide have larger pores (Alberts, 2002).

Our method utilised used the denaturing and reducing SDS-PAGE technique. We aimed to separate our protein by mass (as had been demonstrated previously in the work on myometrial cells with PLCL1 (Chapter 1 Figure 1.10). SDS denatures and binds proteins making them negatively charged, the amount of negative charged added to each protein is proportional to the size of the protein in KDa. The addition of DTT aids in the denaturing process by disrupting the secondary protein structure by destroying di-sulphide bonds in the proteins (Alberts, 2002).

65µl of each sample was placed in a labelled 1.5ml Eppendorf tube. 25µl of SDS sample buffer and 10µl of DDT (100mM) was added to each Eppendorf. Samples were vortexed for 10 seconds and incubated at 95°C for 5 minutes. The DTT and heat disturb the secondary, tertiary and quaternary structure of the proteins.

Proteins were resolved on Mini-PROTEAN®TGX™ Precast Gels according to the manufacturer instructions. Samples were loaded alongside 8µl of pre-stained molecular weight marker. 40µl of sample was added to each lane corresponding to approximately 25µg of total protein.

When voltage is applied, the SDS bound proteins migrate through the gel towards the positively charged electrode. Proteins with a larger mass travel more slowly through the gel due to the sieving effect of the gel. We anticipated that PLCL1 at ~130KDa would travel slowly and remain near the top of the gel and β-actin at ~30KDa travels well in the gel and reach near to the bottom of the gel.

Gels were ran at 120V for 1 hour 15 minutes or a little longer until the dye had migrated to the bottom of the gel. At completion of the run, the cell was disconnected and cassette removed.

Western Blotting was used to transfer proteins onto a nitrocellulose membrane by Western. Cassettes were opened using the opening lever as per the manufacturer instructions. The plates were pulled apart and the gels is then gently removed from the cassette and placed on to nitrocellulose transfer membranes. Filter papers, blotting pads and nitrocellulose membranes were soaked in transfer buffer prior to assembly.

The following sequence was then placed on the cathode; two blotting pads, one filter paper, one gel, one nitrocellulose membrane, one filter paper, one blotting pad, one filter paper, second gel, one nitrocellulose membrane, one filter paper, two blotting pads. At all times it was ensured that the sequence was soaked in transfer buffer. Each time, following placement of the filter paper, the sequence was pressed using a roller to eliminate any air bubbles which may have been present. The anode plate was fitted. A current of 230mA was created perpendicular to the gel for 1 hour 50 minutes. This allowed the migration of the negatively charged proteins through the gel towards the anode and therefore deposited the proteins on to the nitrocellulose membrane.

The nitrocellulose membrane was then blocked by incubation for 1 hour at room temperature with 5% non-fat dried milk to prevent any non-specific binding of antibodies to the surface of the membrane.

Membranes were washed in TBS-T several times and treated with the primary antibodies overnight at 4°C. The next day the membrane was washed three times for 15 minutes on the rocking machine with TBS-T. The membrane was incubated for 1 hour at room temperature with the HRP-conjugated secondary antibodies raised against the primary antibodies followed by three washes in TBS-T for 15 minutes.

We utilised a luminol based substrate to produce a chemiluminescent signal. In the presence of HRP and a peroxidase buffer, luminol oxidises and forms an excited state product that emits light. X-ray film is then used to detect the chemiluminescent signal. The substrate was prepared, 1 ml for each blot to ensure that each blot was completely wet and did not dry out. The blots were incubated for 1 minute with the substrate. The blots were then removed from the substrate and placed in a plastic membrane protector made from plastic wrap. All air bubbles were removed. The blot was then imaged using a film. Several sheets of film were used to try and determine the optimal exposure time.

3.2.4 Immunocytochemistry (ICC):

3.2.4.1 Cytospin:

Following sample preparation as per the BD SurePath™ protocol, 1ml of PBS was added to each sample. For processing in the cytospin centrifuge the optimal cell density required was 1 x 10^6 cells per ml. All samples remained on ice during processing.

Each sample was counted using a haemocytometer. The haemocytometer and cover-slip were cleaned using alcohol and the haemocytometer moistened and cover slip affixed. 10µl of the cell suspension was pipetted at the edge of the cover slip and allowed to run under. The sample was then visualised under the light microscope and the number of cells in each of the four corner squares counted using a cell counter. Cells were included in the count if they were crossing the edge of the upper or left border of the corner squares and excluded if they were crossing the edge of the lower or right border of the corner squares to ensure accuracy and consistency from sample to sample. The number of cells per ml was calculated as follows:

$$No.of\ \frac{cells}{ml} = mean\ no.of\ cells\ in\ a\ corner\ square\ x\ dilution\ factor\ (1)x\ 10^4$$

Samples were then re-spun in the centrifuge to repellet the sample and less PBS added if the cell concentration was too sparse, or diluted further if the cell concentration was too dense.

Samples were processed using the Thermo Shandon Cytospin 4. This uses centrifugal force to deposit a monolayer of cells in a defined area on a glass slide. 100µl of each sample were loaded cytofunnels with white filter cards and a charged glass slide. Each patient sample was loaded into 4 cytofunnels to allow the production of 4 glass slides per sample. This was to allow for ICC for PLCL1, CD117 (negative control), a sample in which no primary antibody was used and one spare slide. The cytospin was set at 400RPM for 6 minutes. The glass slides were then removed from the cytofunnel and filter paper, allowed to dry and the samples were then ready for immunocytochemistry.

3.2.4.2 Immunocytochemistry:

Samples had been previously fixed in the BD SurePath™ media. We used the NovoLink Max Polymer Detection system for ICC at room temperature.

The methodology described as per the manufacturer instructions was adapted following consultation with our laboratory immunohistochemistry expert Mr Sean James. Instead of using TBS for the washing steps, TBS-T was used to ensure that the cell membranes were disrupted, allowing access to the intracellular cell compartments.

For immunocytochemistry, samples were washed in TBS-T for 5 minutes. To prevent any endogenose peroxidase activity from causing a false positive in our sample, peroxidase block was added to each slide for 5 minutes followed by two washes with TBS-T for 5 minutes each. Protein block is added to the slides for 15 minutes to reduce non-specific binding of the primary antibody and polymer, followed by two washes with TBS-T for 5 minutes each. Each sample then had the primary antibody (PLCL1, CD117 or no primary antibody). CD117 was chosen as the negative control as this receptor tyrosine kinase is a protein which is known not to be present in cervical epithelial cells. This was followed by two washes in TBS-T for 5 minutes. Slides were incubated with post primary block for 30 minutes to enhance penetration for the polymer reagent and again washed in TBS-T for 5 minutes. Then Novolink polymer was added for 30 minutes (this recognises mouse and rabbit immunoglobulins and therefore detected any tissue bound to the primary antibodies) followed by two washes in TBS-T for 5 minutes with gentle rocking to ensure all excess Novolink polymer was removed. DAB working solution was added for 5 minutes allowing the development of peroxidase activity to produce a visible brown precipitate at the antigen site. Finally slides were rinsed in water, counterstained with hematoxylin,

rinsed for a further 5 minutes and dehydrated using alcohol and xylene. The final slides were mounted with cover slips for examination under the light microscope

3.2.5 Enzyme-Linked Immunosorbant Assay (ELISA):

An ELISA is an analytical biochemical assay which is plate based and is devised to detect and quantify compounds including proteins. The PLCL1 ELISA utilised in our experiments was an indirect assay. This is demonstrated in Figure 3.1.

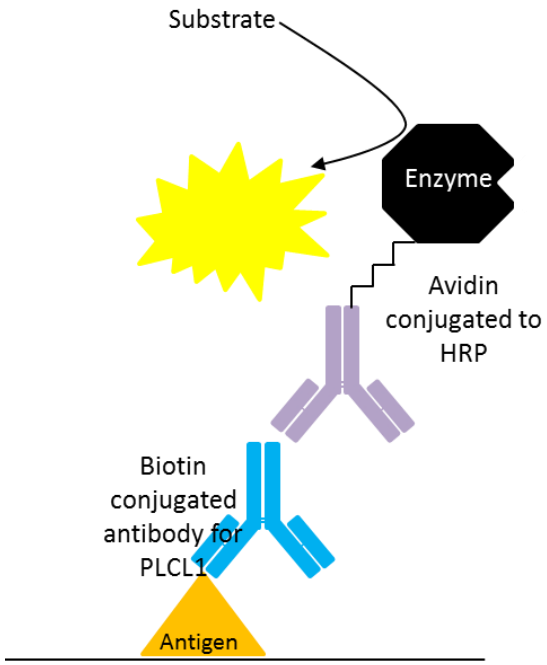

Figure 3.1: An indirect ELISA Assay. In our assay, the plate wells were coated with immobilised human PLCL1 antibody. When the sample was added to the well, any PLCL1 will bind to the immobilised antibody. Our primary antibody was a biotin conjugated antibody for PLCL1 and avidin conjugated HRP acted as the secondary antibody. The substrate from the ELISA kit was then added and the colour developed. The amount of colour is proportional to the amount of PLCL1 present in the sample. The colour development is stopped and the optical density of each well is determined using a microplate reader set to 450nm.

Human inactive phospholipase C-like protein 1 (PLCL1) ELISA Kit (CUSABIO) was used to determine the presence of and quantify PLCL1 in our samples (cervical cell lysates) as per the manufacturer protocol. Following initial experiments, the protocol was modified. The standard was reconstituted using RIPA buffer instead of the sample diluent provided in the ELISA kit to produce the stock solution. The serial dilutions of the standard were then also made using RIPA buffer in the place of the sample diluent.

Each cervical cell sample was processed in triplicate using the 96 well plate provided. As was performed for the total protein quantification for each sample, all results were analysed using Microsoft Excel. The CV was calculated for each of the triplicates as described earlier in this Chapter in section 3.2.3.3.

3.3 Initial Experimental Results:

3.3.1 Background:

The initial experiments aimed to answer the first questions:

1. What solution to immerse the cells in following collection with the cytobrush sampler and store in prior to sample processing?
    a. This needed to not interfere with the structure of the PLCL1 protein
    b. Allow the subsequent development of a method of removal of cervical mucus from the sample prior to total protein and PLCL1 quantification
2. How to separate the cells from the cervical mucus?

Utilisation of ThinPrep® vials filled with 20ml of Preservcyt® solution led to problems with separation of cervical cells and cervical mucus in the samples. Large amount of mucus were

seen in the conical centrifuge tubes following centrifugation mixed with the cell pellet. It was consequently decided that an alternative method of sample collection was required - the BD SurePath™ system was utilised. This method appeared to remove the cervical mucus. The mucus became stuck in the density gradient medium during the centrifugation process and was removed with the supernatant.

3.3.2 Results from Initial Experiments:

The Figures 3.1 and 3.2 demonstrate the initial results obtained following sample process using the BD SurePath™ system.

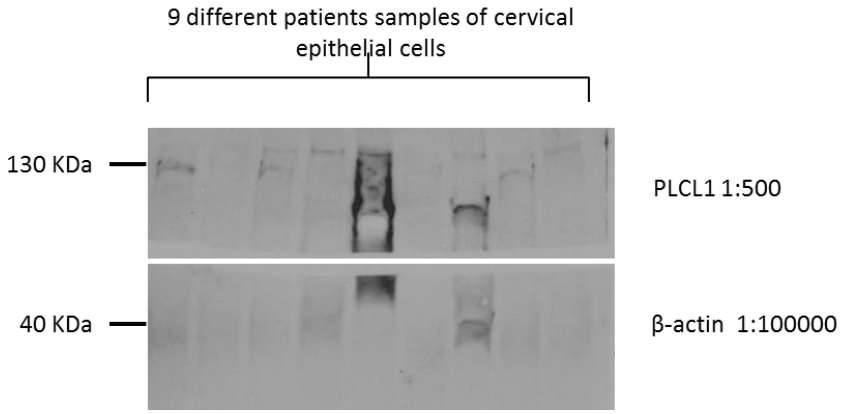

Figure 3.2: Western blot analysis of PLCL1 from total cell lysates from 9 samples of patient cervical epithelial cells collected during pregnancy. Sample were prepared following the BD SurePath™ Pap Test Process Workflow.

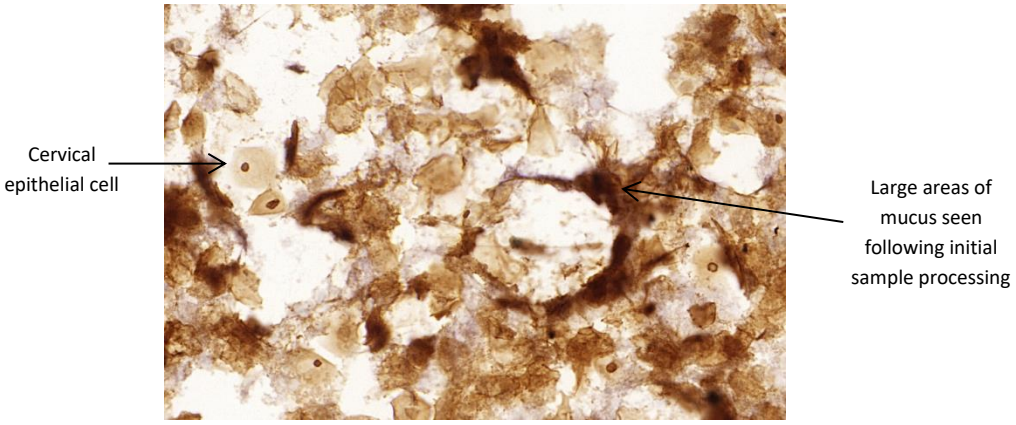

Figure 3.3: Immunocytochemistry of PLCL1 from patient sample (PID 132) of cervical epithelial cells collected during pregnancy. Samples were prepared following the BD SurePath™ Pap Test Process Workflow. Image taken using Pannoramic Viewer, 20x magnification

The immunocytochemistry results obtained (shown in Figure 3.3) demonstrated that using the BD SurePath™ protocol still did not lead to the removal of all of the cervical mucus from our samples. This could have influenced the movement of the proteins during the SDS-PAGE and therefore affect the results obtained (Figure 3.2). We were also concerned about whether we were adequately lysing the cervical cells.

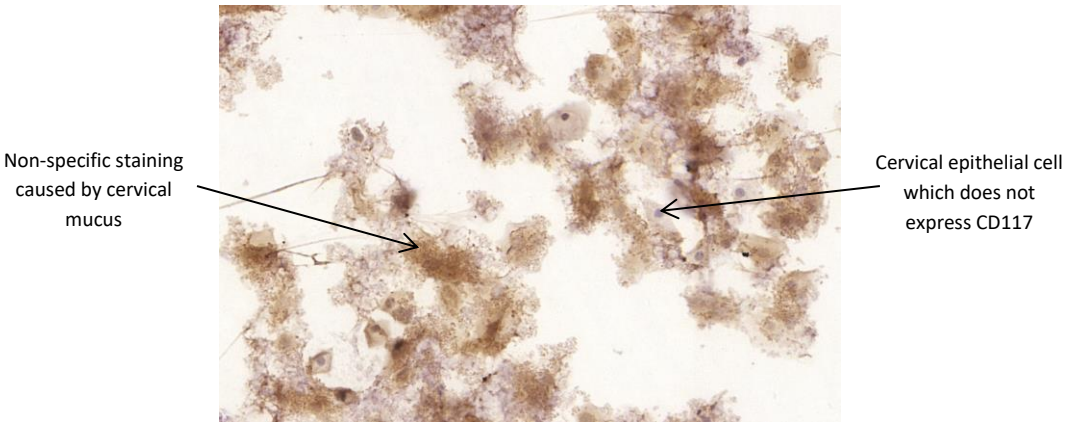

Figure 3.4: Immunocytochemistry of CD117 from patient sample (PID 132) of cervical epithelial cells collected during pregnancy. Samples were prepared following the BD SurePath™ Pap Test Process Workflow. Image taken using Pannoramic Viewer, 20x magnification.

Figure 3.4 demonstrated some of the further problems that inadequate removal of the mucus had when interpreting immunocytochemistry results. As mentioned, CD117 is known not to be present in cervical epithelial cells, however the presence of cervical mucus led to non-specific staining of slides.

3.3.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR):

3.3.3.1 Background:

Polymerase chain reaction (PCR) is a process in which a single DNA molecule can be amplified into millions of copies. There are three steps, denaturation, annealing and extension. During denaturation, the sample is heated to separate the two strands, then during annealing, primers are allowed to bind to flanking regions of the target DNA. Finally,

DNA polymerase extends the 3' end of each primer along the target DNA. The three steps are repeated exponentially producing millions of copies of the target.

RT-PCR is a technique which can be used to quantify mRNA. It can be used on very small samples. Initially the RNA is reverse transcribed into complementary DNA (cDNA) by reverse transcriptase. The cDNA can then act as a template for the quantitative PCR reaction. It can be performed in one or two steps, in the one step process the reverse transcriptase and PCR are combined in the same tube, in the two step process they are performed separately.

Real time RT-PCR is the technique of collecting data throughout the PCR process as it occurs, it combines amplification and detection into a single step and allows the initial quantity of the target to be accurately determined. Reactions are characterised by the point in time or PCR cycle where the target amplification is first detected, this value is referred to as cycle threshold (C_t) (Wong & Medrano, 2005). The amount of DNA is measured after each cycle using a florescent dye (SYBR green). The signal is in direct proportion to the number of PCR products. Data are collected in the exponential phase of the reaction and used to determine the quantity of the target in the initial sample.

3.3.3.2 RNA Extraction and Sample Preparation:

Samples of epithelial cervical cells were collected from patients as per the protocol described in Chapter 2, Section 2.3.11. Total RNA was extracted from the samples using RNA STAT-60 following sample processing through the density gradient medium following the SurePath Process WorkFlow. 400µl of ice-cold RNA STAT-60 was added to each sample and transferred to nuclease-free Eppendorf tubes. 80µl of chloroform was added to each

sample. Each sample was vigorously vortexed and stored at -80°C until the assay was performed. RNA from the samples was separated by centrifugation (16000g, 30 minutes at 4°c), the top aqueous phase was transferred to a second Eppendorf tube containing 200µl ice-cold isopropanol. Samples were vortexed and stored overnight to allow precipitation. The sample were then centrifuged at 16000g for 15 minutes at 4°C and the pellet washed twice in 75% ethanol/25% nuclease free water. The supernatant was then removed and samples allowed to air dry to eliminate all ethanol for 15 minutes. Dried pellets were suspended in 20µl of TE buffer and RNA absorbance determined by absorbance at 260nm on NanoDrop® ND-1000 spectrophotometer

3.3.3.3 cDNA Reverse Transcription:

1µg of total RNA was reverse transcribed using Quantitect Reverse Transcription Kit as per the manufacturers' instructions and the resultant cDNA used for RT-PCR.

3.3.3.4 RT-PCR:

cDNA was assayed in triplicate using the SYBR Green Master MIX PCR reagents and specified primers. They were quantified on an automated 7500 real-time PCR system from which cycle threshold (C_t) values were obtained. Any variance in cDNA input was nullified by normalising against the expression of L19 (a 60S ribosomal protein) reference gene. The primer sequences were as follows for PLCL1, sense 5' GCA GCA GCA TCA TCA AGG 3' anti-sense 5' GCT GCT GAA AGA CAC GGT TT 3' and for L19, sense 5' GCG GAA GGG TAC AGC CAA 3' anti-sense 5' GCA GCC GGC GCA AA 3'.

3.3.3.5 Results from RT-PCR:

Five samples were chosen for processing, sample identification numbers 276, 279, 280, 281, 282. Two myometrial samples were also included as controls as this method had been used previously by our group successfully to quantify PLCL1 mRNA levels. The results obtained are shown in Figure 3.5.

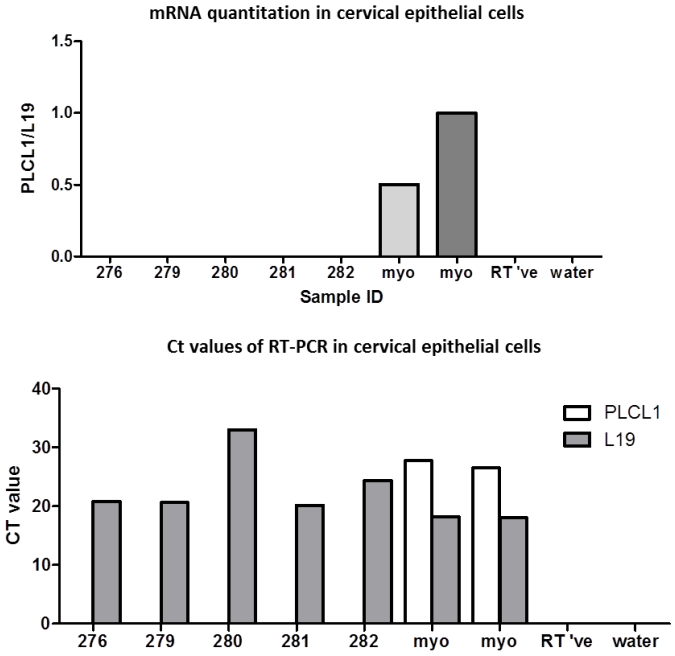

Figure 3.5: PLCL1 mRNA in cervical epithelial samples obtained (276, 279, 280, 281 & 282) two myometrial samples were also used as controls. PLCL1 mRNA was undetermined in our samples as can be seen.

It is likely that the reason we could not obtain PLCL1 mRNA levels in our samples is because the majority of the cells collected are cervical squamous epithelial cells which are dead. However to confirm this, a trypan blue exclusion protocol was performed to determine cell viability.

3.3.4 Trypan Blue Exclusion:

Cell viability was determined by the trypan blue dye exclusion assay on three samples of cervical epithelial cells (samples with ID numbers 117, 139 and 157). Cells were suspended in PBS containing 0.2% (v:v) trypan blue and counted using both an automated cell counter with trypan blue detection (LUNA Automated Cell Counter, Logos Biosystems) and manually using a Neubauer approved haemocytometer. The trypan blue assay works on the principle that viable cells with intact membranes exclude the dye and remain clear, whereas non-viable cells cannot and will appear blue. The percentage of viable cells was calculated as

$$\frac{number\ of\ blue\ cells}{total\ number\ of\ cells} \times 100\%$$

The results are shown in Figure 3.6.

