The Role Of the Endocannabinoid, Anandamide In Parturition And In The Prediction Of Preterm Labour

A thesis submitted for the degree of

Doctor of Medicine

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Abstract

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Preterm birth is responsible for 75% of the perinatal mortality and morbidity. There is a trend towards an increase in its prevalence. Until now screening and prevention programmes have not been that successful due to limited knowledge of the basic molecular mechanisms of labour. As a result the predictive markers of preterm birth are not that effective raising the need for novel biomarkers.

The endogenous endocannabinoid *N*-arachidonoylethanolamine (anandamide; AEA) acts as a ligand for cannabinoid receptors CB_1, CB_2,and non-CB_1 and non-CB_2 receptors. There is lack of knowledge on the role of anandamide in pregnancy and labour in humans. However the fact that smoking of exogenous cannabinoids such as marijuana is associated with preterm birth suggests a role for the endocannabinoids in labour. Plasma AEA was shown to increase 3.7 fold with labour.

The aim of this thesis was to develop an improved method for the measurements of plasma AEA and to investigate further its role in labour and to measure its level in a cohort of women at high risk of delivering preterm.

This led to the development of UPLC-MS/MS method of measuring plasma AEA. In women undergoing induction of labour, plasma AEA level was found to be elevated 1.5 fold in active labour (1.82±0.87nM) in comparison to levels in the non-labouring phase (1.20 ± 0.57nM) ($P<0.0001$).

In women at high risk of delivering preterm the plasma AEA levels were significantly elevated in those who delivered within 6 weeks of sampling compared to women who delivered at term ($P=0.01$). A plasma AEA level of 1.10nM, predicted preterm delivery at <37 weeks of gestation with a sensitivity of 66.6% and specificity of 81.8%.

Plasma AEA levels were not found to be significantly elevated in women delivering preterm amongst women presenting with threatened preterm labour. However a small sample size could have contributed to this result. The mean plasma AEA level was significantly elevated in women who had a positive fetal fibronectin test when compared to those who tested negative suggesting a relationship between the two.

These data provide additional evidence that plasma AEA plays a significant role in labour and it could be used as a predictor of preterm delivery.

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My special thanks to my husband who had always been there for me. He has helped me push my limits and has encouraged me to achieve goals. My love to my son who by being so attached has helped me unwind at times of stress.

It is a pleasure to thank Dr Mark Finney and Dr Patricia Lam for their support, co-operation and companionship in trying to understand and make the mass spectrometer work. My sincere thanks goes to Dr Timothy Marczylo who helped me understand the working of the mass spectrometer in a better way and without his help it would have been a real struggle to complete the study.

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Appendix 1: Publications arising from the Thesis

Ultra Performance Liquid Chromatography Tandem mass Spectrometry Method for the Measurement of Anandamide in Human plasma.

Analytical Biochemistry, Volume 380, Issue 2, September 2008, Pages 195-201.

Patricia M.W.Lam, Timothy H. Marczylo, Mona El-Talatini, Mark Finney, Vijaianitha Nallendran, Anthony H. Taylor, Justin C. Konje

1. *A solid* phase method for the extraction and measurement of anandamide from multiple human biomatrices.

Analytical Biochemistry, Volume 384, Issue 1, January 2009, Pages 106-113.

Timothy H. Marczylo, Patricia M.W. Lam, Vijaianitha Nallendran, Anthony H. Taylor, Justin C. Konje.

2. The plasma levels of the endocannabinoid, anandamide, increase with the induction of labour.

British Journal of Obstetrics and Gynaecology, Volume117, Issue 7, June 2010, Pages 863-869.

Nallendran.V, Lam.P, Marczylo.T, Bankart.M, Taylor.A, Taylor.D, Konje.J

Appendix 2: Presentations arising from the Thesis

Poster Presentations:

1. Role of anandamide in labour.

Annual Scientific Meeting of the Society for Gynaecologic Investigations, San Diego USA, March 2008.

2 Prediction of preterm labour among asymptomatic high risk patients using plasma anandamide levels.

Annual Scientific Meeting of the Society for Gynaecologic Investigations, Glasgow UK, March 2009.

Appendix 3: Ethics documentation, Patient information and Consent forms

Bibilography

List of abbreviations

AA	Arachidonic acid
ACTH	Adrenocorticotrophin hormone
AC	Adenylate cyclase
AE	Amniotic epithelium
AEA	Anandamide
AEA-d_8	Octa deuterated anandamide
AEA-Na^+	Sodium adduct of anandamide
AEA-H^+	Hydrogen adduct of anandamide
AF	Amniotic fluid
AMT	Anandamide membrane transporter
AP-1	Activator protein-1
ARM	Artificial rupture of membranes
ATP	Adenosine triphosphate
AUC	Area under the curve
BEH	Bridged ethylene- silicon hybrid particles
BMI	Body mass index
BV	Bacterial vaginosis
C	Carbon
Ca^{2+}	Calcium
CAP	Contraction associated proteins
cAMP	Cyclic adenosine monophosphate
CB1	Cannabinoid receptor1
CB2	Cannabinoid receptor 2
CD80	Cluster of differentiation 80
c/EBP	Ccaat enhancer binding protein
CI	Confidence interval

COX-1	Cyclo-oxygenase-1
COX-2	Cyclo-oxygenase-2
COX-3	Cyclo-oxygenase-3
$cPLA_2$	Cytosolic Phospholipase-A_2
CRE	Cyclic adenosine monophosphate response element
CRH	Corticotrophin releasing hormone
CRH-BP	Corticotrophin releasing hormone binding protein
CRP	C-reactive protein
CTL	Connective tissue layer
Cx-43	Connexin-43
Cx-26	Connexin-26
D	Decidua
DHEA-S	Dehydroepiandrosterone sulphate
DNA	Deoxyribonucleic acid
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELBW	Extreme low birth weight
ELISA	Enzyme-linked immunosorbent assay
EP-1	Prostaglandin E receptor-1
EP-2	Prostaglandin E receptor-2
EP-3	Prostaglandin E receptor-3
ERα	Oestrogen receptor-α
ERβ	Oestrogen receptor-β
ES^+	Positive electron spray ionization mode
FA	Focal adhesions
FAAH	Fatty acid amide hydrolase
FAK	Focal adhesion kinases

FDA	Federal drug administration
FFN	Fetal fibronectin
FIGO	International Federation Of Gynaecology and Obstetrics
GABA	Gamma amino butyric acid
GAG	Glycosaminoglycan
GBS	Group B streptococci
GIFT	Gamete intra fallopian transfer
GPR-17	G protein coupled receptor-17
GPR-55	G protein coupled receptor-55
GRB-2	Growth factor receptor bound protein
H^+	Hydrogen
HDAC 3	Histone deacetylase 3
HPLC-MS	High performance liquid chromatography mass spectrometry
HPLC-MS/MS	High performance liquid chromatography tandem mass spectrometry
HUAM	Home uterine activity monitoring
ICAM-1	Intercellular adhesion molecule-1
IGF-1	Insulin dependent growth factor-1
IGF-2	Insulin dependent growth factor-2
IGFBP-1	Insulin dependent growth factor binding protein-1
phIGFBP-1	Phosphorylated insulin dependent growth factor binding protein-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-1β	Interleukin-1β
IIS	Innate immune system
IQR	Inter quartile range
ITP_3	Ionosine-triphosphate
IVF	In-vitro fertilization

JNK	c-Jun N-terminal kinases
K^+	Potassium
kV	Kilovolts
LLETZ	Large loop excision of transformation zone
Ln (IDI)	Natural log of the predicted induction to delivery interval
LOD	Limit of detection
LOQ	Limit of quantification
LPD	Luteal phase defect
LPE	Liquid phase extraction
MAPK	Mitogen activated protein kinase
mER	Membrane oestrogen receptor
MLC20	Myosin light chain 20
MLCK	Myosin light chain kinase
mM	Millimole
MMP	Matrix metalloproteinases
MMP-1	Matrix metalloproteinases-1
MMP-2	Matrix metalloproteinases-2
MMP-3	Matrix metalloproteinases-3
MMP-8	Matrix metalloproteinases-8
MMP-9	Matrix metalloproteinases-9
mPR	Membrane progesterone receptor
MRM	Multiple reaction monitoring mode
MR score	Mass restricted score
mRNA	Messenger ribonucleic acid
MRM	Multiple reaction mode
M/Z	Mass to charge ratio
NADA	N- arachidonoyl dopamine
NAPE	*N*-arachidonoyl phosphatidyl ethanolamine

NF-κB	Nuclear factor- kappa B
NHS	National health service
NK cells	Natural killer cells
nM	Nanomoles
NMDA	*N*-methyl-D-aspartate
NOS	Nitric oxide synthase
nPR	Nuclear progesterone receptor
OR	Odds ratio
OTR	Oxytocin receptor
P	Probability
PCA	Protein kinase A
PGD_2	Prostaglandin D_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGE_2	Prostaglandin E_2
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PGDH	Prostaglandin dehydrogenases
PGFR	Prostaglandin F receptor
PGHS-1	Prostaglandin synthase-1
PGHS-2	Prostaglandin synthase-2
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PMOL	Picomole
pPROM	Preterm premature rupture of membranes
PR-A	Progesterone receptor A

PR-B	Progesterone receproor B
PR-C	Progesterone receptor C
PTB	Preterm birth
PTD	Preterm delivery
PTFE	Poly tetra fluoroethylene
QALY	Quality adjusted life year
r^2	Regression estimate
RCT	Randomized control trial
RDS	Respiratory distress syndrome
ROC	Receiver operating curve
RR	Relative risk
RSD	Relative standard deviation
RT-PCR	Reverse transcriptase polymerase chain reaction
RU-486	Mifepristone
SD	Standard deviation
SELDI-TOF-MS	Surface enhanced laser desorption/ionization time of flight mass spectrometry
SEM	Standard error of the mean
SIR	Selected ion recording mode
$sPLA_2$	Secretory Phospholipase A_2
SPE	Solid phase extraction
SROM	Spontaneous rupture of membranes
T	Trophoblast
THC	Tetrahydrocannabinol
TIMP	Tissue inhibitor of matrix metalloproteinases
TLR	Toll like receptors
TNF-α	Tumour necrosis factor-α
TRβ1	Thyroid hormone receptor beta-1

UK	United Kingdom
UPLC	Ultra performance liquid chromatography
USA	United States of America
VEGF	Vascular endothelial growth factor
VLBW	Very low birth weight
WHO	World Health Organization
2-AG	2- Arachidonoyl glycerol
11β-HSD	11β- Hydroxy steroid dehydrogenase

Chapter 1

Introduction

1 Introduction

Preterm birth (PTB), according to the World Health Organization (WHO), is defined as delivery of an infant between 20 and 37 weeks of gestation, though the accepted lower limit of gestation varies in different countries (Goldenberg, et al., 2008).

The incidence of preterm delivery (PTD) according to NHS maternity statistics of England in 2002-2003 was 7%. In the USA, the reported PTD rate was 12-13% and in Europe, the reported rate was 5-9% (Goldenberg, et al., 2008).

PTB is of major public health concern. It is responsible for 75% of perinatal mortality and two thirds of these deaths occur in babies born before 32 weeks of gestation (Slattery and Morrison, 2002). PTB is also associated with 75% of neonatal morbidity and long term neurological-developmental problems, respiratory and visual dysfunction (Wen et al., 2004). In addition, there is a huge financial burden on the health care system in providing intensive care treatment to these preterm infants.

The rate of PTD has been rising in most industrialized nations. In the USA, the rate increased from 9.5% in 1981 to 12.7% in 2005 (Goldenberg et al., 2008). Despite advancing knowledge of the risk factors and mechanisms related to preterm labour (PTL), efforts at preventing PTD have not been very successful. It is thought that an improved understanding of the physiological pathways that regulate uterine contractions and cervical effacement during labour will help in the development of novel intervention strategies to prevent PTD.

1.1 Parturition

Following conception the fetus develops within the uterus. The uterus is composed of two components, very different in both structure and function - the corpus uteri and the cervix. The corpus uteri is composed of mainly smooth muscles which remain quiescent for most part of gestation before contracting with the start of labour. The cervix is composed mainly of collagen and remains uneffaced, which means it is long and tightly closed during most of pregnancy to support the growing fetus.

Within the uterine cavity the fetus grows inside the amniotic cavity which is filled with amniotic fluid (AF). The amniotic cavity is lined by the amnion and the chorion. The amnion and chorion together constitute the fetal membranes. These play an active role in labour (Calder.,2000). The feto-placental unit is shown in Figure 1.1.

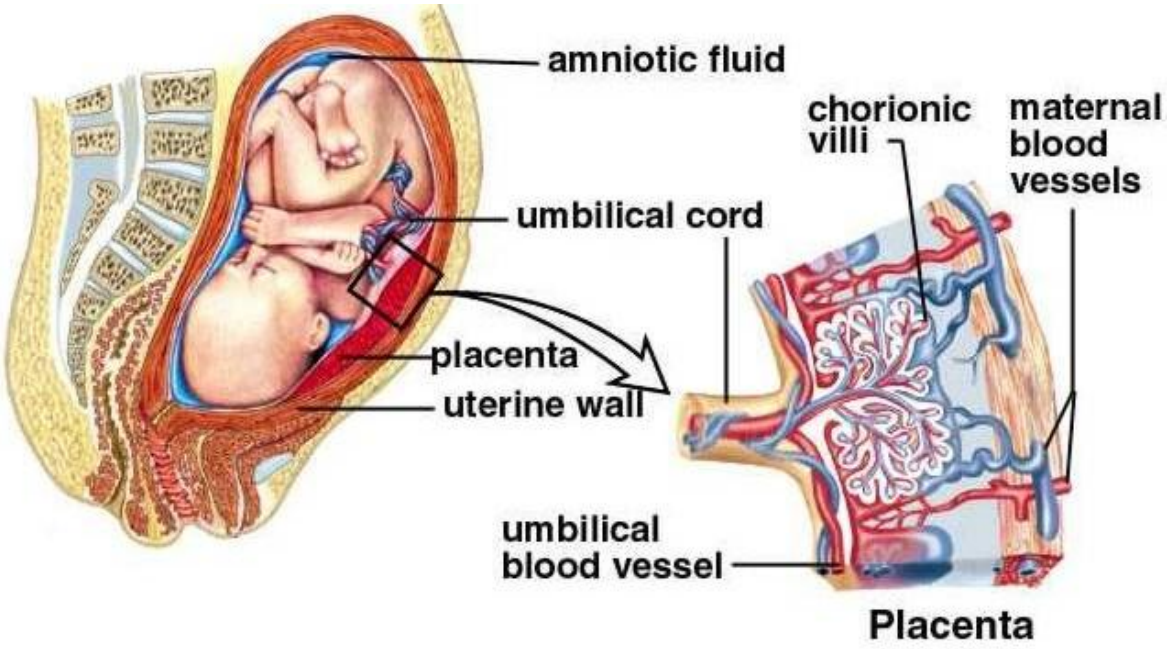

Figure: 1.1. Fetoplacental unit with uterine and intrauterine structure. Reproduced from www.tutorvista.com.

Human pregnancy usually culminates in a series of physiological events known as ‘labour’ which leads to the delivery of the fetus. Several hormones called uterotonins, such as oxytocin and some prostaglandins (PG) play an active role in labour by activating the uterus and cervix; these will be described in detail in later sections.

Clinically labour is defined as regular uterine contractions associated with effacement and dilatation of the cervix resulting in the delivery of the fetus followed by expulsion of the placenta (Calder, 1999). It usually starts at any time between 37 and 42 weeks of gestation (Steer, 1999) with 3-10% (Selina and Sabaratnam., 1999) of pregnancies delivering after 42 weeks of gestation and another 5-10% delivering preterm (delivery less than 37 weeks) (Steer, 1999)

Labour has traditionally been divided into two defined phases; latent and active phase (Garfield et al., 1998) (Figure 1.2). The quiescent phase during pregnancy is followed by the latent phase when the myometrium becomes spontaneously active and excitable. In addition, there is softening and effacement of the cervix along with changes in the fetal membranes (Garfield et al., 1998). During the active phase, the uterus contracts in response to stimulation by uterotonins; the cervix dilates and there is rupture of the fetal membranes. This phase of labour is clinically divided into three stages. The first stage starts with the onset of regular and painful uterine contractions and ends at full cervical dilatation. The second stage starts from full cervical dilatation and lasts up to delivery of the fetus and the third stage starts with the end of second stage and finishes with the delivery of the placenta. Involution of the uterus postpartum corresponds to phase 3 of parturition (Stirrat, 1997)). The changes that occur within the uterus, cervix and fetal membranes with the onset of parturition are explained in the next section.

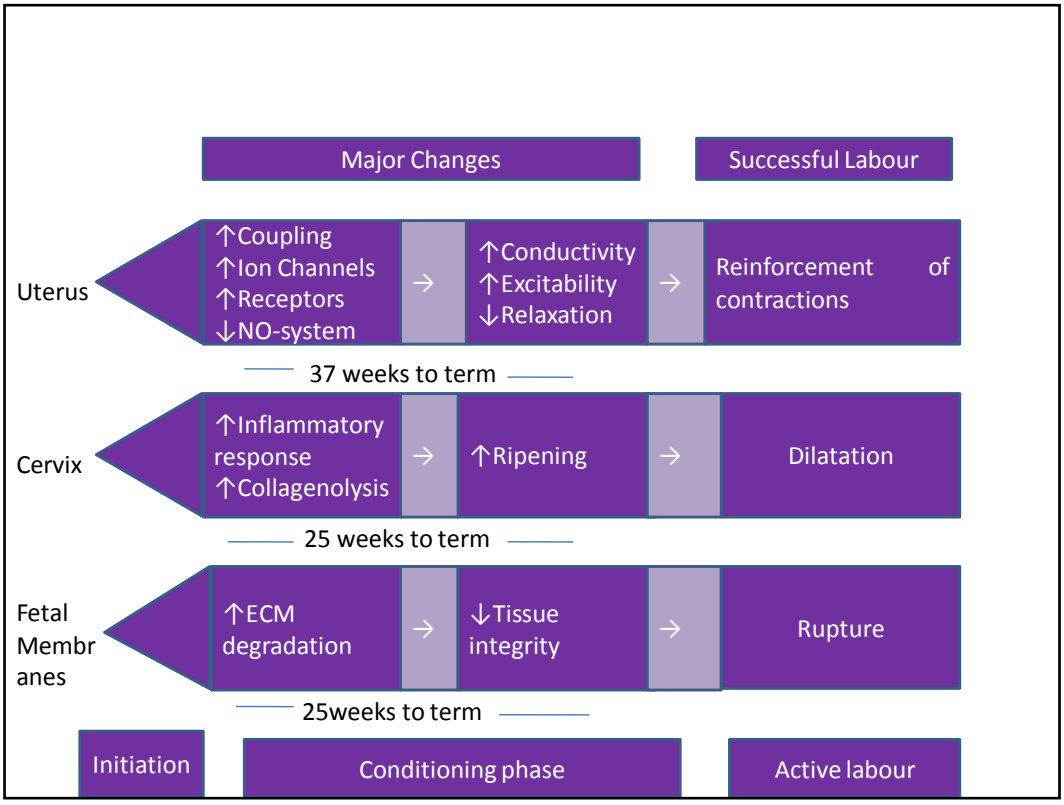

Figure: 1.2. Model of parturition. Figure reproduced from Garfield, et al., 1998.
The model shows the changes in the uterus (myometrium), cervix and extracellular fetal membranes during the 'conditioning' and 'active' phase of parturition.

1.1.1 Changes in the Uterus, Cervix and Fetal Membranes

1.1.1.1 Uterus

The uterus remains quiescent during most part of pregnancy. The contractions that do occur are of low amplitude and are not synchronized. Myometrial quiescence is maintained by the activity of various substances such as progesterone, prostacyclin (PGI_2), relaxin, parathyroid hormone related peptide and nitric oxide. Activation of the G proteins involved in the relaxation pathway leads to an increase in intracellular cyclic adenosine mono phosphate (cAMP) and activation of protein kinase A (PKA), which inactivate myosin light chain kinase (MLCK) (Figure 1.4) (Smith et al., 1998). With the onset of labour there is an increase in the rhythmic contractions of the myometrium.

An understanding of myometrial contractility requires a basic knowledge of the myometrium. The myometrium is composed of billions of myometrial cells that lie adjacent to each other in tight bundles. Muscle contraction is brought about by the sliding action of the myofilament proteins actin and myosin over each other. For this to happen, myosin needs to be activated by phosphorylation through the action of the enzyme MLCK (Sanborn, 2001). Following phosphorylation of the myosin light chain, Mg^{2+}ATPase hydrolyses adenosine triphosphate (ATP) bound to the myosin providing the energy for formation of cross-bridges between the myofilaments. This results in the sliding of the actin filament over myosin leading to contractions. Calmodulin, a cytoplasmic protein is essential for the activation of MLCK, which in turn, requires calcium for its own activation (Smith, 2007).

The uterine contractions are controlled by an electrical event called an action potential. This depolarizes the membrane potential leading to calcium entry through ion channels and contraction (Smith et al., 1998) During most part of pregnancy, myocytes maintain a relatively high interior electronegativity which prevents generation of action potential and there are very minimal gap junctions leading to poor electrical contraction coupling across cells (Figure 1.3) (Smith, 2007). With the onset of labour binding of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and oxytocin to cell surface receptors opens ligand-gated membrane calcium (Ca^{2+}) channels, whilst also releasing Ca^{2+} from the sarcoplasmic reticulum (Smith, 2007). The resulting decrease in electronegativity opens voltage-gated Ca^{2+} channels allowing rapid entry of calcium into cells and leading to membrane depolarization (Figure 1.5). Also,

binding of oxytocin to its receptor leads to activation of $G\alpha_q$ proteins linked to phospholipase C (PLC). Activated PLC, in turn, activates protein kinase C (PKC) and releases ionositol triphosphate (ITP_3). PKC activates myosin light chain kinase and ITP_3 releases calcium from intracellular stores. The stretching of myometrium resulting from fetal growth can also activate contraction through the action of mitogen activated protein kinase (MAPK) (Figure 1.6) (Smith, 1998).

With the onset of labour there is also an increase in the expression of a group of proteins termed 'contraction-associated protein' (CAP) (Smith, 2007). There are three types of CAP: (i) those that enhance the interaction between actin and myosin thereby increasing the strength of contractions, like structural proteins that form the membrane Ca^{2+} channels; (ii) those that increase the excitability of individual muscle cells such as receptors for oxytocin and PGs and (iii) those that promote the intercellular connectivity such as gap junction protein-connexin-43 (Cx-43), which promotes the spread of electrical activity and enables synchronous contractions (Smith, 2007).

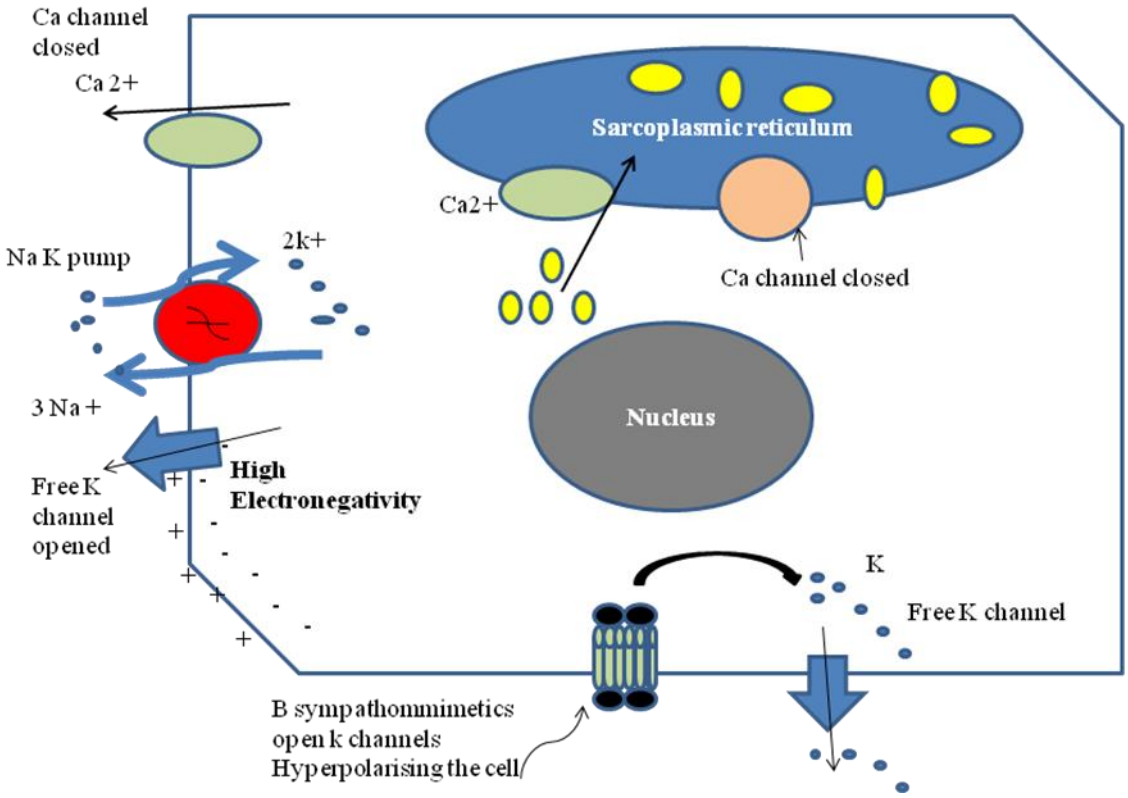

Figure: 1.3. Hyperpolarized refractory state of the myometrial cell. Figure modified from Smith, 2007.

The sodium-potassium pump maintains negative intracellular electrical potential. Free potassium (K^+) channels which are both voltage-and calcium (Ca^{2+})-regulated increases the potential difference across cell membrane preventing depolarization. As a result, Ca^{2+} influx into the cell from the extracellular region and the sarcoplasmic reticulum is prevented. β-sympathomimetics which activate the free K^+ channels, promotes myometrial relaxation.