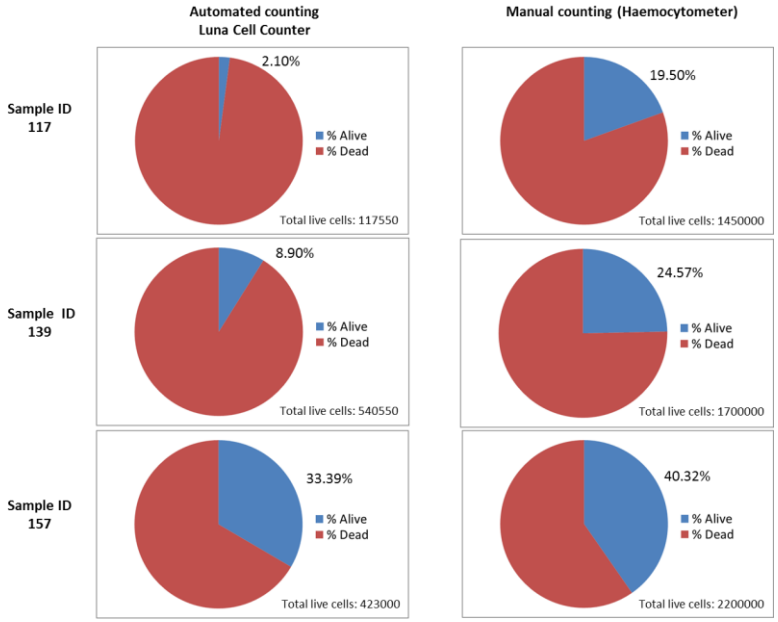

Figure 3.6: Results of trypan blue exclusion assay demonstrating that the majority of cells were dead in our samples.

As expected, the trypan blue exclusion assay demonstrated that the majority of the cells in our samples were dead, explaining the results obtained from our RT-PCR experiments. We therefore decided to focus on optimisation of our protocol to ensure all mucus was excluded from the samples, achieving optimal cell lysis, and optimal quantification of PLCL1 protein in our samples.

3.4 Further optimisation:

We needed to improve our method further to remove all mucus from the samples prior to further analysis. A literature review demonstrated that there is a lot of current research looking at inflammatory markers and cell types in sputum samples from patients with chronic lung disease. As with our cervical samples, these need to be separated from large

amounts of mucus. Previous studies have demonstrated that the use of Sputolysin® with sputum significantly improved the total cell counts obtained and subsequent cytospin quality (Efthimiadis *et al.*, 1997; Louis *et al.*, 1999).

Sputolysin® Reagent is a mucolytic which can be used to homogenise sputum, it was developed for the isolation of epithelial cells, bacteria, fungi and yeast from sputum. Following liaison with the cytology department at UHCW laboratory, they identified a protocol from a previous study looking at sputum samples to aid us with the development of our processing described below.

Additionally, we tested the use of 40µm Falcon cell strainers and whether their use helped to minimise the amount of sputum which remained in our cervical cell samples after processing. Figures 3.7 and 3.8 demonstrate the same sample processed using Sputolysin® Reagent and processed using Sputolysin® Reagent and 40µm Falcon cell strainer.

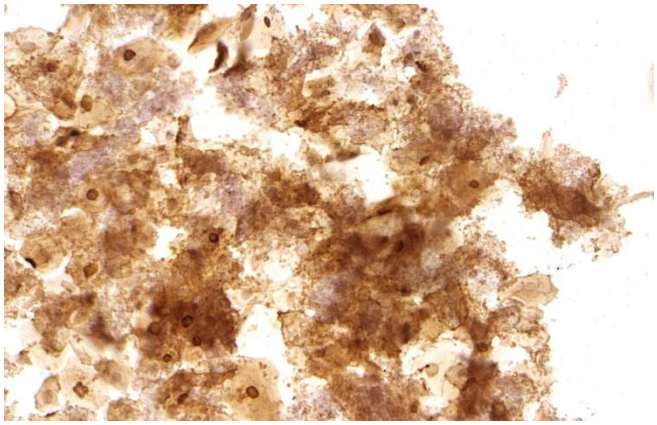

Figure 3.7: Immunocytochemistry of PLCL1 (from patient sample PID 132) of cervical epithelial cells collected during pregnancy. Samples were prepared following the BD SurePath™ Pap Test Process Workflow and Sputolysin® Reagent. Image taken using Panoramic Viewer, 20x magnification.

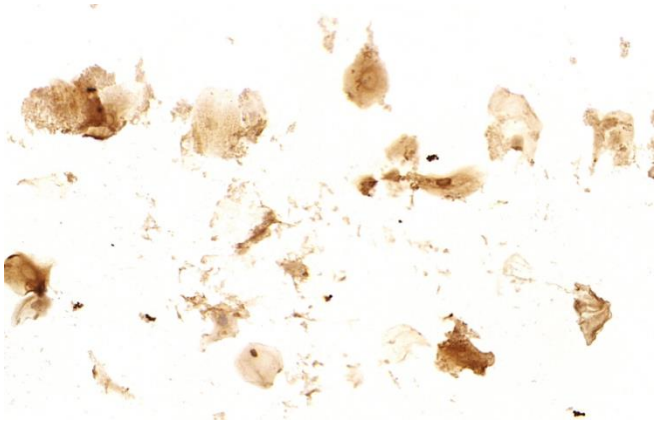

Figure 3.8: Immunocytochemistry of PLCL1 from patients sample (PID 132) of cervical epithelial cells collected during pregnancy. Samples were prepared following the BD SurePath™ Pap Test Process Workflow and Sputolysin® Reagent and 40µm Falcon cell strainer. Image taken using Panoramic Viewer, 20x magnification.

Utilising these additional components we developed the following method for processing our cervical cell samples prior to PLCL1 quantification using the PLCL1 ELISA.

As explained in Section 3.2.2, samples were collected by the study team using the cytobrush, immersed in 10ml SurePath® Preservative Fluid, capped, labelled, flash frozen in liquid nitrogen and stored in the Arden Tissue Bank prior to further sample processing. Samples were processed using the Prepstain® Density Reagent as described in Section 3.2.2, resulting in a pellet of cervical cells at the bottom of the centrifugation tube. This sample was kept on ice. Prior to addition of the Prepstain® Density Reagent centrifuge tubes were individually weighed and this documented. Following processing, the tube and cell pellet were reweighed, to allow determination of the pellet weight. The Sputolysin® Reagent was diluted as per the manufacturer instructions. A specified volume of diluted Sputolysin® Reagent was added to each pellet (two times to weight of the cell pellet in volume). A minimum volume of 150µl was added. Each sample was vortexed for 30

seconds and the mixture allowed to stand at room temperature for 15 minutes as per the manufacturer instructions (this was the only part of the process not performed on ice). Following this, samples were passed through a 40µm Falcon cell strainer and centrifuged at 1500rpm for 5 minutes at 4°C, the supernatant was discarded. Cell pellets were washed in 1ml cold PBS for 5 minutes, transferred to 2.0ml Eppendorfs tubes and the centrifugation process repeated at the same settings. The supernatant was removed and cell pellets stored on ice. The samples used for ICC were then processed as per Sections 3.2.4.1 and 3.2.4.2. Results are shown in Figure 3.9.

For subsequent ELISA and protein quantification methods, cell lysis was required. A combination of three methods of cell lysis were needed to lyse the cervical epithelial cells adequately and release intracellular PLCL1. 0.5ml of RIPA buffer was added to each cell pellet. This was then homogenised on ice using Polytron system PT 1200E Manual disperser for one minute. Finally the sample was sonicated 3 minutes on ice.

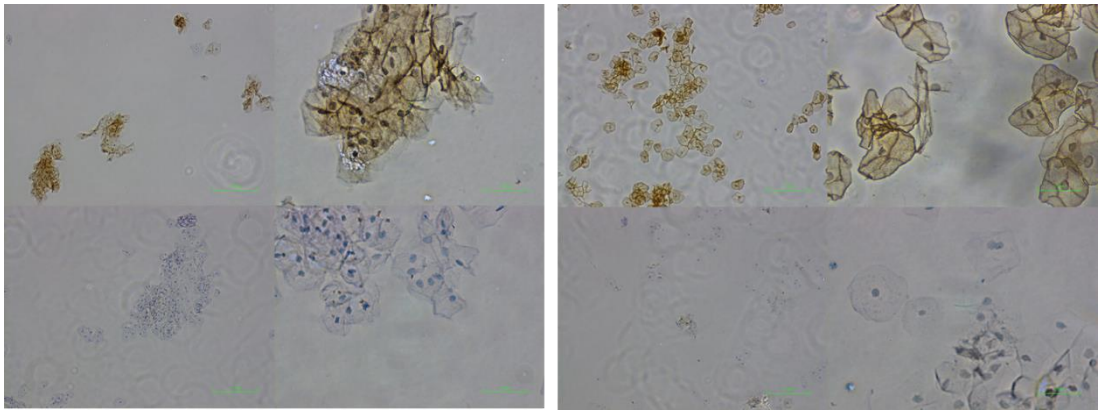

Figure 3.9: Immunocytochemistry of PLCL1 and control (PLCL1 above, control below) from two patient samples (PID 117(left) & 157(right)) of cervical epithelial cells collected during pregnancy. Samples were prepared following the process explained in Sections 3.2.4.1 and 3.2.4.2. Images were taken at 10x (right) and 40x (left) magnification.

Each sample was then processed following this protocol. The total protein per sample determined using the Bradford method (Section 3.2.3.3). PLCL1 was then quantified using the ELISA (Section 3.2.5). Sample results were then expressed as PLCL1/protein concentration in pg/mg.

A decision was made to exclude patient samples with a total protein concentration of <0.3mg/ml from further analysis. This is because samples with low concentrations of total protein had spectrophotometric absorbances outside of the optimal exponential phase of the standard curve, meaning interpolation of the total protein concentration could be potentially inaccurate. This is discussed further in Chapters 4, 5 and 6.

Chapter 4

4. Induction of labour

4.1 Background:

The process of induction of labour is an attempt to artificially initiate labour. This process is commenced after the gestation of fetal viability at a time when there is no evidence of active labour. A patient may be induced for maternal or fetal reasons, or sometimes both, when interrupting the pregnancy is thought to be safer for the mother or the baby than allowing gestation to continue. Induction of labour is common in the UK. In 2004-2005 one in five deliveries were following induction of labour. When labour was induced pharmacologically, 67% of women had a normal vaginal delivery, 15% of women had an instrumental delivery and 22% of women had an emergency caesarean section (The National Institute for Health and Care Excellence, 2008). The aim of the induction of labour process is to achieve a safe vaginal delivery

Common indications for induction of labour include, prevention of prolonged pregnancy (postdates induction of labour), pre-labour rupture of membranes at term, fetal growth restriction, multiple pregnancy, advanced maternal age and women with medical co-morbidities which could either harm themselves or their baby (for example, diabetes mellitus, pre-eclampsia / pregnancy induced hypertension and obstetric cholestasis).

Previous studies have demonstrated that the risk of stillbirth and perinatal mortality increases postdates as is illustrated in Figure 4.1. For this reason the current NICE recommendations are that all women are offered induction of labour between 41-42 weeks gestation (The National Institute for Health and Care Excellence, 2008).

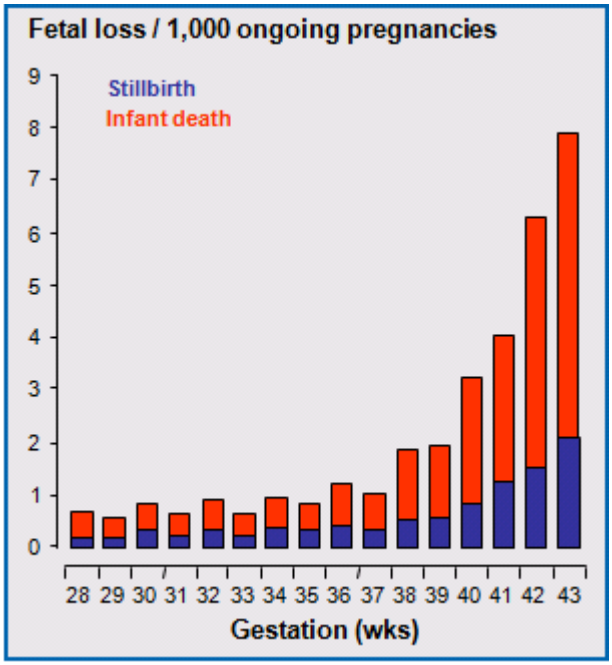

Figure 4.1: Rate of stillbirth and infant mortality with gestation. A retrospective analysis of over 170000 births from 1981-1991 was performed by Hilder, Costeloe & Thilaganathan demonstrating the rate of stillbirth and infant mortality per 1000 total or live births as a proportion of ongoing pregnancies at the given gestation. This illustrates a better estimate of the risk of mortality faced by a fetus and infant at each week of gestation, using the number of ongoing pregnancies as the denominator (not total births at each week of gestation). This analysis demonstrated above clearly shows the risk of stillbirth increases significantly in prolonged pregnancy (Hilder et al., 1998).

Historically, success of the induction of labour process evaluated outcomes of patients undergoing induction of labour and comparing these outcomes to those of women who laboured spontaneously. As would be expected, this led to inappropriate conclusions about the risks of induction of labour particularly as the indication for induction of labour often independently increased the risk of caesarean section.

Gülmezoglu and colleagues conducted a Cochrane systematic review and meta-analysis in 2012 evaluating the benefits and harms of induction of labour at term or post term in

comparison to awaiting spontaneous labour or later induction of labour. This review included 22 randomised controlled trials involving 9383 women. The analysis identified one perinatal death in the induction of labour group and thirteen perinatal deaths in the expectant management group. They therefore calculated that the number needed to treat to benefit with induction of labour to prevent one perinatal death is 410 (Gülmezoglu *et al.*, 2012).

Several observational studies have reported increased caesarean section rates in women undergoing induction of labour. Studies more recently compare induction of labour with expectant management (Gibson & Waters, 2015). A population based retrospective cohort study of singleton pregnancies delivered after 37 weeks gestation was published in 2012. This study included data from over one million women, with approximately 26% undergoing the induction of labour process for elective or medical indications. It concluded that when compared to expectant management, elective induction of labour from 37-41 weeks gestation is associated with a reduction in perinatal mortality and did not lead to a reduction in rates of spontaneous vaginal delivery (Stock *et al.*, 2012).

The Canadian Multicentre Post-Term Pregnancy trial was performed to determine the effects of induction of labour on perinatal and neonatal mortality when compared with expectant management. Secondary analyses looked at the effects of induction of labour in this group on the rate of delivery by caesarean section. 3418 women were included in the study, interestingly this study demonstrated a significantly higher caesarean section rate in the expectant management group (24.5%) when compared to the induction group (21.2%), this was reported to be the results of a reduction in fetal distress in the induction of labour group (Hannah *et al.*, 1992). These findings were further supported by a more recent systematic review and meta-analysis published in 2014. It demonstrated in women with

intact membranes, induction of labour was associated with a moderately but statistically significant reduction in the risk of caesarean section (Wood *et al.*, 2014).

Furthermore, secondary outcomes of the HYPITAT and DIGITAT trials demonstrated that induction of labour when compared to expectant management was not associated with increased rates of caesarean section (Bernardes *et al.*, 2016).

4.2 Methods of Induction of Labour:

Induction of labour has been documented in the literature for approximately one hundred years with a variety of methods utilised. Initially only surgical methods were used, with the introduction of medical methods in 1931. Castor oil was the first used medical compound for induction of labour but this caused several undesirable side effects. The use of oxytocin began in 1942. The method of oxytocin administration has also changed with time. Prostaglandins became available for use in induction of labour in the 1980's (Nabi *et al.*, 2014).

Both pharmacological and mechanical methods are currently used in the process of induction of labour for cervical ripening prior to oxytocin use. Pharmacological methods aim to mimic natural processes that occur in the cervix, with mechanical methods aiming to stimulate natural endogenous prostaglandin production (Connolly *et al.*, 2016). There are several pharmacological methods of induction of labour currently available including prostaglandin E_2 (PGE_2), oxytocin, misoprostol (PGE_1) (both oral and PV) and isosorbide mononitrate. There are also to several mechanical methods available (single and double balloon catheters and osmotic dilators).

Different methods have different advantages and disadvantages. The main advantages and disadvantages are the rates of vaginal delivery within 24 hours, and the risk of uterine hyperstimulation respectively, contradicting factors in the aim of induction of labour, achieving a safe vaginal delivery.

Currently the most favoured method for induction of labour is pharmacological induction with vaginal PGE_2. Unless there are clinical reasons not to use this, it is administrated as a gel, tablet or pessary. The choice of method for induction of labour should take into account the reason for the labour being induced, how urgent the delivery needs to be, the mother's obstetric and past medical history, maternal choice, national guidance and local protocols (Alfirevic *et al.*, 2016).

4.3 Current Guidance and Clinical Practice:

The current regime for induction of labour as recommended by NICE is illustrated in Figure 4.2 (The National Institute for Health and Care Excellence, 2008). Initially, each patient is assessed to determine how favourable her cervix is for the induction of labour process. A vaginal examination is performed to assess the dilatation, length, position and consistency of the cervix and the station of the fetal head. This assessment gives a Bishop score and was developed by Edward Bishop in 1964 (Bishop, 1964). A Bishop score of 8 or more is used to indicate that the cervix is ripe/favourable. Table 4.1 demonstrates the original Bishop scoring system published in 1964.

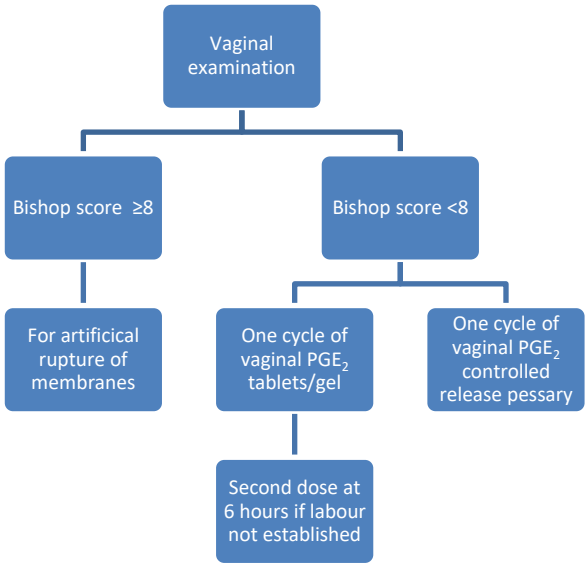

Figure 4.2: Current NICE guidelines for induction of labour. If Labour is not established after the treatment described here, this is defined as failed induction of labour. A further attempt can be made to induced labour following discussion between an obstetrician and the patient (The National Institute for Health and Care Excellence, 2008).

	Bishop Score			
Cervix	0	1	2	3
Position	Posterior	Middle	Anterior	
Consistency	Firm	Medium	Soft	
Effacement (%)	0-30	30-60	60-70	80
Dilatation (cm)	0	1-2	3-4	5-6
Station	-3	-2	-1-0	+1-+2

Table 4.1: Dr Edward Bishop's method of pelvic scoring as described in (Bishop, 1964).

Since 1964 the Bishop score has undergone a few changes and the current scoring system used is shown in Table 4.2.

	Bishop Score			
Cervix	0	1	2	3
Cx Position	Posterior	Central	Anterior	
Consistency	Firm	Medium	Soft	
Length (cm)	3	2	1	0
Dilatation (cm)	0	1-2	3-4	5-6
Station to spines	-3	-2	-1	0+

Table 4.2: The current Bishop Scoring system used to assess patients who are undergoing the induction of labour process. As can be seen from comparison to Table 4.1, cervical length has replaced the previously described cervical effacement and the scores received for station of the presenting part have altered slightly. Cx = cervix.

As discussed in Chapter 1, Section 1.7, prior to the onset of labour, the cervix undergoes a process a remodelling and ripening so that it can dilate in response to myometrial contractions. The cervix is composed primarily of fibrous connective tissue, collagen, elastin, fibroblasts and a small amount of smooth muscle (Irani & Foster, 2015). The firmness of the cervix is the result of the presence of collagen (Types I and III). Collagen is the predominant protein of the extracellular matrix, and in particular it is thought that its directionality may determine the cervix's ability to withstand the considerable forces which are put upon it in pregnancy (Nott *et al.*, 2016b).

In pregnancy the cervix helps to maintain the growing fetus within the uterus, and protects the fetus from ascending infection from the lower genital tract. Prior to the onset of labour, there are multiple changes which occur involving matrix metalloproteinases, hylauranic acid, prostaglandins, changes to the water content, changes to collagen concentration and structure (a reduction in the crosslinking between the helices) and increases in vascularity

(Gibson & Waters, 2015; McCarthy & Kenny, 2014). These changes decrease the structural integrity of the cervical tissue and result in increased compliance, allowing for cervical dilatation. The process of cervical ripening is at least in part mediated by changes in the expression of key enzymes and proteins involved in collagen synthesis (Irani & Foster, 2015).

It is recognised that PGE_2 administration can lead to ripening of the cervix in a patient whose cervix was previously unfavourable. Prostaglandin E_2 has been demonstrated to lead to numerous changes in the cervix including dilatation of the small vessels, an increase in IL8 release and increases in collagen degradation mediated by increased chemotaxis for leukocytes (Gilstrop & Sciscione, 2015; McCarthy & Kenny, 2014).

There currently is no doubt in the literature surrounding the benefits of induction of labour in reduction of perinatal mortality in the groups discussed, however prediction of its success remains an area in which there is further work to be done.