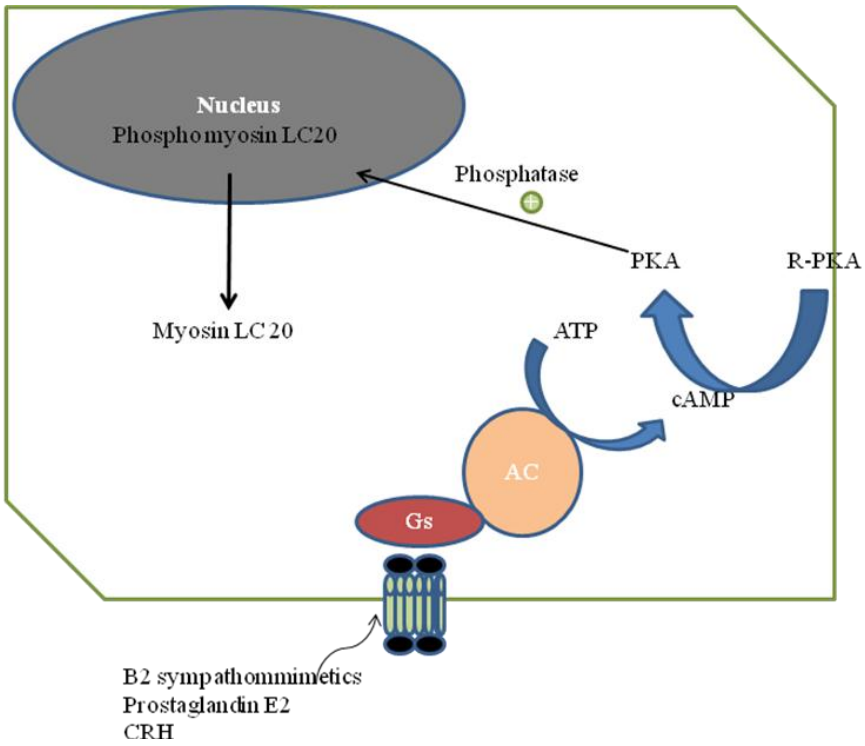

Figure: 1.4. Biomolecular changes within the myometrium in a relaxed state. Figure modified from Smith, 2007

Myometrial relaxants such as β_2-sympathomimetics act through receptors associated with $G\alpha_2$ proteins, activate adenylate cyclase (AC) located in the cell membranes, leading to the formation of cyclic adenosine mono phosphate (cAMP). cAMP activates Protein kinase A(PKA). PKA inactivates Myosin light chain kinase (MLCK).

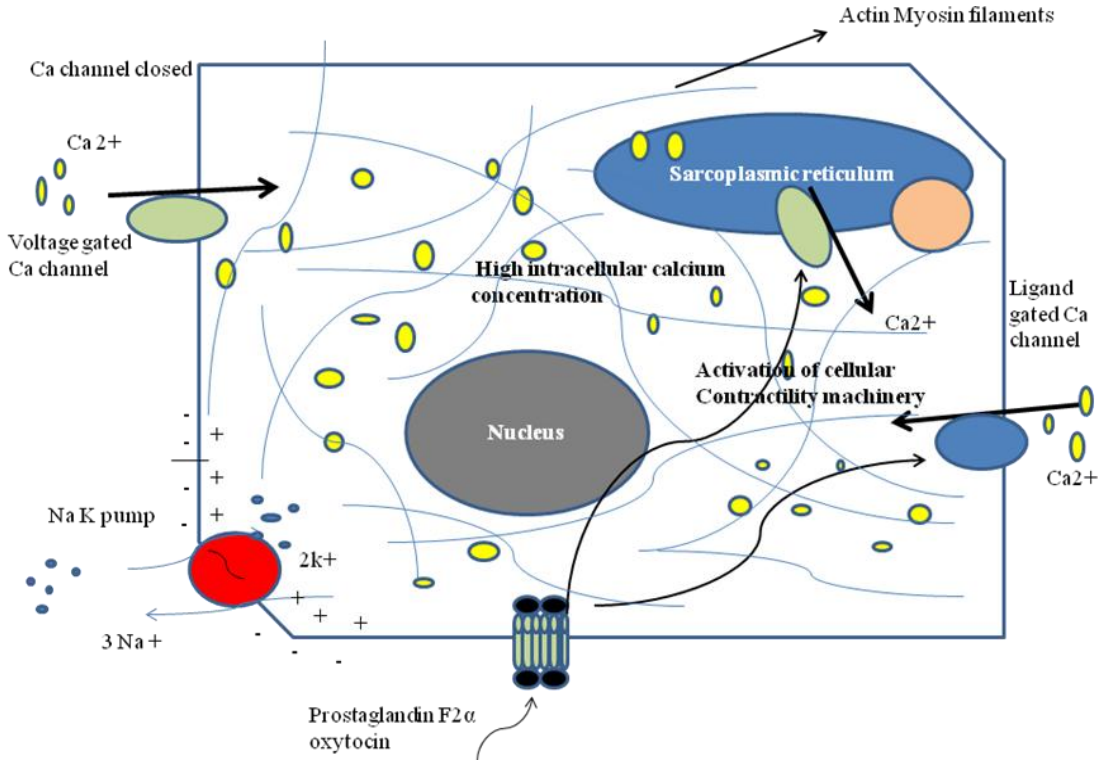

Figure: 1.5. Depolarized contracted status of the myometrial cell. Figure modified from Smith, 2007

Binding of prostaglandins and oxytocin to cell surface receptors leads to activation of ligand-gated calcium (Ca^{2+}0 channels and the release of Ca^{2+} from the sarcoplasmic reticulum causing influx of Ca^{2+} into the cells. The resultant decrease in electronegativity opens up voltage-gated Ca^{2+} channels causing a rapid influx of Ca^{2+} into the myocyte and ultimately to activation of the cellular contractile machinery.

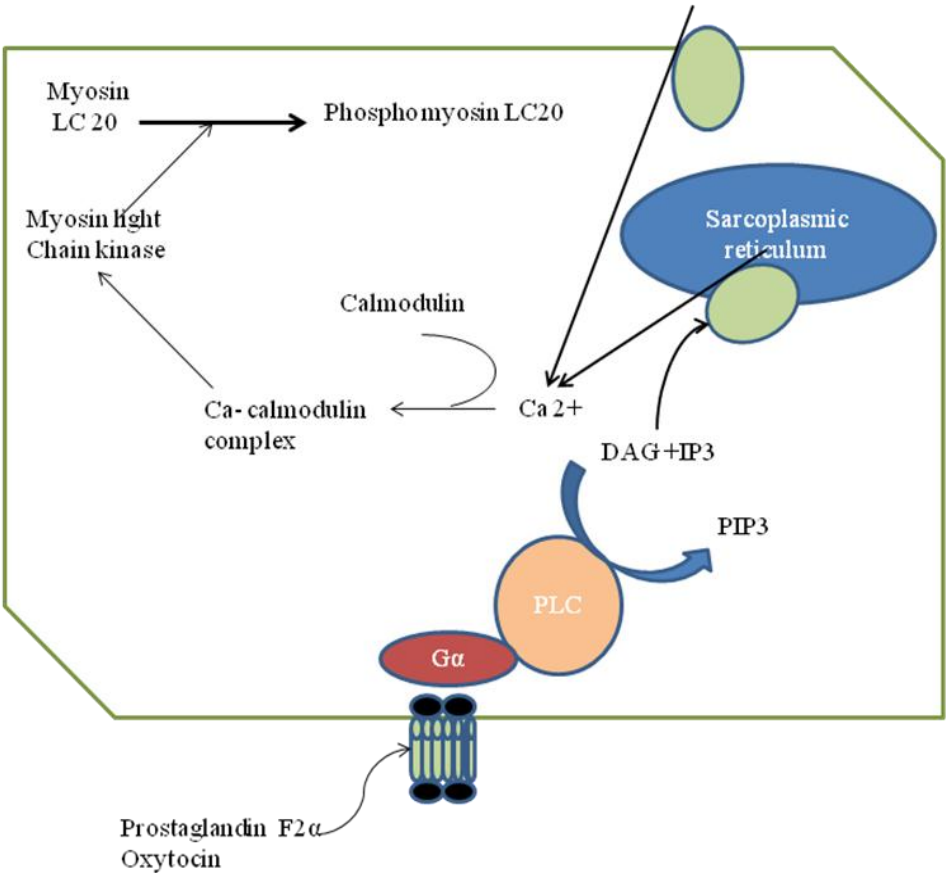

Figure: 1.6. Contraction of Uterine Myocyte. Figure modified from Smith, 2007
Activation of receptors by oxytocin and PGs leads to activation of Gα proteins, which in turn activates Phospholipase C (PLC). PLC acts on phosphoinositol-tri-phosphate (PIP3) and releases ionositol-triphosphate (IP3), which in turn, causes release of Ca^{2+} from the sarcoplasmic reticulum. Ca^{2+} and calmodulin complex together and that complex activates myosin light chain kinase (MLCK) which phosphorylates the myosin light chain (myosin LC20) ultimately leading to contraction.

1.1.1.2 Cervi x

During most of pregnancy the cervix is firm, long and closed. During the conditioning phase the cervix becomes soft and shortens, a process known as cervical ripening (Garfield et al., 1998). Cervical ripening is an active biophysical process and occurs independently of uterine contractions.

The cervix is predominantly made up of connective tissue, which is mainly collagen with small amounts of elastic tissue and very small amounts of muscle fibres. The collagen is made up of dense regular fibrils arranged in parallel bundles held together by cross-links with few interspersed mast cells and other cellular elements like fibroblasts (MacKenzie, 2006). The ground substance is composed of a proteoglycan complex. The proteoglycan complex consists of core proteins with glycosoaminoglycan (GAG) side chains that are attached to hyaluronic acid chain. The main GAGs in the cervix are dermatan sulphate and chondroitin sulphate (MacKenzie, 2006).

With advancement of pregnancy there is an increase in vascularity of the cervix, fibroblasts become secretory and there is an increase in the number of inflammatory cells, such as leucocytes and macrophages, which infiltrate into the stroma along with an increase in the water content induced by the actions of hyaluronic acid. There is a reduction in the collagen cross connections and a relative increase in glucuronic acid containing GAG, heparin sulphate, which is less binding to the cellular structures. There is a breakdown of collagen by collagenases/ matrix metalloproteinases (MMPs) produced by fibroblasts and polymorphonuclear leucocytes and there is destruction of elastin by leucocyte elastase. PGs and cytokines, such as interleukin-8 (IL-8), platelet activating factor, monocyte chemotactic factor and nitric oxide are all thought to be the main initiating factor(s) for cervical ripening (Garfield et al., 1998). During this process the junction between fetal membranes and decidua breaks leading to the release of tissue adhesive protein, fetal fibronectin (FFN), into the vagina and into its secretions (Smith, 2007). All these changes results in decreasing cervical resistance and dilatation of the cervix in response to uterine contractions.

1.1.1.3 Fetal membrane activation

In most pregnancies, labour begins at term in the presence of intact fetal membranes that usually rupture towards the end of first stage of labour. In 10% of term pregnancies and 30% of preterm deliveries, however, labour is preceded by rupture of membranes known as term spontaneous rupture of membranes (SROM) and preterm prelabour rupture of membranes (pPROM) respectively (McLaren et al., 1999). Given the implications of pPROM it is important to understand some of the physical and biological properties of fetal membranes.

Fetal membranes are extra embryonic tissues that are critical for the normal progression of pregnancy. They are composed of two main layers, an inner amnion and an outer chorion. This membrane is adherent to the underlying decidua. The normal organization and microscopic anatomy of fetal membranes is shown in the Figure 1.7. The innermost layer is amniotic epithelium (AE), which is in direct contact with amniotic fluid. This overlay the basement membrane, underneath which is the compact layer. Beneath this is the fibroblast layer which has abundant mesenchymal cells. The underlying spongy layer is rich in proteoglycan and can imbibe water. This allows it to slide over the underlying chorion. The chorion is composed of extracellular matrix (ECM) and cytotrophoblast (Bryant-Greenwood, 1998).

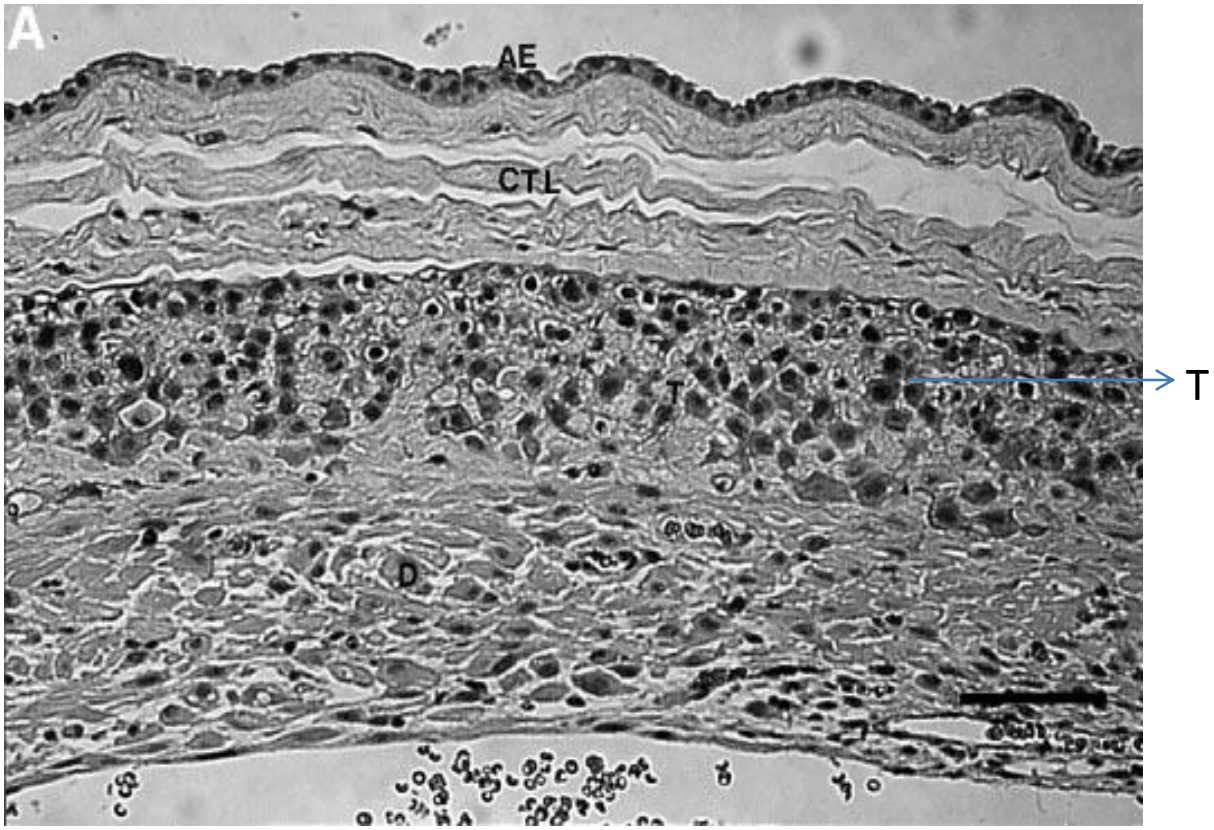

Figure: 1.7. Histologic section of the mid zone of human amnion and chorion. Reproduced from McLaren et al.(1999).
The amniotic epithelium (AE) rests on the amniotic basement membrane. The connective tissue layers (CTL) are compact, spongy, fibroblast and reticular layer, underneath this is the trophoblast (T) and the decidual (D) layer.

Fetal membranes need sufficient strength and elasticity to withstand stretching so that by term they double their size and withstand the effect of vigorous fetal movements. The tensile strength of the chorio-amnion increases up to 20 weeks of gestation and then plateaus until the 39th week after which it falls dramatically (Kendal-Wright, 2007). Thus the force of uterine contraction required to rupture the membranes decreases as gestational age advances.

The main extracellular component of the fetal membranes, collagen type I and type III with small amount of type V, VI and VII provide the tensile strength (Bryant-Greenwood.,1998), whilst elasticity is due to the presence of elastic fibres and fibrillin based microfibrils (Bryant-Greenwood., 1998). In addition fibronectins and laminin are present.

Before the onset of labour at term a 'zone of altered morphology' develops in fetal membranes. This area corresponds to the part of the fetal membrane overlying the cervix (McLaren et al., 1999). In this zone, total thickness of the fetal membrane is comparatively less when compared to other areas (McLaren et al., 1999). It exhibits an increase in the CTL together with a decrease in the thickness of the decidual and cytotrophoblast layers. This zone develops due to extracellular remodeling and apoptosis and represents an area of inherent weakness which ruptures normally during labour (Moore et al., 2006). This area is stretched maximally by the expanding uterine contents and is also exposed to the inflammatory mediators arising from the cervix and fetal membranes (McLaren et al., 1999).

These changes are thought to be brought about by a combination of physical stretch and a programmed cellular remodeling caused by changes in collagen type and content (Moore et al., 2006). These changes are followed by cellular apoptosis. The collagen changes are induced by matrix metalloproteinases (MMP). MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9 have been shown to be responsible for the cleavage of collagen in the fetal membranes (Moore et al., 2006). The inhibitors of these enzymes, tissue inhibitor of matrix metalloproteinase (TIMPs), are also produced by the same cells that produce MMP. The ratio of the MMP to TIMP determines the degree of collagen degradation (Moore et al., 2006).

Activation of these enzymes could be caused by physiological autocrine and paracrine factors like relaxin. Relaxin is a hormone produced by decidual cells (Bryant- Greenwood., 1998). It has receptors on the decidual and cytotrophoblastic cells. *In- vitro* studies have shown that relaxin can induce the production and expression of MMP-1, MMP-3 and MMP-9 when added to fetal membrane explants (Qin et al., 1997). Thus relaxin can trigger the activation of a cascade of enzymes within fetal membranes leading to rupture. Pathological triggers like infection can also lead to release of various cytokines which in turn can activate the MMP. Thus both autocrine and mechanical signals can lead to loss of "cell-matrix" contacts and induction and activation of MMP. This is illustrated in Figure 1.8.

In addition, many surfactant proteins (lipo-protein complexes produced by the developing fetal pulmonary alveolar cells), inflammatory cytokines, cortisol and corticotrophin releasing hormone (CRH) CRH levels increase in the AF with advancing gestation, leading to increased production of prostaglandins E_2 (PGE_2) in the amnion. The PGE_2 produced cannot activate the myometrium because the chorion underlying the amnion secretes prostaglandin dehydrogenase (PGDH) during most of pregnancy. PGDH catalyses the metabolism of PGs and thus prevents the PG from reaching the decidua and myometrial layer. However, as gestation advances there is a decline in the production of PGDH, leading to PG-dependent activation of the myometrium and decidua (Challis, 2000).

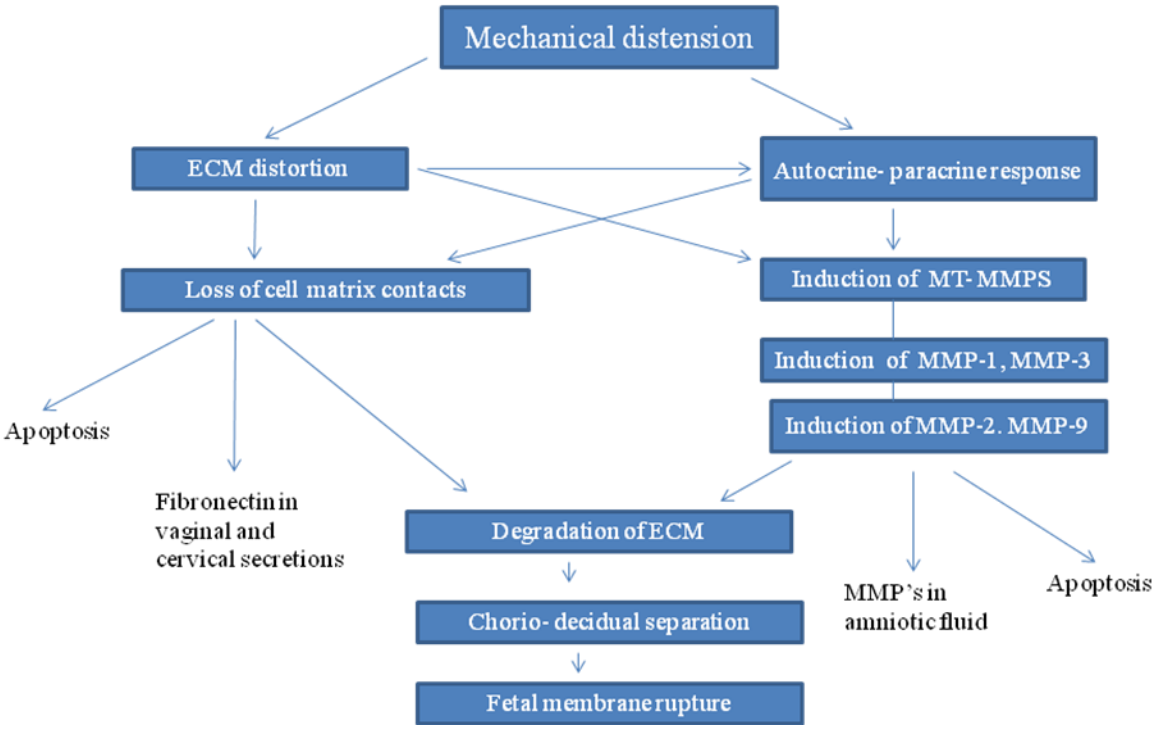

Figure: 1.8. Hypothesis of extracellular matrix and hormonal signaling in fetal membrane rupture. Reproduced with modification from Bryant-Greenwood, 1998.
Mechanical distension results in extracellular matrix (ECM) distortion in the fetal membranes and activates the autocrine and paracrine systems. This ECM distortion leads to loss of cell-matrix contact and ultimately leads to cellular apoptosis and release of FFN into vaginal secretions. Activation of autocrine and paracrine responses leads to activation of MMP leading to degradation of extracellular matrix and membrane rupture.

1.1.2 Factors involved in the initiation of parturition

1.1.2.1 Activation of fetal hypothalamic-pituitary-adrenal axis

In sheep, activation of the fetal hypothalamic-pituitary-adrenal axis and increased cortisol production by the fetal adrenal gland is the primary trigger of parturition (Smith, 2007). Cortisol stimulation of cytochrome $P450_{c17}$ enzymes in the placenta leads to increased production of androgens from progesterone and these androgens are converted to estrogens under the influence of the P450 aromatase enzyme. Estrogens are essential for the production of CAP and regulatory enzymes essential for uterine contractility. CAP has been discussed in section 1.1.1.1. Estrogens increase the concentration of receptors for oxytocin, the synthesis of connexin-43 at gap junctions and production of PGE_2 and $PGF_{2\alpha}$. Thus oestrogen promotes strong and co-ordinated uterine contraction (Weiss, 2000). Progesterone on the other hand inhibits uterine contractility and inhibits gap junction formation. Progesterone stimulates nitric oxide synthetase, down regulates PG production, development of Ca^{2+} channels and oxytocin receptors. It also increases TIMP in the cervix (Weiss, 2000). Thus, an altered estrogen/progesterone ratio tips the balance towards initiation of parturition.

In humans, the role of the fetal hypothalamic-pituitary-adrenal axis is not as well defined as it is in the sheep and may not be dependent upon the fetus at all. For example, the mean gestational age at delivery in 29 human anencephalic pregnancies without polyhydramnios was 39.6 weeks, identical to that of normal singleton pregnancy (Bernal et al., 2010). This demonstrates that parturition can occur in humans without a functional pituitary-adrenal axis. However, stress and cortisol release in the mother is associated with PTB (Hobel, 2004).

1.1.2.2 Uterine Stretch and activation of parturition

Stretch increases the level of mRNA encoding Cx-43 and oxytocin receptor (OTR) in the rat myometrium (Lye et al., 1998). The insertion of 3mm vinyl tube into one uterine horn of a non-pregnant ovariectomised rat led to an increase in Cx-43 mRNA when compared to controls. Labour did not increase the expression of CAP genes in the non-gravid horns of unilaterally pregnant rats, despite the normal hormonal changes. However, insertion of a 3mm tube resulted in the expression of CAP genes. On the other hand, stretch did not induce the expression of CAP genes on day 20 before

the onset of labour. These data suggest that endocrine signals are necessary but not sufficient to induce CAP gene expression and that myometrial stretch is a requirement. Indeed, the endocrine milieu of pregnancy prevents stretch-induced changes in the myometrium.

Lye et al., (2001) suggests a model of myometrial growth in which estrogen-induced myocyte proliferation early in pregnancy is mediated through insulin dependent growth factor 1(IGF-1) and epidermal growth factor (EGF) creating a pool of myometrial cells. As pregnancy proceeds, fetal growth induces myometrial stretch which causes myometrial hypertrophy in the presence of progesterone. This stretch induced uterine growth prevents activation of CAP genes. With the fall in the progesterone levels, or following functional progesterone withdrawal (discussed in section 1.1.2.3), myometrial hypertrophy is curtailed, even though fetal growth continues. This, in turn, increases the expression of CAP genes and brings about myometrial activation (Lye et al., 2001). The molecular mechanism by which stretch induces CAP gene expression is not fully evident. However there are preliminary studies suggesting involvement of transcription factors *c-fos, c*-Jun *N*-terminal kinases (JNK), and MAPK in the activation of Cx-43 expression (Lye et al., 2001).

Little is known about how myometrial cells sense mechanical signals. The pathways transducing stretch signals could include changes in ion channel activity, activation of cell surface receptors, signaling associated with matrix/integrin interactions, deformation of the cell cytoskeletal network and/or growth factor release (Sadoshima et al., 1993). The cellular consequences of the stretch signal include changes in gene expression especially the cellular proto-oncogenes, increased contractility and cellular growth and matrix synthesis (Lye et al., 2001).

Myometrial stretch induces activation of ion channels especially Ca^{2+}, Na^{+} and K^{+} channels causing membrane depolarization which enhances propagation of electrical and mechanical activity within uterine smooth muscles (Lye et al., 2001).

Focal adhesions (FA) are sites of contacts between cells and ECM. FA mainly consist of transmembrane proteins called integrins, the cytoplasmic region of which is linked to the cytoskeleton

while the extracellular domains are adherent to ECM molecules such as collagen, FFN and laminin. Through these interactions FA play a role in cellular changes such as repair, remodeling and formation of new cells. In addition FA has important signaling functions. The proteins at these FA sites have domains that mediate specific protein-protein interactions, sites of serine, threonine and tyrosine phosphorylation and contain tyrosine kinases such as focal adhesion kinases (FAK) and c-Src. FAKs can bind and phosphorylate paxillin which can result in vinculin binding. Thus signaling proteins are linked to structural proteins. An adaptor protein, growth factor receptor-bound protein (GRB-2) can bind to FAK and activate Ras-MAPK leading to changes in gene expression. Thus FA may be the sensors that cells use to transfer mechanical signals into biochemical signals that mediate cell growth and contractions (Lye et al., 2001).

In the myometrium, there is an increase in FAK activity towards the end of pregnancy and a decrease in activity with the onset of labour. Progesterone prevents the attenuation of FAK activity and blocks the onset of labour. FAK activity during pregnancy helps with the cellular remodeling associated with myometrial hypertrophy and decreased activity during labour, stabilizes FA and increases the strength of myocyte-ECM interactions leading to optimal shortening of myocytes with the contractions (Lye et al., 2001). Thus stretch appears to be an important factor in myometrial activation.

1.1.2.3 Steroid hormonal control of parturition: Progesterone withdrawal

Progesterone is a pro-gestational agent which promotes myometrial quiescence. In contrast oestrogen promotes uterine contractility. The contractile status of the myometrium and timing and the initiation of parturition is thus determined by the balance between the levels of these two hormones.

Labour is initiated in most species by treatment with progesterone antagonists (Mesiano and Welsh, 2007) and natural parturition is preceded by a fall in the level of circulating progesterone in many mammals. There is also an increase in the estrogenic activity at the time of parturition which is mediated by an increase in circulating oestrogen levels in most species. However, in human pregnancy the level of circulating progesterone does not fall in labour (Mesiano and Welsh, 2007).

Progesterone

Progesterone affects myometrial contractility through both genomic and non-genomic pathways. The genomic actions are mediated through nuclear progesterone receptors (PR) of which there are 3 types: PR-A, PR-B and PR-C. PR-B is the principal mediator of progesteronic action and the PR-A acts mainly through repressing the action of PR-B (Mesiano and Welsh, 2007). PR-B acts by modulating the expression of genes encoding the contraction-associated proteins (CAPs). Progesterone decreases the levels of OTR and $PGF_{2\alpha}$ receptors. It also increases the metabolism of PGs by activating PGDH. It also prevents the occurrence of co-ordinated uterine contractions by inhibiting the expression of Cx-43. Progesterone also promotes the activity of cAMP /PKA in myometrial cells. cAMP/PKA inhibits the myometrial contraction by inhibiting PLC/Ca^{2+}pathway (Mesiano and Welsh, 2007).

In humans the administration of progesterone into the AF decreased the frequency of spontaneous contractions and decreased the responsiveness of myometrium to oxytocin (Mesiano et al., 2007). These effects were rapid and sometimes lasted for days suggesting that progesterone has non-genomic actions. In women at high risk of PTL, the administration of progesterone 100mg daily by vaginal suppository or 17- hydroxyl progesterone caproate intra-muscular injections starting at 16 weeks gestation prevented PTD and improved neonatal outcome (da Fonseca et al., 2003; Meis et al., 2003). Women treated with vaginal progesterone suppositories responded to β mimetic tocolytics compared to women who received placebo (tocolytics are medications used for stopping uterine contractions). This effect was seen immediately after the administration of progesterone (da Fonseca et al., 2003). This response to β mimetic has not been universally reported and has been argued that this effect was through genomic action.

Non-genomic action is mediated through membrane progesterone receptors (mPR) which are linked to the intracellular signaling system, through activation of src/MAPK intracellular signaling pathway by ligand activated nuclear progesterone receptors (nPR) and progesterone interaction with neuro-transmitter and peptide hormone receptors–e.g. gamma amino butyric acid ($GABA_A$) and the oxytocin receptor (Mesiano and Welsh , 2007).

mPRs are structurally related to G-protein coupled receptors and are connected to inhibitory G protein (Karteris et al., 2006). Once activated they cause a decrease in intracellular cAMP levels and enhance contraction. However, it has been found that mPRs also increase the transcriptional activity of PR-B and therefore promote relaxation. Thus, throughout pregnancy progesterone acts through PR-B and mPR to promote uterine relaxation (Karteris et al., 2006). With the progesterone withdrawal non genomic action prevails through mPR, promoting contraction.

With the onset of parturition there is a fall in progesterone levels in most species. This has been shown to be due to decreased production in the placenta in sheep or the regressing corpus luteum, as shown in rats (Mesiano and Welsh, 2007). The mechanism by which functional progesterone withdrawal initiates parturition is not clear. The fact that Mifepristone (RU-486), a nuclear progesterone receptor antagonist, administration initiates parturition at any stage of human pregnancy suggests that inhibition of nPR mediated genomic action is important in the initiation of human parturition.

In humans, there is no reduction in the systemic level of progesterone prior to parturition. Human parturition involves some form of functional progesterone withdrawal. Proposed mechanism(s) include sequestration of free active progesterone by a circulating binding protein, intracrine inactivation of local progesterone bioactivity by myometrial cells, production of endogenous progesterone antagonists and decreased myometrial progesterone responsiveness mediated by change in the levels of specific nPR or nPR co-activators/ co-repressors (Mesiano and Welsh., 2007).

The PR-A/PR-B ratio determines the genomic progesterone responsiveness. The ratio at 30 weeks of pregnancy was reported as 0.5, increasing to 1 at term and increasing further to 3 at the time of active labour. Further there was an increase in another truncated progesterone receptor called PR-C at the level of fundus. This receptor also represses the transcriptional activity of PR-B (Condon et al., 2006).

The functional progesterone withdrawal could also be mediated by inhibition of the nPR interaction with target deoxyribonucleic acid (DNA). There is decrease in the co-activators like cAMP response element binding protein and steroid receptor co-activators 2 and 3. This reduction in co-activators

decreases the histone acetylation which closes the chromatin around the progesterone response element, making it inaccessible to the nPR transcriptional complex.

Oestrogen

The administration of 17-β oestradiol to term non-labouring pregnant women has been shown to result in the onset of uterine contractions and increase in responsiveness to oxytocin. Pinto et al., (1967) showed that these effects could be reversed by the administration of progesterone (Mesiano and Welsh, 2007). In rhesus monkeys administration of androstenedione led to increased placental production of oestrogen and resulted in uterine contractions and PTB. Inhibition of aromatase activity eliminated this response. However, when oestradiol alone was injected it did not result in contractions (Mesiano and Welsh, 2007). Thus it has been thought that the local production of oestrogen in the utero-placental circulation is important in the parturition process.

In most species, the onset of parturition is associated with an increase in oestrogen levels along with functional progesterone withdrawal. However, in humans the oestrogen levels start to increase from around mid-gestation and continue to rise gradually until term (Tulchinsky et al., 1972). Thus, the myometrium is unresponsive to oestrogen through most part of pregnancy but not during parturition when it is functionally responsive to oestrogen. Oestrogen responsiveness is determined by the presence of ERα and ERβ receptors and the mER. It has been shown that levels of ERα in the term non-labouring myometrium are very low and the levels increase with the onset of parturition (Mesiano and Welsh, 2007). It has also been shown that ERα levels are closely related to the levels of PR-A/B ratio. Progesterone mediates the decrease in ERα levels through PR-B (Leavitt et al., 1987). Thus functional progesterone withdrawal is associated with a rise in PR-A which leads to a rise in ERα levels, which in turn increases the myometrial responsiveness to oestrogen. Any pathological event (stretch, inflammation) that increases PR-A, will thus result in the onset of the parturition cascade.

Thus, progesterone maintains pregnancy by promoting myometrial relaxation while oestrogen stimulates uterine contraction and both together play an important role in the initiation and timing of parturition.

1.1.2.4 Corticotrophin Releasing Hormone (CRH)

The length of gestation and timing of parturition has been proposed to be determined by the presence of a "CRH placental clock" with evidence that maternal concentrations of CRH at mid-gestation were higher in women who subsequently delivered preterm and lower among women who delivered post term (McLean et al., 1995, 1999). Moreover, CRH levels were higher in women who presented with threatened PTL and subsequently delivered within 24-48 hours when compared to women who delivered at term (Warren et al., 1992). All these data suggest that CRH could be an initiating factor for human parturition. Normally, maternal plasma CRH levels increase exponentially from mid-gestation, peaking at the time of delivery (McLean and Smith, 2001). The activity of CRH during most of gestation is low due to its binding to CRH-binding protein (CRH-BP) until 36 weeks of gestation when the level of CRH-BP begins to fall. How this comes about has been the subject of intense research. For example, Challis et al *(*2000) suggested that CRH is involved in a series of positive feedback loops at multiple levels to initiate parturition. The first loop operates between the placenta and fetus where placental CRH enters the fetal circulation and stimulates dehydro-epi-androsterone sulphate (DHEA-S) production from the fetal adrenals and corticotrophin production from the fetal pituitary. However, the stimulation of the fetal pituitary is also inhibited by the maternal cortisol that also enters the fetal circulation. To overcome this, a mid-gestational increase in the activity of 11β-hydroxysteroid dehydrogenase in the placenta prevents the maternal cortisol from reaching the fetus. Simultaneously, the fetal hypothalamic-pituitary-adrenal axis is activated leading to increased adrenocorticotrophin hormone (ACTH) production and fetal cortisol, which then cause maturation of fetal organs such as the lungs and kidneys and synchronizes fetal maturation with the initiation of labour (Smith, 2007). Fetal cortisol and DHEA-S enter the placenta, where they stimulate the production of further CRH and act as a substrate for production of oestrogen, respectively.

A second positive loop exists in the amniotic compartment, where CRH stimulates PG production by the decidua, amnion and chorion (Challis et al., 2000). The PGs further stimulate the production of CRH. These feedback loops are shown in Figure 1.9.

There is also evidence that CRH, through activation of AC and increasing intracellular myometrial cAMP levels, causes myometrial relaxation (Grammatopoulos et al., 1999). In addition, there are several forms of CRH receptors in the myometrium; CRH can activate multiple classes of G proteins, giving rise to two possible outcomes where activation of Gα causes myometrial relaxation, whereas activation of Gq results in myometrial contraction (Challis et al., 2000).

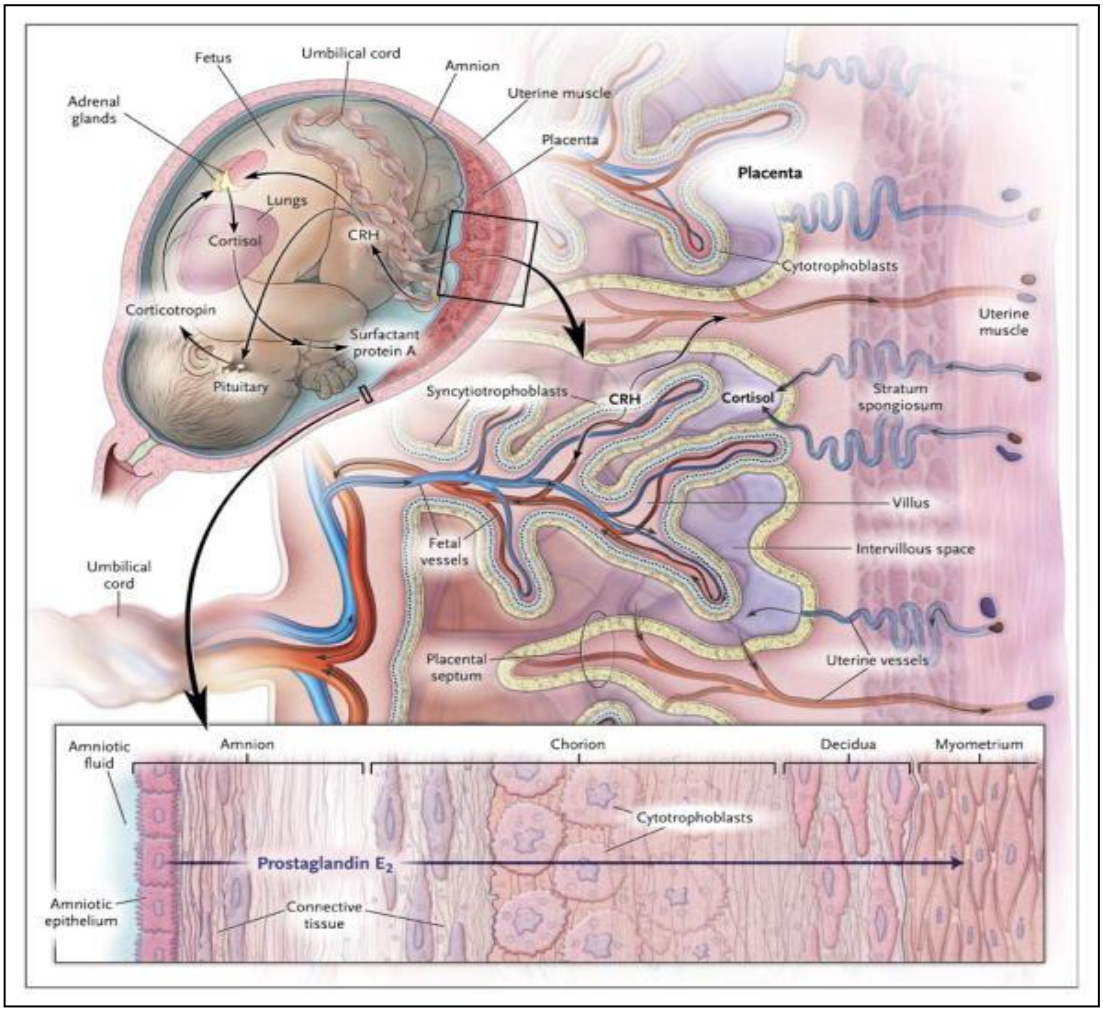

Figure: 1.9. Maternal-Fetal Interactions. Reproduced from Smith, 2007.

Feedback loops between maternal and fetal circulations exist which are important in the initiation of parturition. The placenta is the main source of Corticotrophin releasing hormone (CRH) and this is released into the intervillous space. From there CRH enters the fetal circulation through the umbilical vein. CRH in the fetus stimulates the fetal pituitary to secrete fetal Adreno corticotrophin hormone (ACTH) which in turn stimulates the fetal adrenal synthesis of fetal cortisol. Fetal cortisol stimulates fetal lung maturation and secretion of surfactant protein A. Surfactant protein A enters into the amniotic fluid and stimulates the production of cyclooxygenase 2 (COX-2) and the synthesis of PGE2 from amnion and chorion (Mendelson and Condon, 2005). The PGs at term can pass through the chorion and decidua and stimulate the underlying maternal myometrial cells to synthesize additional COX-2 and PGF2α (Lindstrom and Bennett, 2003).

1.1.2.5 Cytokines

It is increasingly recognized that inflammation has a role in parturition. Cytokines are signaling protein molecules that are secreted by a variety of cells including the immune system. Cytokines play an important role in mediating the effects of inflammation and immune-modulation.

The pro-inflammatory cytokines, such as IL-6, IL-8, interleukin-1β (IL-1β) and tumour necrosis factor alpha (TNF-α) are elevated in the AF, fetal membranes, decidua, myometrium and serum of pregnant women with signs of infection (Goldenberg et al., 2000). Labour without any associated infection is also associated with a rise in the inflammatory markers IL-8 and IL-1β in the amnion, choriodecidua and myometrium. This shows labour, whether associated with or without infection, is marked by a rise in the level of inflammatory cytokines.

The main cytokines in the uterus are IL-6, IL-1β and TNF-α. Macrophages are the main producers of these cytokines and the decidua contains the maximum amount of these cytokines. Other cells that can produce cytokines include natural killer (NK) cells, decidual cells, T lymphocytes, endothelial cells and trophoblasts. All these cytokines stimulate translocation of NF-κB into the nucleus leading to the transcription of other inflammatory genes (Lindstorm et al., 2004).

Cytokines play a role in the various steps of parturition including, cervical ripening, membrane rupture and myometrial contractility (Orsi and Tribe, 2008). During cervical ripening infiltration of the cervix by neutrophils is mediated by IL-8 (Keelan et al., 2002). Degranulation of the neutrophils leads to the release of collagenases especially matrix metallo-proteinase-8 (MMP-8) and in conjunction with local PGs acts to decrease the total concentration and type of collagen (Kelly, 2000).

Cytokines are associated with many of the key steps in the production of PGs. Both IL-1β and TNF-α regulate and stimulate the expression of prostaglandin synthase 2 (PGHS2) and PG synthesis (Christiaens et al., 2008). In mice lacking IL-1 and TNF-α receptors, introduction of *Escherichia coli* was associated with lowered prostaglandin synthase1 (PGHS1) mRNA. Both IL-1β and TNF-α are also known to increase the level of 15-hydroxyl prostaglandin dehydrogenase (PGDH), the key

enzyme involved in the degradation of PGs (Christiaens et al., 2008). IL-6 also up regulates the expression of the prostaglandin F receptor (PGFR) (Christiaens et al., 2008).

The cytokines IL-1β and TNF-α also stimulate the expression of vascular endothelial growth factor (VEGF) (Christiaens et al., 2008). VEGF is abundant in decidual cells and their levels are comparatively higher in the chorio-decidua from women in spontaneous PTL than in term labour. It plays a key role in the decidual proliferation and in the accumulation of white blood cells within the decidua and thus augments the inflammatory response during labour (Christiaens et al., 2008).

Cytokines are involved in the regulation of other CAP expression like oxytocin receptors (OTR), CX-43 and inducible nitric oxide synthases (iNOS) (Christiaens et al., 2008). Pro-inflammatory cytokines inhibit the expression of 11β-hydroxy steroid dehydrogenase (11β-HSD) in the placenta, the enzyme involved in the conversion of active cortisol into inactive cortisone (Christiaens et al., 2008). This results in an increased level of cortisol in the fetus. Placental cortisol increases placental CRH, which is associated with the onset of parturition.

Cytokines through their action on PGs increase the level of MMP-2 and MMP-9 and also decrease the levels of the TIMP-1 (Christiaens et al., 2008). MMP promotes leucocyte migration into the decidua, increasing the release of cytokines and catalyses the conversion of pro IL-1β into active IL-1β (Christiaens et al., 2008).

PR-C, an isoform of progesterone receptor is incapable of binding to the DNA and dimerizes with PR-B and thus decreases its transcription (Condon et al., 2006). IL-1β has been shown to increase the level of PR-C through its action on NFκβ and thus diminishes the effectiveness of progesterone's action (Christiaens et al., 2008).

In addition, there is recent evidence from the mouse parturition model that surfactant protein A secreted by fetus stimulates macrophages in the amniotic cavity. The macrophages translocate to the

uterine wall eliciting an inflammatory response which leads to the initiation of parturition (Mendelson et al., 2005).

In spite of all this evidence, unequivocal data substantiating the role of cytokines in the initiation of human parturition are, however, lacking, as the rise in inflammatory mediators can well be a consequence of normal labour.

1.1.2.6 Oxytocin and Oxytocin Receptor (OTR)

Oxytocin was previously assumed to be the initiating factor for parturition as administration of oxytocin induces labour which is indistinguishable from spontaneous labour (Blanks and Thornton, 2003)

Plasma levels of oxytocin were found to be elevated during labour in rabbit, sheep, cow, goat, rhesus monkey, pig and humans (Blanks and Thornton, 2003). Oxytocin secretion during labour is pulsatile and is maximal at the time of fetal expulsion. Most human studies confirm that oxytocin is increased during the expulsive phase of labour (Fuchs et al., 1991; Burd et al., 1987). In studies that contradict these results there could have been 3 factors which could have contributed to these findings; Lack of rapid sampling strategies, failure to use oxytocinase inhibitors and failure to prevent antibodies cross-reacting with oxytocin precursors giving spurious high results (Blanks and Thornton, 2003).

The afferent stimulus for this pulsatile release in oxytocin during the expulsive phase comes from distension of the cervix. The nerve impulses from the cervix stimulate the nucleus tractus solitarius in the supraoptic and paraventricular nuclei of the hypothalamus, which in turn causes the release of oxytocin from the posterior pituitary. This is called the Ferguson reflux (Blanks and Thornton, 2003). This has been well studied in a cross- circulation study in sheep. The proximal jugular vein of one sheep was connected to the distal jugular vein of the other. Fetal expulsion in the first sheep leads to contraction of mammary glands in the second sheep (Blanks and Thornton, 2003).

The evidence for the involvement of oxytocin in the initiation of labour is controversial. In sheep and rhesus monkey there is a nocturnal increase in the uterine contractions prior to the onset of labour (Blanks and Thornton, 2003). These contractions were brought about by maternal oxytocin and inhibited by oxytocin antagonist (Owiny et al., 1992). In the rhesus monkey this pattern was reproduced by infusion of androstenedione into maternal circulation. This resulted in onset of labour and preterm delivery (Mecenas et al., 1996). The androstenedione was infused to mimic the diurnal release of fetal DHEA-S, which is the source of placental production of estrogen. This created a circadian pattern of estrogen mediated oxytocin release. This DHEA-S mediated effect was prevented by aromatase inhibitors and was not mimicked by estrogen infusion (Nathanielz et al., 1998). This suggests that hypothalamic mediated release of oxytocin release was not involved in the process and local metabolism of oestrogen is involved in this process. Thus, these experiments suggest that oxytocin is integral in the pathway linking fetal maturation with myometrial activation.

Evidence for a paracrine source of oxytocin in labour is proven by the finding of oxytocin mRNA in intrauterine tissues in humans. The levels of mRNA are increased in amnion, chorion and decidua during labour (Chibbar et al., 1993). Oestrogen is likely to mediate the formation of local oxytocin as estrogen increases oxytocin synthesis in explant cultures and this effect is inhibited by tamoxifen (Chibbar et al.,1995). Chorion and decidua can metabolize the oxytocin and thus efficacy of paracrine oxytocin in stimulating the adjacent myometrium is dependent on the balance of oxytocin production and catabolism (Mitchell and Wong, 1995).

The role of oxytocin in the initiation of labour was questioned following the observation of the normal onset of labour in oxytocin deficient (-/-) mice (Nishimori et al., 1996). However, in mouse, parturition is preceded by a raise in the plasma levels of $PGF_{2\alpha}$ which induced luteolysis and progesterone withdrawal, leading to the initiation of labour. The importance of PGs in mice parturition is evidenced by absence of labour in $PGF_{2\alpha}$, cytosolic Phospholipase A_2 ($cPLA_2$) and COX-1 null mice (Blanks and Thornton, 2003). Surgical or pharmacological luteolysis in these animals restores progesterone withdrawal and the onset of labour. Thus, both oxytocin and PGs are not indispensable for labour once progesterone withdrawal is initiated.

Labour is initiated normally on the day of delivery in COX-1/ oxytocin null mouse (Gross et al., 1998), however, labour is prolonged in some mice over a number of days. Oxytocin has a luteotrophic action which is unmasked in the double knockout mice (Gross et al., 1998). Infusion of oxytocin into the oxytocin null and wild-type mice on day 15.5 elicited variable responses according to the concentration (Imamura et al., 2000). At lower concentrations, gestation was prolonged whereas higher concentration resulted in parturition. The onset of parturition was not associated with luteolysis and was mainly due to its uterotonic action. There was also a reciprocal relationship between the oxytocin receptor mRNA levels in the ovaries and uterus. On day 15, the receptor levels were the highest in the ovaries and declined to a low level on day 19 when uterine levels of receptors increased. Thus, oxytocin seems to be important in the timing of the onset of parturition.

Uterine oxytocin receptors have been found to increase in number towards the end of pregnancy in a number of species including humans (Blanks and Thornton, 2003). The receptors are found in both myometrium and uterine epithelium and decidua. In the myometrium, oxytocin induces uterine contractions. In the decidua, there is evidence that oxytocin increases PG secretion. In explant cultures, oxytocin stimulates the secretion of PGE_2, $PGF_{2\alpha}$ and leukotrienes in contrast to the amnion where PGE_2 is the main product. This effect occurs through an increase in PLA_2, COX-1 and COX-2. The importance of oxytocin receptors is further proven by the effectiveness of the oxytocin antagonist, atosiban, in preventing PTL (Goodwin et al., 1994).

1.1.2.7 Prostaglandin (PG)

It is thought that PGs play a central role in human labour, because they appear to stimulate myometrial contractions and promote cervical ripening (Lindstrom and Bennett, 2003). PG biosynthesis involves three main steps as shown in Figure 1.10.

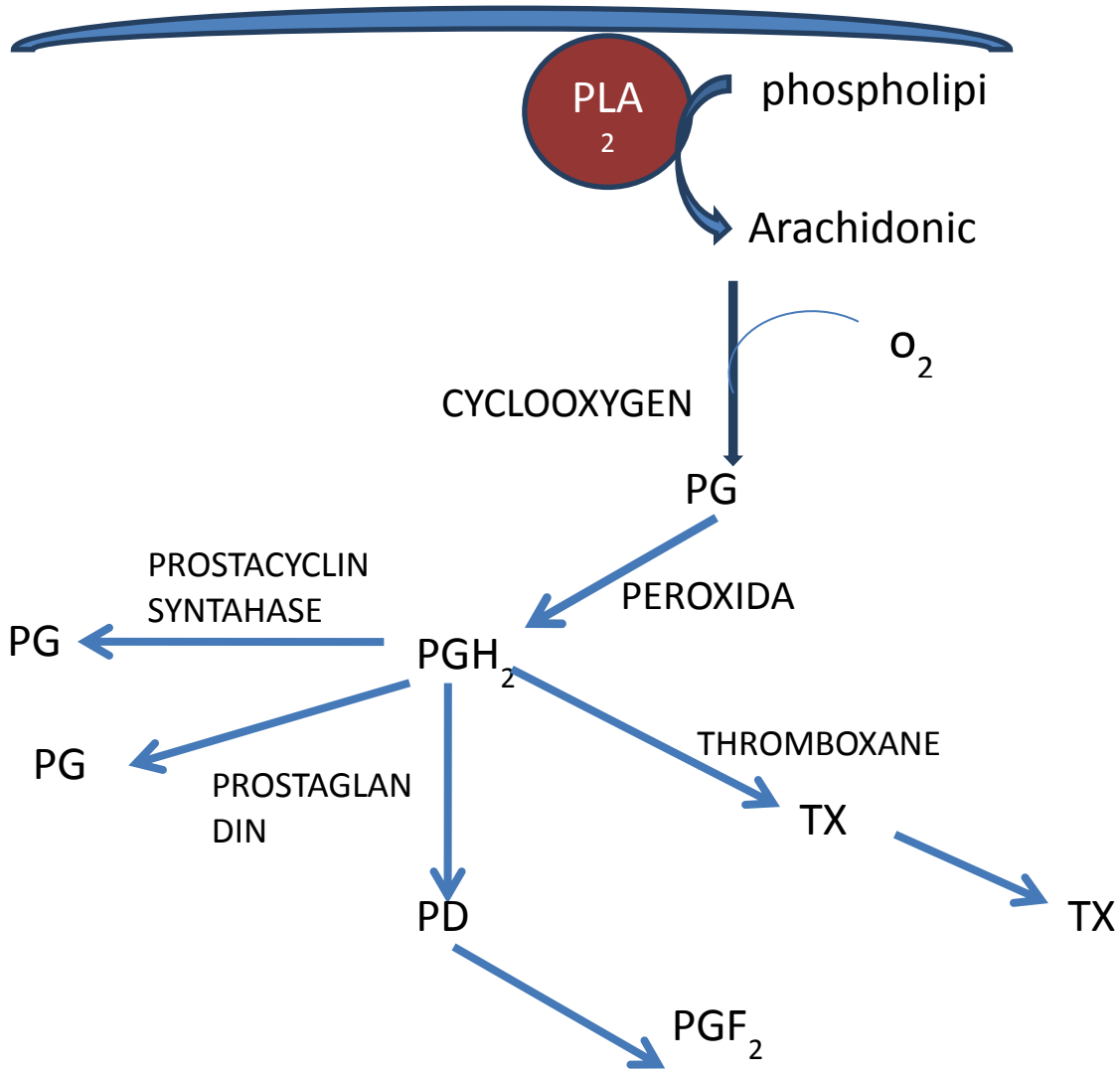

Figure 1.10. Pathway of synthesis of Prostaglandins

(1) First arachidonic acid (AA) is released from membrane phospholipids through the action of phospholipase enzyme PLA_2.
(2) AA is then converted in a rate limiting step into PG intermediate prostaglandin H_2 ($PGH_{2,}$) catalyzed by the enzyme COX. There are 3 isoforms of COX: COX-1, COX-2 and COX-3 of which COX-2 is the inducible form that is primarily involved in parturition.
(3) PGH_2 is finally converted to $PGE_{2,}$ $PGF_{2\alpha,}$ Prostacyclin (PGI_2), PGD_2 and thromboxane by specific prostaglandin synthetase enzymes. PGE_2 and $PGF_{2\alpha}$ are inactivated by enzyme prostaglandin dehydrogenase (PGDH) (Lindstrom and Bennett, 2003).