4.4 Predicting the outcome of induction of labour – Bishop Score:

One of the challenges of inducing labour is predicting which patients will have success (Hatfield *et al.*, 2007). Success of the induction of labour process has long been thought to be dependent upon the favourability of the cervix at the start of the process. In 1966, Burnett published the findings of his study, stating that parous women with a Bishop score of ≥6 had a 90% chance of vaginal birth within 6 hours, if women had a Bishop score of less than 6, their labour was less predictable (Burnett, 1966). Burnett used a simplified version of Bishop's original pelvic scoring system which is similar to that system used today. The

Bishop score was developed for the assessment of low risk parous women with cephalic presentation.

Several retrospective publications and a Cochrane review have evaluated the Bishop score and concluded that women with a more favourable cervix as assessed using the Bishop scoring system have a lower rate of caesarean section, but women with both high and low Bishop scores have a higher rate of vaginal delivery when compared to expectant management (Gülmezoglu *et al.*, 2012; Laughon *et al.*, 2012; Le Ray *et al.*, 2007).

There are also many other confounding factors which are difficult to adjust for when trying to develop a prediction model for the chance of a successful induction of labour. These include parity, estimated fetal weight and gestational age, maternal BMI, maternal age and medical co-morbidities (Gibson & Waters, 2015).

The use of the Bishop Score in the decision making process about whether to induce a patient remains controversial. In 2005, a prospective study of over 1300 women reported high caesarean section rates in nulliparous women with a Bishop score of five or less (Vrouenraets *et al.*, 2005). However, Kolkman *et al* in 2013 performed a systematic review looking at the use of Bishop Score as a predictor of success of induction of labour. They concluded that a Bishop score of 4-6 is a poor predictor of the success of induction of labour and therefore should not be used in the decision making process about whether to induce or not (Kolkman *et al.*, 2013). Additionally, Bernardes *et al* published their secondary analyses of the HYPITAT and DIGITAT studies, they concluded that concerns about increased risk of failed induction of labour in women with a Bishop score of 3-6 seem unwarranted (Bernardes *et al.*, 2016).

4.4.1 Criticisms of Bishop Score:

As discussed above, although a high Bishop score does predict a high probability of a normal vaginal delivery within 24 hours, particularly in a parous woman, the implications of a lower Bishop score are not so clear.

There are also other criticisms of the Bishop score a method of assessing favourability of the cervix. The supravaginal part of the cervix which is hidden above the anterior fornix of the vagina and bladder, this component of the cervix may not be able to be assessed if the external os of the cervix is closed, this can lead to inaccurate estimates of cervical length. This is particularly a problem as effacement of the cervix begins superiorly at the level of the internal os.

The Bishop score is a subjective measurement so making comparisons between different healthcare professional's assessments difficult and may include a margin of error. This can then lead to difficulties combining the results of studies in systematic reviews and meta-analyses and provide accurate recommendations about benefits and risks.

Patient discomfort may affect the scoring, many patients find vaginal examinations painful and therefore accurate assessment of the cervix can be difficult.

Consequently, assessing favourability of the cervix via other methods and the subsequent success of induction of labour has been a topic of many systematic reviews. Other methods currently in the research stages include, the use of transvaginal cervical length scanning and the use of the biomarkers used to aid prediction of preterm labour, fFN and phIGFBP-1.

4.5 Predicting the Outcome of the Induction of Labour Process - Cervical length:

Several studies have examined the role of transvaginal cervical length scanning prior to starting the induction of labour process as an aid to predict outcome. Firstly as with the prediction of preterm labour (discussed in Chapter 1, Section 1.8.2 and Chapter 5.1.1) it can be difficult to combine the results of studies looking at the use of cervical length as a predictor of successful induction of labour or studies comparing cervical length to Bishop score as a predictor of induction of labour. Significant heterogeneity exists between the studies in terms of, the induction strategies used, the definitions of "successful" induction of labour, and cut offs utilised for cervical length measurements. The populations used are also heterogeneous and therefore it is difficult to combine the findings of different studies.

Nevertheless, several studies have been published in the literature recently examining these roles. In 2007, a systematic review and meta-analysis by Hatfield, Sanchez-Ramos & Kaunitz was published, their analysis did not support the use of transvaginal ultrasound scanning of cervical length as a diagnostic tool to predict the outcomes of induction of labour. Their paper highlighted the significant heterogeneity between studies previously carried out and used in their meta-analysis. A variety of induction methods were used, studies had different primary outcomes and different cervical length cut-offs (Hatfield *et al.*, 2007).

Since this meta-analysis, several other studies have been completed. Cubal *et el* published the results of their prospective observational study in 2013. This included 206 women over a period of one year. They concluded that both Bishop Score and cervical length measured by transvaginal ultrasound are good predictors of successful induction of labour in

nulliparous women with neither being superior, a cut-off cervical length of 30mm was used (Cubal *et al.*, 2013).

Groeneveld *et al* published the results of a longitudinal study in 2010, the aim of this study was to compare Bishop Score with transvaginal cervical length prior to induction of labour as predictors of mode of delivery within the subsequent 96 hours. This was a small study of 100 patients, 66 nulliparous and 44 parous with a variety of indications for induction of labour. They concluded that transvaginal cervical length was not a good predictor of success of induction of labour in nulliparous or multiparous women. Bishop score was found to be a good predictor in nulliparous women. The authors postulated that one reason for their findings which were contradictory to some of the proceeding studies could be that they included women with a variety of indications for induction of labour between 37-42 weeks gestation whereas other studies with have identified cervical length measurement as a predictor of success of induction have used women being induced for postdates only (Groeneveld *et al.*, 2010).

Although this is an interesting point, it could be argued that there is less ambiguity surrounding the decision for induction of labour the group of women being induced for postdates with good evidence that the risk to the fetus increases with increasing gestation, therefore what is the value in a test about prediction of success on when there are very limited other options available to the patient in this clinical scenario. However, in other women being induced there may be less definitive evidence and therefore the benefit of a test may be greater.

A recent Cochrane Review by Ezebialu and colleagues examined methods for assessing pre-induction cervical ripening. The review compared Bishop score to other methods of

preinduction cervical assessment including transvaginal ultrasound measurements, IGFBP-1 and fFN. This only identified two randomised controlled trials which met their criteria, involving 234 women. These two studies did not show a superiority of one method over another in terms of the main outcome when comparing Bishop Score and transvaginal cervical length scanning (Ezebialu *et al.*, 2015).

The use of transvaginal cervical length scanning prior to induction of labour in clinical practice remains to be determined. Currently it appears we are a long way off utilising this method to aid our decision making process and for obtaining more information for patient counselling about the induction process and individualised outcomes.

4.6 Predicting the Outcome of the Induction of Labour Process - Biological Markers:

Both fFN and pIGFBP-1 have been studied as methods of assessing success of induction of labour. In a prospective study, 193 nulliparous women were tested for pIGFBP-1 in their cervicovaginal fluid as a predictor of successful induction of labour at term (defined as vaginal delivery and vaginal delivery within 24 hours). A positive test corresponded to a level of 10 micrograms/L in the sample obtained. 19% of women tested positive for pIGFBP-1, it was found to be moderately predictive of vaginal delivery and vaginal delivery within 24 hours (AOR, 5.5 and 4.9 respectively) and was also found to be better tolerated than Bishop Score (Vallikkannu *et al.*, 2016).

In the last 20 years fFN has been looked at as a marker for successful induction of labour. Initial work demonstrated fFN in the cervicovaginal secretion yielded a high probability of

successful induction of labour with a significantly ($p<0.0001$) shorter duration from the start of the induction process to delivery (Ahner *et al.*, 1995).

Further studies have been carried out, some demonstrating an association between fFN and increasing levels of success and others not. Crane in her systematic review concluded that although fFN did predict successful induction of labour, it was not demonstrated to be superior to Bishop Score for prediction (Crane, 2006).

Currently, no test has been identified with good quality evidence to suggest its widespread use in patients who are undergoing the induction of labour process. Further work is required to determine the potential of these tests (in isolation or in combination) and their role if any in clinical practice.

4.7 Outpatient vs Inpatient Induction of Labour:

Current NICE guidelines advocate that some women can use PGE_2 for cervical ripening in the outpatient setting (The National Institute for Health and Care Excellence, 2008). Many obstetric units are hesitant about using this option due to an inability to predict when uterine contractions may commence, leading to concerns surrounding unrecognised fetal hypoxia (Amorosa & Stone, 2015).

Several studies have aimed to assess maternal and fetal safety by examining both maternal and fetal outcomes of inpatient verses outpatient cervical ripening. As can be seen in Table 4.3, none of the studies have identified that outpatient cervical ripening increased the rate of adverse outcomes.

Study	Study type	Number of women in study	Outcomes
(Biem *et al.*, 2003)	RCT	300	No differences in maternal or fetal outcomes
(Salvador *et al.*, 2009)	Retrospective cohort study	1300	No difference in fetal outcomes assessed by 5 minute Apgar score and admission to NICU
(Farmer *et al.*, 1996)	Prospective cohort	76	Study not aimed to assess safety but no adverse outcomes in outpatient group
(Wilkinson *et al.*, 2015)	RCT	827	No difference in labour complications

Table 4.3: Studies assessing fetal and maternal safety of outpatient induction of labour using PGE_2. All studies demonstrated no difference in maternal and fetal outcomes when comparing outpatient and inpatient induction of labour.

In 2015, the results of a randomised controlled trial of outpatient versus inpatient cervical ripening (OPRA) were published (Table 4.3)(Wilkinson *et al.*, 2015). This study examined the benefits of outpatient induction of labour in relation to both psychosocial aspects and cost effectiveness. The biological plausibility behind the study was that if women were rested at home and had not had one to several sleepless nights in hospital, they would be better prepared to be able to cope with the demands of labour and this may lower the threshold for intervention. Inclusion criteria for the study were uncomplicated term pregnancies, either being induced for postdates or social reasons with a normal CTG, women were randomised in the study post administration of PGE_2 gel. The conclusion of the study was that there was no clinical advantage or disadvantage in outpatient PGE_2 cervical ripening, there was no significant difference in oxytocin use, rate of caesarean section or vaginal delivery within 24 hours (Wilkinson *et al.*, 2015).

Prior to this large study, Kelly, Alfirevic & Ghosh had concluded that with the current data available, although no adverse events in the outpatient groups were recorded it is not

possible to determine if the intervention of induction of labour is safe in the outpatient setting due to a lack of number of studies investigating this (Kelly *et al.*, 2013), therefore further work is needed.

(Biem *et al.*, 2003) reported a greater patient satisfaction with outpatient induction of labour process, with 56% patient satisfied in the outpatients setting compared to 39% in the inpatient group (Biem *et al.*, 2003).

Although it is clear that patient safety should not be compromised by attempts to save costs, the OPRA study did consider the financial implications of outpatient versus inpatient cervical ripening. They concluded that the overall findings were not significant but there was a trend towards cost saving with outpatient induction of labour, of approximately $156 less, even when including the costs of a specialist clinic for the process (Wilkinson *et al.*, 2015).

Sharp, Stock and Alfirevic surveyed clinical leads for obstetrics in 210 NHS hospitals in the UK about outpatient induction of labour, 164 units responded, 27 of which currently offered an outpatient of induction of labour service. This survey demonstrated a growing desire to provide this service, following a robust selection criteria for suitability. They did however close by mentioning that it was of concern that very few units have a process of assessing the woman and her fetus once she had gone home (Sharp *et al.*, 2016)

With appropriate patient selection of low risk pregnancies, we postulated that there could be a role for expanding the use of outpatient cervical ripening, particularly if clinicians could be reassured that a patient is very unlikely to experience the onset of contractions in the outpatient setting and therefore eliminating concerns about undiagnosed fetal hypoxia.

If we could develop a test which could be used to help to screen women for those who are sensitive to prostaglandins and therefore experience contractions soon after administration of PGE_2 and those who will not, we may be able to improve patient satisfaction and outcomes for some women who require induction of labour.

Additionally with increasing rates of induction of labour, if we could develop a test which may be used to assess or give further information about the likelihood of a successful induction of labour then this could be used as part of the counselling of women in their antenatal care when making decision about whether to opt for induction of labour or expectant management when the indication is not absolute. This may be particularly useful for the group of women who are on therapeutic anticoagulation as would allow appropriate stoppage of medication minimising the time off treatment and therefore minimising risk.

4.8 Hypothesis for PLCL1:

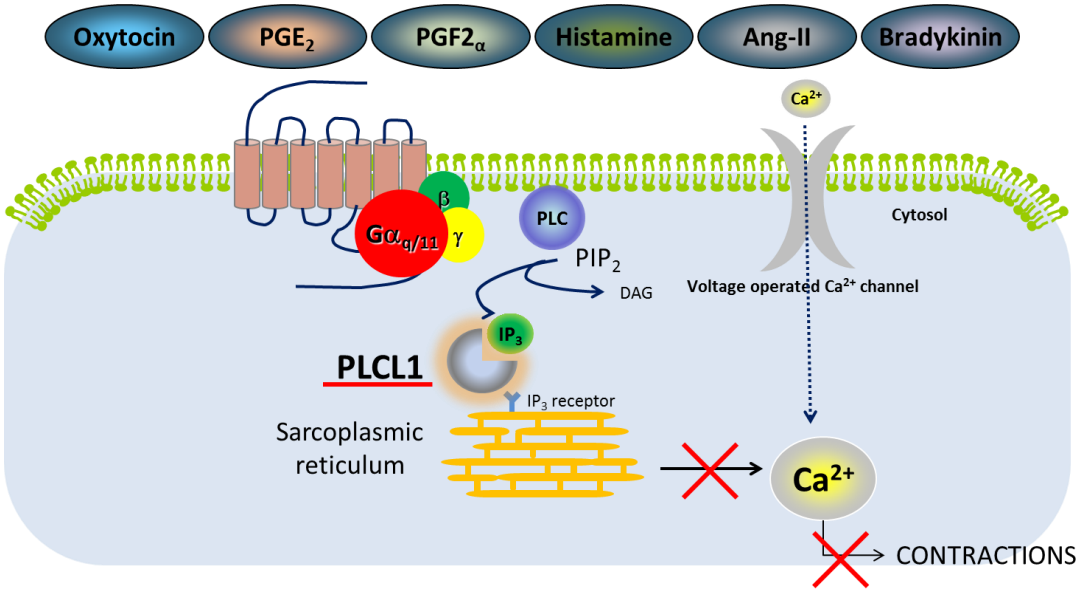

Figure 4.3: PLCL1 uncouples prostaglandin signalling to contractions by chelating IP3 thereby preventing calcium Ca^{2+} release from the SR. Ca^{2+} release from the SR is required for smooth muscle contractions via the Ca^{2+}-calmodulin, mMLCK, myosin actin filament interaction pathway(Brighton et al., 2014).

We hypothesised that high cervical expression of PLCL1 would indicate lack of responsiveness to prostaglandin during induction of labour at term. We aimed to answer the question, can PLCL1 levels help to predict the duration and efficacy of the induction of labour process with prostaglandin and oxytocin?

4.9 Process of induction of labour at our University Teaching Hospital:

All women (nulliparous and parous) who attend our University Teaching Hospital for induction of labour on the antenatal ward are assessed as shown in Figure 4.4. All patients who attend for induction of labour are booked prior to their attendance to the antenatal

ward, either from the hospital antenatal clinic, the community antenatal clinics or from the labour ward triage area. The process of induction of labour is discussed with each patient by the clinician or healthcare professional involved in their care prior to their attendance, along with the risks and benefits of the process.

If the patient consents to the induction of labour process they are advised of a date and time to attend the antenatal ward. On arrival to the antenatal ward, routine observations are checked (temperature, pulse, blood pressure, oxygen saturations and a urine dipstick). Each patient is commenced on the CTG to monitor fetal well-being and their antenatal and obstetric history is reviewed by a clinician or midwife on the ward. Currently at our University Teaching Hospital, we do not offer outpatient induction of labour. All patients who are in the process of induction of labour are admitted as inpatients until the delivery of their baby.

When patients attended the antenatal ward for induction of labour they were approached by myself and offered a patient information leaflet about the study (Appendix 2). Written consent was obtained to take part in the study. Following this, a sterile speculum examination was performed prior to vaginal examination to assess the cervix. To minimise inter-observer error for a subjective assessment all patients were examined by myself, cervical swabs taken and Bishop score calculated. A sample of cervical cells was obtained from each participant using a cervical cytobrush as per the study protocol and explanation given in Chapter 2. This was then snap frozen in liquid nitrogen and processed as per the protocol to determine the amount of PLCL1/protein for each patient as explained in Chapter 3. Several samples were utilised in optimisation experiments.

Data was collected on a CRF for each patient (sample shown in Appendix 8). This included demographic information about each patient and information about the induction of labour process and their labour and delivery.

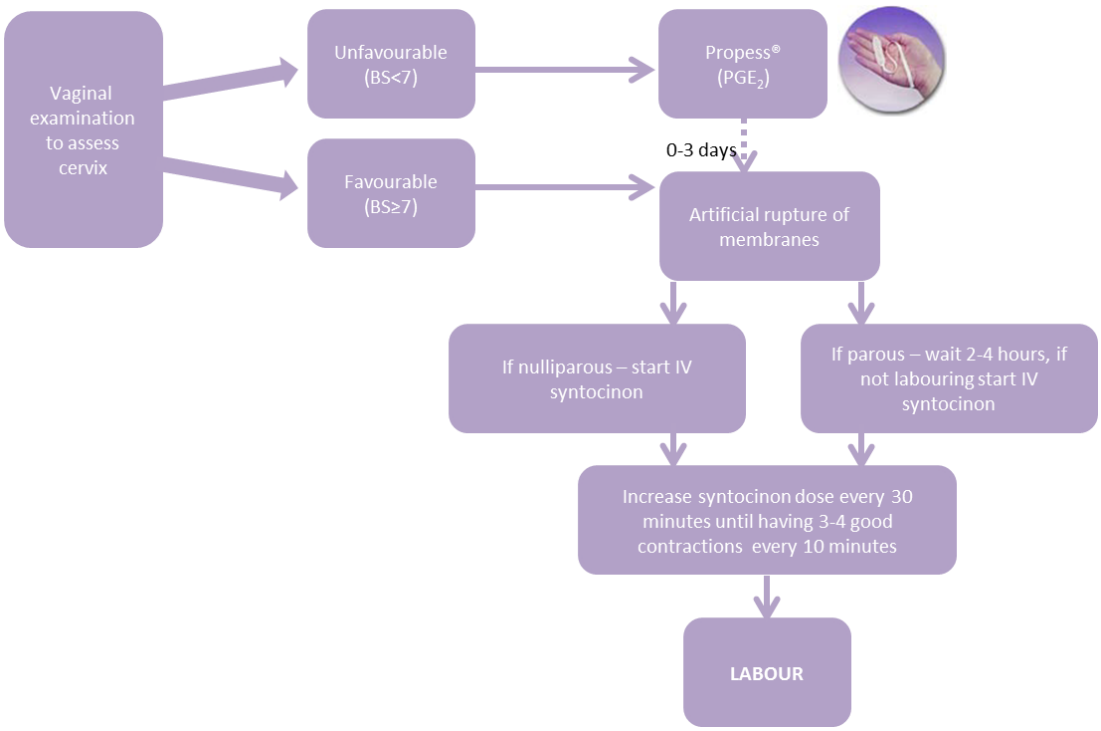

Figure 4.4: Process of induction of labour on the antenatal ward at our University Teaching Hospital. If a patients' Bishop score is <7, she receives a Propess® pessary. This is placed in the posterior fornix of the vagina next to the cervix. It remains in for 24 hours unless the patient goes into labour or there are concerns about the fetus. The Bishop Score is then reassessed, if it remains <7, the pessary is left in situ for 8 further hours. If after 32 hours, the Bishop score remains <7, the patient is discussed with a senior obstetrician. If the obstetrician advises and the patient is in agreement, a further Propess® pessary is inserted into the posterior fornix for 24 hours and the Bishop Score then reassessed. Once the patient has a Bishop score of ≥7 they then await a bed on the labour ward. Once a bed is available they are transferred to the labour ward and have an artificial rupture of membranes (ARM). Nulliparous women are commenced soon after ARM on a syntocinon infusion. Parous women are allowed 2-4 hours to await contractions following ARM. If the parous women do not go into labour, they are then also commenced on a syntocinon infusion. The rate of the syntocinon infusion is

increased every 30 minutes until the patient has 3-4 strong contractions per ten minutes. 10 units of syntocinon are diluted in 49ml of normal saline 0.9%, the infusion is commenced at a rate of 0.6ml per hour. It is increased following the trust protocol. There is continuous electronic fetal monitoring during this process.

4.10 Results:

4.10.1 Demographic data:

One hundred patients attending the antenatal ward for induction of labour consented to take part in Prediction of Inappropriate Myometrial Function (PIMF) between March 2014 and June 2015. Their demographic data are demonstrated in Table 4.4.

Demographic	
Gestational age (weeks) (range)	37+0 - 41+5
Age at booking (years) Mean +- SD (range)	28.9 $\pm$ 5.81 (18-42)
BMI (Kg/m^2) Mean +- SD (range)	28.1 $\pm$ 5.99 (16.9-46.6) BMI for 2 patients not known
Parity	
Nulliparous	47
Parous	53
Ethnicity	
White British	75
White other	10
Asian	8
Black British	3
Black African	3
Not known	1
Smoking status	
Never	57
Exsmoker	19
Smoker	20
Not known	4

Table 4.4: Demographic data of the 100 patients attending for induction of labour to the antenatal ward who consented to take part in our study.

Induction of labour was undertaken for a variety of reasons. Indications for induction of labour are shown in Table 4.5.

Reason for induction of labour	Number of patients
Postdates (T+9 - T+12)	27
Recurrent reduced fetal movements/reduced fetal movements at term	25
Fetal growth restriction	17
Small for gestational age	5
Pre-eclampsia (mild)/Pregnancy induced hypertension	4
Gestational diabetes mellitus	3
Large for gestational age	3
Obstetric cholestasis	2
Previous stillbirth	2
Spontaneous rupture of membranes at term	2
Other	10

Table 4.5: Indications for induction of labour in women recruited to study. Patients induced for large for gestations age had experienced a previous shoulder dystocia or a previous third degree tear. Patients who were induced following spontaneous rupture of membranes at term, one patient did not labour for 24 hours following SROM and therefore was induced, the second patient was Group B Streptococcus positive and therefore began the induction process on presentation to the department with spontaneous rupture of membranes. Other indications for induction of labour included the following; raised maternal age, late booking of pregnancy (in late second trimester), mild polyhydramnios, previous SGA baby, postural orthostatic tachycardia syndrome. Three patients were induced for maternal request due to 1. pruritic urticarial papules and plaques of pregnancy, 2. severe back pain, 3. severe symphysis pubis dysfunction. One patient had a history of borderline raised anti-cardiolipin antibodies and recurrent miscarriage, one patient has JKA antibodies and therefore induction was planned for when blood was available in case of haemorrhage.