Phospholipase A_2 (PLA_2)

There are two main types of PLA_2 enzymes: the secretory ($sPLA_2$) and cytosolic forms ($cPLA_2$). The PLA_2 is widely distributed within the intrauterine tissues. Both types may be involved in phospholipid metabolism within the uterus but the cytosolic form seems to have a non-redundant role as $cpla_2^{-/-}$ knock-out mice fail to undergo labour at term and eventually deliver small, non-viable litters (Uozumi et al., 1997). $cPLA_2$ is principally seen within the amnion and the $sPLA_2$ is seen within the term placenta. Studies have shown variable results regarding the levels of PLA_2 in tissues with the onset of labour. Studies report contradictory results regarding changes to the level of both cytosolic and secretory levels in the chorio-amnion with the onset of labour (Skannal et al 1997; Munns et al., 1999; Bennett et al., 1994). Similarly, in the myometrium Skannal et al., (1997) found no difference in the levels of both $cPLA_2$ and $sPLA_2$ in the lower uterine segment myometrium between preterm and term states or with the onset of parturition. However, Slater et al., (2000) found an increase in $sPLA_2$ in the upper and lower uterine segments with gestational age and with the onset of labour.

PLA_2 are activated in response to numerous stimuli including cytokines, neurotransmitters, endotoxins and hormones (Lindstrom and Bennett, 2003). $cPLA_2$ is activated by phosphorylation by various protein kinases including tyrosine kinases and MAPK. Once activated $cPLA_2$ is transferred to the nuclear membrane in the presence of Ca^{2+} where it exerts its action. Increased transcription of PLA_2 gene can be brought about by TNFα, IL-1β and interferon γ activity. The rat $cPLA_2$ gene contains a NFκβ and activator protein-1 (AP-1) binding sites within the promoter region.

The $sPLA_2$ synthesis and secretion is induced by the inflammatory cytokines IL-1β, IL-6, TNF-α and cAMP elevating agents. The promoter region has a binding site for NFκβ and seems to be involved in the transcription initiation by IL-1β and TNF-α.

Cyclo-oxygenase (COX)

There are 3 isoforms COX-1, COX-2 and COX-3. They both catalyse the conversion of AA into intermediate metabolite PGH_2. COX-1 is constitutively present in numerous tissues. Whereas COX-2 is not normally present and is induced by cytokines and growth factors (Lindstrom and Bennett, 2003).

Studies in COX-1 knockout mice show that COX-1 is involved in luteolysis and initiation of labour. There is no relevance of this in human parturition due to the fundamental difference in the parturition process. COX-2 knockout mice do not ovulate and have impaired blastocyst implantation and therefore studies on labour were not possible. In humans there is an increase in the expression of COX-2 gene, COX-2 mRNA levels and enzyme activity in the amnion tissues at term and with the onset of labour. There is no change in COX-1 levels. Similar changes occur in chorio-decidua. There are no changes in COX activity within the placenta. There has been discrepancy in the outcome of the studies relating to COX-2 levels within the myometrium depending on the site of myometrium sampled and time duration between sampling and onset of labour.

COX-2 gene expression is induced by inflammatory cytokines IL-1β and TNF-α, oxytocin, CRH and platelet activating factor. cAMP levels has been shown to both up-regulate and down- regulate the gene. The anti-inflammatory cytokine IL-10 down-regulates COX-2 transcription. The promoter region of human COX-2 gene has NFκβ, cAMP and c/EBP response elements.

Prostaglandin Dehydrogenases (PGDH)

PGDH is mainly located in the chorion, with low levels present in the decidua and amnion (Cheung et al., 1992). Thus chorion acts as a functional barrier preventing transport of PGs synthesized in the fetal membranes to gain access to myometrium and decidua during most of gestation (Nakla et al.,1986). The levels of PGDH in the chorion have been found to be reduced in a few women with PTL (Van Meir et al.,1997).

PGDH levels have been found to be regulated by cytokines. IL-1β and TNF-α have been found to reduce the PGDH activity and this is opposed by anti- inflammatory cytokine IL-10. This phenomenon

could be associated with term labour. The PGDH promoter region contains CRE/AP-1/Ets response elements and its expression is regulated by hormones; progesterone maintains and cortisol decreases the level of PGDH. This action of progesterone is through changes in transcription involving cAMP levels.

Thus PGs seem to play a key role in parturition. In spite of this, the study of human parturition is still replete with a number of uncertainties and further research into other molecules that might play a role in the biomolecular control of parturition is important. Such knowledge will probably increase our understanding of the regulatory mechanisms involved in parturition.

Disruption of the timing of parturition due to various reasons will end in preterm delivery, which is associated with complications to the neonate. The next few sections will deal with the aetiology, pathophysiology and complications of PTB.

1.2 Preterm Birth

PTB is defined as birth before 37 completed weeks of gestational age. This is the official definition endorsed by the WHO and International Federation of Gynaecology and Obstetrics (FIGO).

1.2.1 Epidemiology of PTB

As stated in section 1, PTB is one of main clinical/obstetrical problems facing the modern clinician. PTB occurs for the following three main reasons,

1.iatrogenic/induced (Usually for maternal or fetal indication)

2.spontaneous labour with intact membranes and

3.pPROM.

About 30-35% of preterm births are iatrogenic/induced, 40-45% follows spontaneous PTL and 25-30% arises after pPROM, defined as spontaneous rupture of the membranes at least 1 hour before the onset of contractions before 37 weeks gestation. PTL is defined as the presence

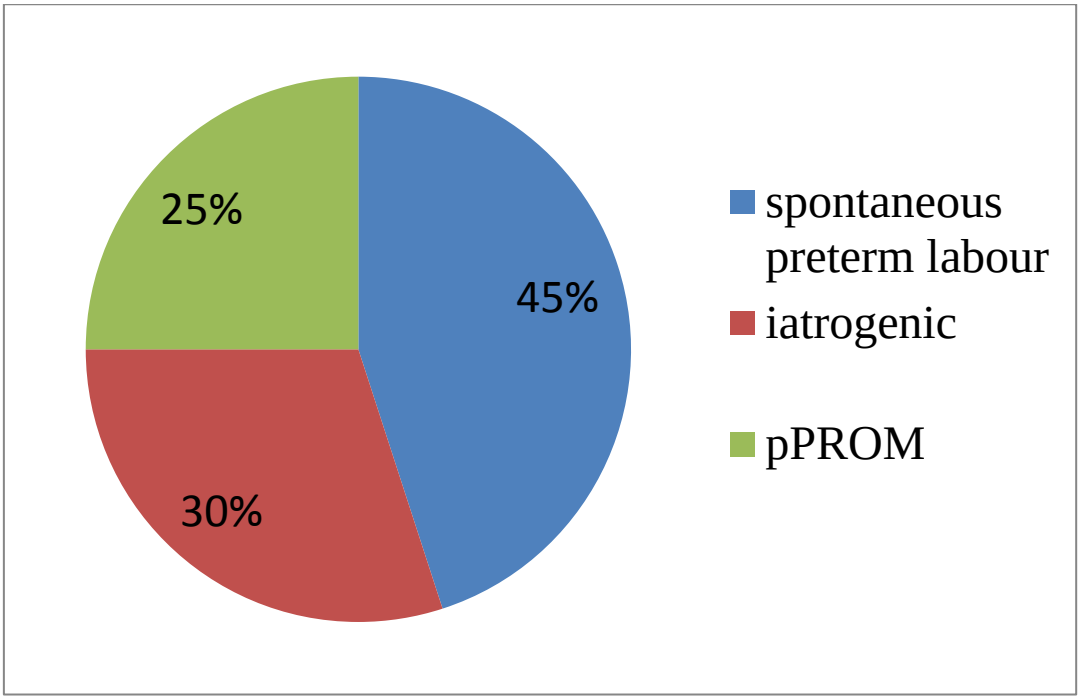

Figure 1.11. The proportion of the main contributors to preterm birth.

of 3-4 regular uterine contractions in 10 minutes accompanied by cervical changes in the absence of ruptured membranes before 37 completed weeks of gestation. (Figure 1.11)

PTB can also be classified according to the gestational age at which the delivery occurs. About 5% of PTBs occur at less than 28 weeks (extreme prematurity), about 15% occur between 28-31 weeks gestation (severe prematurity), about 20% between 32-33 weeks (moderate prematurity) and most (60-70%) between 34-36 weeks of gestation (near term).

1.2.2 Prevalence and trends in the incidence of preterm delivery

In Europe, the PTB rate is 5.5-11% in 2004 (http://www.europeristat.com) whereas in USA the rate is 11.99% in 2010 (http://www.cdc.gov/nchs/data). With the exception of France the incidence of PTD has increased in developed countries (Cano et al., 2001). In the USA, the rate increased from 9.5% in 1981 to 12.7% in 2005. In Denmark, a population based study showed that the PTB rate increased by 22% between 1995 and 2004. The main increase in Denmark was seen in low risk primiparous women; the PTB rate increased from 3.8% to 5.7%. The increases in iatrogenic births for maternal and fetal indications were the major contributing factor towards this rise. An increase in multiple gestations arising from an increase in the number of fertility treatments was the other reason for the increasing trend in PTB rates.

Similar trends have been reported for a Canadian University Hospital from 1978 to 1996 (Kramer et al., 1998). PTB rates increased from 6.6% to 9.8% for birth at 34-37 weeks gestation, from 1.7 to 2.3 % at 33-34 weeks gestation, and from 1.0 to 1.2 % at < 32 weeks. Obstetric interventions, rising maternal age and low educational status of the mother were the main relevant risk factors.

Assisted conception is also a major contributor to increased PTB rates. In a Swedish study, 30% of the 5856 successful IVF pregnancies ended in PTB (Bergh et al., 1999). The singleton IVF pregnancies themselves are inherently at increased risk of PTD although the reason behind that increase is unclear. The underlying infertility problem, the higher serum relaxin levels associated with the use of gonadotrophins, trauma, bleeding and infection associated with the use of catheters for embryo

transfers and increasing medical intervention are some of the factors that have been associated with the increased risk (Berkowitz et al., 1994). The multiple pregnancies that arise from assisted conception further increase the risk of PTD (discussed in p44).

Many studies and review articles have justified why PTB is regarded as a major problem in perinatal medicine. This is because it is associated with a number of medical, social and economic consequences which cause a huge burden on society with lifelong financial implications for medical services and the families of those affected. In the next section these consequences are discussed.

1.2.3 Neonatal complications

Incomplete adaptation of the fetus to the extra-uterine environment is the main reason for the increased morbidity and mortality associated with PTB (Cano et al., 2001). The most important factor that determines the outcome of the infant delivered preterm is the gestational age at the time of birth. Other factors influencing the outcome include antenatal administration of steroids, initiation of assisted ventilation at resuscitation, surfactant replacement therapy and management in a tertiary neonatal centre with modern ventilators.

1.2.3.1 Perinatal Mortality

Mortality varies with the gestational age at delivery. PTBs can be considered as either late, (>32 weeks of gestation) or early (<32 weeks gestation); late PTBs being five times more common than early PTBs (Saigal and Doyle, 2008). The neonatal mortality for late PTBs is about 4 times higher than for term births (4.1 vs 0.9 per 1000 live births) (Engle et al., 2008). Few babies delivered before 28 weeks gestation survived before the advent of assisted ventilation in 1970s (Saigal and Doyle, 2008). With increasing usage of antenatal corticosteroids, assisted ventilation and usage of surfactant in the neonatal intensive care unit the survival rate of preterm babies born before 28 weeks of gestation has improved since 1990. For example, the survival rate of extreme low birth weight infants (ELBW) (<1000gm) has increased three fold from 25% in 1979-1980 to 73% in 1997 (Saigal and Doyle, 2008).

The results of the EPICURE 2 study showed that the survival rate was 51%, 47% and 67%, respectively for infants born at 22-23, 24, and 25 weeks of gestation (Lacovidou et al., 2010).

The outcome for preterm babies also varies according to the level of neonatal care provided. Regionalization of care, which encompasses transfer of sick infants from district general hospitals to tertiary centres and transfer of high risk mothers to a perinatal centre before delivery, has improved outcome. Nevertheless, there are both short- and long-term complications associated with PTB.

1.2.3.2 Short term complications

Intrapartum hypoxia and birth trauma associated with the delivery of very low birth weight babies are important contributing factors to perinatal morbidity and mortality.

In the immediate neonatal period, the risks include respiratory distress syndrome (RDS), apnoea, necrotizing enterocolitis, intracranial haemorrhage, convulsions, periventricular leucomalacia, septicaemia, hypoglycaemia, hypothermia, jaundice, kernicterus, and feeding difficulties (Engle et al., 2008).

1.2.3.3 Long term Complications

The brain, lungs and eyes are more susceptible to the injuries arising out of PTB leading to high rates of long-term neurological, respiratory and ophthalmic complications.

1.2.3.3.1 Neurodevelopmental sequelae

Cerebral palsy, mental retardation and sensory impairments are some of the neuro-developmental disorders experienced by preterm infants (Saigal and Doyle, 2008). The estimated degree of problem varies with different studies, but roughly 25% of the survivors of very early PTBs experience substantial neurological morbidity. However, because there has been a substantial improvement in neonatal survival rates, the absolute number of cases of cerebral palsy has increased. Preterm infants also experience minor neuromotor dysfunction and poor coordination.

Several studies have shown that during infant development there are problems in other areas such as cognitive deficits, underachievement and grade failures at school. These deficits were apparent in children without neurosensory impairments and normal intelligence quotient and there was an increased risk of behavioral problems such as attention deficit hyperactivity disorder, which was increased by 2.6 to 4 times in very early preterm infants. Additionally, preterm infants have emotional problems and have traits such as shyness, unassertiveness and social maladaptation, and are often anxious and withdrawn. In most studies, very low birth weight infants(VLBW) had lower rate of educational achievements, employment, and independent living than normal birth weight controls when they became young adults (Saigal and Doyle, 2008).

1.2.3.3.2 Other sequelae

Up to 40% of VLBW survivors have bronchopulmonary dysplasia, which leads to recurrent hospital admissions in early childhood (Saigal and Doyle, 2008). Retinopathy of prematurity continues to be one of the morbidities experienced by infants born less than 26 weeks gestation and hearing impairments occur in 3-5% of VLBW infants. Hearing impairment has a detrimental effect on acquisition of language skills and learning at school (Saigal and Doyle, 2008).

1.2.3.4 Socioeconomic Consequences

Prematurity generates a huge social impact on the individual, parents, siblings and society in general. Parents of preterm infants feel a deep sense of loss, grieving for the perfect baby they had wished for, and this is often manifested as anger or guilt. They also undergo the emotional trauma of being in constant fear that their baby is going to die or develop signs suggestive of poor neuro-developmental outcome. This psychological distress is greatest in the first month of the infant's life and persists during the first two years of life. There is also family disruption caused by the mother having to care of the neonate for long periods of time often separated from the family and the father having to undertake work adjustments to take care for existing siblings. For a family with a surviving premature infant with physical or developmental disabilities the psycho-social burden is particularly great. Such

children demand increasing attention in terms of time and effort, to such an extent that the care of other siblings is sometimes affected and partners feel ignored.

Society contributes to the costs of the resources that are required to provide medical care and welfare resources to families with preterm infants. Neonatal intensive care contributes to the direct cost of prematurity with an association between the scale of costs and the degree of prematurity. Neonatal care, however, seems to be cost effective as those neonates who die tend to do so in the first few days and contribute very little to the overall expenditure (Gill, 2001). In 1998, 40% of the total Australian budget of 145 billion dollars was used for the care of babies who weighed<2500g at birth and 10% on the 0.4% of babies who weighed <1000g at birth. Though neonatal intensive care is expensive, the quality adjusted life year (QALY) outcome is encouraging in that there is an inherent value for money in terms of quality of life year generated (Gill, 2001).

The indirect costs that arise from the long term health care and the educational needs of surviving preterm infants persist through childhood and adult life. Lewit et al. undertook a comprehensive assessment of post-neonatal expenditure in the USA and found that in the first year, the care of preterm infants led to an incremental cost of $15,000 per infant above the cost for the care of a normal surviving infant. Later costs for healthcare and education require an additional $10,000 per infant.

In view of the health and socioeconomic consequences of prematurity, it is extremely important to prevent preterm birth and take steps to improve neonatal outcome. In order to achieve this it is important to know the risk factors for preterm birth and understand the pathophysiology of preterm parturition.

1.2.4 Risk factors

Identification of risk factors is helpful in identifying the women are at significantly higher risk of delivering preterm and advocating preventive measures.

1.2.4.1 Demographics

PTB rates among black African-American and Afro-Caribbean populations have been consistently high at 16-18% compared to 5-9% in white women. Black women are also 3-4 times more likely to have very early preterm birth even after controlling for other confounders. The exact reasons behind this increased risk are unknown but an obvious one that has been suggested is genetics, especially as prematurity is known to 'run in families' (Berkowitz et al., 1994; Goldenberg et al., 2008)

Other maternal demographic characteristics of significance include low socioeconomic and educational status, extremes of age and single marital status (Slattery et al., 2002). Teenage girls between the ages of 13 and 17 years are at higher risk of delivering preterm when compared to women delivering from the 20-24 year age group, after controlling all confounding factors (RR 1.9) (Fraser et al., 1995). Older teenage mothers between 18 and 19 years were also at increased risk (RR 1.5) (Fraser et al., 1995).

Working long hours and undertaking hard physical labour under stressful conditions are also associated with an increased risk, although the level of physical activity does not consistently correlate with rate of preterm birth (Goffinet, 2005). In the EUROPOP study, the odds of delivering preterm among women who worked more than 42 hours per week was 1.33; women who stood for more than 6 hours was 1.26 (Saurel-Cubizollles et al., 2003).

Low BMI is also associated with a high risk of PTD. The rate of PTD among women with a BMI < 19 was 16.6% when compared to a rate of 8.1% among women with a BMI between 25 and 29.9 (Hendler et al., 2005). Obesity increases the risk of congenital abnormalities like neural tube defects which are associated with preterm delivery. Obesity also predisposes to the development of diabetes mellitus and hypertension both of which increase the risk of iatrogenic PTB (Saigal and Doyle., 2008).

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1.2.4.2 Past obstetric history

A previous PTB increases the risk of PTB by 15-50% depending on the gestational age and the number of the previous PTDs. The rate of PTB in women with 1 prior PTB is 14.3% and in women with 2 previous PTBs it is 28.1% (Slattery et al., 2002). The risk is inversely proportional to the gestational age of the previous birth.

An inter-pregnancy interval of less than 6 months is also associated with a 2-fold increased risk of PTB. Maternal depletion of important nutrients and failure of the uterus to return to its original state before another pregnancy are some of the possible explanations put forward for the high risk associated with short inter-pregnancy interval (Goldenberg et al., 2008), although these remain unproven.

Women with one or two prior spontaneous miscarriages have not been shown to be at higher risk when compared to those who have not had prior miscarriages. However, the risk following 3 or more miscarriages is variable ranging from 1.5 to 3.3 (Berkowitz et al., 1994). Cervical weakness arising from the trauma to cervix at the time of dilatation and curettage could be one of the possible explanations behind this increased risk. There is no evidence for a definite association between prior induced abortion and PTB, however, the procedure which requires dilatation of the cervix and curettage might be associated with an increased risk (Berkowitz et al., 1994).

1.2.4.3 Pregnancy characteristics

Multiple pregnancies account for only 2-3% of all pregnancies but may be responsible for up to 15-20% of all PTBs (Goldenberg et al.., 2008). Because nearly 60% of twins are born preterm and nearly all of the higher order multiple gestations end in preterm delivery, it is thought that uterine over distension and subsequent myometrial stimulation activates the final terminal pathway of labour prematurely ending in PTD (Romero et al., 2006).

Singleton pregnancies conceived after *in-vitro* fertilization (IVF) and Gamete Intra Fallopian Transfer (GIFT) are at a 20% higher risk of PTD than normal pregnancy. This risk is attributed to the underlying infertility factor, increased medical intervention, uterine abnormality, cervical trauma and disturbed implantation (Slattery et al., 2005). The risk of PTD for twin pregnancies conceived after IVF is similar to that of other twin pregnancies due to the lower incidence of monozygotic pregnancies (Slattery et al., 2005).

Vaginal bleeding, oligohydramnios or polyhydramnios, previous cervical surgery, uterine abnormalities, and maternal medical disorders such as diabetes, hypertension and thyroid diseases are all associated with an increased risk of PTB (Goldenberg et al., 2008). In a systematic review and a meta-analysis performed on obstetric outcomes following conservative treatment of cervical intraepithelial neoplasia, cervical conization was significantly associated with a RR of PTD at <28, 32 and 37 weeks of gestation 2.59, 2.78 and 5.33, respectively. The risk associated with large loop excision of transformation zone (LLETZ) was 1.2 and 1.70 for PTD <32 and <37 weeks of gestation respectively (Arbyn et al., 2008; Kyrgiou et al., 2006).

Psychological and social stress is associated with a 2-fold increased risk of PTB. The association between maternal depression and PTB persists after controlling for confounding factors such as smoking or drug or alcohol abuse (Goldenberg et al., 2008). The RR of PTB among women with pregnancy related anxiety was 2.1; women with negative life events was 1.8 and women with perception of racial discrimination was 1.4 (Goffinet, 2005). There is some evidence to show that stress through its interaction with CRH could lead to increased PTB. C-reactive protein (CRP) is also elevated in women with stress indicating a possibility of involvement of inflammatory pathways in the mechanism of causation of PTB (Goldenberg et al., 2008).

Smoking increases the risk of PTB by 2-fold. Nicotine and carbon monoxide in the cigarette smoke are potent vasoconstrictors that can cause uteroplacental insufficiency, increasing the risk of iatrogenic PTB. Smoking also increases the systemic inflammatory response and through this pathway can increase spontaneous PTBs (Goldenberg et al., 2008). Substance abuse has been associated with

increased risk of PTD but the actual contribution of risk from substance abuse is confounded by other risk factors such as socio-economic status. Most studies show an increased risk with cocaine and heroin abuse (Slattery et al., 2005).

Intrauterine infections account for 25-40% of PTBs (Goldenberg et al., 2008). Intrauterine infection is more commonly associated with PTL at an early gestational age. At 21-24 weeks, 66.7% of the spontaneous PTBs are associated with histological chorioamnionitis compared to 10% at 34-36 weeks of gestation. Usually low virulent microorganisms such as mycoplasma species are commonly reported in the amniotic cavity. This explains the reason behind the chronic nature of intrauterine infections and frequent absence of overt clinical signs of infection. The other organisms that are found include *Streptococci agalactiae, Escherichia coli, Fusobacterium, Gardnerella vaginalis and Bacteroides* (Romero et al., 2006).

Bacterial vaginosis (BV), a disorder caused by a change in the microbial ecosystem of the vagina, where there is an increase in the number of anaerobic microorganisms when compared to aerobic organisms, is clinically diagnosed by the presence of a profuse vaginal discharge, fishy odour, pH greater than 4.5 and presence of clue cells in the discharge. Clue cells are vaginal epithelial cells that have a distinctive stippled appearance due to being covered with bacteria. Nugent's criteria uses gram stained smears to measure the number of lactobacillus and other anaerobic organisms like *Mobiluncus* and *Bacteroides* (Denney et al., 2009).

Alteration in the vaginal eco-system causes irritation of vagina and stimulates the mucosal immune system, which causes the release of inflammatory cytokines and PGs leading to PTB. BV is associated with a 1.5 to 3-fold increased risk of PTB (Honest et al., 2004). BV infection present before 16 weeks poses the greatest risk for PTB as the odds ratio (OR) increases from 1 to 7.55 (Guaschino et al., 2006).

The association between other genital infections and PTB is not clear. There is a probable association between *Chlamydia trachomatis* (Andrews et al., 2006), *Treponema pallidum, Neisseria Gonorrhea*

and *Trichomoniasis* infections and PTB (Moodley et al., 2000). Other systemic maternal infections such as pyelonephritis, asymptomatic bacteriuria, pneumonia, appendicitis also predispose to PTB (Goldenberg et al., 2008) and specific infections such as periodontal disease are associated with PTB (Xiong et al., 2005).

Periodontal disease, a chronic gram-negative anaerobic infection of the gums and the tooth support structures) is associated with PTB. The endotoxins released by the bacteria in the plaque formed around the tooth and gum line is possibly the inciting factor for PTB. The extensive tissue damage resulting from endotoxins released from bacteria results in host inflammatory and immune responses in the gum. These inflammatory mediators could enter the circulation and result in activation of the pregnant uterus. A meta-analysis of 17 peer-reviewed observational studies have shown odds of preterm low birth weight among pregnant women with periodontitis was 2.83 (95% CI: 1.95-4.10, P<0.0001). There was significant heterogeneity among studies and the better quality studies showed lower association strength and therefore the results have to be interpreted with caution (Vergnes et al., 2007).

1.2.5 Pathophysiology:

The pathological processes that cause PTB include intrauterine infection, uterine ischaemia, uterine overdistension, abnormal allogenic recognition, allergic-like reaction, cervical disease and endocrine disorders (Figure 1.12) (Romero et al., 2006).

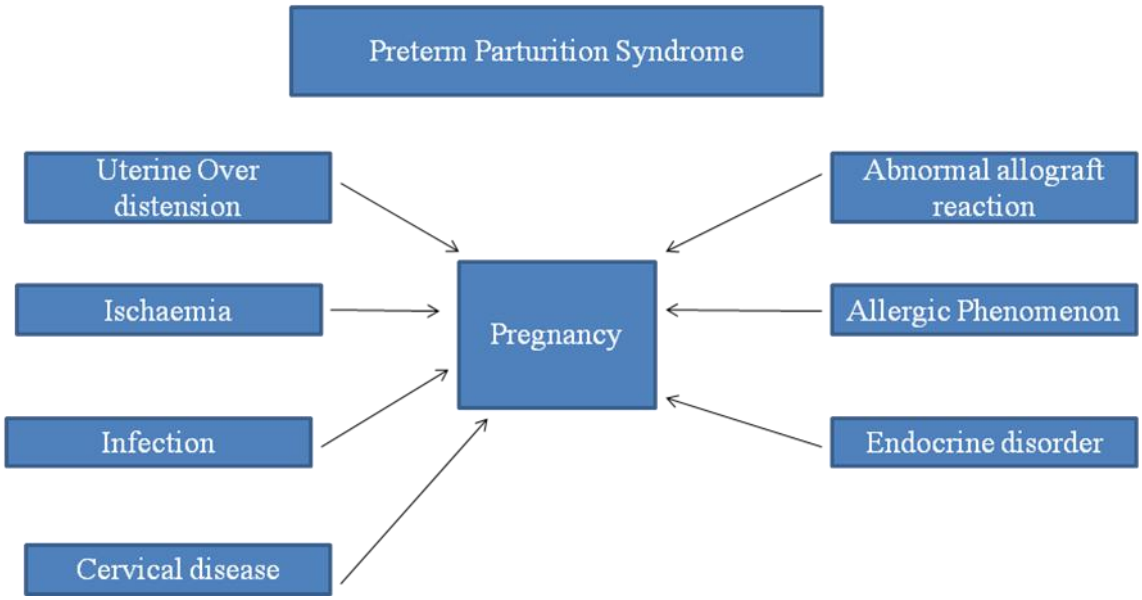

Figure 1.12. Pathological processes that are implicated in the preterm parturition syndrome. Reproduced from Romero et al., 2006.

Infection and inflammation are the most frequent cause for PTB and is the only pathological process to have a firm causal link with PTB. In the non-inflammatory group of PTB the most common lesion found in the placenta are vascular lesions. Uterine overdistension is the pathogenetic factor behind increased risk of PTL among women with multiple pregnancy and polyhydramnios. The other pathological factors include cervical incompetence, abnormal allograft and allergic phenomenon and progesterone insufficiency.

1.2.5.1 Infection:

As discussed in section 1.2.4.3, infection accounts for 25-40% of PTBs. Most of these intrauterine infections are subclinical in nature. The prevalence of infection varies with the clinical presentation and gestational age at the time of presentation. The lower the gestational age at the time of presentation, the higher the frequency of positive amniotic fluid (AF) cultures. For example, in one study 12.8% of women who presented with PTL had positive AF cultures and this rose to 22% in women who deliver preterm. The incidence of positive AF culture is 32.4% in women presenting with pPROM (Romero et al., 2006).

Using fluorescence *in-situ* hybridization techniques, bacteria were demonstrated in the fetal membranes in 70% of women who underwent elective caesarean section at term. This suggests that the presence of bacteria alone is not sufficient to cause PTL. The infection needs to elicit an inflammatory response in the maternal or fetal system to induce PTL (Romero et al., 2006).

Once microorganisms enter the system they are recognized by the innate immune system (IIS) and a host response is elicited to prevent tissue invasion and microbial proliferation. Toll like receptors (TLR) are a type of pattern recognition receptors of IIS that are capable of recognizing and binding to patterns of molecular structures present on the surface of microorganisms. Once bound, TLR activates NF-κB, which in turn leads to production of cytokines, chemokines and antimicrobial peptides. They also induce the expression of CD-80 and CD-86, which along with microbial antigens are presented by major histocompatibility complex class II proteins in dendritic cells and macrophages and activate naïve CD4 T-cells to initiate an adaptive immune response. As described earlier, major cytokines that are involved in preterm parturition include IL-1, TNF-α and IL-8. These cytokines can induce the production of PGs by the amnion, decidua and myometrium. TNF-α can stimulate the production of MMPs, which play an important role in cervical ripening and membrane rupture. Other cytokines IL-6, IL-16, IL-18, colony-stimulating factors, macrophage migration inhibition factor and monocyte chemotactic protein-1 are also known to be associated with preterm parturition. In fact IL-6 levels are elevated in the plasma of fetus with systemic inflammation and these fetuses have higher rates of neonatal sepsis, pneumonia, intraventricular haemorrhage and necrotizing enterocolitis. In women

with pPROM, elevated fetal IL-6 can predict onset of PTL irrespective of the AF inflammatory status suggesting that fetal inflammation can initiate labour. Thus there is accumulating evidence for the role of cytokines in the stimulation of the cascade of events leading to parturition (Romero et al., 2006).

In addition infection can inhibit chorionic PGDH in the chorion increasing the transit of PG into the myometrium. Infection can also lead to increased production of CRH by the fetal hypothalamus leading to increase in fetal adrenal cortisol production. Cortisol in turn increases PG production from the placenta (Goldenberg et al., 2000). These pathways are shown in Figure 1.13.

Gene-environment interactions seem to determine the susceptibility and the outcome of infection. For example, in a study by Macones et al., (2004), women who carried the TNF-α2 allele and had BV had an OR of 10 for PTB whereas women who were either sole carriers of TNF-α2 allele or BV infection were not at high risk for PTD.

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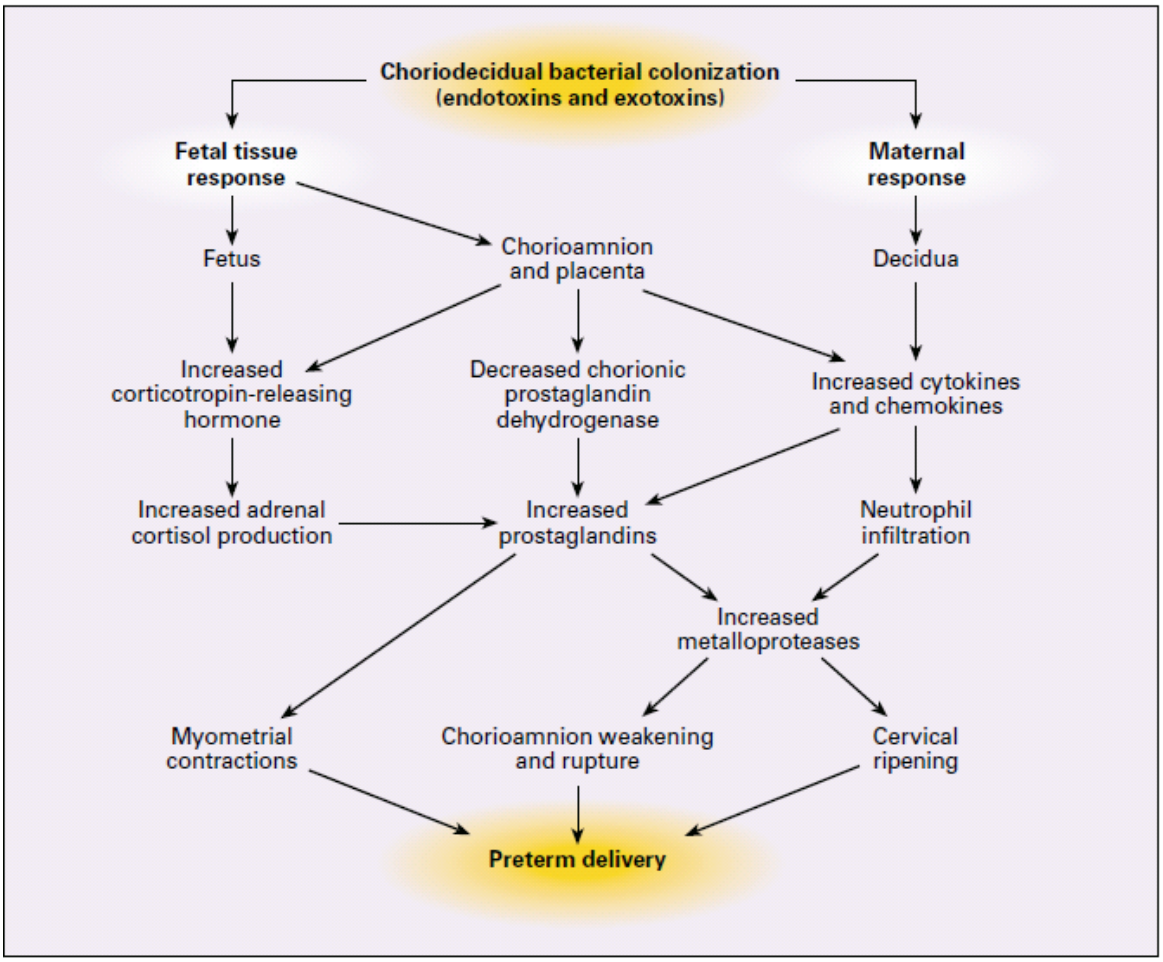


Figure 1.13. The mechanism of activation of common pathway of parturition by infection. Reproduced from Goldenberg et al., 2000.

The current understanding regarding the mechanism of PTB induced by infection is that bacterial infections of the choriodecidual space and their products exotoxins and endotoxins leads to release of cytokines and chemokines which activate the decidua and fetal membranes. These in turn stimulate PG synthesis and release, neutrophil chemotaxis and activation leading to release of MMP. The PGs stimulate uterine contractions whereas the MMPs promote cervical ripening and membrane rupture.

1.2.5.2 Uteroplacental ischaemia

Most women in spontaneous PTL have inflammation of the placenta but the remaining do not show any signs of inflammation. Among the non-inflammatory group, fetal and maternal vascular lesions are the common pathological lesion identified in the placenta. The maternal vascular lesions include failure of the physiological transformation of the myometrial segment of the spiral arteries, atherosis and thrombosis of the spiral arteries. Fetal lesions include a decrease in the number of arterioles in the villi and fetal arterial thrombosis (Romero et al., 2006).

The exact mechanism by which uteroplacental ischaemia activates the pathway of parturition is not clear but a role for the renin-angiotensin system has been postulated (Romero et al., 2006). Uterine ischaemia can induce the production of uterine renin and angiotensin II, which can stimulate myometrial contractility directly or indirectly through the production of PGs, alternatively, severe uterine ischaemia leading to decidual necrosis and haemorrhage could be involved because this leads to the production of thrombin, which can also activate the pathway of parturition (Romero et al., 2006).

1.2.5.3 Uterine over distension:

As stated earlier (section 1.2.4.3) PTB is more common in multiple pregnancies and pregnancies complicated by polyhydramnios compared to singleton pregnancies. Uterine stretch is the common inciting factor in both these scenarios and therefore stretch might initiate parturition.

During most of pregnancy, uterine distension resulting from the growth of the fetus and placenta does not cause a raise in intra-amniotic pressure. This is because progesterone induces myometrial relaxation. Progesterone also inhibits the stretch-induced expression of the CAP. However, as pregnancy advances the uterus reaches a finite size when the myometrium can no longer relax to accommodate the increased volume occupied by the fetus and placental tissues, consequently, stretch somehow overcomes the inhibitory influence of progesterone leading to an increase in myometrial contractility, PG release and expression of Cx-43 and OTRs. The effect of stretch on the myometrium

of twin pregnancies was investigated in an in-vitro study and there was no evidence of changes in $G_{s\alpha}$,Cx-43, Cx-26, EP1, 3 and 4 receptors (Lyall et al., 2002). The author's conclusion was that the methods by which stretch induces contraction is complex and requires further investigations.

1.2.5.4 Cervical disorders

Cervical weakness is defined as the inability of the cervix to retain the pregnancy due to premature cervical ripening in the absence of uterine contractions (Althuisius et al., 2002). There is no objective diagnostic test for this condition and it is therefore traditionally diagnosed from a patient's past obstetric history and therefore cannot be used as a predictor in primigravid women. Women with cervical weakness usually present with a history of recurrent second trimester losses and early PTDs (Althuisius et al., 2002). The preterm loss is usually preceded by a painless cervical dilatation and bulging of the membranes giving a feeling of vaginal pressure followed by membrane rupture. There may be no associated vaginal bleeding, the fetuses are born alive and labour duration is often shorter when compared to the normal population.

Although the concept of cervical weakness is widely accepted, its precise role in PTD is changing. Short cervix in the mid trimester is thought to be an expression of a spectrum of cervical diseases that include:

1. loss of cervical connective tissue after a cervical surgery, such as conization,
2. a congenital disorder, such as cervical hypoplasia from exposure to diethylstilbestrol,
3. intrauterine infection,
4. suspension of progesterone action and
5. a cervical disorder with the clinical presentation of cervical insufficiency (Romero et al., 2006)

Biomechanical testing of women with true cervical incompetence has shown low strength and high extensibility in the cervix (Bauer et al., 2007), with hydroxyproline extractability and collagenolytic activities being increased in the cervical biopsies of women with cervical incompetence.

There are two possible mechanisms by which cervical weakness could cause PTD. These are: (1) exposure of the choriodecidual space to vaginal pathogens that results in abnormal colonization of the fetal membranes leading to activation of the inflammatory cascade events that precipitate parturition, and/or (2) loss of mechanical support in the cervix that can lead to membrane rupture and the release of PGs that activate the terminal pathway of parturition.

1.2.5.5 Hormonal disorders

Progesterone is required for pregnancy maintenance and functional progesterone withdrawal is important for parturition. It is thought that some pathological states with diminished progesterone function can initiate parturition.

Luteal phase defect (LPD), a disorder defined as either a defect in progesterone secretion by the corpus luteum or a defect in endometrial response, is commonly implicated in the aetiology of infertility and recurrent miscarriage (Potdar and Konje., 2005). A retrospective study found that 31.2% of patients with LPD, who did not receive progesterone supplementation, delivered preterm when compared to 13.7% in women who received progesterone supplementation (Check et al., 1992).

Functional progesterone deficiency is also thought to be one of the mechanisms through which intrauterine infection stimulates parturition. Infection is associated with an increase in cytokines such as IL-1β, which can stimulate expression of the nuclear transcription factor NF-κB. NF-κB, in turn, can repress progesterone activity resulting in preterm parturition (Romero et al., 2006).

It is important to identify women who are at high risk of delivering preterm as appropriately applied interventions in high risk women may help in the prevention of PTD. The biophysical factors which help in identifying the high risk women are discussed in the next section.

1.2.6 Predictors of PTB

Several factors have been scrutinized as potential predictors of PTB and these have been grouped into the following categories (Goldenberg, et al., 2003) which are;

1. demographic and behavioral characteristics of the mother such as age, race, parity, alcohol, tobacco and drug abuse, and previous pregnancy complications,
2. current pregnancy complications such as bleeding and polyhydramnios,
3. maternal nutrition and psychosocial characteristics,
4. extra-uterine infections such as BV, other genital infections and periodontitis,
5. bio-physical characteristics such as cervical length and/or funneling as measured by ultrasound, effacement and dilatation of the cervix assessed by digital examination and uterine contraction frequency,
6. biochemical tests using various body fluids such as FFN, IGFBP-1, urocortin, CRH from various biomatrices and
7. newer developments.

1.2.6.1 Traditional risk scoring systems

Scoring systems based on demographic factors, maternal nutritional status, psychosocial and past obstetric history, classify between 4% and 30% of women as high risk for PTD. From this high risk group, only 15-62% of total PTBs will arise (Creasy, et al., 1980, Mercer et al., 1995). Moreover, the scoring system is not applicable to primigravidas who constitute 45% of pregnant women. In addition, interventional studies aimed at reducing PTB based on this traditional risk scoring system have not been successful (Goffinet, 2005). However, past history of PTB remains the single strongest predictor of PTD with a relative risk (RR) of 6-8. Screening tests combining the traditional and the latest risk factors might therefore be more useful in identifying the ‘at risk’ patients.

1.2.6.2 Cervical FFN

The glycoprotein, FFN, is a major component of the ECM of the decidua basalis, AF and placental tissues (Lockwood et al., 1991), and is frequently seen in cervical secretions of women up to 20 weeks

of gestation. This is due to the incomplete fusion between decidua and the fetal membranes. However, its presence in the late second and third trimester is highly suggestive of disruption of the choriodecidual interface brought about by a mechanical or an inflammatory damage effect on the membranes or placenta and thus, the presence of FFN is a high risk factor for subsequent PTB (Leitich et al., 2003).

FFN has been measured from cervical or vaginal secretions using a number of methods including an ELISA assay, a solid-phase immunogold assay or by a dipstick membrane immunoassay. Based on a large cohort study, a FFN concentration >50ng/ml in samples is now considered as positive test result (Kurtzman et al., 2009). In clinical use, contamination of the sample with maternal blood, sampling within 24 hours of intercourse and preeclampsia may reduce the accuracy of the result (Honest et al., 2002), making this a problematic test.

A meta-analysis in 2003 by Leitich et al., showed that among asymptomatic women the sensitivity and specificity of the test in predicting PTD <37 weeks was 52%, and 53 %, respectively and at <34 weeks gestation was 85% and 89%, respectively indicating that the test was better for early preterm prediction. Furthermore, among asymptomatic women, serial sampling done in high risk women was more effective than screening low risk women. The best sensitivity rates were obtained by testing symptomatic women for delivery within 7 days of the test when the sensitivity and specificity rates were 77 % and 87% in predicting PTD.

A systematic review of the accuracy of cervicovaginal FFN test in predicting risk of spontaneous PTB carried out on 64 primary articles; 28 studies in asymptomatic women and 40 studies in symptomatic women, with a total of 26,876 women (Honest et al., 2002). Among asymptomatic women the study showed that the likelihood ratio for PTB at less than 34 weeks gestation for a positive result was 4.2 and negative result was 0.78; the post test probability for a positive test result was 13.7. The likelihood ratio for PTB<37 weeks was 2.94 for a positive result and 0.52 for a negative result; the post test probability following a positive result was 29. The results were not affected by the risk status, the type of test, repetition of the test and gestation at testing.

Among symptomatic women, the best result was obtained for the risk of delivery within 7-10 days of having a test. The likelihood ratio for a positive and a negative test result were respectively 5.42 and 0.25. The post test probability following a positive result was 14.4. The ROC curve area for prediction of PTB within 7-10 days of testing among symptomatic women was 0.84. This result was most accurate when compared to prediction of PTB at <34, <37 weeks gestation (ROC area 0.77; 0.71). The authors also explained the clinical usefulness of FFN testing. With the use of FFN test, 17 women will need to be treated to prevent 1 case of RDS at 31 weeks whereas 109 women will need to be treated to prevent 1 case of RDS without the use of FFN (Honest et al., 2002).

Both studies have therefore concluded that the most effective use of FFN is in symptomatic women with threatened PTL to determine the risk of PTB within 7-10 days of presentation. This enables clinicians to make a rational approach to decision making regarding inpatient admission, administration of antenatal steroids and *in-utero* transfer of women. This test is thus considered to be the gold standard test for prediction of PTB, but as stated has its limitations.

1.2.6.3 Insulin like growth factor binding protein-1 (IGFBP-1)

Insulin like growth factor-1 (IGF-1) and its homologue IGF-2, are insulin like polypeptides that stimulate cellular proliferation, differentiation and metabolism (Rutanen, 2000). The interaction between IGF's and their receptors is modulated by six high binding affinity proteins (insulin like growth factor binding proteins IGFBP-1 to 6). IGFBP-1 is believed to regulate acute changes in serum IGF levels and to modulate the action of IGF at the cellular level. The exact role of IGF system in human reproduction is unclear but there is evidence that it could be involved in implantation and placentation, as well as in fetal growth (Martina et al., 1997).

IGFBP-1 is produced mainly by the decidualized endometrium (Rutanen et al., 1985) and the liver (Lembet et al., 2002). AF contains high amount of IGFBP-1 (Drop and Hintz, 1984) which is non-phosphorylated whereas the IGFBP-1 (phIGFBP-1) in decidua is. Tissue destruction in the lower uterine segment secondary to either contraction or infection-induced proteolysis may cause leakage of phIGFBP-1 into the cervix. Thus, the presence of phIGFBP-1 in cervico-vaginal secretions is used to

predict PTL and the effectiveness of phIGFBP-1 as a predictor of PTB has been evaluated in few studies.

Among asymptomatic women, the sensitivity of the test in predicting PTB <37 weeks of gestation varied between 22-40% and the specificity was between 82-94%. The positive predictive value was 11-14% while the negative predictive value was between 94-98% (Kekkei et al., 2001; Paternoster et al., 2007; Altinkaya et al., 2009).

Among symptomatic women the sensitivity of cervico-vaginal phIGFBP-1 was best for the prediction of PTB within 7 days of the test (93.8%). The sensitivity for predicting PTB <37 weeks varied between 46 to 97%. The specificity, positive predictive and negative predictive values for PTB <7 days was 79-94%, 56-83% and 94-97%, respectively. The specificity, positive predictive and negative predictive values for PTB <37 weeks of gestation were 68-94%, 41-94% and 88-95%, respectively (Kekkei et al., 2001, Kurkinen-Raty et al.,2001, Lembet et al., 2002, Paternoster et al., 2007, Altinkaya et al., 2009, Tanir et al., 2009).

Although early studies suggest that the test accuracy is similar to that of FFN, more studies to confirm the usefulness of this test are required. The phIGFBP-1 test has some additional advantages over the FFN test in that level of phIGFBP-1 is not affected by semen or urine, but it is affected by the presence of blood and the cost of phIGFBP-1 test is much lower than that for FFN (Paternoster et al., 2007).

1.2.6.4 Urocortin

Urocortin is a 40-amino acid neuropeptide belonging to the CRH family, which binds both type 1 and type 2 CRH receptors with great affinity, and is expressed mainly by gestational tissues such as amnion, chorion, decidua, trophoblast and myometrium. It is measurable in both the maternal and fetal circulations, showing stable concentrations in maternal plasma from the first to third trimesters of pregnancy (Florio et al., 2007).

Urocortin is known to increase myometrial contractility in *in-vitro* studies by directly enhancing myometrial contractility by augmenting the myometrial contractile response to PGs (Petraglia et al., 1999) and directly activating the MAPK signaling pathways that regulate myometrial contractility (Grammatapoulos, 2007). Urocortin is also known to increase MMP-9 levels and thus may be one of the bio-molecules involved in rupture of membranes (Li and Challis, 2005).

Plasma urocortin has been investigated as a predictor of PTD in patients with threatened PTL. One cohort study investigated the level of urocortin in 85 women admitted either with spontaneous PTL with ruptured membranes. The levels of urocortin were significantly elevated in women who delivered <34 weeks, and the levels were significantly higher in women who delivered <7 days from the time of sampling than those who delivered after 7 days. The PTD rate in the study group was 35%. The sensitivity and specificity of the urocortin assay were 80% and 100% respectively, whilst the positive predictive value was 100% and negative predictive value was 90% (Florio et al., 2007).

A prospective cohort study of 41 women who delivered preterm and 41 women who delivered at term did not, however find any difference in the level of urocortin in the AF in the two groups (1.55 ± 0.63ng/ml *vs.* 1.6 ± 0.49ng/ml; P=ns) (Iavasso et al., 2009).

A retrospective case control study measuring urocortin, CRH, oestriol, cortisol and DHEA-S levels in the AF of 130 healthy women undergoing mid trimester amniocentesis found that the urocortin levels were much lower in the women who subsequently delivered preterm (0.50 ± 0.07 ng/ml *vs.* 0.90 ± 0.26 ng/ml; P<0.001) (Torricelli et al., 2009).

Urocortin, thus, seems to be a promising marker in the prediction of PTD; especially as the high positive predictive value in the study by Florio *et al*.2007 seems to be very useful. This study had a 30% prevalence rate of PTD; however, other studies with low prevalence rates are giving contradictory results (Ivasso et al., 2009; Torricelli et al., 2009). Obviously, further, larger studies using different risk groups need to be performed before a conclusion about the usefulness of urocortin in predicting PTB can be made.

1.2.6.5 Corticotrophin releasing hormone (CRH)

CRH and its role in parturition have been discussed in section 1.1.2.4.

Placental CRH is produced in the syncytiotrophoblast, decidua and fetal membranes and may be the placental clock that determines the onset of labour. CRH has also therefore been tested for its usefulness as a predictive factor for PTB in asymptomatic and symptomatic pregnant women.

The placental clock hypothesis proposed by McLean et al. suggests that a placental clock is set in the early stages of pregnancy that triggers the onset of parturition after a predetermined duration of gestation, and progresses at different rates in individual pregnancies with some pregnancies programmed for early or late delivery (McLean et al., 1995). Many factors may affect the speed of the placental clock including genetic predisposition and pathological events in the mother and the fetus (McLean and Smith, 2001). This theory is supported by many cross sectional and longitudinal studies which have shown elevated CRH plasma concentrations in patients with PTL and levels have been shown to be elevated up to 11 weeks prior to onset of PTL.

Another prospective study by McLean et. al. in 1999, studied 860 women from the second trimester until term. The effectiveness of risk factor score, plasma CRH level and plasma alpha-fetoprotein in predicting women destined to deliver preterm were compared individually and as a combination of three factors. The study showed that CRH was able to predict 24% of all PTDs, alpha-fetoprotein predicted 25% and risk factor score predicted 27% of all PTDs for a false positive rate of 5%. The combination of all three factors predicted 37% of all PTDs and 50% of all idiopathic PTL (McLean et al., 1999).

A number of other studies have been conducted in low risk and high risk asymptomatic populations. The odds of delivering preterm in women with elevated CRH varied between 0.98-6.8 (Berkowitz et al., 1996; Wadhwa et al.,1998; Hobel et al., 1999; Leung et al., 2001).

The advantage of CRH in predicting PTB is that it identifies at risk pregnancies as early as 11 weeks before the onset of PTL. This allows time for any available effective interventions. However, risk associated with raised CRH is variable in different studies and is not high, suggesting that in itself this is not a good biomarker for predicting PTB.

Among symptomatic women, studies have shown an association between raised CRH level and risk of PTD (Campbell et al., 1987; Wolfe et al., 1988; Kurki et al., 1991). In a study involving 91women with threatened PTL, the sensitivity, specificity, positive and negative predictive values of delivering <37 weeks were 39%, 90%, 67% and 75% respectively (Campbell et al., 1987). In the 1991 study by Kurki et al., the odds of delivering preterm was 2.8 among women with raised CRH levels. The largest of the studies measuring CRH in 94 symptomatic women gave a positive likelihood ratio of 3.9 and a negative likelihood ratio of 0.7 (Coleman et al., 2000).

More robust studies in carefully selected groups of women might give us a clearer idea about the usefulness of CRH in the prediction of PTD.

1.2.6.6 Markers of Infection and Infection Screening

Studies in low risk populations have shown that BV increases the risk of PTD (RR 1.4-6.9) (McGregor et al., 1990, Hillier et al., 1995). These findings were confirmed in a metanalysis (Flynn et al., 1999). However, antibiotic treatment of BV does not prevent PTD, as shown in a Cochrane review of 15 trials, which showed that antibiotic therapy in pregnancy was highly effective in eradicating BV (OR 0.21), but was not associated with a significant reduction in the risk of PTD (birth <37, 34, 32 weeks) (OR 0.87, 1.22, 1.14). There was also no benefit in women with history of prior PTB (McDonald et al., 2005). In conclusion, the available evidence does not favor routine screening and treatment of BV in the low risk, asymptomatic, pregnant population.

Chlamydia is associated with a twofold increased risk of PTB at less than 37 weeks of gestation and a threefold increased risk of PTB less than 35 weeks (Andrews et al., 1996). There is no evidence for

routinely screening low risk women for this infection, but treatment is necessary once the infection is identified to prevent consequences such as neonatal opthalmitis.