4.10.2 Observational Data from Women Undergoing Induction of Labour in Our Study:

67% of women who underwent induction of labour from our study achieved a normal vaginal delivery. 20% of women required an instrumental delivery, an overall vaginal delivery rate of 87%. 13% of women required a caesarean section. Of these patients who required a caesarean section, one patient required a category 3 caesarean section for unsuccessful induction of labour. Six patients required category 1 caesarean sections in the first stage of labour for suspected fetal distress, four patients required category 2 caesarean sections for failure to progress in the first stage of labour. One patient required a category 1 caesarean section at full dilatation following an abnormal fetal blood sampling result and one patient required a category 2 caesarean section for failure to progress in the second stage of labour. These rates are very similar to those reported in the NICE induction of labour guideline (The National Institute for Health and Care Excellence, 2008).

47 nulliparous women took part in the study. Of this group, 22 had normal vaginal deliveries, 15 had instrumental deliveries and 10 required caesarean sections. 53 parous women took part in the study. Of this group 45 had normal vaginal deliveries, five required instrumental deliveries and three required caesarean sections.

Table 4.6 summarises the observational data collected about the patients who consented to take part in PIMF who required induction of labour for the reasons explained above.

Delivery data			
Number of Propess® pessaries required	All	Nulliparous	Parous
0	27	5	22
1	43	24	19
1+	19	15	4
2	11	3	8
Number of women who delivered within 24 hours of Propess® insertion	25/73	10/42	15/31
Bishop score at beginning of induction of labour process	All	Nulliparous	Parous
0-3	32	19	13
3-6	43 (two women did not receive Propess®)	23	20
≥7	25	5	20
Delivery within 24 hours of Propess® insertion compared to Bishop score at start of induction of labour process	All	Nulliparous	Parous
0-3	11/32	4/19	7/13
3-6	14/43	6/23	8/20
Mode of delivery compared to Bishop score at start of induction of labour process	0-3	4-6	≥7
Normal vaginal delivery	19	28	20
Instrumental delivery	7	9	4
Caesarean section	6	6	1

Table 4.6: Data about the labour and delivery of women in our study. Propess pessaries were given as per our teaching hospital guideline shown in Figure 4.4. The exception to this was two women with as Bishop score of <7, who did not receive a Propess® pessary as per their consultants request, instead they awaited ARM on labour ward. Women who did not receive Propess® were excluded from the delivery within 24 hours rates.

As can be seen from Table 4.6, more than one third of women who received Propess® delivered within 24 hours of administration. This included almost half of parous women and one quarter of nulliparous women delivering within 24 hours. Although our sample size is small, there was only a small difference in rates of delivery within 24 hours when you compare this to favourability of the cervix as assessed by Bishop Score. Again as can be seen the rates of normal vaginal delivery, instrumental delivery and caesarean section were similar when comparing initial Bishops score of 0-3 and 3-6. 80% of women with a Bishop score of ≥7 at the start of the process had a normal vaginal delivery with 96% achieving a vaginal delivery.

4.11 Processing of Samples from Induction of Labour Patients:

100 samples were collected from patients attending for induction of labour, they were utilised as shown in Figure 4.5.

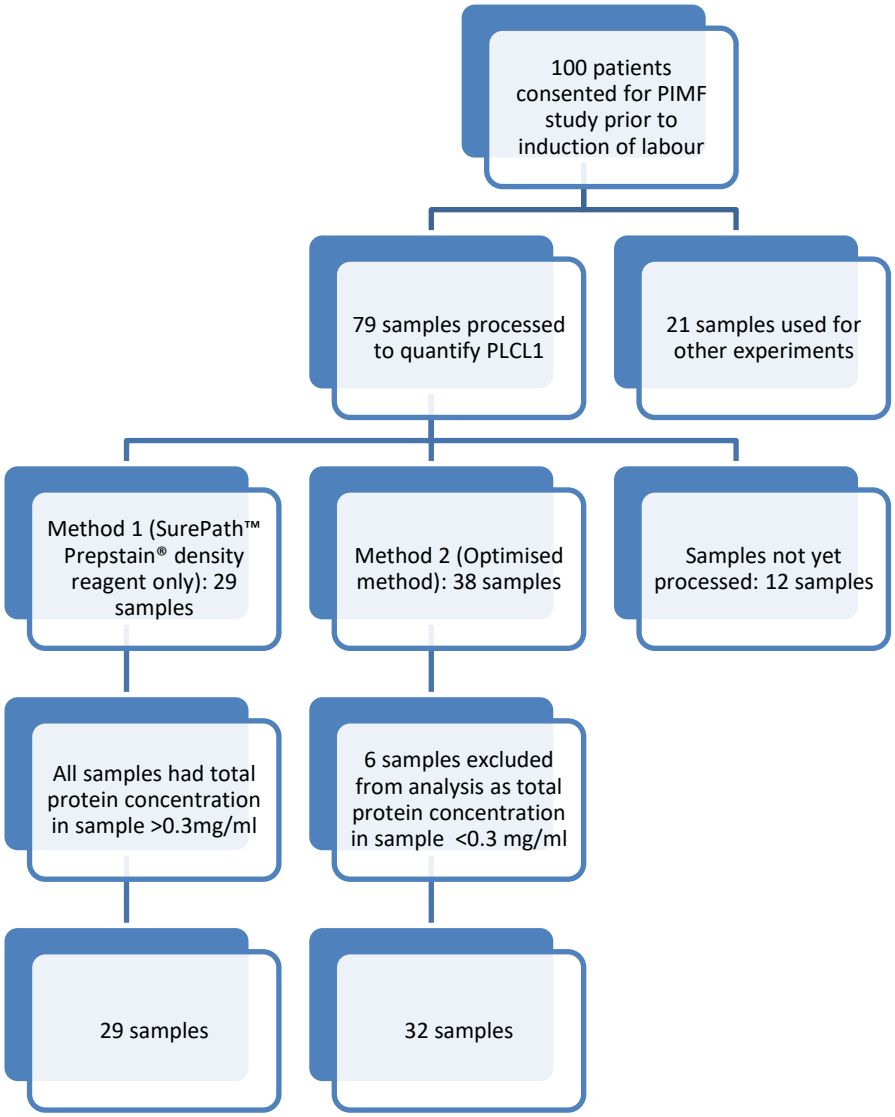

Figure 4.5: Summary of processing of cervical samples from patients who attended for induction of labour. Of the 100 samples collected, 21 samples were used in other experiments including SDS PAGE/Western blotting, Cytospin and immunocytochemistry and RNA quantification (RT-PCR). The first 29 samples were processed using the SurePath™ Prepstain® Density reagent only. Samples from then onwards were processed as per the optimised method described in Chapter 3. Note no samples from induction of labour patients were collected in ThinPrep® vials.

4.12 Preliminary data:

Initially 5 samples were processed following collection, this analysis indicated that PLCL1 in cervical epithelial cells may predict time between induction and delivery as shown in Figure 4.6

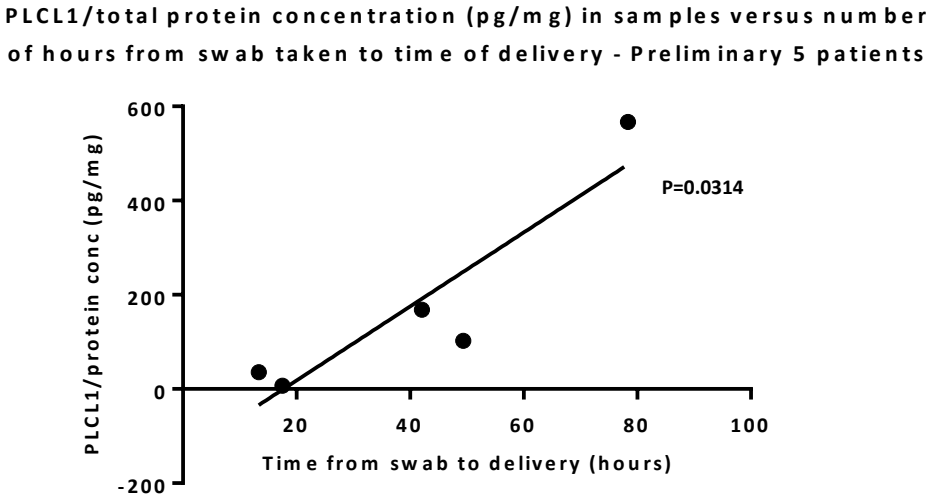

Figure 4.6: Initial results from 5 cervical epithelial samples collected for induction of labour patients. Samples were processed following the SurePath™ Prepstain® Density reagent method as described in chapter 3. All samples had a total protein concentration >0.3mg/ml. The PLCL1/total protein concentration was calculated. This is shown above versus the total number of hours from when the swab was taken to delivery.

This demonstrated a statistically significant relationship (p=0.0314). We then proceeded to recruit a larger cohort of patients to examine this relationship in more detail.

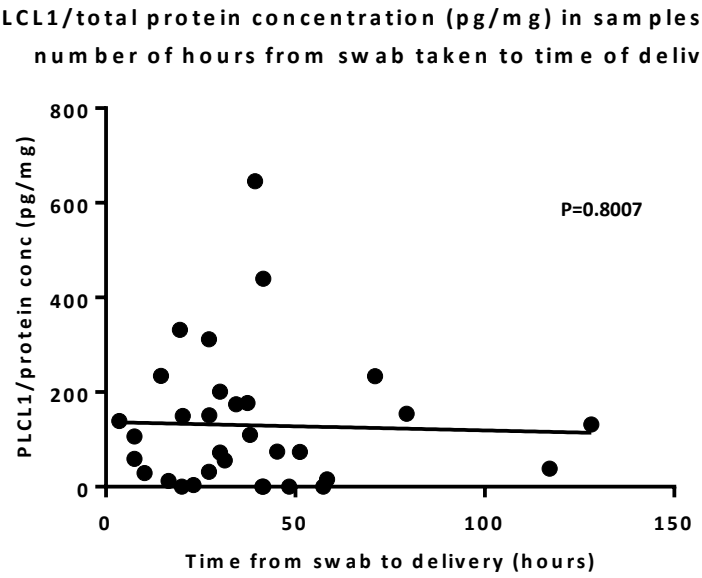

Figure 4.7: Results from 32 samples taken from patients attending for induction of labour versus number of hours of the induction process. Samples processed following the optimised method. Comparing PLCL1/protein concentration (pg/mg) with number of hours from sample of epithelial cervical cells taken to delivery.

As can be seen in Figure 4.7, further samples did not demonstrate a relationship between PLCL1/protein concentration (pg/mg) and duration of the induction of labour process. However, many of these patients following administration of Propess® and achieving a Bishop score of ≥ 7 had to wait before a bed was available on labour ward to enable them to have an ARM. 15 patients laboured with Propess® and therefore were taken to labour ward without a delay, however 17 patients waited an average of 16 hours before being transferred to the labour ward. Figure 4.8 demonstrates a comparison of PLCL1/protein concentration (pg/mg) with number of hours from sample of epithelial cervical cells taken to delivery, excluding the number of hours delay to attend labour ward for ARM plus or minus initiation/augmentation of labour with syntocinon. This is making the presumption that the cervix is not changing during the hours each patients waited to go to labour ward for their ARM.

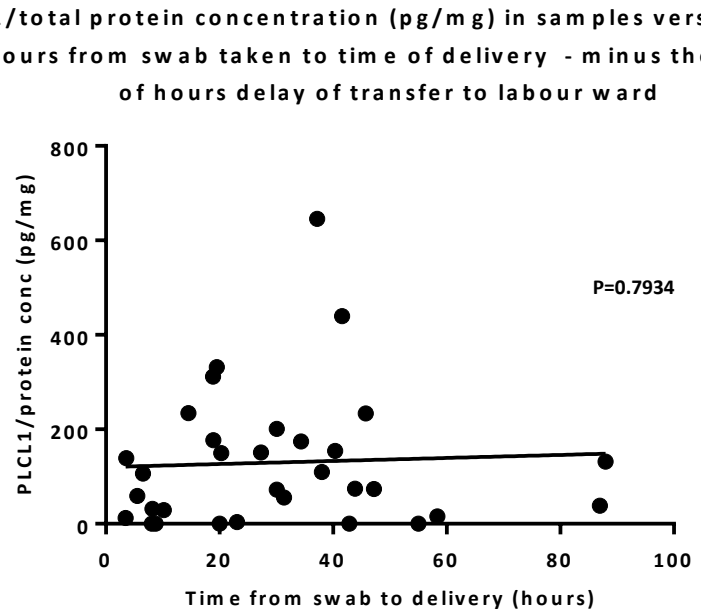

Figure 4.8: 32 samples taken from patients attending for induction of labour versus number of hours of the induction process, excluding delays. Samples processed following the optimised method. Comparing PLCL1/protein concentration (pg/mg) with number of hours from sample of epithelial cervical cells taken to delivery excluding the number of hours each patient had to wait to be transferred to labour ward for ARM.

Again, this did not demonstrate an ability of PLCL1/protein concentration to predict the duration of induction of labour.

We then wanted to identify if PLCL1 level could determine the efficacy of the cervix to respond to prostaglandins. As discussed in Chapter 1, Figure 1.11, previous work by our group (Brighton, 2015), has demonstrated that in primary myometrial cell lines, upregulation and downregulation of PLCL1 changes prostaglandin E_2 mediated calcium signalling. The results of this analysis are demonstrated in Figure 4.9.

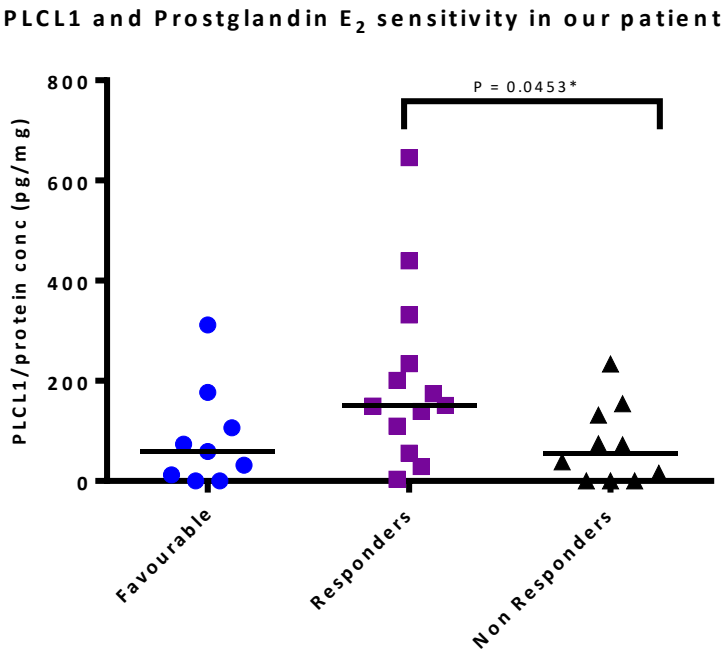

Figure 4.9: Sensitivity to prostaglandin E2. PLCL1/total protein concentration (pg/mg) in three groups. Favourable, those not requiring Propess® as suitable for ARM as per the hospital protocol. Responders were defined those with signs of active labour after Propess® insertion. Non responders were defined as those with no signs of active labour following Propess® for 32 hours. Patients were categorised into responders and non responders by two observers who reviewed the patient notes independently. Signs of active labour were defined as regular contractions leading to cervical dilatation or spontaneous rupture of membranes.

There was a significantly higher PLCL1 level in the patients who were in the responder group when compared to the non responder group. This was the opposite of what we hypothesised from the laboratory data.

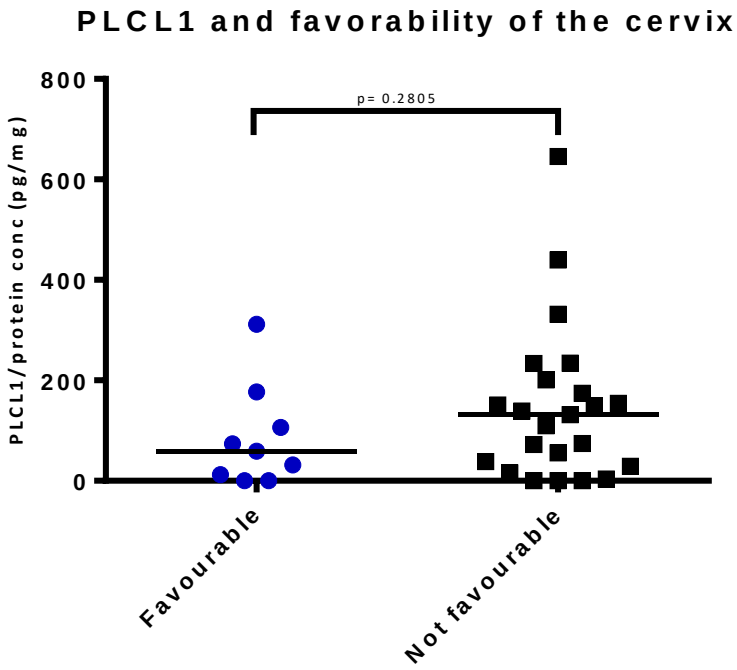

Figure 4.10: Is there a relationship between PLCL1 level and favourability of the cervix. All women who had a Bishop Score pf ≥7 at the beginning of the induction process were classified as favourable as per the hospital protocol shown in Figure 4.4. All women with a Bishop score of less than 7 were classified as not favourable and therefore require Propess® as per the induction of labour protocol.

There was not a statistically significant difference in PLCL1 level when comparing those patients who were favourable and suitable for ARM to those who were not. This indicates that PLCL1/protein concentration does not correlate with Bishop score at the beginning of the induction of labour process.

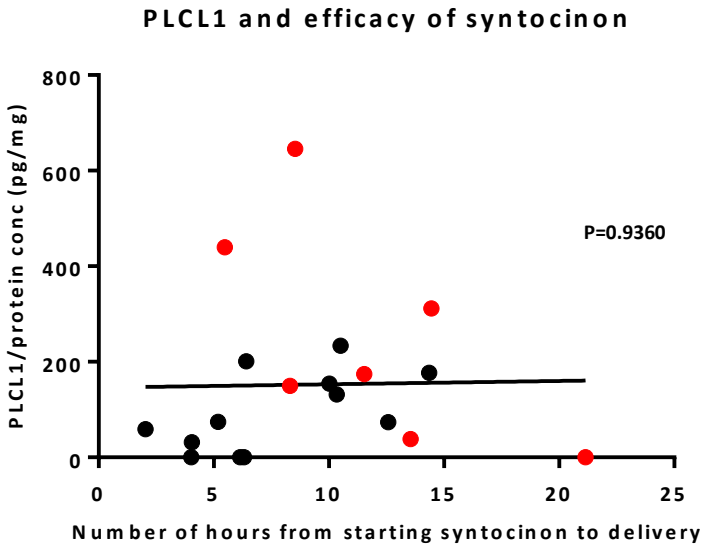

Figure 4.11: PLCL1 and efficacy of syntocinon. A comparison of PLCL1/protein concentration (pg/mg) to number of hours from starting exogenous syntocinon to delivery. Patients who delivered vaginally are shown in black. Patients who delivered by caesarean section are shown in red.

Finally we wanted to assess if PLCL1/protein concentration could predict the efficacy of syntocinon during labour. As can be seen from Figure 4.11, initial results demonstrated no relationship. However patients who had a caesarean section had an inaccurate duration of labour, therefore we exclude these patients from this analysis, this demonstrated the relationship shown in Figure 4.12.

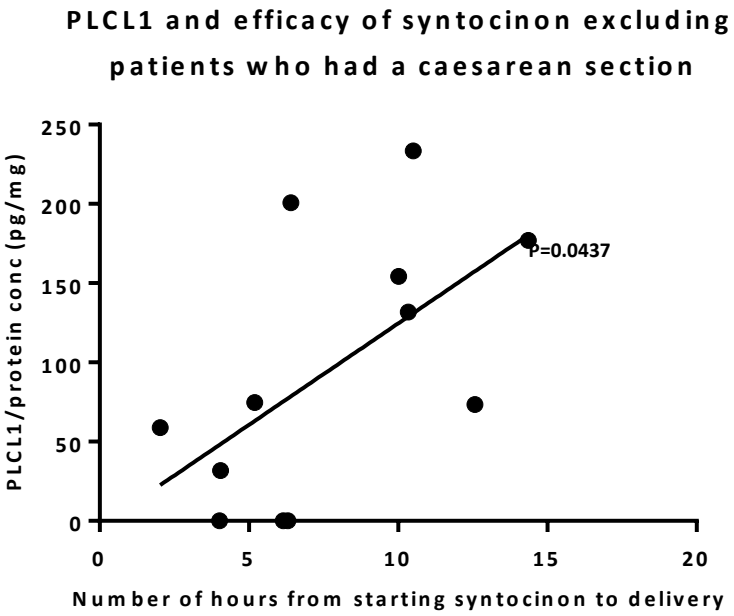

Figure 4.12: PLCL1 and efficacy of syntocinon, excluding caesarean section patients. A comparison of PLCL1/protein concentration (pg/mg) to number of hours from starting exogenous syntocinon to delivery excluding patients who had a caesarean section.

When the patients who had a caesarean section were excluded, a statistically significant relationship (p=0.0437) between PLCL1/ protein concentration (pg/mg) and the number of hours from commencing exogenous syntocinon to delivery is demonstrated suggesting that PLCL1 can predict efficacy of syntocinon. Our numbers are small here, possibly further work is required.