Five cohort studies have demonstrated an association between *Trichomoniasis* infection and PTD, pPROMs and low birth weight (Grice, 1974; Hardy et al., 1984; Minkoff et al., 1984; Riduan et al., 1993; Meis et al., 1995). A Cochrane review of the efficacy of antibiotic treatment of this infection however, did not find any benefit with one trial (Kulmezoglu and Azhar, 2011) and another study found an increased PTD rate among cases treated with metronidazole (RR 1.8) (Klebanoff et al., 2001).

There is some evidence from a large Austrian RCT trial showing that treatment of candidiasis is effective in reducing PTB rate, where 4429 asymptomatic pregnant women were screened and treated for BV, trichomoniasis and candidiasis. There was a PTB rate of 3% in the treatment group compared to 5% in the control group. The maximum benefit was found in women treated for candidiasis, where spontaneous PTB occurred in 8/289 (0.02%) women and in 22/29 (0.07%) women with candidiasis in the control group (OR 0.35) (Kiss et al., 2004).

The association of Group B haemolytic streptococcal (GBS) infection with PTB is conflicting in different studies (McKenna and Iams, 1998; Romero et al., 1989; Gibbs et al.,1992; Gibbs et al., 1997). The largest study has in fact shown an association (OR-1.5) (Reganet al., 1996). However erythromycin treatment of GBS colonized women did not find an improvement in the perinatal outcome (Klebanoff et al., 1995). The study by Romero et al (1989) showed that there was a significant association between asymptomatic GBS bacteriuria and PTD.

A systematic review on periodontitis did not find significant evidence to recommend routine screening and treatment of periodontitis in pregnant women (Vergnes et al., 2007). So, at the moment there is not enough evidence to recommend routine screening and treatment of genital infections and periodontitis, unless symptomatic, in both low risk and high risk pregnant populations.

Other infectious markers, such as intercellular adhesion molecule-1 (ICAM-1) in cervico-vaginal secretions has been tested in symptomatic women and the likelihood of delivering preterm within 3 and 7 days of testing positive was 12.4 and 7.4 (Marvin et al., 2000). The predictive ability of ICAM-1 was independent of that of FFN, and therefore, these tests could be combined to get a better sensitivity.

Defensins are antimicrobial peptides that increase in biological fluids with microbial invasion (Schneider et al., 2005). Using a combination of 3 serum tests: alpha–fetoprotein, granulocyte colony stimulating factor and defensins, Goldenberg et al. (2001) was able to detect 80.5% of PTBs with an OR of 14.7 and specificity of 78.1%.

Vaginal defensins have been studied with BV in a cohort study in predicting PTB at <32, 34 and 37 weeks. Vaginal fluid specimens were obtained between 24-29 weeks gestation in asymptomatic women and higher vaginal defensin levels were associated with a higher risk of delivering <32 weeks. Hazard ratios for defensin levels between 1-2.8μg/ml, 2.8-8.2μg/ml and >8.2μg/ml were 1.7, 2.4 and 3.1, respectively (Balu et al., 2003)

1.2.6.7 Cervical measurements

1.2.6.7.1 Digital examination

Digital examination is the traditional method of assessment of the cervix, where a gloved finger is used to 'feel' the cervix. The main problem with manual examination is that only the vaginal portion of the cervix can be assessed, unless the cervix is fully dilated. Moreover, this method suffers from poor reproducibility in between examiners and thus biased. Transvaginal ultrasonography is superior to digital examination in the prediction of PTD.

1.2.6.7.2 Ultrasound examination of the cervix

Transvaginal ultrasound is increasingly used to assess the cervix, because it provides a clear and consistent view of the cervix and has been proven to be acceptable for patients (Berghella et al., 2003). Trans-abdominal ultrasound, however, may be unreliable, because cervical length can increase due to

an over-filled bladder and visualization of the cervix may be difficult due to abnormal maternal habitus, cervical position and obscuring effect of fetal parts (Berghella et al.,2003). In normal practice, the transvaginal ultrasound probe is placed in the anterior fornix with the women's bladder empty. The appropriate sagittal section of the cervix is obtained by locating the triangular echo dense area at the external os and the v-shaped notch at the internal os and line of echo density or echolucency in between the two. The measurement is done over a period of 3 minutes to allow time for the development of funneling and to demonstrate dynamic changes in cervical length due to uterine contractions. Cervical length is measured along the line made by the interface of the mucosal surfaces, with calipers placed at the internal and external os. Three measurements of cervical length are obtained and the shortest measurement recorded. Undue pressure on the cervix that might artificially increase the length is avoided by first obtaining a satisfactory image of the cervix and withdrawing the probe until the image blurs and finally reapplying enough pressure to restore the image (Berghella et al., 2003). The main problem with this technique is that funneling is described differently by different sonographers and reproducibility of funneling is very low (Berghella et al., 2003).

1.2.6.7.2.1 Asymptomatic women

Among asymptomatic women, the risk of PTD increases with decrease in the cervical length. In the meta-analysis done by Honest et al. (2003), 18 studies on asymptomatic women with singleton gestations were included. The results were classified according to the following 3 criteria: gestation at the time of testing, criteria of the test (various cervical lengths) and the outcome (gestational age at delivery). An outcome at 34 weeks gestational age was considered to be more clinically relevant as three-quarters of neonatal mortality and one-half of long term neurological impairment occurs in this group of gestation.

Testing before 20 weeks using a cervical length of 25mm gave a positive likelihood ratio of 6.29 for PTD at <34 weeks gestation, which increased the pretest probability from 4.1 % to 21.2%. A shorter cervical length of 20mm gave a positive likelihood ratio of 14.47.

The presence of funneling does seem to be equally effective in predicting PTD. A study tested the effectiveness of funneling at <20 week gestation and a positive test increased the likelihood ratio for PTD at <34 weeks gestation by a factor of 26.81, and the accuracy of the test varied with the criteria used between 20-24 weeks. The positive likelihood ratio varied between 3.10 and 7.97 (Honest et al., 2003).

Clinically, ultrasound cervical length testing before 20 weeks and using a shorter cervical length cut-off is safer and more efficacious as interventions can be directed at the women in whom it would be most beneficial. However, the sensitivity of this test is very low for it to be an effective screening test, and therefore future research with a larger sample size will be needed to get sufficient power to test the efficacy of intervention. The results will be more reliable if it is tested in a more homogenous population, who might have similar underlying pathological processes leading up to PTD.

1.2.6.7.2.2 Threatened preterm labour

Among symptomatic women there were 11 studies. All studies were conducted at >20 weeks of gestation. The positive likelihood ratio for PTD <37 weeks varied between 1.5-12.83 depending on the criteria of cervical length. A cut off of 25mm gave a positive likelihood ratio of 4.45 and a negative likelihood ratio of 0.42. At 30mm the positive likelihood ratio was 2.15 and the negative likelihood ratio was 0.32.

Among this group of women around 30% end in PTD. Choosing a longer cervical length will be most useful in this group as the sensitivity will be around 80-100% using this criterion. Using a cut off of 25mm the sensitivity is around 60-65% and therefore an increased number of women who will deliver preterm will be missed.

1.2.6.7.3 Combination of cervical length and the FFN test

Cervical length measurement has been compared with other predictors, such as the FFN test. There were 3 studies on symptomatic women. In the first study by Tsoi et al., (2006), both cervical length

and FFN were tested in women presenting with PTL between 24 and 36 weeks of gestation. A cervical length of <15mm was taken as the criteria. A cervical length <15mm had a positive predictive value of 51.4% in predicting delivery within the next 7 days, whereas a positive FFN test gave a positive predictive value of 21.2%.

In the study by Rozenberg et al., (1996) a cervical length of <26 mm and FFN testing were used and shown to be equally effective. A positive FFN test had a sensitivity of 70%; specificity of 69.6%, positive predictive value of 14.2% and a negative predictive value of 86.6%. A cervical length of <26mm predicted PTD with a sensitivity of 75%; specificity of 73.2%, a positive predictive value of 50% and a negative predictive value of 89.1%. Furthermore, these authors combined the tests and found that if either a cervical length <26mm or a postive FFN was taken as a positive test, the predictive values were: sensitivity 90%; specificity 60.7.6%; positive predictive value 45% and negative predictive value 94.4%. If the test criteria were both the presence of cervical length <26mm and a positive FFN test, the sensitivity became 55%; specificity 82.1%, positive predictive value 52.4%; and a negative predictive value of 83.6%.

In the study by Gomez et al. (2004), both the tests were performed in symptomatic women presenting between 22 and 35 weeks of gestation. A cervical length <15mm had a positive predictive value of 56.7% in predicting delivery within the next 7 days, whereas a positive FFN test gave a positive predictive value of 34.6%. According to the authors, 22% of women testing positive for FFN test and cervical length 15-30mm will deliver within 7 days of test, 35.7% of women with cervical length <15mm with a negative FFN test will deliver preterm, whereas if both cervical length length was <15mm and FFN test was positive, 75% delivered preterm.

Therefore, in a clinical situation with limitation of resources, cervical length can be the main stay of screening women with threatened PTL. As the negative likelihood ratio is 0.4, the pre-test probability of delivery within 7 days will be decreased to post test probability of 5.2 %. Therefore, women with a cervical length >15mm will not need any further testing. FFN testing can be reserved for women with a cervical length <15mm.

Among asymptomatic women the study by Heath et al., (2000) showed that a cervical length <15mm was a better predictor of PTD when compared to positive FFN test when tested at 23 weeks of gestation in singleton pregnancies. The outcome was PTD before 33 weeks of gestation. The RR of delivery with a short cervix was 46.2, whereas with a positive FFN test the relative risk was 8.1. At a cervical length of 25mm both cervical length and FFN test were equally effective. This was compatible with the outcome of the study by Goldenberg et al (1998), which showed that the OR of delivery before 32 weeks of gestation was 9.8 for a positive FFN test and 8.7 for a cervical length<25mm.

1.2.6.8 Home Uterine Activity Monitoring

PTL is thought to arise from a coalescence of small uterine contractions into larger contractions, which then generates cervical changes (Iams et al., 2003). The usage of tocolytics to prevent PTL is not found to be effective (Goldenberg and Rousse, 1998). One of the reasons for the failure of tocolysis is believed to be the late administration of the tocolytics after PTL is established (Iams, 2003).

The March of Dimes PTB prevention multicentre trial was conducted to test the efficacy of education to self-recognize symptoms of PTL through self-palpation of contractions and frequent visits by the healthcare team in reducing the PTB rate. The results were not encouraging. Two large RCTs did not find any difference in the rate of PTBs between women who were monitored with a tocodynamometer and woman who were not monitored (CHUMS trial, 1995; Dyson et al., 1998).

Home uterine activity monitoring (HUAM) prediction study was a large prospective observational study of uterine contraction frequency in 274 women with either previous PTD or bleeding in the second trimester in the current pregnancy and 32 women with no risk factors, in which, 106 women delivered before 37 weeks. Those who delivered before 35 weeks had more contraction frequency when compared to those who delivered after 35 weeks, but the efficacy of contraction frequency monitoring in predicting PTD was low, as the sensitivity was only 10% and the positive predictive value was 25% at 22 week of gestation (Iams et al., 2002). Uterine activity monitoring is therefore not a good predictive test for PTB.

1.2.6.9 Proteomics

Proteomics is the study that encompasses knowledge of the structure, function and expression of all the proteins in the biochemical context of an organism in a given moment. Proteomics studies the interplay of multiple distinct proteins and their roles as part of a larger network (Buhimschi et al., 2006). Proteomics use tools to separate and identify proteins in biological samples and uses computational algorithms to obtain further information about the proteins. Techniques such as 2-dimensional gel electrophoresis, fluorescence 2-dimensional difference gel electrophoresis and mass spectrometry (MS) are some of the techniques used, although surface-enhanced laser desorption/ionization quadrupole time of flight mass spectrometry (SELDI-TOF-MS) is now considered to give the highest accuracy (Buhimschi et al., 2006). This technique results in multidimensional separation of proteins and in addition to detecting and quantifying proteins it can also quantify post translational modification in proteins (Buhimschi et al., 2006).

Proteomics has been used to identify and quantify changes in the proteins in the AF of women with threatened PTL and PPROM (Vuadens et al., 2003) where gel electrophoresis of AF of women with pPROM found 2 proteins to be differentially expressed. These were found to be agrin and perlecan.

Buhimschi et al. (2005) performed a proteomic analysis of AF from women with imminent preterm birth or pPROM. They used the SELDI-TOF technique to analyse the samples and identified 4 proteins which were present in women with evidence of intra-uterine inflammation. Based on the number of markers that were present, they devised scoring system called ‘mass-restricted (MR) score’. A score of 0-2 indicated absence of and 3-4 indicated the presence of inflammation. These 4 proteomic markers were identified to be defensin-1, defensin-2, calgranulin-A and calgranulin-C (Buhimschi et al., 2005).

The clinical usefulness of the MR score was tested in a population of women with twins and those who underwent cervical cerclage for cervical weakness. In this, 30% of the women with cervical weakness but no control subjects had an MR score of 3-4 (Weiner et al., 2005). In addition, a new proteomic biomarker was identified which was recognized to be α and β haemoglobin. Women with

both haemoglobin and calgranulin in the AF had the shortest interval to delivery period (Catalin et al., 2006). It thus appears that this rapidly developing field will be critical in the identification of useful biomarkers that will help in early diagnosis, prediction and monitoring the impact of treatment in the future.

Thus a number of markers have been tested as predictors of PTD. However none of them are completely effective in identifying at risk women. So there is a need to test novel molecules as biomarkers. Anandamide (AEA), an endocannabinoid is one such marker.

1.3 Endocannabinoid biochemistry

Cannabis has been used in medical practice since early human civilization. Narcotic effects of hemp were known to the Egyptians (Piomelli et al., 2003) and the information about the psychotropic properties of cannabis was transferred to British scientists through Napoleonic troops. Cannabis was first used in medicine in Britain in the mid nineteenth century by O'Shaughnessy following his visit to India where it was used in multiple conditions such as rabies, epilepsy and muscle spasms (Hirst et al., 1998), whilst Cannabis was found in the pelvic region of a young girl who died during childbirth in 3rd century AD (Hirst et al., 1998). This shows that knowledge about the therapeutic value of cannabis in malfunctions of childbirth has been recognized since ancient times.

Despite this early knowledge, elucidation of the active principle of cannabis that had the medicinal value took a long time. The active chemical constituent of plant cannabis, delta-9 tetrahydrocannabinol (Δ^9-THC) was finally isolated and its structure was elucidated in 1964 (Mechoulam and Gaoni., 1965). The molecular mechanism of action was a mystery until the late 1980s. Initial assumption of lack of stereoselectivity led to the belief that Δ^9-THC acted by molecular perturbation of the lipid layer of the cell membrane (Mechoulam et al., 1998). However, research showed that minor modifications in the structure of the Δ^9-THC molecule led to significant changes in activity; for example, the introduction of a methyl group next to the C-4 position but not next to C-2 position totally eliminated the activity of Δ^9-THC (Mechoulam et al., 2008). This experiment could not

be explained by a non-specific mode of action of Δ^9-THC and paved way for the identification of the cannabinoid receptor in the rodent brain (Devane et al., 1988) along with the discovery of the endogenous ligands for these receptors called endocannabinoids. AEA was the first of this group of endogenous ligands to be discovered (Devene et al., 1992).

The endocannabinoid system is now considered to be composed of the cannabinoid receptors, endocannabinoids and the proteins used for their synthesis and inactivation.

1.3.1 Endocannabinoid Receptors

Following the discovery of the first cannabinoid receptor, Herkenham et al (1990) showed the distribution of the receptors to be heterogeneous with the greatest concentrations being found in basal ganglia, hippocampus and the cerebellum. Subsequently, two types of cannabinoid receptors, called CB1 and CB2 were identified.

CB1 receptors were first cloned from rat cerebral cortex (Matsuda et al., 1990). They have since then been recovered mainly from tissues of the central nervous system that correlate with the observed psychoactive effects of cannabinoids, including impairment of cognition, memory, learning and motor coordination (Demuth et al., 2006). In the central nervous system, CB1 receptors are mainly localized in the olfactory areas, the cortex, hippocampus, cerebellum, basal ganglia and spinal cord. There are relatively fewer receptors in the thalamus, hypothalamus and brain stem (Herkenham et al., 1990; Glass et al., 1997; Tsou et al., 1998). CB1 receptors have also been identified in some peripheral tissues such as the spleen and tonsils (Galiegue et al., 1995), small intestine (Pertwee et al., 1996a), urinary bladder (Pertwee and Fernando, 1996), vas deferens (Pertwee et al., 1996b), sympathetic nerve terminals (Ishac et al., 1996; Vizi et al., 2001) and in small levels in adrenal gland, heart, prostrate, uterus and ovary (Galiegue et al., 1995).

The CB2 receptor was initially cloned from human promyelocytic leukaemia cells (Munro et al., 1993). Overall this receptor shares only 48% amino acid sequence homology with the CB1 receptor and 68% homology in the membrane spanning region. CB2 receptors are mainly localized in the

peripheral tissues and have been isolated in the spleen (Schatz et al., 1997), in tonsil and on immune cells (Galiegue et al., 1995; Schatz et al., 1997).

There is evidence to suggest the presence of a non-CB1, non-CB2 cannabinoid receptor. Cannabinoids have also been shown to act through the G-protein coupled receptors GPR55 and GPR19 (Brown, 2007) and there is also evidence that some of their actions are mediated by activating peroxisome-proliferator-activated receptor (Sun et al., 2006). Thus, the cannabinoid receptors belong to the superfamily of G-protein coupled receptors which have seven transmembrane spanning domains and an extracellular N-and an intracellular C-terminus, with several potential glycosylation sites.

Activation of the cannabinoid receptors leads to several signal transduction pathways which include:

1. Inhibition of AC activity, which is dose-dependent, stereoselective and pertusis–toxin sensitive (Devane et al., 1988). This leads to reductions in cAMP levels and reductions in PKA mediated short- and long-term effects.
2. Activation of MAPK signaling leading to regulation of cell functions, such as growth, differentiation and apoptosis (Demuth et al., 2006).
3. Modulation of ion channels:
    - Inhibition of voltage-gated P-, Q-and N-type Ca^{2+} channels
    - Stimulation of inwardly rectifying G-protein coupled K^{+} Channels (De Petrocellis et al., 2004).
4. Stimulation of phosphatidylinositol 3-kinase and intracellular Ca^{2+} mobilization through activation of PLC-γ (De Petrocellis., 2004).

1.3.2 The Endocannabinoid, AEA

As stated above, the first endocannabinoid to be discovered *N*-aracidonoyl ethanolamide, was named "AEA" after the Sanskrit word for bliss, 'ananda' (Devene et al., 1992). This was followed by the discovery of numerous other endocannabinoids, including 2-arachidonoyl glycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995), 2-arachidonyl-glyceryl ether (Noladin; Bisogno et al.,

2000), *O*-arachidonoyl- ethanolamine (virhodamine) (Huang et al., 2002) and *N*-arachidonoyl – dopamine (NADA) (Porter et al., 2002), amongst others.

AEA is an amide of AA and belongs to the class of lipid called *N*-acyl ethanolamines. The structure of AEA is shown in Figure 1.14.

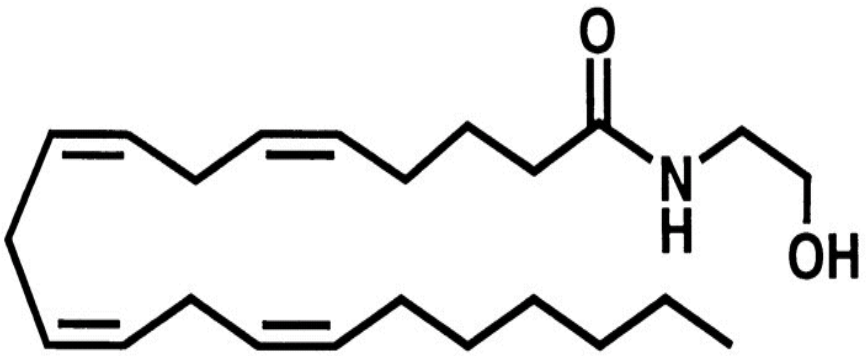


Figure: 1.14. The chemical structure of Anandamide (AEA)

1.3.2.1 Synthesis of AEA

AEA is synthesised by the enzymatic hydrolysis of *N*-arachidonoyl-phosphatidyl ethanolamine (NAPE). The enzyme that catalyses this hydrolytic reaction is a specific phospholipase D (PLD). It is selective for NAPEs with low affinity for other membrane phospholipids (Di Marzo et al., 1994) (Figure 1.15). NAPE is synthesized by the transfer of AA from phosphatidyl choline to phosphatidyl ethanolamine, a process catalysed by the enzyme *N*-acyl transferase (De Fonseca et al., 2005).

An alternative route of synthesis of AEA, that does not involve NAPE-PLD, has been recognized (Liu et al., 2006; Leung et al., 2006). This is a two-step process; first is the generation of phosphoanandamide through the enzymatic cleavage of NAPEs by the action of PLC. This is followed by dephosphorylation of phosphoanandamide into AEA by phosphatases, such as some forms of tyrosine phosphatases. This mode of synthesis is mainly prevalent in macrophages following stimulation by bacterial endotoxins .

Other possible routes of synthesis include sequential cleavage of the two sn-1 and -2 acyl groups of NAPE, catalysed by α/β- hydrolase 4. This is followed by phosphodiesterase mediated hydrolysis of glycerophospho-AEA to AEA (De Petrocellis and Di Marzo, 2009). Another mechanism involves the conversion of NAPE to 2-lyso-NAPE by a soluble form of phospholipase A2, followed by hydrolysis to AEA through the action of lyso-phospholipase D (De Petrocellis and Di Marzo., 2009).

AEA is not thought to be stored in resting cells but is synthesised and released from cells "on demand" (Di Marzo et al., 1998). The stimuli for synthesis could be physiological such as neuronal depolarization or activation of the ionotropic glutamate *N*-methyl-*D*-aspartate (NMDA).

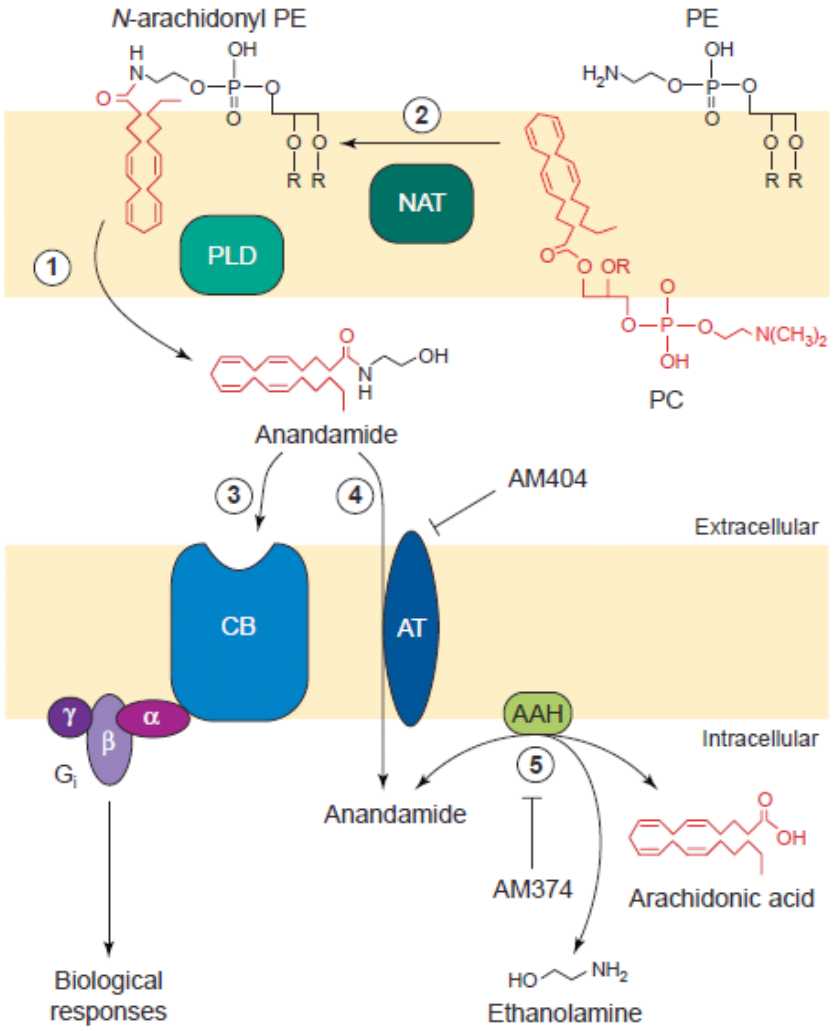


Figure 1.15. Synthesis and degradation of AEA. Figure reproduced from Piomelli et al., 2000.

(Step 1). AEA is synthesised by hydrolysis of *N*-arachidonoyl phospahatidylethanolamine (NAPE) which is catalysed by phospholipase D (PLD). (Step 2). *N*-acyl transferase catalyses the formation of NAPE by transfer of arachidonic acid to phosphatidylethanolamine from phosphatidylcholine. (Step 3). AEA stimulates the G protein coupled receptors on neighbouring cells. (Step 4). AEA actions are limited by transfer into cells through carrier mediated transport. (Step 5). Once transported into cells, AEA is hydrolysed into arachidonic acid and ethanolamine by action of enzyme fatty acid amide hydrolase (FAAH).

receptors, or nicotinic α7 neuronal receptors, or stimulation of metabotropic receptors of neurotransmitters such as dopamine, glutamate and acetylcholine (De Fonseca et al., 2004). Pathological stimuli such as bacterial lipopolysaccharides can also stimulate the synthesis of AEA (Di Marzo and Petrosino, 2007).

AEA has been isolated from various tissues including the brain, kidney, testis, skin, spleen, plasma, heart, breast cancer cells and mouse uterus (Facci et al., 1995; Felder et al., 1996; Cadas et al., 1997; Deutsch et al., 1997; Koga et al., 1997; Schmid et al., 1997a, stella et al., 1997a; Kempe et al., 1996).

Following synthesis, AEA is immediately released into the extracellular milieu via a mechanism which is still unknown but could probably be through the same putative membrane transporter that is involved in the cellular reuptake of AEA (Di Marzo et al., 1994; Beltramo et al., 1997; Bisogno et al., 1997). AEA produces a spectrum of effects similar to that produced by Δ^9-THC. These include increased appetite, reduced blood pressure, weakness of muscles (Smith et al., 1994), euphoria followed by drowsiness (Di Marzo et al., 1998), depersonalization, decreased memory recollection (Castellano et al., 1997) and reduced perception of auditory and visual stimulus. In addition, it exhibits analgesic (Mechoulam et al., 1995), anticonvulsive, antiemetic and hypothermic properties similar to that of Δ^9-THC.