4.12 Conclusions:

The results are not consistent with our hypothesis generated from laboratory data. Our hypothesis was that PLCL1 could uncouple prostaglandin signalling from myometrial contractions and therefore clinically we would have anticipated an increased amount of

PLCL1 would result in a lack of sensitivity to induction of labour with prostaglandin E_2. This was not demonstrated in our results.

We did show that the PLCL1 level indicated sensitivity to oxytocin, as those with less PLCL1 delivered more quickly after the initiation of oxytocin. This is consistent with our hypothesis that PLCL1 uncouples oxytocin signalling from myometrial contractions.

There are several reasons which may contribute to the inconsistencies between our hypothesis and our results. One of the limitations of our laboratory work was the detection range offered by the ELISA kit used. The minimum detectable level of PLCL1 is 11.7 pg/ml as stated by the kit instructions. This could have led to a problem with our samples. As explained in chapter three, some of our sample concentrations were very small and therefore we excluded samples with a total protein concentration of <0.3mg/ml from further analysis. Many of our samples were likely to have PLCL1 levels towards the lower range of what can be detected. An example of the standard curve generated from the PLCL1 ELISA kit is demonstrated in Figure 4.13.

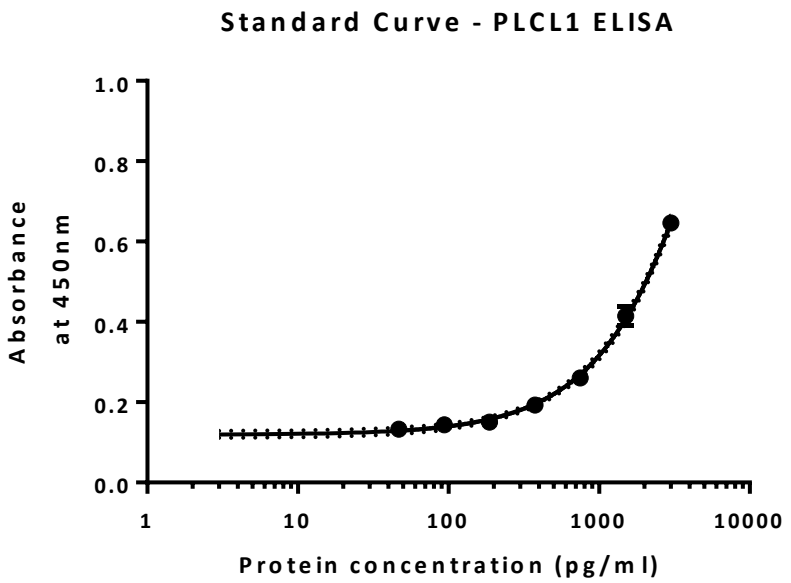

Figure 4.13: An example of the standard curve generated from the ELISA kit. Standard was reconstituted using RIPA buffer as the diluent and serial dilutions performed as per the manufacturers instructions. The optical density was determined following completion of the experience and a standard curve generated using GraphPad Prism.

As can be seen from Figure 4.13, a small difference in optical density generated from the samples can lead to a considerable difference in interpolated protein concentration of each sample. Our results may be improved using a more sensitive ELISA kit in which we can use the exponential phase of the standard curve.

During the process of labour the roles of the cervix and myometrium vary considerably. As discussed, the cervix undergoes a process of remodelling and ripening to allow it to distend and dilate with the onset of labour. Conversely the myometrium is concerned with contraction and retraction with the aim of expulsion of the fetus. Could our differing results obtained from our cervical samples in relation to sensitivity to prostaglandins reflect this? In a favourable patient a hypothesis leading on from these results could suggest that in the cervix, a higher level of PLCL1 is expected in patients who go on to labour quickly as the

higher level prevents the cervix from contracting, indirectly facilitating better cervical dilatation.

Furthermore, the process of initiation of labour still remains elusive, involving multiple signalling pathways and therefore could be considered to be hugely complex in comparison of augmentation of labour using synthetic oxytocin. An over simplification of the process with regards to our hypothesis may account for our unexpected results in terms of sensitivity to prostaglandins.

Chapter 5

5. Preterm Labour, Prediction and Prevention

5.1 Background:

5.1.1 Prediction:

As discussed in Chapter 1, preterm labour is a huge challenge in the practice of obstetrics with vast numbers of babies being born preterm each year worldwide, leading to high rates of infant mortality and morbidity. Prediction remains difficult due to the complex multifactorial nature of preterm birth.

There is no "gold standard" test available at present for the prediction of preterm birth. Although much progress in this area is being made with current studies focusing on the currently available tests, both in isolation and combination for the different patient groups, improvements are still needed. More accurate prediction will enable better allocation of resources to those at highest risk and more appropriate administration of, corticosteroids to reduce respiratory distress syndrome and other complications of prematurity, magnesium sulphate for neuroprotection, as well as relocation of patients to units with sufficient services to care for preterm infants. All interventions are aimed to reduce the burden of preterm delivery.

The biomarkers currently used in obstetric practice, fFN and phIGFBP-1 are clinically useful for their negative predictive value, particularly in the symptomatic group of patients. Nevertheless, increasing numbers of studies are reporting their use in the asymptomatic population (Abbott *et al.*, 2015; Hezelgrave *et al.*, 2016; Vandermolen *et al.*, 2016).

Transvaginal cervical length scanning is also clinically useful (See Chapter 1, Sections 1.8.2.1.1, 1.8.2.1.2 and 1.8.2.7).

NICE suggest a role for cervical length scanning in women who present to the labour ward in suspected preterm labour with a closed cervix over 30 weeks gestation. If the cervical length is more than 15mm, the patient is unlikely to be in preterm labour. With regards to biochemical markers used for the prediction of preterm delivery, the guidance suggests the use of quantitative fFN as a diagnostic test (with a cut of level of 50ng/ml indicating a positive result) in women who are more than 30 weeks gestation who cannot have or will not consent to a transvaginal cervical length scan (The National Institute for Health and Care Excellence, 2015)

Asymptomatic patients with a short cervix are at a higher risk of spontaneous preterm birth. The role of transvaginal cervical length scanning as a screening test as part of the fetal anomaly screening programme (FASP) 18+0-20+6 ultrasound scan has been explored. However, although a short cervix would lead to the recommendation of closer surveillance of the pregnancy due to an increased risk of preterm labour, a “normal” length cervix cannot reassure clinicians that the patient is not at risk of preterm birth (Barros-Silva *et al.*, 2014). Increasingly, in the research setting a combination of fetal fibronectin measurements and transvaginal cervical length are being explored as a prediction tool (Jwala *et al.*, 2016; Kuhrt *et al.*, 2016; van Baaren *et al.*, 2015).

In the asymptomatic high risk population the most common method utilised for screening those at risk of preterm delivery is transvaginal cervical length scanning. A recent survey published in the British Journal of Obstetrics & Gynaecology in 2014 highlighted significant heterogeneity in current clinical practice in specialist preterm labour clinics for

asymptomatic high risk women (Sharp & Alfirevic, 2014). Transvaginal cervical length scanning is used for risk stratification, however cut offs vary from clinic to clinic, with the majority of clinics (59%) using <25mm to initiate treatment, however some use <15mm (9%) (Sharp & Alfirevic, 2014). A further dimension to consider when measuring cervical length is how it changes with increasing gestation, as discussed in Chapter 1. Some studies have demonstrated that for more accurate prediction of preterm birth, gestation needs to also be accounted for when screening for preterm birth and using this test. Some clinics in the UK utilise centile charts for risk stratification which take into account gestational age (14%) (Sharp & Alfirevic, 2014). Clinics then use the 3rd centile as a cut off for risk stratification and initiation of intervention (Goodfellow *et al.*, 2016).

Currently there are no data published in the UK population for normal cervical length throughout pregnancy. A large prospective study of cervical length measurements was undertaken in the French low risk population over three years. This identified changes in cervical length across gestation and resulted in the publication of centile charts (Salomon *et al.*, 2009). This data has been validated in the UK population and therefore has been suggested to be a useful reference range to determine when preventative treatments should be offered (Goodfellow *et al.*, 2016).

If preterm labour can be predicted, there are evidence based guidelines to prevent or delay its onset.

5.2 Prevention:

5.2.1 Current National Guidelines:

NICE published guidance about Preterm Labour and Birth in November 2015 (The National Institute for Health and Care Excellence, 2015). Professionals are expected to consider this guidance alongside individual's needs and preferences. In the prevention of preterm birth, this guideline suggests to offer;

- Prophylaxis (consisting of vaginal progesterone or prophylactic cervical cerclage) to women with a history of preterm birth or mid-trimester loss (16-34 weeks gestation), and who have a cervical length of less than 25mm (measured between 16-24 weeks gestation)
- Prophylactic vaginal progesterone to women who have a cervical length of less than 25mm (measured between 16-24 weeks gestation) and no history of preterm birth or mid-trimester loss

And to consider;

- Prophylactic cerclage for women who have a cervical length of less than 25mm (measured between 16-24 weeks gestation) who have had previous preterm pre-labour rupture of membranes or a history of cervical trauma
- Rescue cerclage in women 16+0-27+6 weeks gestation with a dilated cervix and exposed intact fetal membranes (The National Institute for Health and Care Excellence, 2015)

Further guidance about cervical cerclage in the prevention of preterm birth is covered in the Royal College of Obstetricians & Gynaecologists (RCOG) Green-top guideline No 60, Cervical Cerclage. Cervical cerclage is a widely used intervention for the prevention of

preterm birth, despite the absence of a validated population for whom it is been demonstrated to provide a benefit (Royal College of Obstetricians and Gynaecologists, 2011c). It reports several indications for the use of cervical cerclage including obstetric history, ultrasound findings and rescue cerclage. Several types of cerclage techniques exist including McDonald (transvaginal), Shirodkar (high transvaginal) and transabdominal (either performed laparoscopically or via a laparotomy). A summary of the indications for cervical cerclage as suggested by the guideline are shown in Table 5.1.

Type of indication	Recommended if
History indicated	- Three or more previous spontaneous preterm births and/or second trimester losses
Ultrasound indicated	- One or more spontaneous preterm birth or second trimester loss with a transvaginal cervical length <25mm before 24 weeks gestation - Consider – in women undergoing sonographic surveillance due to a history of spontaneous preterm birth or second trimester loss with cervical shortening with increasing gestation
Rescue cerclage	- Individualised decision taking into account gestation at presentation

Table 5.1: RCOG Green-top guideline No. 60 recommendations about the use of cervical cerclage in pregnancy (Royal College of Obstetricians and Gynaecologists, 2011c).

As discussed, the normal cervical length changes with gestational age. The above guidelines do not take this into detailed consideration.

5.2.2 Preterm Prevention Clinic at our University Teaching Hospital:

Although in 2011 a Cochrane Review suggested that there was no clear evidence that specialised antenatal clinics reduce the number of preterm births, they did conclude that there was a lack of evidence. It was therefore difficult to detect differences between

groups when looking at the outcomes of perinatal death and extreme preterm birth in the specialist clinic versus the standard antenatal clinic (Whitworth *et al.*, 2011). Overall, other specialist antenatal clinics have clearly demonstrated improvements in clinical outcomes.

The preterm prevention clinic at our university teaching hospital was set up in December 2013. The aim of this clinic was to improve patient care in our region for patients at a higher risk of preterm labour. The risk of preterm labour in our population when the clinic was established was higher than the national average. The secondary aims of the clinic were to allow recruitment of patients to PIMF and randomised clinical trials in the area of preterm labour.

Prior to the setup of this clinic, patients assessed to be at a higher risk for preterm labour were managed in the general antenatal clinics by a variety of consultants. The gestational age at which they were first seen in the high risk clinics varied considerably. These patients were often not seen until they were well into their second trimester.

The plan for the preterm prevention clinic was to see patients who were at a higher risk for spontaneous preterm labour due to, a history of second trimester loss or preterm labour, a history of cervical surgery including LLETZ or cone biopsies of the cervix, or due to patients have a known congenital uterine anomaly. The target when the clinic was established was to see patients as soon as possible into their pregnancy. We generated a preterm prevention clinic referral proforma (Appendix 10), this was widely distributed to all community midwives in the region to ensure all women were seen as soon as possible into their pregnancy so that a management plan could be put into place, ideally within the first to early second trimester.

Referrals were received for all women from their community midwife or from another healthcare professional who deemed that she met the criteria to attend the preterm prevention clinic. Upon receipt of the referral, an appointment was made in the next available clinic under the guidance of the clinicians involved in providing care in the clinic. An appointment letter was sent out to each patient inviting them to attend the clinic as per the Study Flow Diagram (Chapter 2, Figure 2.1). A patient information leaflet and invite letter (Apppendices 2 and 5) were included with the appointment letter.

At each patient's first appointment to the preterm prevention clinic, their history was reviewed, assessing their risk of spontaneous preterm labour is this pregnancy. The role of the clinic was explained to each patient by the clinician in the clinic. The current guidance about assessment of risk of preterm labour and prevention of preterm labour discussed above was explained. A management plan was then instigated for each patient depending upon their history. This involved surveillance using transvaginal cervical length scanning to allow risk stratification. Individualised care plans were used, some patients had a prophylactic cervical cerclage and some women were commenced on prophylactic vaginal progesterone. Patients were then given appointments 2-4 weekly (based upon history and scan findings) until approximately 28 weeks gestation.

Many women who attended had been seen for a postnatal debriefing appointment with their consultant obstetrician following a previous preterm delivery or mid-trimester loss. A management plan for future pregnancies had often been suggested at this meeting. For women in this situation, a discussion in the preterm prevention clinic took place explaining the current advice and guidance, a joint management plan was agreed between the clinician and the patient for the pregnancy. Some patients therefore had a prophylactic cervical cerclage on the basis of a clinical history consistent with cervical weakness or were

commenced on prophylactic vaginal progesterone outside of the current guidance outlined above and outside of the recommendations of the preterm prevention clinic.

In general, the principles of the clinic were as follows. All women, following review of their past history meeting the criteria for referral to the clinic were offered transvaginal cervical length scanning for surveillance. Initiation of treatment was based upon cervical length measurements, if scanning demonstrated a cervical length <25mm or serial scans demonstrated cervical shortening, patients were offered the options of cervical cerclage and/or vaginal progesterone.

All women who attended the clinic were screened for bacterial vaginosis. If detected, this was treated with 2% clindamycin vaginal cream for 5 days once daily.

When patients attended the preterm prevention Clinic they were approached by myself or the consultant in charge of the clinic to ask if they had considered taking part in the study following receipt of the patient information leaflet and patient invite letter. Written consent was obtained to take part in the study. Following this, a sterile speculum examination was performed prior to transvaginal cervical length scanning to assess the cervix. To minimise interobserver error all patients were examined and samples taken by myself. All cervical length measurements were taken by the consultant in charge of the clinic or by myself under direct supervision. Transvaginal cervical length measurements were taken to the nearest millimetre (mm). A sample of cervical cells was obtained (in addition to the microbiome and Enose swabs) from each participant using a cervical cytobrush as per the study protocol and explanation given in Chapter 2. Samples were then snap frozen in liquid nitrogen and processed as per the protocol to determine the amount of PLCL1/protein for each patient as explained in Chapter 3. Several samples were utilised

in optimisation experiments. Repeat samples were taken at subsequent visits to the clinic throughout gestation with patient consent.

Data was collected on a CRF for each patient (sample shown in Appendix 8). This included demographic information about each patient and information throughout their pregnancy about transvaginal cervical length measurements, evidence of funnelling on scan, interventions instigated if any and finally information about their labour and delivery.

5.3 Prediction of Preterm Birth – A New Test:

We hypothesised that patients with a low PLCL1 level have an increased susceptibility to preterm labour. As discussed in Chapter 1, laboratory studies have demonstrated that PLCL1 inhibits agonist stimulation of myometrial contraction by chelating IP_3. Therefore low levels of PLCL1 make the uterus permissive to the onset of labour, making patients susceptible to endogenous increased levels of oxytocin or prostaglandins and consequently preterm labour.

We aimed to answer the following questions:

- In patients who are at a high risk for spontaneous preterm labour due to a history of preterm labour/mid-trimester loss, a history of cervical surgery or who have a congenital uterine anomaly, can PLCL1 act as a susceptibility marker to preterm labour?
- Laboratory tests have demonstrated *in vitro*, PLCL1 is increased following treatment with progesterone (Chapter 1, Figure 1.12), is this mimicked *in vivo* with patients utilising vaginal progesterone?

5.4 Results:

5.4.1 Demographic data:

Ninety one patients attending the preterm prevention clinic at our University teaching hospital consented to take part in Prediction of Inappropriate Myometrial Function (PIMF) between February 2014 and July 2015, This resulted in 237 cytobrush samples of cervical cells being taken for the study. The demographic data of the patients is demonstrated in Table 5.2.

Demographic	
Age at booking (years)	
Mean +- SD (range)	30.4 $\pm$ 5.16 (19-42)
BMI (Kg/m^2)	27.1 $\pm$ 5.76 (17.7-43.5)
Mean +- SD (range)	BMI for 7 patients not known
Ethnicity	
White British	56
Asian	14
White other	13
Black British	6
Black African	2
Smoking status	
Never	55
Exsmoker	19
Smoker	13
Not known	4

Table 5.2: Demographic data of the 91 patients attending preterm prevention clinic who consented to take part in study.

As explained above, women attended at a variety of gestational ages, with some women attending regularly throughout the second and into the third trimester. The number of

visits depended upon patient history and scan findings in the clinic. The gestation at which patients attended the clinic and took part in the study ranged from 12+0/40 to 31+1/40. Swabs for cervical cells were repeated at each visit independent of the frequency of attendance to the clinic, with patient consent. A summary of gestational age when samples of cervical cells were taken is shown in Table 5.3. The number of cytobrush samples of cervical cells taken per patient is shown in Table 5.4.

Gestation when sample of cervical cells obtained (weeks + days)	Number of swabs for cervical cells taken
12+0 – 15+6	33
16+0 – 19+6	71
20+0 – 23+6	66
24+0 – 27+6	55
≥28+0	12

Table 5.3: Gestation when samples of cervical cells were obtained from high risk patients attending the preterm prevention Clinic. 237 samples of cervical cells were taken from 91 patients attending the clinic.

Number of samples of cervical cells obtained per patient	Number of patients who had samples of cervical cells taken
1	28
2	19
3	21
4	13
5	4
6	6

Table 5.4: The number of samples obtained from each patient who attended the preterm prevention clinic.

Women in our study attended the preterm prevention clinic for a variety of reasons which are shown in Table 5.5.

Indication for attendance to preterm prevention clinic	Number of patients
Previous preterm birth or mid-trimester loss	**69**
Number of previous preterm deliveries/mid-trimester losses	
1	40
2	14
3	6
4	2
5	1
Previous loss of mid-trimester twins	6
Gestation at which preterm delivery/mid-trimester loss occurred (weeks)	
Mid-trimester loss (≤23+6)	45*
24+0 - 27+6	9
28+0 – 31+6	10
≥32+0	12
Not known	23
Had rescue cerclage this pregnancy	2
Previous cervical surgery	22**
Known congenital uterine anomaly	1***

*Table 5.5: Indications for referral to the preterm prevention clinic. Where twins are mentioned in the table above, the number refers to the number of sets of twins * in addition to 45 losses in the mid-trimester, there were further losses of 6 sets of twins in the mid-trimester. ** two patients referred with a history of cervical surgery were subsequently identified following further correspondence of only having cervical biopsies taken and were therefore discharged from the clinic. Two patients with a history of cervical surgery also had a history of preterm birth. *** the patient with a congenital uterine anomaly had a history of three previous preterm births.*

5.4.2 Observational Data from Patients Attending Preterm Prevention Clinic in out Study:

Of the 91 patients who attended the preterm prevention clinic and consented to take part in the PIMF study, 28 went on to deliver preterm or have a mid-trimester loss, 58 delivered at term and 5 patients were lost to follow up, mainly because of transfer of antenatal care to another unit.

Of the 28 who delivered preterm/had a mid-trimester loss, one had a medical termination of pregnancy at 17 weeks gestation following amniocentesis, which diagnosed Trisomy 21 and three were iatrogenic preterm births. Indications for iatrogenic preterm birth were delivery at 36+6 weeks following induction of labour for pre-eclampsia, induction at 33 weeks gestation due to a deterioration in maternal renal function (patient had a kidney transplant) and induction following presentation to the labour ward triage with reduced fetal movements and the diagnosis of an intrauterine death at 33 weeks gestation.

The 24 remaining patients laboured spontaneously. Four had mid-trimester losses, of which all had been referred to the clinic with a history of one or more mid-trimester losses (although one was a history of mid-trimester loss of twins). Three of these women delivered despite transvaginal McDonald cervical cerclage inserted at 12 weeks gestation and the use of vaginal progesterone.

Four patients delivered between 24 and 28 weeks gestation. Three of these women had a history of mid-trimester losses (one of whom also had a history of preterm delivery) and one had a history of two previous LLETZs. One patient had a prophylactic cervical cerclage at 12 weeks gestation and one had an ultrasound indicated cervical cerclage inserted at 22 weeks gestation due to a shortening of cervical length to 8mm, however went on to deliver

at 25 weeks gestation. One was using vaginal progesterone and one did not have any intervention.

Four patients delivered between 28 and 34 weeks gestation. Of this group, one had a history of mid-trimester losses and PPROM, the remaining three all had a history of spontaneous preterm birth (one of whom was known to have a unicornuate uterus).

Finally the remaining twelve patients delivered between 34 and 37 weeks gestation. Of this group, two had a history of mid-trimester losses, six had a history of preterm labour, four had undergone cervical surgery in the past, and one had a history of both preterm labour and cervical surgery.

As discussed above and in Chapter 1, Section 1.8.2.2, clinics utilise a variety of cut offs for cervical length prior to initiation of treatment. In addition, cervical length measurements are known to change throughout gestation. Salomon *et al*, 2009 have produced gestational specific reference ranges for cervical length measurements (Salomon *et al.*, 2009). Figure 5.1 below demonstrates the Salomon *et al*, 2009, gestational specific centiles for cervical length measurements with the measurements of transvaginal cervical length of our patients. Purple dots illustrate the measurements taken from patients who went on to deliver at term. Red dots illustrate the measurements of patients who went on to deliver preterm (<37 weeks gestation). Cervical length measurements were only included in Figure 5.1 if patients met the criteria for appropriate referral to the preterm prevention clinic. Measurements were also excluded if a patient had a prophylactic cervical suture *in situ* or if they had an iatrogenic preterm birth. Blue horizontal lines represent the cervical length cut offs of 25mm and 15mm throughout gestation.