Similar to Δ^9-THC, AEA acts mainly through cannabinoid receptors CB1 and CB2. AEA acts as a partial CB1agonist and a weak CB2 agonist (Bisogno et al., 2005). AEA can also act through non-CB1 and non-CB2 receptors (Di Marzo et al., 2000).

1.3.2.2 AEA transport and metabolism

Once AEA has exerted its effect its action are terminated through its degradation. The first step in this process is transportation into the cells ($t_{1/2}$ < 5min) followed by degradation by the enzyme FAAH (Figure 1.15) into AA and ethanolamine (Glaser et al., 2002; Hillard and Jarrahian, 2003). The mechanism by which AEA is transported into cells is a matter of controversy. Being lipophilic, AEA can diffuse through the plasma membrane if the extracellular concentration is higher than the

intracellular concentration. However, for this process to be rapid there is a need for a controllable and selective mechanism such as a membrane transporter protein or an intracellular enzymatic process capable of reducing the intracellular concentration rapidly.

AEA is indeed thought to be transported via a selective, saturable, temperature-dependent and Na^+-independent 'facilitated transport' mechanism called the anandamide membrane transporter (AMT) (Di Marzo et al., 1994; Maccarrone et al., 1998). However, the protein that makes up the AMT has not been cloned (Bisogno et al., 2005), suggesting that it might not exist.

The enzyme that degrades AEA, FAAH, was originally purified and cloned from rat liver (Cravatt et al., 1996). FAAH has since then been discovered in many tissues including brain, kidneys, testes, lung and spleen (Fowler et al., 2001), but is undetectable in skeletal muscle and heart (Mechoulam et al., 1998). The physiological role played by FAAH in the inactivation of AEA is recognized by the distribution of FAAH close to the localization of CB1 receptors in the brain (Egertova et al.,1998). The brain of FAAH knockout mice contains 15-fold higher levels of AEA when compared to wild type mice (Petrocellis et al., 2004).

The promoter region of FAAH gene has been identified (Puffenbarger et al., 2001; Waleh et al., 2002). The gene seems to be up regulated by progesterone and leptin (Maccarrone et al., 2003a, b), and down regulated by estrogens and glucocorticoids (Waleh et al., 2002).

A new endocannabinoid-like compound, *O*-phosphorylcholine-AEA, has been identified in FAAH null mice. This was a poor substrate for FAAH and was hydrolysed back to the parent compound AEA by the action of the choline-specific phosphodiesterase NPP6. It is not clear how the *O*-phosphoryl choline-AEA was formed and it is not known whether this pathway represents a way of storing and releasing AEA when needed (Mulder and Cravatt., 2006).

In addition, a second FAAH isozyme, FAAH-2, has been cloned in several mammals, apart from mice, and it also inactivates N-acyl ethanolamines. It shares 20% structural homology with FAAH-1

which has 38-fold greater activity in hydrolysing AEA when compared to FAAH-2 (Wei et al., 2006).

AEA could also be metabolized through oxidation by the enzymes belonging to the arachidonate cascade, such as some cytochrome p450 enzymes, lipooxygenases and cyclooxygenase-2 (Petrocellis et al., 2004). The prostaglandin-ethanolamides, also called prostamides, are resistant to hydrolysis and prostamide $F_{2\alpha}$ is known to have pharmacological activities (De Petrocellis et al., 2009). Oxygenation by 12- and 15 lipooxygenases leads to production of hydroperoxy-and hydroxyl-derivatives, while oxidation by cytochrome p450 oxygenases leads to the formation of epoxyeicosatetraenoyl-anandamide. These compounds are known to have functional activity at cannabinoid receptors, but their physiological role(s) remains uncertain.

1.3.3 Role of the Endocannabinoid System

Endocannabinoids have been shown to play a role in health and disease in various systems, such as central nervous system, gastrointestinal, reproductive, immune and skeletal system. Pharmacological studies using Δ^9-THC, selective CB1 and CB2 receptor agonists and antagonists, observation of the CB1, CB2 and FAAH knockout mice and measurement of AEA levels, has given insight into AEA reference levels and their role in physiological and pathological states. For example, AEA acts as a retrograde messenger on presynaptic receptors and through this, regulates the cognitive and emotional functions in the cortex, hippocampus and amygdala. It also reinforces the use of substances of abuse in the mesolimbic system, controls appetite in hypothalamus, controls movement and posture in the basal ganglia and cerebellum and modulates the hypothalamo-pituitary-adrenal axis (Di Marzo and Petrosino, 2007). Aberrant endocannabinoid activity has been noted in hepatic fibrosis, Parkinson's disease, Alzheimer's disease, ulcerative colitis and eating disorders (Di Marzo and Petrosino, 2007).

As a result of this knowledge, the endocannabinoid system has been manipulated in the treatment of various disease conditions. For example, Rimonobant, a CB1 receptor antagonist was once licensed

to treat obesity (Jonsson et al., 2006) and Sativex, a pharmaceutical agent based on cannabis extract has been marketed for neuropathic pain in Canada.

1.3.4 THC, Endocannabinoids and Pregnancy

1.3.4.1Animal work

Animal studies in rats, guinea pigs, hamsters and rabbits demonstrated embryotoxicity and teratological malformations following exposure to cannabis extracts (Park et al., 2004). However, the dosage of cannabinoids used in these studies was well beyond the range normally used by humans. Exposure of Δ^9-THC in early pregnancy caused miscarriage in Rhesus monkeys (Asch et al., 1986) and chronic exposure over a 5 year period resulted in increased reproductive loss in the form of miscarriages, resorptions, fetal deaths and neonatal deaths (Sasernath et al., 1978). In mice there was increase in the incidence of intrauterine deaths and reduced fetal weight following exposure to Δ^9-THC (Park et al., 2004).

1.3.4.2 Human work

Marijuana is one of the most commonly used illicit drugs in the UK and USA and the estimates of cannabis use in pregnancy vary between 10-20% (Park et al., 2004). Similar to that observed in animal models, marijuana use in pregnancy has been associated with fetal growth restriction (Zuckerman et al., 1989), placental abruption, preterm birth, stillbirths, congenital abnormalities and miscarriages (Conner, 1984; Hatch and Bracken, 1986; Felder and Glass, 1998)

Δ^9-THC at concentrations similar to that in the serum of cannabis users has been found to inhibit the proliferation of cytotrophoblast cells, with the expression of the transcription factor thyroid hormone receptor–β1 (TRβ1) and the transcriptional corepressor, histone deacetylase 3 (HDAC3) being affected by Δ^9-THC (Khare et al., 2006). The effect of Δ^9-THC on pregnancy thus could be due to its effect on genes that modulate growth, apoptosis and ion exchange pathways.

In humans, RT-PCR studies have demonstrated the presence of CB1 and CB2 receptors in the human myometrium (Dennedy et al., 2002); human placenta (Kenney et al., 1999, Park et al., 2003) and

fetal membranes (Park et al., 2003). Similarly FAAH activity has been demonstrated in human uterine epithelial cells and in the placenta (Park et al., 2003). AEA has been quantified in human reproductive fluids such as mid-cycle oviductal fluid, follicular fluid, AF and seminal plasma (Schuel et al., 2002).

When AEA was administered chronically to rats it prolonged the duration of pregnancy and increased the stillbirth rate (Wenger et al., 1997). This was associated with a decrease in serum luteinising hormone, progesterone and PGF1α and PGF2α.

CB1 receptor mRNA has also been identified in the mouse uterus using northern blot hybridization technique and RT-PCR (Das et al., 1995). Δ^9-THC and AEA inhibited forskolin-stimulated cAMP formation and this action was inhibited by pertussis toxin. This suggests that uterine CB1 receptor is coupled to inhibitory G protein coupled receptor and thereby can cause uterine contraction (Das et al., 1995). In addition steroid receptor functions could be modulated by cAMP (Power et al., 1991). Thus, it can be speculated that AEA can modulate the function of steroid hormones through the cAMP pathway (Power et al., 1991). Since functional progesterone withdrawal is important in the initiation of parturition, AEA could be involved in this process.

The effect of AEA and Δ^9-THC on term non-laboring human myometrium was investigated by Dennedy et al (2004). Both AEA and Δ^9-THC exerted a concentration dependent relaxant effect on the myometriun *in-vitro,* although the myometrium biopsy specimens were taken from the lower uterine segment, a piece of tissue that should be relaxing during parturition, whereas the fundal region biopsy might have behaved differently. This relaxant effect in lower segment samples was thought to be mediated through CB1 receptors and this has been reconfirmed Helliwell et al (2004).

Additionally, AEA levels have been quantified in the maternal plasma in all three trimesters of pregnancy (10 women in each trimester), 22 women in the term non-laboring state and 25 women in the labouring state (Habayeb et al., 2004). The plasma AEA levels fell from 0.89 ± 14nM in the first trimester to 0.44 ± 0.12nM in the second trimester and remained low at 0.44 ± 0.11nM in the third

trimester. The AEA levels then rose to 0.68 ± 0.09nM in the term non-labouring state. The plasma AEA levels then rose further to 3.7 times the level in the term labouring phase to 2.5 ± 0.22nM. This rise in the plasma AEA level in labour has been suggested to arise from a consequence of functional progesterone withdrawal resulting in lowered FAAH levels (Habayeb et al., 2004). There was also an inverse correlation between the plasma AEA level and the time from sampling to delivery and there was also a trend towards increasing plasma AEA levels with increasing duration of contraction. Thus, it could be that AEA has a differential action on the myometrium in the upper segment and lower segments.

1.4 Hypothesis

Since AEA levels have been shown to rise by almost 4-fold prior to the establishment of labour at term and its levels are modulated by progesterone whose functional withdrawal is pivotal to the onset of labour, it is hypothesized that a rise in AEA levels will occur prior to PTL and this can be used as a biomarker to differentiate among women with threatened PTL those who will deliver preterm from those who will deliver at term.

The studies in this thesis were designed to address this hypothesis. Specifically, I aimed to determine

a. A much improved method of measuring plasma AEA than previously described.
b. An alternative form of AEA extraction, the solid phase extraction technique which is equally effective but less time consuming.
c. Whether plasma could be stored for a day prior to extraction without any evidence of decrease in concentration.
d. Whether the plasma AEA levels get elevated in active labour in women after undergoing induction of labour. This will be a much stronger evidence when compared to the previous study (Habayeb et al., 2004) confirming a possible association of AEA with labour.
e. Whether the plasma AEA level is elevated in women who are at high risk of PTD and to investigate its use as a predictor of PTD in this cohort of women.
f. Whether the plasma AEA levels are elevated in women who delivered preterm amongst a cohort of women who presented with PTL. To compare the efficacy of plasma AEA as a predictor of PTD with that of the gold standard FFN test.

Chapter 2

Materials and Methodology

2.1 Introduction:

AEA has been measured in numerous animal and human tissues including blood, plasma, serum, breast milk, seminal, and oviductal fluid using single quadrupole high performance liquid chromatography-mass spectrometry (HPLC-MS) (De vane et al., 1992; Crawley et al., 1993; Felder et al., 1993; Schuel et al., 1994; Schmid et al., 1995; Hillard et al., 1997; Schmid et al., 1997; Schmid et al., 1997; Berrendero et al., 1999; Di Marzo et al., 2000; Derkinderen et al., 2001; Fride and Shohami, 2002; Schuel et al., 2002; Habayeb et al., 2004; Lazzarin et al., 2004). Research on AEA, however, has been limited due to a number of technical difficulties associated with extraction and measurement, especially the lengthy retention time of AEA, with run times lasting from 35 minutes to in excess of 60 minutes per sample (Schuel et al., 1994; Fontana et al., 1995; Berrendero et al., 1999; Schuel et al., 2002). This has severely restricted the number of samples that can be processed at a single point in time.

Recent studies on the measurement of endocannabinoids by high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) in rat brain and human plasma have shown reduced analysis times from 60 minutes down to 7 minutes. However, the studies have not been validated (Giuffrida et al., 2000) and in addition, comparatively high limits of detection have been reported (Maccarrone et al., 2001). Some studies have been based mainly on AEA measurements in brain tissue rather than in plasma (Koga et al., 1995). Although AEA measurements in human plasma have been made, the absolute levels vary considerably between studies, with values ranging from 0.37nM (Giuffrida et al., 2000) to 2.58nM (Schmidt, Brune et al., 2006) and 3.73nM (Maccarrone et al., 2001). This 10-fold variability between studies suggests that plasma AEA concentrations can be difficult to determine routinely and that a robust, validated, analysis method is required.

Although AEA extraction and measurement from human plasma had been undertaken in our laboratory using a Waters 1525 binary liquid chromatography pump interfaced to a Quattro Ultima triple quadrupole mass spectrometer, (i.e. a HPLC-MS technique), the run time was 30 minutes,

which severely restricted the turnover time between samples and consequently the number of samples that could be measured.

It was therefore important to develop a system for measuring AEA in plasma, which would be fast, robust, highly sensitive and reproducible. The Quattro Premier^{TM} XE Tandem Mass Spectrometer (Waters Ltd.; Figure 2.1) selected for this purpose has an Acquity Ultra High Performance Liquid Chromatography (UPLC) system connected in line with a Quattro Premier Tandem Mass Spectrometer (i.e. MS/MS technique). It has the advantage of a high-throughput due to reduced retention time on the column. The reduced retention times are primarily due to the high pressures associated with ultra high performance liquid chromatography columns. The highly sensitive and specific MS/MS technique also gives a precise result.

2.2 Materials and Methods:

2.2.1 Chemicals:

AEA and octa-deuterated anandamide (AEA-d_8) were obtained from Cayman Chemicals (Ann Arbor, MI). Acetonitrile, chloroform, formic acid and methanol (all HPLC grade) and analytical grade ammonium acetate were purchased from Fisher Scientific (Loughborough, UK). HPLC grade water was obtained using a water purification system (Maxima ELGA, ELGA, and High Wycombe, UK). All mobile phases were filtered through polytetraflurothylene (PTFE) filters, 4 to 7mm in diameter, and with pore size of 0.2µm (Waters Ltd., Elstree, UK) prior to use. Saline was obtained from Fannin UK Ltd. (Reading, UK).

2.2.2 Description of the UPLC-MS/MS instrument:

The various components of the machine are shown in Figure 2.1. These are the

1. Acquity UPLC system consisting of a binary solvent manager, a sample manager, a column manager, a tunable photodiode array and a specialized column and the
2. Quattro Premier^{TM} XE, a tandem mass spectrometer consisting of a sample inlet, a vacuum system and a Mass Lynx control system.

2.2.2.1 Acquity UPLC system:

Chromatography is a technique used for separation of compounds dissolved in a liquid mobile phase. The mobile phase along with analyte of interest and other compounds pass through a stationary phase. The different compounds travel through the stationary phase at various speed depending on their level of interaction with the stationary phase. This results in separation of the analyte of interest from the other compounds. Methods in which the stationary phase is more polar than the mobile phase is called the normal phase liquid chromatography and the opposite is called the reversed phase chromatography.

The Acquity UPLC system columns (2.1 x 50mm) are packed with 1.7µm, bridged, ethylene-silicon, hybrid (BEH) particles that perform under high-pressure conditions (up to 1034 bar). This is the stationary phase of the UPLC system. The smaller diameter of the Acquity UPLC columns provides superior resolution, sensitivity and faster run times when compared to the older HPLC columns. For the measurement of AEA the column is maintained at 40°C whilst the samples are maintained at 4°C. This is achieved with the help of the column manager.

The mobile phases are pumped by the binary solvent manager through to the system. The sample is drawn into the needle from the vials in the sample manager and then injected into the sample loop. Injection volume for samples and standards were 7µl with needle overfill. In the sample loop, the sample gets mixed with the mobile phase and then injected into the column. In the column, the analyte (AEA) undergoes chemical interaction with the stationary phase (Silica particle) and then elutes at a particular retention time. The eluate from the UPLC is delivered into the mass spectrometer for further analysis.

2.2.2.2 The Quattro Premier^{TM}XE

The Quattro Premier^{TM}XE is a high-performance tandem quadrupole mass spectrometer (Figure 2.2). The Quattro Premier is connected inline to the Acquity UPLC system *via* a micro bore plastic tubing. This delivers the sample to a z-spray^{TM} ionization source, where the samples are ionized at

atmospheric pressure. The ions travel through a sampling cone into the first quadrupole, where they are filtered according to their mass-to-charge ratio (M/z). The mass-separated ions pass into the T-wave collision cell where they undergo collision-induced decomposition and then pass on to the second quadrupole. The transmitted ions are detected by a conversion dynode, phosphor and photomultiplier detection system and the output signal is then amplified, digitized and passed to the Mass Lynx control system that presents the results in graphical and numerical forms.

Figure 2.1. The UPLC-Quattro Premier XE Mass Spectrometer System. The UPLC-Quattro Premier XE Mass Spectrometer system consists of the UPLC coupled to the Quattro Premier XE mass spectrometer. Solvents (mobile phase) are pumped into the system from the reservoir bottles. The eluate from the UPLC is delivered through the probe into the ionization chamber of the mass spectrometer. The PC based Mass Lynx controls the entire system. The syringe pump aids in the identification and measurement of very costly analytes available in minimal quantities.

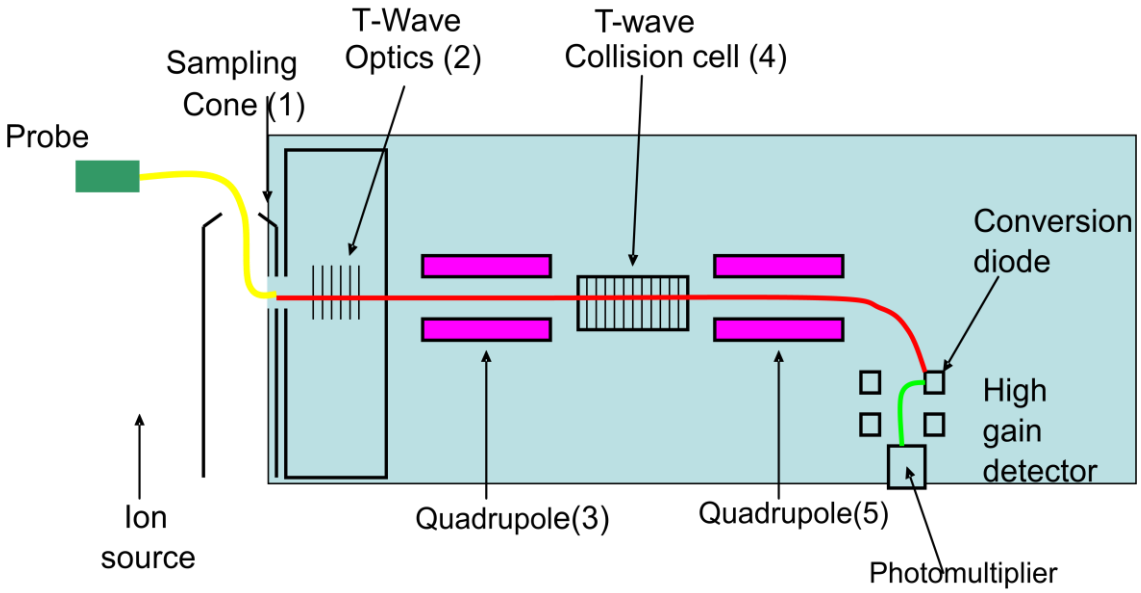


Figure 2.2. Schematic Diagram of a Mass Spectrometer (1) Samples are introduced into the sampling cone and are ionised. (2) Ions are sampled through a series of orifices. (3) Ions are filtered according to their M/z in the first quadrupole. (4) The mass separated ions undergo collision-induced decomposition in the T wave collision cell. (5) The fragmented daughter ions are filtered according to their M/z in the second quadrupole. (6) The ions are detected by the photomultiplier detection system.

2.2.3 Methodology

2.2.3.1 Preparation of standards

AEA and AEA-d_8 stock solutions were prepared by drying original solution under a gentle stream of nitrogen before being reconstituted in acetonitrile at 5mg/ml and 100µg/ml respectively. The stocks were then aliquoted for single use and stored at -20^{o}C. Further dilutions were made in ice-cold acetonitrile on the day of analysis.

2.2.3.2 Extraction of AEA from human plasma Liquid phase extraction (LPE)

At first, extraction was performed using a very simple method provided by Waters in which 20µl of blood was added to 40µl of 0.1M zinc sulphate and 100µl of acetonitrile. After thorough mixing the acetonitrile layer was injected onto the column. Unfortunately, this method of extraction led to inconsistencies in the amount of extracted AEA, a poor calibration curve and a machine system that needed extensive cleaning and significant sample carry over. Therefore, the method of AEA extraction described by Habayeb et al (Habayeb et al., 2004) was used with some very minor variations.

In brief, whole blood (4 ml) was collected in Ethylenediaminetetraacetic acid (EDTA) tubes (Sarstedt Ltd., Leicester, UK) and then immediately transported on ice to the analytical laboratory to be centrifuged at 1200*g* for 30 min at 4 °C to separate plasma from cells. Plasma (2 ml) was then transferred to a 7-ml Kimble scintillation vial (Kinesis, St Neotts, Cambridge, UK). For the purpose of determining extraction efficiency, 2.5 picomole (pmol) of the AEA-d_8 internal standard was added to the 2 ml of plasma prior to thorough mixing with a desktop vortexer. Protein was then precipitated by adding 2 ml of ice-cold acetone followed by mixing of the sample and centrifugation at 1200*g* for 10 min at 4 °C. The supernatant was transferred to a fresh Kimble scintillation vial and the acetone evaporated under a gentle stream of nitrogen gas. Lipid extraction was performed on the remaining mixture by the addition of 2 ml methanol: chloroform (1:2 v/v) followed by gentle mixing by repeated inversion. The sample was centrifuged at 1200*g* for 10 min at 4 °C and the lower organic layer was recovered into a fresh Kimble vial and dried under a gentle

stream of nitrogen before reconstitution in acetonitrile (80 µl). The reconstituted mixture was analysed in the mass spectrometer.

2.2.3.3 Tuning of the Mass Spectrometer

Using a syringe infusion technique, where the samples are injected directly into the MS rather than through the UPLC system, the mass spectrometer was initially tuned to detect AEA (the parent ion) which has M/z of 348.2. Then, the parent ion was fragmented into daughter ions using collision gas, giving rise to 2 daughter ions with a mass to charge ratio of 62.3 and 44.3. This MS-MS technique is a clear advancement over the earlier single MS technique as it is very rare for two compounds that share the same molecular mass to fragment into similar sized daughter ions. This therefore leads to a greater specificity and certainty that the compound being measured is the desired one. Similarly, AEA-d_8 which has a mass to charge ratio of 356.2 and its daughter ions with mass to charge ratio of 70.3 and 52.3 were measured. Fragmentation of the ethanolamine molecule from AEA resulted in these 2 daughter ions.

The parameters that were manipulated to allow optimal detection were.

1. Capillary Voltage - this ionizes the particles from the UPLC by supplying excess charge to droplets.
2. Cone voltage - this helps draw ions into the first vacuum.
3. Cone gas/Desolvation gas - this removes the neutral, non-charged particles from the cone
4. Source temperature/Desolvation temperature – this controls evaporation of the solvent depending on the flow rate.
5. Collision energy, entrance and exit energy - this fragments the parent ion into the daughter ions.
6. Adjustment of each of these parameters produced optimal signals for AEA and AEA-d_8 as shown in Figure 2.3.

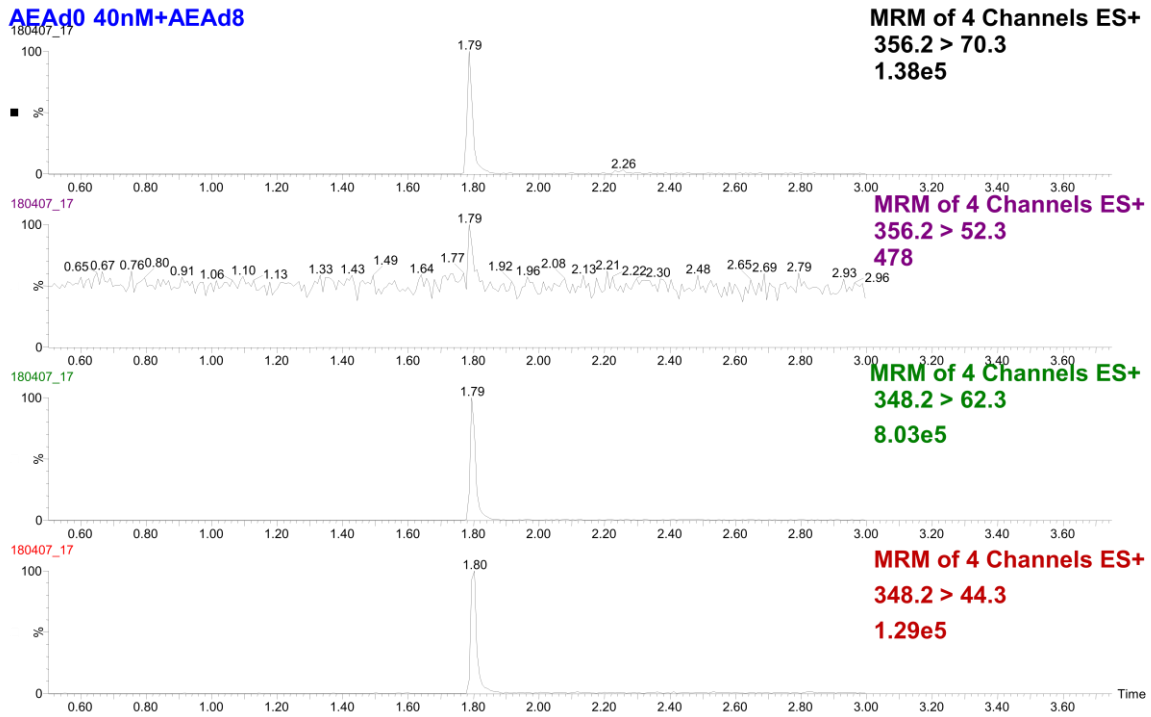


Figure 2.3. Multiple reaction monitoring signal of AEA and AEA-d_8. The two daughter ions of AEA (M/z=348.2) are M/z=44.3 and M/z=62.3 and that of AEA-d_8 (M/z=356.2) are M/z=70.3 and M/z=52.3. MRM stands for multiple reaction mode of analysing sample by the mass spectrometer. M/z is mass to charge ratio. 348.2 is the M/z ratio of AEA and 44.3 and 62.3 are that of the daughter ions, which are produced as a result of collision. 356.2 is the M/z of $AEAd_8$ and 52.3 and 70.3 are that of the daughter ions. ES^+ stands for positive electron spray technique of ionization of the molecules. E5 stands for the intensity of electrons detected by the mass spectrometer. The retention time is 1.8 minutes.

2.2.3.4 UPLC:

The chromatography conditions were also optimized. The mobile phases initially used were:

A- Water and

B- Methanol

The gradient protocol that was initially devised was based on the previous methodology (Bifulco and Di Marzo., 2002; Giuffrida et al., 2000; Habayeb et al., 2004) that had been modified by the engineers at Waters Ltd. The initial run time was for 8.1 minutes, as shown in Table 2.1.