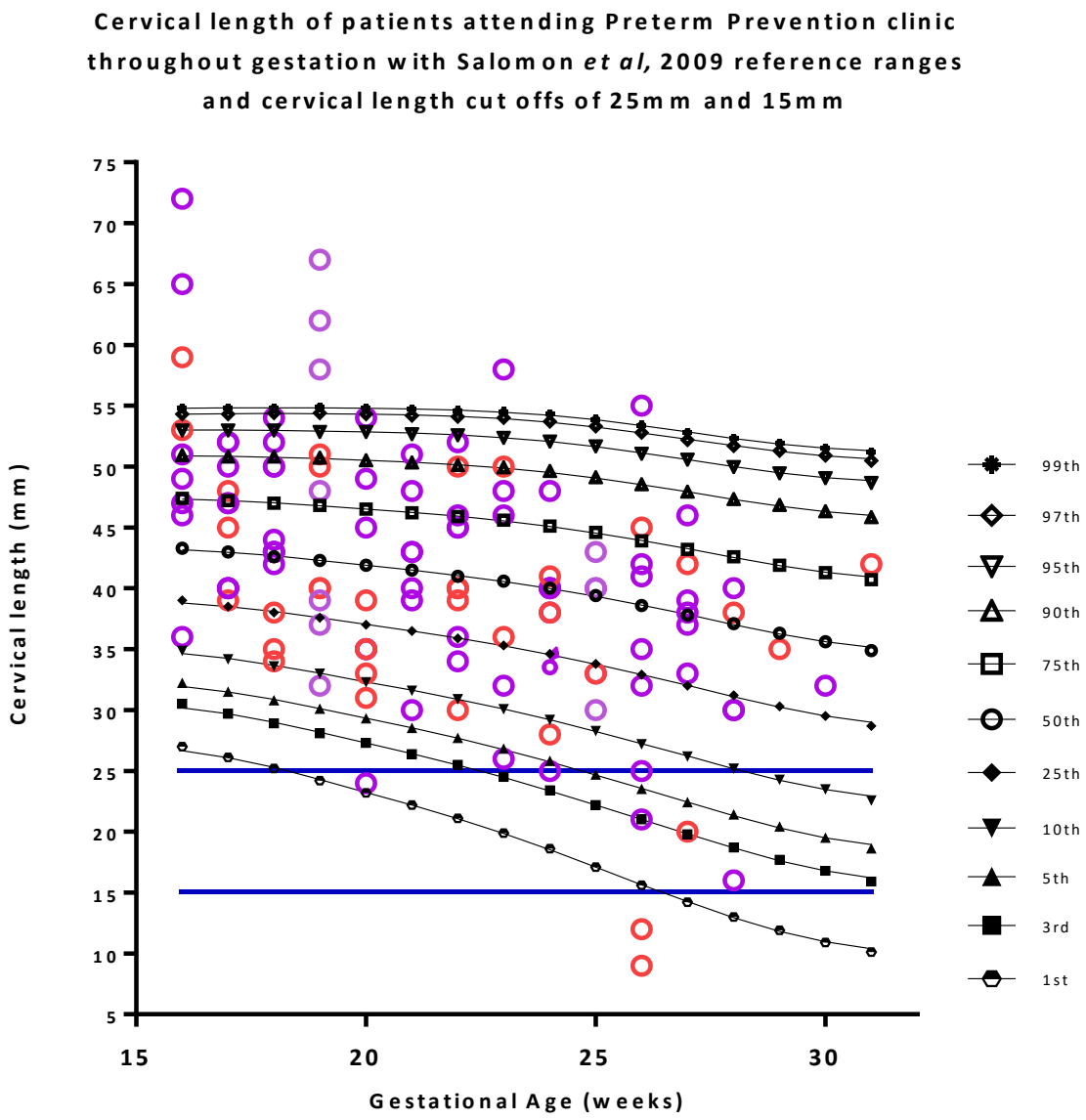

Figure 5.1: Cervical length measurements taken from our high risk asymptomatic women throughout pregnancy during attendance to the preterm prevention clinic. Results shown are those of women who consented to take part in PIMF. Measurements were excluded from the above graph if that patient already had a prophylactic cervical suture in situ. Patients who subsequently had an iatrogenic preterm delivery were excluded from the above data. In addition due to the unclear data about risks from previous preterm delivery/mid-trimester loss of twins, this group was also excluded. Centiles shown in the legend were produced by Salomon et al, 2009 of the low risk French population are illustrated in black (Salomon et al., 2009). The cervical length cut offs utilised throughout gestation of 25mm (as suggest by (The National Institute for Health and Care Excellence, 2015) and

15mm in some clinics are illustrated in blue. Cervical length measurements of our patients are illustrated in purple (if the patient went on to deliver at term ≥37+0 weeks) or in red (if the patient went on to deliver preterm <37+0 weeks). In the same way as Salomon et al, 2009 gestation has been rounded to the nearest week, with ≤4 rounded to the lower week and ≥5 days rounded to the higher week. Cervical length measurements were also excluded if delivery data was not known.

121 transvaginal cervical length measurements from 50 patients who attended the preterm prevention clinic and consented to be in PIMF are illustrated in Figure 5.1, more than one cervical length measurement per patients is illustrated on the Figure.

Figure 5.1 illustrates the cervical length measurements at the gestation they were measured. Of the 50 patients whose cervical length measurements are shown, 17 patients went on to deliver preterm.

Firstly, utilising 25mm as a cut off, as was used in the clinic, during surveillance 6 patients developed cervical shortening to ≤25mm (shown as 8 cervical length measurements on Figure 5.1, two patients has two measurements taken). As can be seen from Figure 5.1, one patient's cervix shortened to 24mm at 20 weeks gestation. This patient was referred to the clinic with a history of one second trimester loss. Following identification of a shortened cervix <25mm, the patient was referred for insertion of a transvaginal cervical cerclage. This patient subsequently went on to deliver at term.

As can be seen from Figure 5.1, at 24 weeks gestation, one patient developed cervical shortening to 25mm. This patient had been referred to the clinic with a history of one previous preterm labour at 27 weeks gestation and one previous mid-trimester loss. As a result of her obstetric history she was using vaginal progesterone throughout pregnancy as

had been agreed in her debriefing follow up from her previous pregnancies. The cervix shortened from 43mm at 18 weeks to 25mm at 24 weeks. The scan was repeated again two weeks later and demonstrated that the cervical length had not changed. The patient went on to deliver at term.

At 26 weeks gestation three patients were identified to have a cervical length of ≤25mm. the first patient had a history of one previous preterm labour and two second trimester losses. At 26 weeks, she had a cervical length of 21mm. The scan was repeated two weeks later as the patient was already using vaginal progesterone pessaries. The cervix had shortened to 16mm (shown on Figure 5.1), however she went on to deliver at term. The second patient who developed cervical shortening at 26 weeks gestation was referred to the clinic with a history of one previous preterm delivery at 25 weeks gestation. Her cervix was 9mm at 26 weeks and she went on to deliver at 30+4 weeks gestation. At 26 weeks she was administered steroids in anticipation of delivery and commenced on vaginal progesterone. Prior to 26 weeks, surveillance scans had shown a cervical length of >25mm, although from 22 to 24 weeks gestation the cervix had shortened from 40mm to 28mm. The third patient who developed cervical shortening at 26 weeks was referred to the clinic with a history of two previous LLETZ procedures. From 23 weeks to 26 weeks gestation, her cervix shortened from 40mm to 12 mm. At 26 weeks she was commenced on vaginal progesterone and administered steroids but went on to deliver at 28+2 weeks gestation.

The final patient who met the cut off of ≤25mm during surveillance was referred with a history of one previous preterm birth following PPROM at 18 weeks and two second trimester losses. In light of her obstetric history she was using vaginal progesterone pessaries throughout pregnancy. At 22 weeks her cervical length was 30mm but this had

reduced to 20mm at 27 weeks as shown on Figure 5.1. She went on to deliver at 32+3 weeks.

If a cervical length cut off of ≤15mm was used, as can be seen from Figure 5.1, one of the three patients who was identified to be high risk using a cut off of 25mm would have been missed. Using the third centile to assess which patients are at a high risk, all three patients identified as being high risk, who went on to deliver at term would have been identified. However, two patients who were assessed to be high risk utilising a cut off of ≤25mm throughout gestation but went on to deliver at term, would not have been stratified as high risk using the third centile. This may have helped to reduce unnecessary increased anxiety levels in an already extremely anxious population. It is difficult to come to any firm conclusions as interventions were put in place in light of the findings and therefore could have influenced the outcomes.

5.5 Processing of Samples from Preterm Prevention Clinic Patients:

237 samples were taken from patients attending the preterm prevention clinic, they were utilised as shown in Figure 5.2.

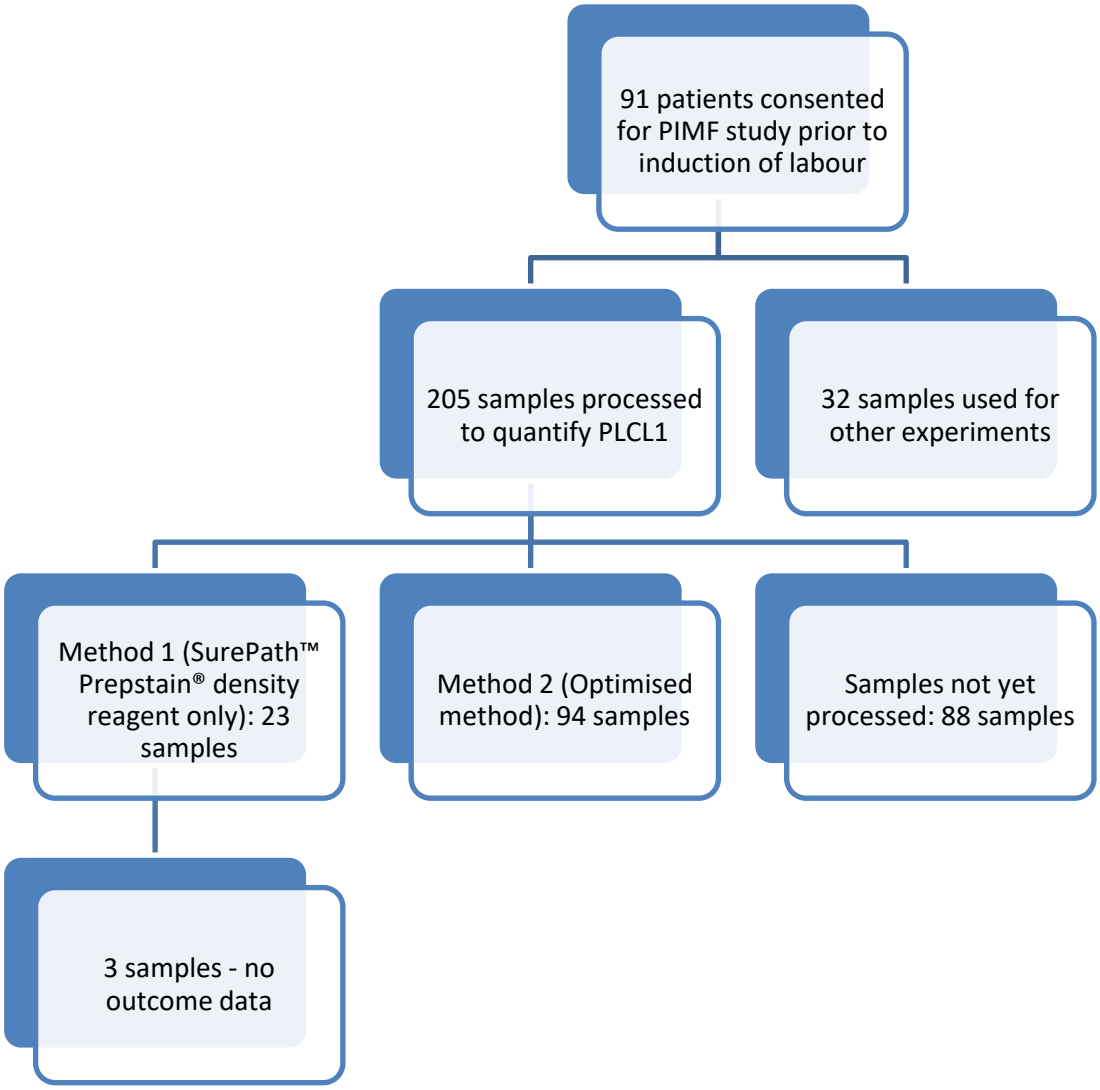

Figure 5.2: Summary of processing of cervical samples from patients who attended the preterm prevention clinic. Of the 237 samples collected, 32 samples were used in other experiments including optimisation experiments, SDS PAGE/Western blotting, Cytospin and immunocytochemistry and RNA quantification (RT-PCR). The first 23 samples were processed using the SurePath™ Prepstain® Density reagent only. Samples from then onwards were processed as per the optimised method described in Chapter 3. Note some samples from preterm prevention clinic patients were collected in ThinPrep® vials, these are included in the 32 samples used for other experiments.

5.6 Results:

In Chapters 3 and 4, we explained that samples from patients who attended for induction of labour with a total protein concentration <0.3mg/ml were excluded from further analysis. The mean $\pm$ SD total protein concentration in the samples obtained from the preterm prevention clinic patients was 0.76 $\pm$ 0.98mg/ml (range 0.01-5.66mg/ml) with 36 samples with a total protein concentration of <0.3mg/ml. It was decided that we could not apply this criteria to these samples because so many had this low amount of protein, but instead we would view results with caution over interpretation. However, 50 samples demonstrated a PLCL1/protein concentration (pg/mg) of 0, which accounted for more than half of the patients with a total protein concentration of <0.3mg/ml. As the assay was a complex multi-step assay, the reason for 0pg of PLCL1 was uncertain as it could be a very low or absent PLCL1 concentration, lack of sensitivity of the ELISA or a laboratory error. Hence all 0pg results were excluded from our analysis because of uncertain validity. Figure 5.3 demonstrates a comparison between PLCL1/protein concentration (pg/mg) of patients who attended the preterm prevention clinic and went on to deliver preterm versus those who went on to deliver at term.

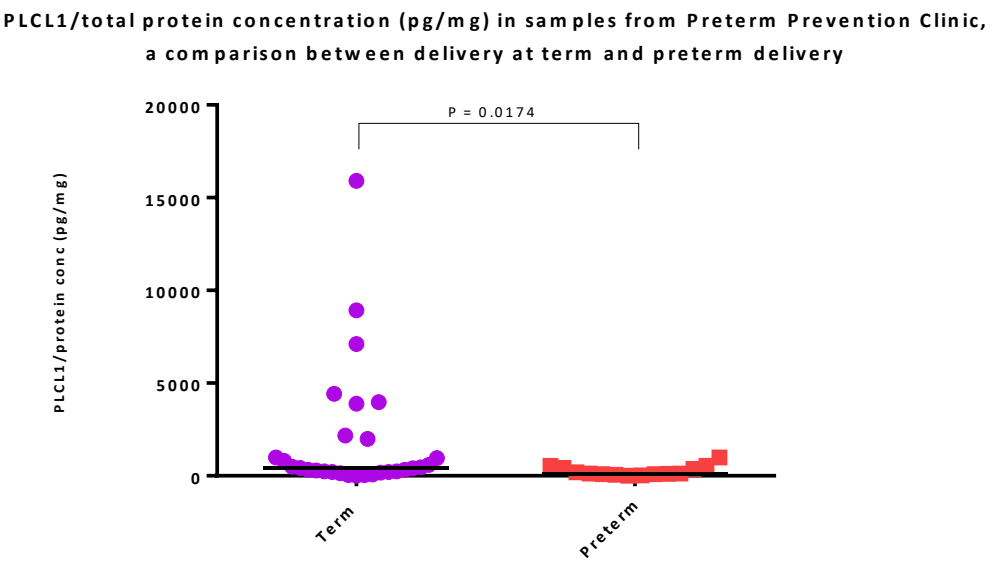

Figure 5.3: Can PLCL1 act as a susceptibility marker for spontaneous preterm labour in high risk asymptomatic high risk women

Figure 5.3 demonstrates the analysis of the data obtained from our patients attending the preterm prevention clinic. Analysis using non-parametric Mann-Whitney test has demonstrated a significant difference between the two patient groups (p=0.0174). This supports our hypothesis that PLCL1 acts as a susceptibility marker for preterm labour. As can be seen from Figure 5.3, there are three results which appear as significant outliers from the term data. Review of total protein concentration of these three samples demonstrated a very low level, hence need to be reviewed cautiously. Even on exclusion of these samples due to concern about multiple factor error when calculating PLCL1/total protein concentration (pg/mg), the relationship remains statistically significant as is illustrated in Figure 5.4.

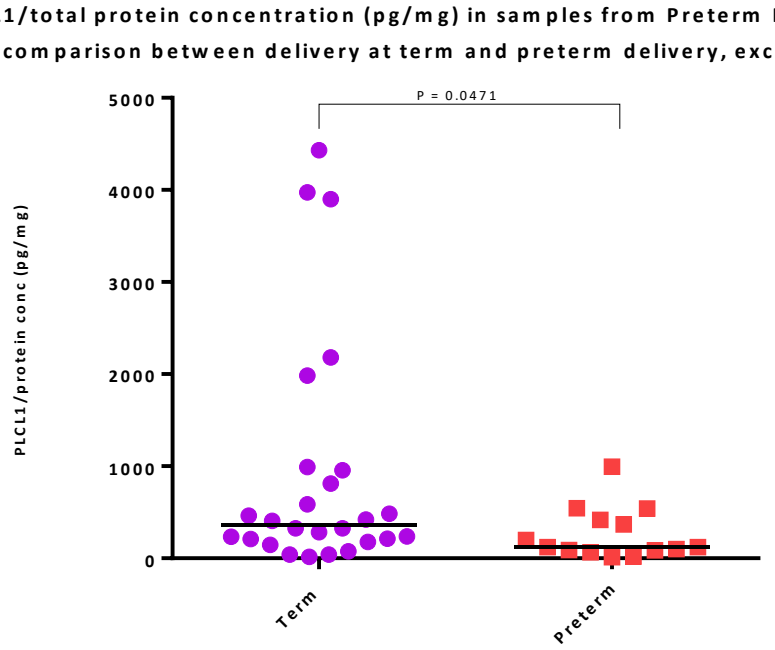

Figure 5.4: Can PLCL1 act as a susceptibility marker for spontaneous preterm labour (excluding outliers) in high risk asymptomatic high risk women. Exclusion of outliers demonstrates the relationship to still be statistically significant.

Further work needs to be undertaken to support our hypothesis, as currently our numbers are small due to difficulties with assay development in the project, and our assay lacking the necessary sensitivity to detect low level of PLCL1 protein. This will be discussed in more detail in Chapter 6.

Our second question was to identify if the laboratory findings about PLCL1 and its responsiveness upon exposure to progesterone *in vitro* could be seen *in vivo*. Does exposure to vaginal progesterone increase PLCL1 protein levels found in the cervical samples? Figure 5.5 summarises the serial samples taken from patients attending the preterm prevention clinic.

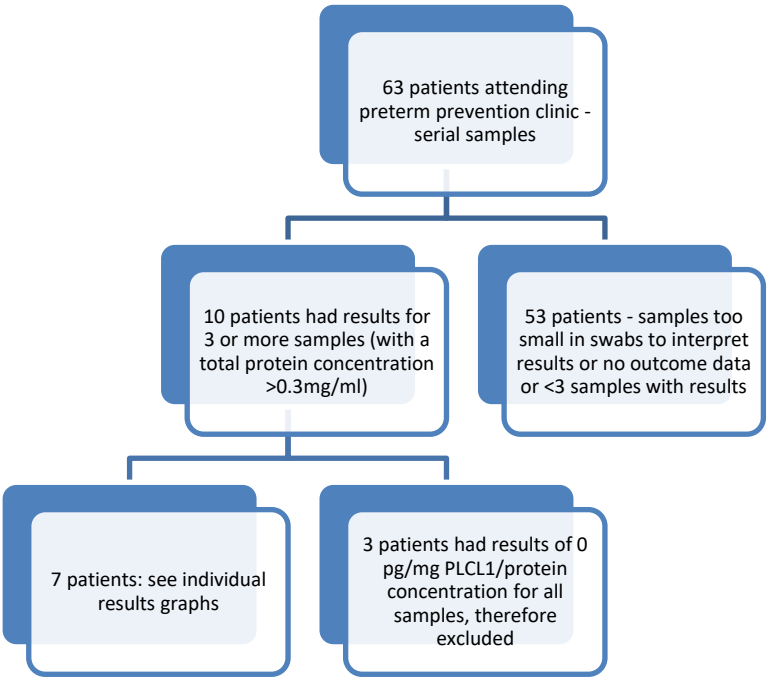

Figure 5.5 Summary of processing of serial samples from each patient attending the preterm prevention Clinic who consented to multiple cervical cytobrush swabs throughout their pregnancy. As can be seen here, 7 patients had serial samples taken from whom results were obtained.

5.6.1 Individual Patients Results From Serial Cervical Cytobrush Samples with Clinical Information:

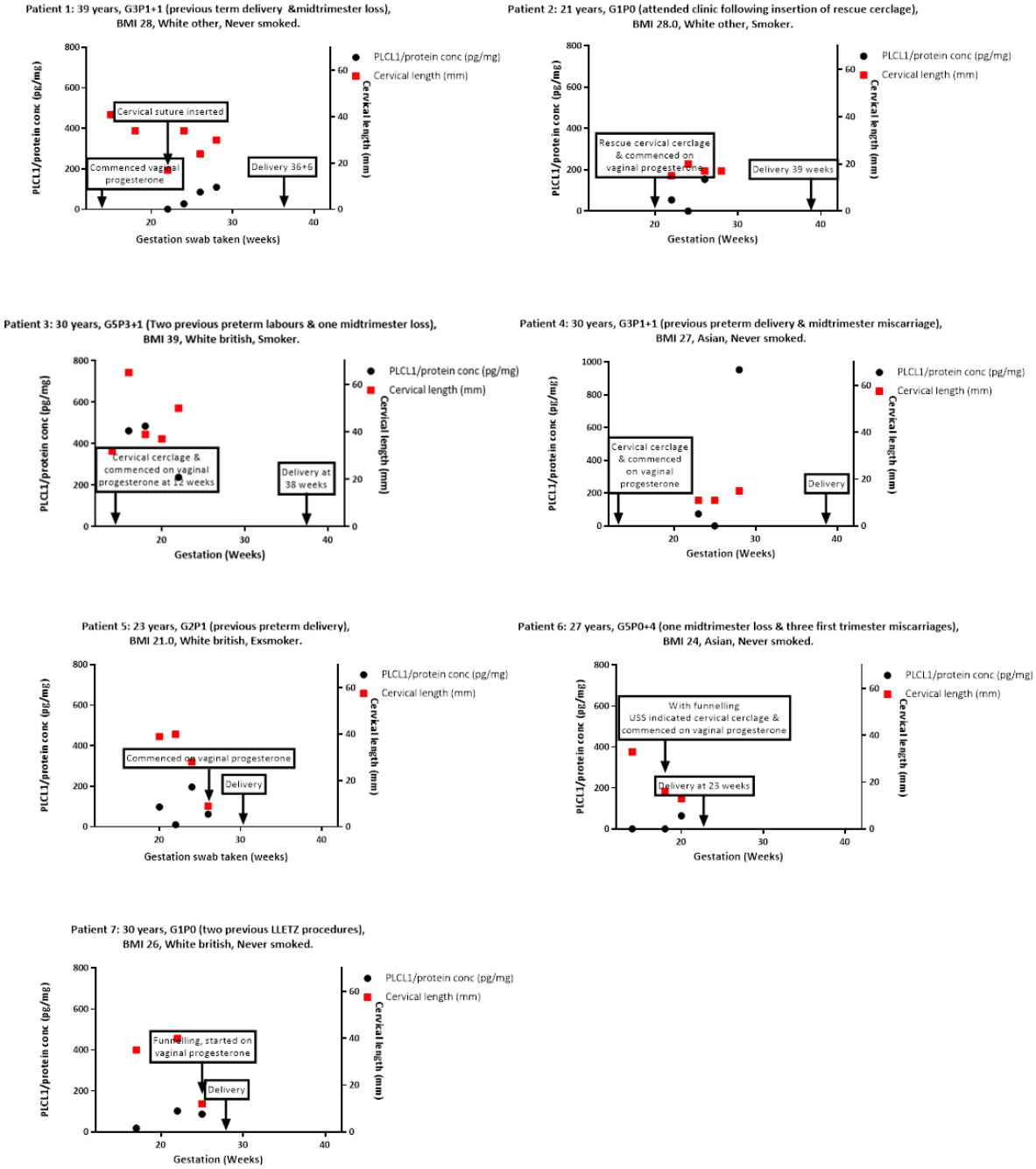

Figure 5.6: Illustration the cervical cytobrush sample results and cervical length measurements of 7 patients who attended the preterm prevention clinic

From Figure 5.6, it can be seen that following administration of vaginal progesterone pessaries, patient 1, 2 and 4 all demonstrated an increase in their PLCL1/protein concentration level 6-14 weeks following it being started. Patient 3 already had high PLCL1 levels. Patients 5, 6 and 7 were all commenced on vaginal progesterone, only a few weeks prior to delivery, could this possibly have been too late to increase PLCL1 levels sufficiently so that it no longer could respond to signalling cascades to initiate labour. Although our number shown here are small, this data could be interpreted to support our hypothesis that PLCL1 levels have been increased *in vivo* in response to vaginal progesterone however, this process takes some time to occur and whether it is biologically plausible could be questioned.

5.7 Conclusions:

The clinical data illustrates that the patients selected were indeed a high-risk group, with 28 out of 91 (31%) delivering preterm. The high-risk nature of the attendees meant that many had no living children or had a child with long term disability due to prematurity. Over 50% of attendees had suffered one or more pregnancy loses from prematurity. In this context the live birth rate of 93% (85/93) was reasonable. There was a significant use of vaginal progesterone at that time as the OPPTIMUM trial recruitment had finished but the trial outcomes had not been published. Hence the clinicians running the clinic thought that the current evidence supported the use of progesterone in women at high risk of preterm birth an issue that is now in doubt following publication of the OPPTIMUM trial (Norman *et al.*, 2016).

In this high-risk group of women, cervical PLCL1 was lower in those that delivered preterm compared to those that delivered at term suggesting that PLCL1 could indeed be a marker

for preterm birth. However this is only a proof of concept study and there are significant limitations with the data.

5.8 Limitations:

As discussed in Chapter 4, there are concerns about our results obtained because of the small size of the sample of cells obtained using the cytobrush technique. The mean sample size obtained from the preterm prevention clinic patients was less than half of the mean size of the samples obtained from the patients who attended for induction of labour (0.76mg/ml versus 1.65mg/ml). On reflection this could be explained by cautious sampling by the study team from this anxious patient group. Due to the complex obstetric history of the majority of the patients attending the clinic, extra caution was taken when obtaining samples. More robust sampling did occasionally lead to some bleeding, we aimed to avoid this as much as possible as we identified that any bleeding heightened anxiety amongst this high risk patient group. The small sample size led to concerns about our results due to the limitations of the sensitivity of the assay used. Furthermore spectrophotometric absorbances from many samples were not within the optimal exponential phase of the standard curves meaning interpolation of both the total protein concentration and PLCL1 concentration was potentially inaccurate.

As explained, due to the small sample size, the significance of the 0pg of PLCL1 is unclear. It is unclear whether this is a true reflection of the cervical cells containing no PLCL1 or as a result of the assay.

Although there was a statistically significant difference in the levels of PLCL1/protein concentration when comparing those patients who went on to deliver at term versus those

who went on to deliver preterm as is shown in Figures 5.3 and 5.4. Several patients who went on to deliver at term did have low levels of PLCL1 in mid-pregnancy. There are biologically plausible reasons for this. Our hypothesis states that PLCL1 is a susceptibility marker for the onset of labour. These patients were followed up regularly in a specialist preterm prevention clinic. We suggest that this may help to reduce the exposure of this group of patients to triggers of preterm labour. That is, that although they were susceptible to preterm labour due to low levels of PLCL1, triggers for preterm labour were screened for in the clinic, for example urinary tract infections. This therefore could prevent activation of proinflammatory pathways which may have triggered labour in susceptible patients.

Further work will be discussed in Chapter 6.

Chapter 6

6. Conclusions:

The overall aim of this project was to determine if a new marker, PLCL1, identified as key in the final signalling pathway for myometrial contraction, could be utilised to help to the predict outcomes of asymptomatic women at high risk for preterm labour, and the outcomes of the induction of labour process.

PLCL1 has been demonstrated to be significantly downregulated with the onset of labour in both the myometrium (Chan *et al.*, 2014) and cervix (Chapter 1, Figures 1.9 and 1.10). It is hypothesised to act to uncouple agonist stimulation of myometrial contractions chelating IP_3, and therefore high levels of PLCL1 should be associated with myometrial quiescence. Over expression of PLCL1 in primary myometrial cells lines inhibits both oxytocin and prostaglandin mediated calcium signalling (Chapter 1, Figure 1.11). Knock-down of PLCL1 enhances both oxytocin and prostaglandin mediated calcium signalling. PLCL1 expression has been demonstrated to be progesterone dependent in the myometrium (Chapter 1, Figure 1.12).

Preterm birth and induction of labour are very important clinical problems in the practice of obstetrics. In preterm labour, the major clinical problem is its accurate prediction, to allow appropriate and timely administration of corticosteroids and magnesium sulphate, and to ensure delivery at a hospital with an appropriate level of special care. Although prevention of preterm birth is also a key area of clinical research, there is currently some debate with regards to the benefit obtained from attempts to prolong pregnancy to promote fetal maturation, versus the risks of continued exposure to a suboptimal or possible hazardous intrauterine environment (Iams *et al.*, 2008).

As discussed in Chapters 1 and 5, current tests for preterm birth have a limited predictive ability and none in present use in clinical practice are based on myometrial quiescence. Similarly, prediction of outcomes of the induction of labour process is an area of unmet clinical need. Patients often experience long hospital stays during this process, leading to huge patient dissatisfaction at great cost to NHS services. As discussed in Chapters 1 and 4, there are limited tests/assessments to predict outcome. Bishop Score, developed in the 1960's remains the most widely utilised.

I aimed to translate novel high quality laboratory science to a clinical application. PLCL1 has many features that suggests that it is regulator of labour and is ideally placed in the signalling cascade to uncouple external signalling from contractions.

We have demonstrated the presence of PLCL1 in cervical epithelial cells taken using the cytobrush technique and developed a method to quantify this protein. Overall analysis of our data collected throughout this project in both groups of patients has demonstrated that PLCL1 is statistically significantly lower at term than throughout the second and early third trimester ($p=0.017$) as shown in Figure 6.1.

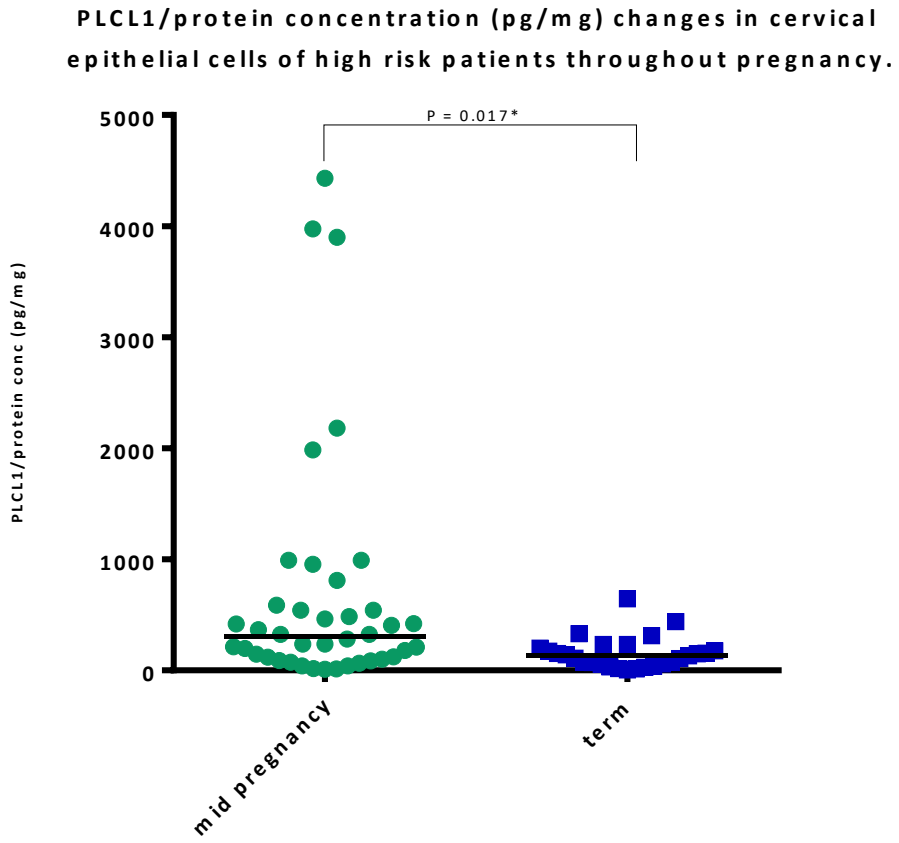

Figure 6.1: A comparison of PLCL1/protein concentration levels (pg/mg) in cervical epithelial cells in the second and early third trimester with samples obtained at term.

Figure 6.1 demonstrates that PLCL1 levels, as expected, decrease toward term. The exact timing of this decrease in relation to the onset of labour has not been established from this project. This will be discussed in the further work section.

Currently we have demonstrated evidence of concept, in that PLCL1 could be used as a susceptibility marker for the onset of labour.

The level of PLCL1 at the start of the induction of labour process did not predict sensitivity of exogenous prostaglandins as hypothesised. However, a statistically significant

relationship was demonstrated between efficacy of syntocinon and PLCL1 level (Chapter 4, Figure 4.12). Possible explanations for these findings were discussed in Chapter 4. The relationship between PLCL1 in high risk asymptomatic women who delivered at term and those who delivered preterm was statistically significant, as hypothesised from laboratory data. The overall findings from this project provide a proof of concept and in support the findings in the laboratory.

6.1 Limitations:

As shown in Chapters 4, 5 and above, our promising work has illustrated that PLCL1 could be important clinically in asymptomatic patients at high risk of preterm labour and with further work may be useful in the induction of labour process. However, limitations of the current project need to be addressed and much more work is needed.

Focusing upon the laboratory work, several challenges were overcome in this project, however there are many areas for improvement. The first challenge was determining if we could detect PLCL1 from epithelial cervical cells taken from pregnant women collected using a cytobrush, then how to separate these cells from cervical mucus, how to lyse the cells and finally how to accurately quantify the protein PLCL1 in samples which were very variable in size and in many cases very small.

The small sample size obtained led to problems with our assay. Following initial experiments, it was determined that all samples with a total protein concentration of less than 0.3mg/ml ideally should be excluded from further analysis as concerns were raised about the accuracy of interpolating total protein results from a standard curve, at the lower portion before it entered the exponential phase. This is illustrated in Figure 6.2. The same

concerns arose with interpolation of PLCL1 results from the generated standard curve (See Chapter 4, Figure 4.13). These two figures demonstrate our sensitivity limit for the assay.

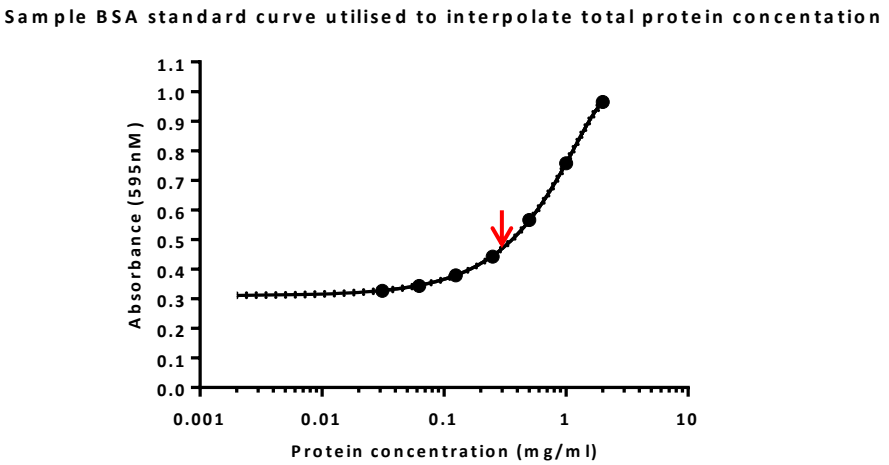

Figure 6.2: A sample curve generated as per the total protein quantification method described in Chapter 3, Section 3.2.3.3 using the Bradford Method. Standard concentrations of bovine serum albumin were made from 0-2mg/ml. As shown by the red arrow (illustrated on the curve at 0.3mg/ml), the exponential phase of the curve begins approximately here. Ideally for accurate protein quantification samples needed to have a total protein concentration greater than this to ensure accurate interpolation from the assay.

Interpatient variability in amount of sample obtained varied considerably with total protein concentrations in all patients sampled ranging from 0.01-6.17mg/ml. As can be seen from Chapter 1, Figure 1.13, there are different levels of expression of PLCL1 in the different layers of squamous epithelium and with the greatest levels of expression in the basal membrane of the cervical epithelium. The variation in total protein obtained may reflect different depths of sampling of the cervical epithelium from patient to patient. This issue needs addressing.

Review of the currently available literature suggested that quantification of an intracellular protein from cervical epithelial cells taken in pregnancy had not previously been performed. Consequently, we needed to develop a method of cell collection, isolation, lysis and quantification. In addition to the sample size varying, the amount of mucus in the samples from patient to patient was also variable with many samples being heavily contaminated (Chapter 3, Figure 3.3). Several methods were explored to try to separate the mucus from the cervical cells until an optimal method was found. A further problem was adequate cell lysis to release our intracellular protein to allow accurate quantification in each sample. Although these problems were overcome, the method developed takes 11 hours and multiple steps which would not be realistic in clinic practice. This method needs improvement in efficiency before it could be considered to be of any use in clinical practice.

We had concerns about the PLCL1 assay sensitivity. Due to the low levels of PLCL1 being detected, again, many of the results were interpolated from the lower end of the standard curve produced, raising some concerns about an increasing margin of error in these samples (Chapter 4, Figure 4.13). The only currently available ELISA kit to quantify PLCL1 uses a polyclonal antibody, this may lead to the target protein binding to more than one antibody molecule on multiple epitopes, this could also influence the accuracy of the quantification of PLCL1 in the samples, a more sensitive monoclonal antibody is required.

6.2 Future Work:

To gain a better understanding of this novel protein and to move forward with this work, I think we need to establish PLCL1 levels in the cervix throughout pregnancy in the low risk population and therefore understand how PLCL1 changes normally pregnancy. From our data it is not clear when PLCL1 levels decline in relationship to the timing of onset of

labour. If indeed it is a susceptibility marker, then this relationship may vary from patient to patient, with some patients becoming susceptible to the onset of labour but not experiencing other stimuli/triggers for some time, and other patients having a decline in PLCL1 level coincide with a stimulus/trigger for the onset of labour and therefore the decline in PLCL1 and onset of labour have a close temporal relationship. Examining PLCL1 in the low risk population would also be important as the pathophysiology of preterm and term labour are known to be different, could the use of PLCL1 as a susceptibility differ in the low risk and high risk population and in the asymptomatic and symptomatic populations as it the case for other biological markers used for prediction.

It would be interesting to investigate if PLCL1 could be utilised with other predictive tests for preterm labour. For example, could PLCL1 and fFN used in combination lead to a better prediction of outcome? We could hypothesise that if PLCL1 level is low and fFN is high, it is likely that the patient will deliver. Biological plausibility behind this is when PLCL1 levels are low, the uterus is permissive to labour, if an adequate stimulus/trigger occurs, could fFN then be utilised to detect if that patient has been exposed to a stimulus/trigger leading to the onset of labour? Much further work is required in this area.

In 2016, van't Hooft *et al* published their work about developing a consensus set of key clinical outcomes for the evaluation of preventative interventions for preterm birth in asymptomatic pregnant women. The aims of this work are to make progress in the field of preterm labour so that data from trials can accurately be compared and combined (van 't Hooft *et al.*, 2016). By eliminating heterogeneity in clinical outcome measures, bias can be minimised. Again in the area of prediction of preterm birth, there is a lack of definitive evidence. As discussed in Chapter 4, there is a variety of monitoring practices offered to asymptomatic high risk patients in the United Kingdom at present (Sharp & Alfirevic, 2014).

With preterm labour being a complex process with many underlying pathological processes, it is highly likely that the mechanisms of preterm labour resulting from a patient with a congenital uterine anomaly versus a history of cervical surgery versus spontaneous preterm labour, are all different. To enable a better understanding the Preterm Clinical Network Database has been established with the plan to capture experience and knowledge on a large scale to provide a valuable source of evidence (Carter *et al.*, 2016).

Moving forward with PLCL1, it may be possible that this susceptibility marker is more beneficial in one specific group at risk of preterm labour than other groups, categorisation of this high risk group of patients attending the preterm prevention clinic, obtaining more samples and analysis of results of these specific groups then may reveal more information about the use of PLCL1 to aid the prediction of preterm birth in the future.

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Appendices

Appendix 1

NHS
Health Research Authority

National Research Ethics Service

NRES Committee West Midlands - South Birmingham
HRA NRES Centre Manchester
3rd Floor
Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7815
Facsimile: 0161 625 7299

14 January 2014

Professor Siobhan Quenby
Professor of Obstetrics
University of Warwick/UHCW
UHCW
Clifford Bridge Road
Coventry
CV2 2DX

Dear Professor Quenby

Study title:	**Prediction of inappropriate myometrial function**
REC reference:	**13/WM/0486**
Protocol number:	**1**
IRAS project ID:	**140732**

Thank you for your letter of 14 January 2014, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Dr Ashley Totenhofer, nrescommittee.westmidlands-southbirmingham@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management

permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		04 January 2014
Evidence of insurance or indemnity	Marsh	26 July 2013
Evidence of insurance or indemnity	Marsh	06 August 2013
GP/Consultant Information Sheets	1	25 October 2013
Investigator CV	Siobhan Quenby	19 October 2013

Investigator CV	Osama Naji	
Investigator CV	Lauren Lacey	
Letter of invitation to participant	1	25 October 2013
Participant Consent Form	3	02 January 2014
Participant Information Sheet	3	02 January 2014
Protocol	2	02 January 2014
REC application	3.5	22 November 2013
Response to Request for Further Information		14 January 2014

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

13/WM/0486	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

Signed on behalf of:
Professor Simon Bowman
Chair

Email: nrescommittee.westmidlands-southbirmingham@nhs.net

Enclosures:	"After ethical review – guidance for researchers"
Copy to:	Dr Peter Hedges - University of Warwick
	Ms Ceri Jones - University Hospital Coventry and Warwickshire NHS Trust
	Dr Lauren Lacey - University Hospital Coventry and Warwickshire NHS Trust
	Dr Osama Naji - University Hospital Coventry and Warwickshire NHS Trust

Appendix 2

THE UNIVERSITY OF WARWICK

Patient information sheet

Title: Is it possible to predict a difficult or preterm labour?
Version 3: 2nd January 2014

We would like to invite you to take part in our research study. Before you decide, we would like you to understand why the research is being done and what it would involve for you. Please read this patient information sheet. If you would like to take part or have any further questions, please contact us on 02476967528 or email s.quenby@warwick.ac.uk. Next time you come for your hospital appointment we will arrange to see you whilst you are there.

What is the purpose of the study?

Preterm delivery (onset of labour and delivery before 37 weeks) is the leading cause of health problems in newborn babies in the United Kingdom, these can have lifelong consequences. To identify women who are at risk, we currently rely on talking to patients about their previous pregnancies and medical problems, but this only allows us to identify 1 in 10 women who actually go into preterm labour. If a patient comes to hospital with symptoms suggestive of preterm labour, there are tests available to help us. But these tests only assure us that a woman is not going into preterm labour and are less useful at warning us about those who are actually in preterm labour. We would like to develop a better test.

Many women do not go into labour on their own and require induction of labour. In some women this is more successful than others. For some women it can take several days to get them into labour, we are also trying to develop a test predict who these women are.

We would like to take cervical (neck of the womb) swabs and do a blood test to look at three different things and see if there are differences in them between women who go into preterm labour, those who go into labour at term (37-42 weeks) and women who do not go into labour on their own.

THE UNIVERSITY OF WARWICK

Why have I been invited?

You have been invited because you are in one of three groups which we are interested in.

Group 1) Patients who are pregnant and have gone into labour at less than 37 weeks in the past, or have had a previous miscarriage at 13-23 weeks.

Group 2) Patients who come into labour ward triage with symptoms which may suggest preterm labour.

Group 3) Patients who they have not gone into labour on their own and are overdue.

Do I have to take part?

It is your choice. If you are happy to take part you will be asked to sign a consent form, of which there are three copies, one for you. You can withdraw at any time you choose.

What will happen to me if I take part?

Group 1:

Visit 1

We will talk to you about our study before your first cervical length scan.

You will be asked about your health and previous pregnancies. Cervical swabs will be taken before your internal scan. We would also like to take a blood test (20mls – 4 teaspoons).

Further visits

We would like to repeat the swabs and blood test when you come for further cervical length scans at the clinic throughout your pregnancy

We would like to look at your medical notes after your baby is born.

Group 2:

We will talk to you about our study. You will be asked about your health and previous pregnancies. Cervical swabs will be taken during your routine internal examination. We would also like to take a blood test (20ml – 4 teaspoons) when your routine blood tests are taken. We would like to look at your medical notes after your baby is born.

Group 3:

We will talk to you about our study. We would like to take swabs from your cervix during your internal examination before the insertion of a tablet to induce labour. We will also ask you about your general health and previous pregnancies. We would like to take a blood test (20ml- 4 teaspoons) with your other routine blood tests. We would like to look at your medical notes after your baby is born.

What are the alternatives for diagnosis or treatment?

If you attend labour ward triage and we are concerned that you are going into preterm labour, we will follow our normal pathway of care. For other patients these are extra tests during your routine examinations.

What are the possible disadvantages and risks of taking part?

There are no disadvantages. Previous studies have shown that this is a safe procedure in pregnancy. There may be some slight discomfort when we examine you to take the swabs and do the blood tests but these examinations will be required anyway, for us to be able to assess you when you come to hospital.

What are the possible benefits of taking part?

We cannot promise the study will help you but what we learn from this study may help improve the treatment of patients with preterm labour in the future.

What happens when the research study ends?

Once we have collected our samples and they have been analysed, if you would like to know the findings of our study we can write to you with the results.

What to do if you are unhappy with your treatment?

If you are unhappy or have any concerns please ask to speak to the researchers who will be happy to help. Contact number: 02476967528. If you remain unhappy and have further concerns please contact:

Patient Advice and Liaison Service
UHCW NHS Trust
Coventry CV2 2DX
Tel: 02476966061 or freephone 0800 028 4203
Email: PALS@uhcw.nhs.uk

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. All information that is collected about you during the course of the research will be kept strictly confidential and accessible only to authorised staff. Your blood samples and swabs will be coded anonymously. Samples will be stored in the tissue bank under the Human Tissue Authority legislation.

With your permission we will keep your contact details and may approach you in the future about further ethically approved studies.

What will happen if I don't want to carry on with the study?

If you withdraw from the study, we will destroy all your identifiable samples

Involvement of the General Practitioner/Family doctor (GP)

Your GP will be advised of your involvement unless you request otherwise.

Will I need to make any extra hospital visits?

No

What will happen to any samples I give?

Your samples will be processed in the laboratory. They will then be stored indefinitely in the tissue bank at the hospital or at the University of Warwick. We do ask for your permission to use any remaining samples for other research in the future. Samples that are no longer needed for research will be destroyed through the normal clinical waste procedures of the University.

THE UNIVERSITY OF WARWICK

What will happen to the results of the research study?

The results will be published in medical literature. We will of course let you know the results.

Who is organising and funding the research?

This work is funded by Biomedical Research Unit, University Hospitals Coventry and Warwickshire NHS Trust. The research is organised and will be conducted according to the legal framework for use of human tissues in research embodied in the Human Tissue Act. This trial will adhere to the Good Clinical Practice (ICH GCP) guidelines. It will be conducted in compliance with the protocol, the Data Protection Act and other regulatory requirements as appropriate.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by NRES Committee West Midlands – South Birmingham.

Contact Details:

If you would like further information or have any concerns about the study, please contact

Professor Siobhan Quenby B. Sc., MBBS, MD, FRCOG
Professor in Obstetrics, Division of Reproductive Health, Clinical Sciences Research Laboratory
Warwick Medical School, University of Warwick
Coventry CV2 2DX
Tel: 02476967528
E-mail: s.quenby@warwick.ac.uk

Patient Advice and Liaison Service
UHCW NHS Trust
Coventry CV2 2DX
Tel: 02476966061 or freephone 0800 028 4203
Email: PALS@uhcw.nhs.uk

Thank you for taking the time to read this information sheet.

Appendix 3

THE UNIVERSITY OF WARWICK

Hospital Number:
Patient Code Number for this project:
Version 3: 2nd January 2014

CONSENT FORM

Title: Prediction of inappropriate myometrial function.

Name of Researchers: Professor Siobhan Quenby, Professor Jan J Brosens, Professor Andy Blanks, Dr Lauren Lacey, Dr Osama Naji

	Please initial
1. I confirm that I have read and understand the Patient Information Sheet Version 3 dated 2nd January 2014 for the above study, I have been given the opportunity to ask questions and discuss this study. I have received satisfactory answers to all my questions.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, and without my medical care or legal rights being affected.	☐
3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from the University of Warwick, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	☐
4. I agree to material collected and stored in this study being used in future studies, subject to Ethics Committee approval.	☐
5. I agree to my GP being informed about my participation in the study	☐
6. I agree to being contacted in the future about further studies	☐

The study has been explained to me by ..... Date.....

Signed..... Date.....

(NAME IN BLOCK CAPITALS).....

Investigator's signature..... Date: .....

(NAME IN BLOCK CAPITALS).....

When completed, 1 for patient, 1 for researcher site file;, 1 (original) to be kept in medical notes

Version 3: 2nd January 2014

Appendix 4

THE UNIVERSITY OF WARWICK

Version 1: 25th October 2013

Professor Siobhan Quenby
Professor of Obstetrics
University of Warwick
Department of Obstetrics and Gynaecology
University Hospitals of Coventry and Warwickshire
Clifford Bridge Road
Coventry CV2 2DX
Email: s.quenby@warwick.ac.uk
Telephone: 02476968657

Dear Doctor

Subject: Prediction of inappropriate myometrial function

As you know, the above-named patient is pregnant and her pregnancy is booked at UHCW NHS Trust.

I am writing to inform you that she has agreed to participate in a research study which is being conducted at University of Warwick and University Hospitals Coventry and Warwickshire NHS Trust.

We are aiming to identify markers which we aim to use to develop a test for preterm labour and dysfunctional labour. We will be taking an endocervical swabs and a blood test during pregnancy or when your patient attends for induction of labour.

Her care will otherwise be entirely unchanged.

I enclose the Information Sheet that we have given her.

Yours sincerely,

Professor S Quenby,
Principal Investigator

Appendix 5

THE UNIVERSITY OF WARWICK

Professor Siobhan Quenby
Professor of Obstetrics
University of Warwick
Department of Obstetrics and Gynaecology
University Hospitals of Coventry and Warwickshire
Clifford Bridge Road
Coventry CV2 2DX
Email: s.quenby@warwick.ac.uk
Telephone: 02476968657
Version 1: 25th October 2013

Dear Madam,

Subject: **Is it possible to predict a difficult or preterm labour?**

We are inviting you to take part in the above study at University of Warwick and University Hospitals Coventry and Warwickshire NHS Trust.

We would like to investigate whether we can develop a test to predict preterm labour (onset of labour before 37 weeks) and other problems with labour.

I enclose a Patient Information Sheet which we would be very grateful if you would read

If you would like to take part please could you contact us on 02476967528 or email s.quenby@warwick.ac.uk.

Yours sincerely,

Professor S Quenby,
Principal Investigator

Appendix 6

University Hospitals Coventry and Warwickshire NHS Trust

Research & Development Department
Head of RD&I: Carl Jones - Tel: 024 7696 6069
RD&I Operations Manager: Tammy Holmes – Tel: 024 7696 6196
RD&I Divisional Finance Manager: Chris Moore - Tel: 024 7696 6198
Deputy RD&I Divisional Finance Manager: Joanne Treadwell - Tel: 024 7696 6199
RD&I Business Manager: Natasha Willeman - Tel: 024 7696 6197
Research Governance Associate: Isabella Patria - Tel: 024 7696 6202
RD&I Assistant: Hannah Williamson - Tel: 024 7696 4995
Research Portfolio Development Manager: Deborah Griggs - Tel: 024 7696 6196

University Hospital
Clifford Bridge Road
Walsgrave
Coventry
CV2 2DX

Tel: 024 7696 4000
Fax: 024 7696 6056
www.uhcw.nhs.uk

17 January 2014

Professor Siobhan Quenby
Professor of Obstetrics
University Hospitals Coventry & Warwickshire NHS Trust
Clifford Bridge Road
Coventry, CV2 2DX

Dear Siobhan

Study Title: Prediction of inappropriate myometrial function

Thank you for submitting the above study for consideration by the Research, Development & Innovation (RD&I) Office, in line with new regulations governing our approval of local sites from 1st April 2009.

I am pleased to inform you that your study has been approved. The documents approved for use in this study are listed below:

Document	Version	Date
Protocol	1	25/10/2013
Participant Information Sheet	1	25/10/2013
Consent Form	1	25/10/2013
Participant Letter of Invitation	1	25/10/2013
GP / Consultant information sheet / letters	1	25/10/2013
REC Application Form		13/11/2013

It is a condition of approval that, should changes be made to any of the above documents, they should be submitted to the Ethics Committee & RD&I for approval. An annual progress and safety report should be submitted to RD&I, the first report is due on 17 January 2015.

If any Serious Unexpected Suspected Adverse Reaction, USAR, Clinical Adverse Event or Protocol Breach arise from your study you must inform the RD&I Department immediately, and complete a DATIX Clinical Adverse Event Form.

Your project may be subject to ad hoc audit by our department to ensure these standards are being met.

Your research is covered by NHS indemnity as set out in HSG(96)48.

May I take this opportunity to remind you that, as a researcher, you must ensure that your research is conducted in a way that protects the dignity, rights, safety and well-being of participants. Trust RD&I Approval assumes that you have read and understand the Research Governance Framework and accept that your responsibilities as a researcher are to comply with it, the Data Protection and Health & Safety Acts.

The Trust wishes you every success with your project.

Yours sincerely

Isabella Petrie
Research Governance Associate

cc: Lauren Lacey

Appendix 7

Biomedical Research Unit in
Reproductive Health
News-Obstetrics

University Hospitals Coventry and Warwickshire NHS Trust

THE UNIVERSITY OF WARWICK

Spring 2014 edition

The **Biomedical Research Unit** at University Hospital, Coventry aims to increase reproductive health in pregnancy, at birth and beyond. The focus lies on the development and testing of novel interventions to safeguard the health of women and their babies at all stages of pregnancy. This newsletter aims to introduce you to us and make you aware of our clinical trials and research projects. If you would like more information, please contact us on 02476 967528 or visit our website www2.warwick.ac.uk/fac/med/research/reproductive/

Meet our clinical team

Professor Siobhan Quenby is the Director of the Biomedical Research Unit. Jane Hillen, Yvonne Nunn, Debbie Bullen, Lyndsey Prue & Nicola Flint make up our talented team of Research Midwives involved in our clinical trials and studies. Together with Professor Jan Brosens and Dr Lauren Lacey, Professor Quenby also runs a dedicated Implantation Research Clinic with the aim of establishing novel treatments and interventions for women suffering IVF failure and miscarriages.

From left to right: MW Nicola Flint, Dr. Lauren Lacey, Dr. Dawn Adamson, Professor Jan Brosens, MW Yvonne Nunn, Professor Siobhan Quenby, MW Jane Hillen, Kerri Geraghty, MW Debbie Bullen, MW Lyndsey Prue. Also in the team: Dr. Sadia Malik, Miss Neelam Engineer.

Implantation clinic

Miscarriage and IVF treatment failure are very distressing events. In most cases, no apparent cause is found. Our studies have shown that poor preparation of the lining of the womb is a major cause of reproductive failure.

Importantly, we are able to examine the preparation of the lining with of a simple womb biopsy. We have developed a test that is based on the distribution of specialised immune cells (uterine natural killer cells) in the lining of the womb to identify women at increased risk of pregnancy failure.

Furthermore, we are developing new protocols to prevent pregnancy failure caused by inadequate preparation of the lining of the womb.

For more information on the uterine natural killer cell test call 02476 967528. The test currently costs £360.

Obstetric Cardiology Clinic

Dr Dawn Adamson, consultant cardiologist and Professor Quenby have set up a specialist obstetric cardiology antenatal clinic to care for women with heart problems in pregnancy. These problems can exist prior to pregnancy or develop in pregnancy.

We are currently reviewing our cases from the last five years so we can give you up to date information about our services.

For more details please call 02476 965673.

Biomedical Research Unit in
Reproductive Health

Trials in the Biomedical Research Unit

There are many clinical trials which the team are involved in recruiting women for. Below is a summary of these trials. You may be invited to take part in one of these in your pregnancy.

Prediction of inappropriate myometrial function
Preterm delivery (onset of labour and delivery before 37 weeks) is the leading cause of health problems in newborn babies in the UK.
We are developing a test that to better predict preterm labour or who will not go into labour.

PRiDE: Are nutrients and vitamins in pregnant women linked with diabetes?
Diabetes in pregnancy is common. Increasing maternal age, higher body weight, a family history and ethnic minority groups are at a higher risk. Are other factors such as low vitamin B12 also risk factors?

EMPiRE: Seizures and the effects of anti-epileptic drugs during pregnancy are thought to affect those born to mothers with epilepsy.
There is very little research on this and we plan to find the anti-epileptic drug monitoring method that is best for pregnancy.

PREP: Can we predict the onset of pre-eclampsia in pregnant women?
In 1% of pregnant women pre-eclampsia occurs before 34 weeks of pregnancy and causes more serious complications for mother and baby. Can we predict this?

Post natal depression study
This aims to identify women who are at risk of postnatal depression.

Our recent publications

* Elevated Periimplantation Uterine Natural Killer Cell Density in Human Endometrium Is Associated With Impaired Corticosteroid Signaling in Decidualizing Stromal Cells. (J Clin Endocrinol Metab. 2013 September).
* Uterine plasticity and reproductive fitness. (Reprod Biomed Online. 2013 July).
* LRH-1: orphaned, adopted and needed for pregnancy. (Nat Med. 2013 August).
* Deficiency in Clonogenic Endometrial Mesenchymal Stem Cells in Obese Women with Reproductive Failure – a Pilot Study. PLOS ONE (Dec 2013).
* Assessment of Myometrial Transcriptome Changes Associated with Spontaneous Human Labour by High Throughput RNA-seq. Exp Physiol. (Nov 2013)
* Elevated Fetal Adipsin/Acylation-Stimulating Protein (ASP) in Obese Pregnancy: Novel Placental Secretion via Hofbauer Cells. J Clin Endocrinol Metab. (Aug 2013).

Appendix 8

Case Report Form – Group 1- Preterm labour clinic

Patient ID for Study ____ Patient hospital number _____

Eligibility to take part

Signed written consent form Yes ☐ No ☐

Date of signed consent form .....

Gestational age..... weeks

Exclusion criteria

Multiple pregnancy Yes ☐ No ☐

Abnormal smear Yes ☐ No ☐

Last smear done.....
Last smear result.....
Smear not done as: Too young ☐ Declined cervical screening ☐

Is patient eligible for study? **YES** ☐ **NO** ☐

Member of staff filling in details.....

Patient information

Age..... Pre-pregnancy weight.....

Height..... Ethnicity.....

Smoking status

Current smoker Number per day.....
Ex-smoker
Never smoked
Unknown

Did patient conceive through assisted conception? Yes ☐ No ☐

Obstetric history

Parity .....

Does patient have a cervical suture in situ Yes ☐ No ☐

Is patient using progesterone pessaries Yes ☐ No ☐

Does patient have a known congenital uterine anomaly Yes ☐ No ☐

Previous pregnancies

Pregnancy	Date of delivery	Gestation at delivery	Birth weight	Mode of delivery	Complications in pregnancy	Other

Medical History

Medical problems.....

Medications.....

Allergies.....

Has the patient taken any antibiotics during pregnancy? Yes No

If Yes – When and which antibiotics.....

.....

Any further problems in this pregnancy of significance

.....

.....

Date of Preterm labour clinic: / / Time of swab..... (24hr clock)

Cervical length (mm).....

Speculum examination findings

Vulva & vagina .....

Cervix .....

Other .....

Was the cervix clearly seen? Yes ☐ No ☐

Were the following swabs taken

HVS/ECS (for C & S and BV) Yes ☐ No ☐

Endocervical cytobrush swab for study Yes ☐ No ☐

Endocervical swabs for study Yes ☐ No ☐

Were bloods taken for study Yes ☐ No ☐

Was there any bleeding after the swab was taken Yes ☐ No ☐

Were the tests repeated during the pregnancy Yes ☐ No ☐

Date / /
Cervical length.....mm

Were the following swabs taken

Endocervical cytobrush swab for study Yes ☐ No ☐

Endocervical swab for study Yes ☐ No ☐

Were bloods taken for study Yes ☐ No ☐

Were the tests repeated again during the pregnancy Yes ☐ No ☐

Date / /

Cervical length.....mm

Were the following swabs taken

Endocervical cytobrush swab for study Yes ☐ No ☐

Endocervical swabs for study Yes ☐ No ☐

Were bloods taken for study Yes ☐ No ☐

Intrapartum information

Date of delivery: / /

Gestation at delivery (weeks):

Mode of delivery:

Normal vaginal delivery ☐ Instrumental delivery ☐ Caesarean section ☐

Indication for MOD.....

Did the patient receive magnesium sulphate Yes ☐ No ☐

Did the patient have any other tocolysis Yes ☐ No ☐
Details.....

Duration of first stage of labour (4cm-fully dilated) (hours)

Was labour augmented in 1st stage Yes ☐ No ☐
Details.....

Rupture of membranes
Spontaneous ☐ Artificial ☐ (Indication.....)

Duration of second stage of labour (minutes)

Was labour augmented in 2nd stage Yes ☐ No ☐
Details.....

Duration of third stage of labour (minutes)

Did patient have an active or physiological third stage of labour Active ☐ Physiological ☐

Estimated blood loss (ml) .....

Other problems in labour.....

.....

Pre-delivery Hb (g/L) .....

Analgesia used in labour

None ☐ TENS machine ☐ N_2O_2 ☐ H_2O ☐

Pethidine ☐ Epidural ☐ Spinal ☐

Appendix 9

Warwick Medical School

Prof Siobhan Quenby
Reproductive Health
Warwick Medical School
University of Warwick
Coventry
CV4 7AL
United Kingdom

6th November 2013

Project Title: Prediction of Inappropriate Myometrial Function
Chief Investigator: Siobhan Quenby
Our Ref: REGO-2013-567

Dear Professor Quenby,

I confirm that the University of Warwick will act as Research Sponsor for the above project, in accordance with the Department of Health's Research Governance Framework for Health and Social care (2005), and, where appropriate, the Medicines For Human Use (Clinical Trials) Regulations (2004).

Any researcher involved in the project is required at all times to comply with the University of Warwick's Research Code of Practice.

Best wishes

Graham Hewitt
Research Ethics and Governance Manager

Dean's Office & Professional Support Services
Warwick Medical School
A010 Medical School Building
The University of Warwick
Coventry
CV4 7AL

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W: www.warwick.ac.uk/wms

THE UNIVERSITY OF WARWICK

Appendix 10

Referral Form for Preterm Prevention Clinic

Tel: 02476 967528

<table>
<tr><td>Referral date:</td><td>Obstetrician:</td></tr>
<tr><td rowspan="2">Affix patient label here</td><td>Contact number for patient:
Email address (if preferred)
.....</td></tr>
<tr><td>Gestation age at referral:</td></tr>
<tr><td>Indication for referral (please circle as appropriate)</td><td>EDD by scan:</td></tr>
<tr><td>History of preterm labour at <34/40</td><td>Down's screening result.....</td></tr>
<tr><td>History of second trimester miscarriage suggestive of cervical incompetence</td><td rowspan="3">Obstetric history
G.....P.....
Number of term deliveries (>37/40).....
Number of preterm deliveries (<37/40).....
Number of second trimester miscarriages.....</td></tr>
<tr><td>History of LLETZ
- size of tissue excised:.....mm</td></tr>
<tr><td>Known uterine anomaly
- Details.....</td></tr>
<tr><td>Short cervix on transvaginal scan
- cervical length.....mm</td><td>Other details</td></tr>
<tr><td>Name of person referring patient:</td><td>Contact number</td></tr>
<tr><td>Appointment:</td><td>Interpreter required: Yes/No
Language.....</td></tr>
</table>

All referrals should be sent to the BRU (opposite ward 24). Once received, we will send an appointment to the patient, either by telephone or in the post
Patients should be made aware that the clinics are run on Wednesday afternoons every 2-3 weeks currently