Previous analytical HPLC-MS methods used sodium [Na^+] adducts of AEA as the target ion for measurement, since this gave the highest peak (Habayeb et al., 2004) after chromatography. The Na^+ adduct is thought to be produced from the Na^+ ions within the system found as a contaminant released from glass. Both the Na^+ and the hydrogen adducts [proton, H^+] for AEA were both identified during the initial tuning, however, the $[AEA+Na]^+$ adducts were refractory to fragmentation into measurable daughter ions. Therefore, the daughters ions of the protonated adduct $[AEA+H]^+$ were used to optimise the system further.

To improve the measurement obtained from protonated adducts, thorough cleaning of the glassware was used to minimise Na^+ and other contaminant. Furthermore, 0.1% formic acid and 2mM ammonium acetate were added to the mobile phases to promote protonation of AEA to the hydrogen adduct. The final mobile phase therefore consisted of:

A- 2mM ammonium acetate with 0.1% formic acid in water

B- Acetonitrile containing 0.1% formic acid

Table 2.1. Gradients of the mobile phase used initially in the ULPC.

Time (min)	Flow (ml/min)	Mobile phase A (%)	Mobile phase B (%)
0	0.600	25	75
5	0.600	0.0	100
8	0.600	0.0	100
8.1	0.600	25	75

Mobile phase A=Water and Mobile phase B=Methanol

Previous methods had used chloroform: methanol mixture to reconstitute the dried AEA for injection into the UPLC, however, Waters advised that chloroform should be avoided in the UPLC-MS/MS system, as it would damage the delicate plastic tubing. Therefore, acetonitrile was used to reconstitute the AEA and to produce the calibration curves as AEA is soluble in acetonitrile and because it matched the original mobile phase.

Solvent standards were prepared using AEA and AEA-d_8 and calibration curves were prepared as described in section 2.4.1. The calibration curves were then used to measure AEA in plasma extracts.

2.2.4 Problems in method development

A number of problems were encountered during method development. These were:

A- Peak tailing (Figure 2.4)

Various experiments were conducted to improve the shape of the peaks such as changing the gradient of flow and the rate of flow in the UPLC to reduce peak tailing. In addition, changes were made in the different parameters of the mass spectrometer; the dwell time was reduced from 0.1 to 0.05 seconds. This increased the number of scans that the MS undertook in a given time, which during the peak for AEA increased from 12 to 20 scans. Multiple injections of AEA standards demonstrated that a range of capillary voltages from 0.6 to 3.0kV produced different shaped peaks and intensities, however, 0.6kV was found to provide the optimal signal. Similarly, flow rates of 0.5-1.0ml/min were investigated but 0.7ml/min yielded results with the greatest peak height and best signal-to-noise ratio (the ratio of the amplitude of the signal from the analyte to that of the background noise) and improvements in peak tailing as shown in Figure 2.3.

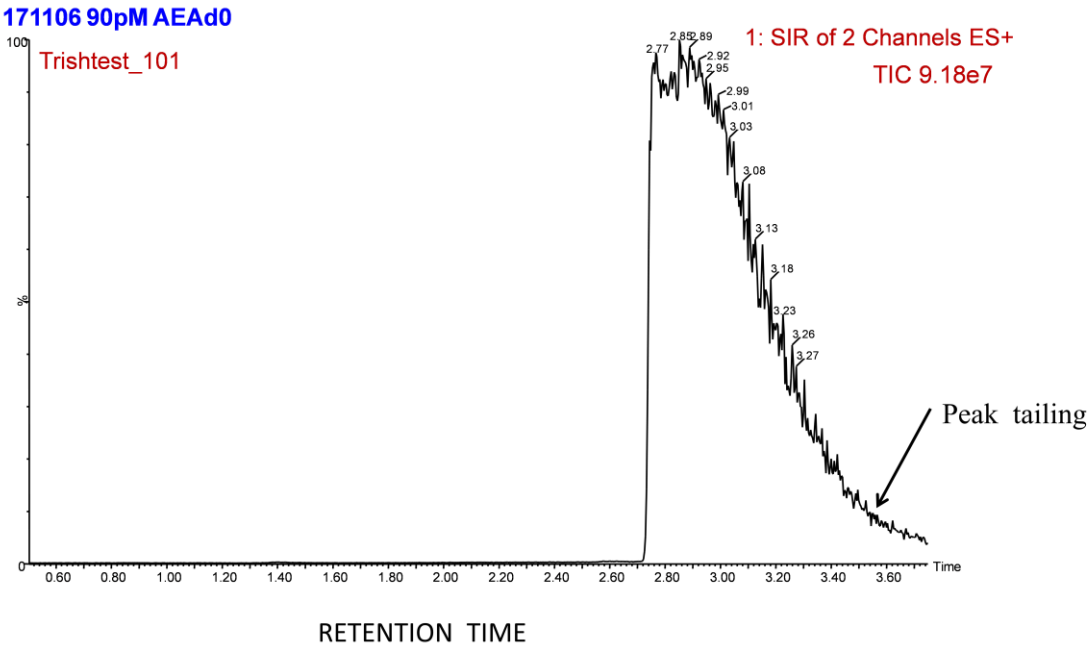


Figure 2.4. SIR graph of AEA showing the peak tailing off. SIR stands for selected ion recording mode of mass spectrometer, where the quadrupole allows a single selected ion with a particular M/z ratio to pass through to the detector. Note the very rapid initial detection of AEA.

B-Carryover of AEA

Concentrations of AEA in excess of 20nM injected into the column were shown to carry over into the subsequent injection of blank mobile phases. So, significant levels of AEA were detected in the chromatograms when a 'blank' sample of acetonitrile was injected into the system. This effect is known as 'carry over' (Figure 2.5). Therefore, lower concentrations of AEA were used to prevent the column from being overloaded. To ensure that the system remained clean and in good condition, an overnight wash of the column with methanol, a stronger needle wash, and the routine application of an ethanol wash of the entire system after every injection of AEA were used. This helped to overcome the problem of sample carryover and system contamination.

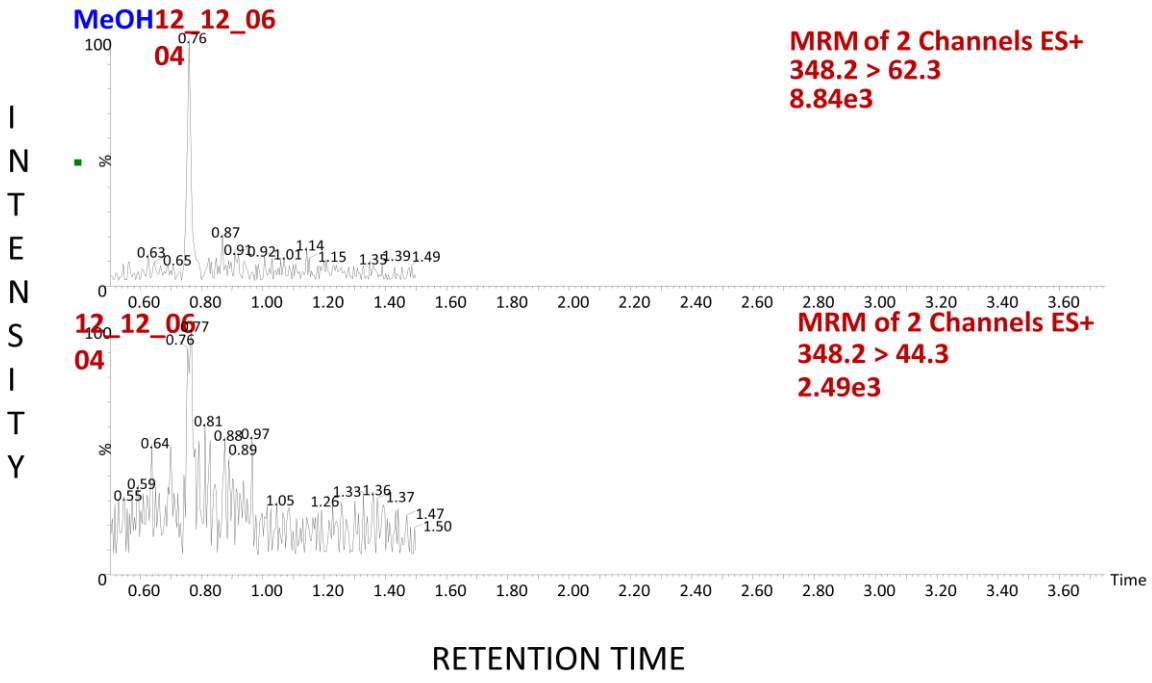


Figure 2.5. MRM elution profile showing carryover of AEA. The elution profile obtained after injecting a blank methanol sample into the column. The profile demonstrates a peak at 0.76seconds for daughter ions with m/z 62.3 and m/z 44.3 of AEA-$_{d0.}$ The peak is from AEA as it is detected at the same retention time as AEA and the mass spectrometer is set at the MRM mode to detect the daughter ions of AEA

C-High Background noise

Initially, a high background noise was encountered. This was solved by instigating a regimen of regular cleaning of the glassware and the machine and by using good quality solvents that were filtered and replaced daily.

D-Poor response for AEA-d_8

There was a large difference in the detector response for similar concentrations of AEA-d_8 and AEA. So, the tuning for AEA-d_8 was re-examined. Three daughter ions for AEA-d_8 were found with mass to charge ratio of 62.3, 63.3, and 70.2. The daughter ion with m/z 63.3 gave the maximum response of 102785 (area under the curve) and was similar to those produced by AEA in similar concentrations whereas the response from daughter ion 62.3 and 70.2 were only 72194 and 4845 respectively. Therefore this daughter ion was used in subsequent measurements.

	Daughter ion (70.2)	Daughter ion (62.3)	Daughter ion (63.3)
Response	4845	72194	102785

The lower response of the AEA-d_8 that was originally present necessitated a higher concentration of AEA-d_8 to be added to the plasma samples to ensure its detection. Once other parts of the method had been optimized, 2.5 pmoles of AEA-d_8 were added instead of 25 pmoles. This reduced the error caused by the auto-conversion of a small percentage of AEA-d_8 to AEA. At the same time, the volume of AEA-d_8 added was increased from 4µl to 40µl. This reduced the pipetting errors and increased reproducibility.

2.3 Validation of the UPLC-MS/MS analysis method for the measurement of plasma AEA

The UPLC–MS/MS method was validated according to Federal Drug Administration (FDA) guidelines (www.fda.gov). Linearity for the assay was determined using 15 eight-point standard curves (1.66 to 133fmol of AEA on the column) and linear regression analysis.

Consistency of the retention time was investigated after 20 injections of 133fmol of AEA. Accuracy, defined as the deviation of the observed concentration from the expected concentration was calculated for solvent containing three different concentrations of AEA representing 3.33, 6.65 and 133fmol on the column (20 samples).

Precision defined as repeatability of the measurement, was calculated after 20 repeat injections of 19, 0.95, and 0.237nM AEA standards in acetonitrile (133, 6.65, and 1.66 AEA on column). Limits of quantification and detection were defined as AEA responses which yielded a signal to noise ratio, without smoothing, of greater than 10 and 3, respectively. These were calculated for AEA-d_8 extracted from both plasma and saline. The limits of quantification for AEA were determined only from saline because of the presence of endogenous AEA present in all plasma samples.

AEA and AEA-d_8 were both eluted from the UPLC and detected by the tandem mass spectrometer at 1.67 ± 0.0009 min after injection and had an observed relative standard deviation (RSD) of 0.05% for the retention time after 20 injections. Retention time was consistent over the lifetime of the column which represented in excess of 9000 injections. Linearity, as derived from 15 calibration curves was described by the equation:

Response ($y=(2.48 \pm 0.14)$ [AEA]nM + (0.004 ± 0.04) with a mean r^2 value of 0.999.

This response was linear over the non-extracted concentration range of 1.66 to 133fmol on the column, which is equivalent to 0.23–19nM of AEA

Precision for the 20 injections of the 19nM AEA standard, equivalent to 133fmol AEA on the column had a mean of 19.00 ± 0.70nM and a relative standard deviation (RSD) of 3.7%. This was the best precision described for AEA. Similarly, precision for 20 injections of 0.24nM (1.66fmol) and 0.95nM (6.65fmol), which resemble the lower AEA concentrations observed in plasma yielded mean values of 0.238 ± 0.009nM and 0.945 ± 0. 046 with RSD values of 3.9% and 4.8%, respectively.

For non-extracted AEA and AEA-d_8, the limit of quantification (LOQ) were 0.22fmol on the column (a signal to noise ratio >10) and the limit of detection (LOD) were 0.055fmol on the column (signal to noise ratio >3). These represented significant improvement over the previous best LOD. The LOD and LOQ for AEA-d_8 extracted from 2ml of plasma were 18.75fmol/ml and 25fmol/ml, respectively. Likewise, AEA and AEA-d_8 extracted from saline yielded LOD and LOQ values of 0.78fmol/ml and 6.25fmol/ml respectively. On the column, the accuracy for 3.33fmol of AEA was 97.5 ± 9.5%, 98.5 ± 6.1% for 6.65fmol and 104.5 ± 3.2% for 133fmol.

2.4 Description of the Final Method

The UPLC-MS/MS system comprised an Acquity UPLC system connected in line with a Quattro Premier tandem mass spectrometer (Waters Ltd., Elstree, UK) as previously described in section 2.2.2. Analytes were quantified using tandem electrospray MS in positive-ion mode (ES^+). The mass spectrometer tuning parameters which gave the best signals for AEA, allowing precise quantification of the analyte were a capillary voltage of 0.6kV, a cone voltage of 18V, a source temperature of 120^{o}C, a desolvation temperature of 440^{o}C, a cone gas flow of 49L/h, and a desolvation gas flow rate of 800L/h. The entry, collision and exit energies were –2, 17, and –17 eV, respectively. Product ions were monitored in multiple reaction monitoring mode (MRM).

The column used was an acquity UPLC BEH C_{18} (2.1x 50mm) maintained at 40^{o}C. The mobile phases were A (2mM ammonium acetate containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid).

The final gradient which gave the optimum separation of AEA and the best peak shape is shown in Table 2.2. The total runtime was 4 minutes and both AEA and AEA-d_8 were eluted from the UPLC and detected by the MS/MS at 1.67 ± 0.0009 minutes after injection. Samples were maintained at 4ºC. Injection volume for each samples and standards were 7µl with overfills.

Table 2.2 Final gradient of the mobile phase in the UPLC system

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	80	20
0.5	20	80
1.5	0	100
3.5	80	20

Mobile phase A=2mM ammonium acetate and 0.1% formic acid in water and Mobile phase B=acetonitrile with 0.1% formic acid.

2.4.1 Development of calibration curves:

Eight point calibration curves were performed in triplicate (Figure 2.6) and AEA peaks were integrated using Masslynx software version 4.1 (Waters Corp., Milford, MA). Quanlynx software (Waters Corp.) quantified the concentration of AEA using a linear regression model whereby calibration curves of concentration against relative response could be calculated from the equation

$$\text{Relative response (y)} = \frac{\textit{Peak area}}{\textit{(IS area)/ [AEA- } d_8\textit{]}}$$

Where y=mx +c

M=gradient of the calibration curve

C=intercept

X=concentration of AEA

IS=peak area of the AEA-d_8 internal standard

[AEA-d_8]=concentration of the internal standard.

Peak area=peak area of AEAd_0.

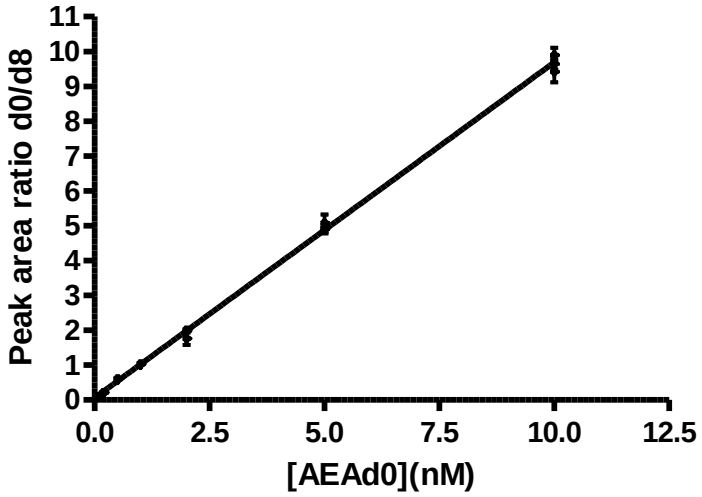


Figure 2.6. Calibration Curve in acetonitrile. The concentrations of AEA and the mass spectrometer response for each standard can be fit to a straight line, using linear regression analysis to create calibration curves.

2.5 Validation of Plasma AEA Levels in Pregnancy

2.5.1 Introduction

In order to validate this method, the plasma AEA levels in pregnant women during the three trimesters of pregnancy and in labour were measured and compared to data previously obtained and published by the Endocannabinoid Research Group (Habayeb et al., 2004).

2.5.2 New Method

In order to replicate the experiment, we conducted the study in 57 pregnant women; 7 were in the first trimester, 14 in the second trimester, 8 in the third trimester, 8 in the term non-labouring group and 8 in term labouring group.

The levels of AEA (Mean ± SEM) during the first three trimesters remained static at 0.90 ±0.10nM, 0.90 ± 0.08nM and 1.14 ± 0.11nM respectively. Thereafter the levels rose to 1.27 ± 0.13nM in the term non labouring women and further rose significantly to 2.2 ± 0.31nm in the term labouring women (Table 2.3) (Figure 2.8).

Table 2.3. Plasma AEA levels (Mean ± SEM) in the various groups

Patient Group	Number of women	Plasma AEA (nM)
1st trimester	7	0.89 ± 0.10
2nd trimester	14	0.90 ± 0.08
3rd trimester	8	1.14 ± 0.11
Term non-labouring	8	1.27 ± 0.13
Term labouring	8	2.2 ± 0.31

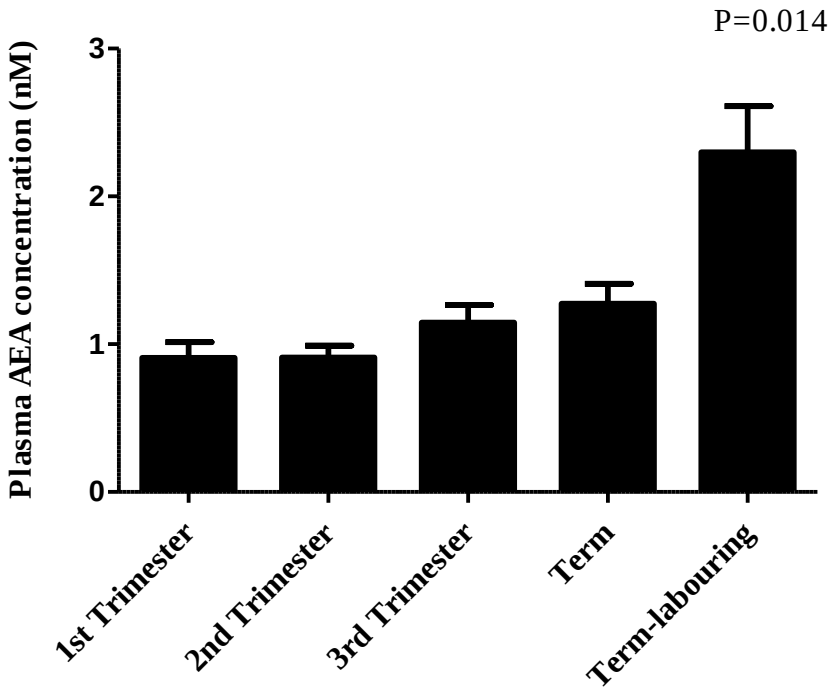


Figure 2.8. Comparison of the plasma AEA concentrations in pregnancy, term non-labouring and labouring state. Data are presented as the Mean ± SEM. Pairwise comparison was performed using two-tailed paired Students' *t*-test with Welch's correction for uneven variances (P=0.0147, Term labouring compared to term non-labouring plasma AEA levels).

2.6 Studies on storage:

Since the timing of human birth is unpredictable, it was anticipated that blood collection from the women with threatened preterm labour could occur at anytime during the day or night. This would inevitably lead to practical difficulties in conducting the collection and processing of blood samples. The easiest solution would have been to store plasma for later processing. So, the effect of storage on plasma AEA levels was investigated.

For this, duplicate blood samples were obtained from 14 volunteers. One of the samples was processed immediately and plasma AEA levels measured. The other sample was also centrifuged at the same time, but the recovered plasma was stored at -80ºC for 24 hours. This was then allowed to thaw on ice and processed for AEA level measurements.

The data indicated that plasma AEA levels in the fresh and the stored plasma did not vary significantly. The mean plasma AEA level in the freshly processed samples was 1.42 ± 0.48nM compared to 1.44 ± 0.48nM in the stored samples (P=0.729; Student's paired *t*-test) (Figure 2.9).

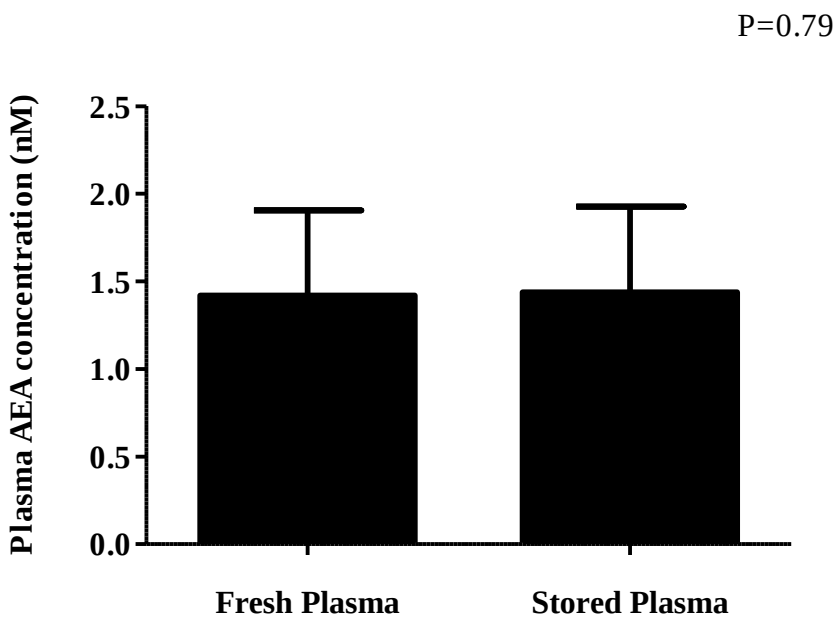


Figure 2.9. Comparison of plasma AEA level between fresh and stored plasma. The plasma AEA concentration (Mean ± SD) in fresh plasma 1.42 ± 0.48nM was not significantly different from mean plasma AEA concentration of 1.44 ± 0.48nM in stored plasma (Students paired‘t’ test; P=0.79).

2.7 Solid Phase Extraction vs. Liquid Phase Extraction:

2.7.1 Introduction

As the extraction of plasma AEA using the LPE method described by Habayeb et al (2004) is tedious and laborious, it was decided that the efficacy of a solid phase extraction (SPE) method to measure AEA concentration should be investigated. The SPE technique separates the analyte from impurities by utilizing the affinity of solutes dissolved in liquid (mobile phase) for a solid (stationary phase) through which the sample is passed. Reversed phase SPE is normally used to separate non-polar compounds from polar ones and so this method was used. The stationary phase of the reversed phase SPE is usually composed of silicon particles with a hydrocarbon side-chain and it is this side-chain that interacts with non-polar compounds, like AEA.

2.7.2 Materials and Method

2.7.2.1 Material

The Oasis HLB 1cc syringe shaped cartridge (Waters Ltd) was used for this experiment. This was recommended by Waters chemist. The cartridge contains a water-wettable reversed phase sorbent particle measuring 30μm each and is mounted on to an extraction manifold, as shown in Figure 2.10. The manifold is connected to a vacuum pump and the application of a vacuum speeds up the extraction process by pulling the sample and washing solutions through the stationary phase.

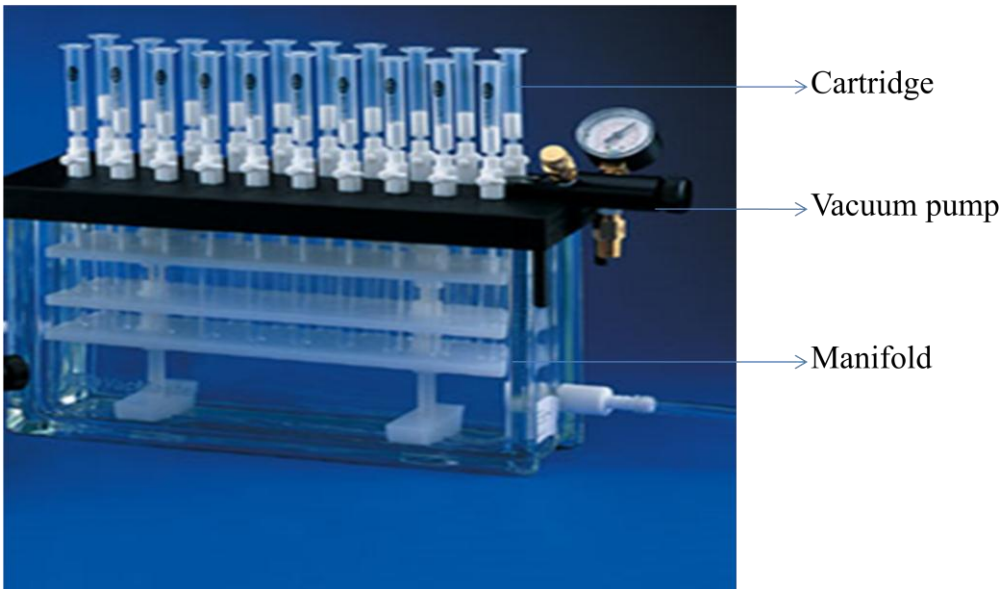


Figure 2.10. Solid Phase Extraction Manifold. 10-15 cartridges containing the stationary phase through which the sample is introduced, is held by the manifold. The sample tubes collect the analyte eluting from the cartridges. The vacuum pump applies vacuum to assist with sample travel through the stationary phase.

2.7.2.2 Method

To estimate sample recovery, 2.5pmol/ml AEA-d_8 internal standard was added to plasma (0.5ml) and for those samples where volumes were less than 1ml, deionized water was added to bring the final volume to 1ml. All samples were then thoroughly mixed on a bench top vortexer and subsequently centrifuged at 16,000*g* for 5min at 4°C.

Meanwhile, the Oasis HLB 1cc cartridge (Waters Ltd.) was preconditioned and equilibrated with 1ml of methanol and 1ml of H_2O *via* the vacuum manifold (Waters Ltd). Samples were then slowly introduced onto the cartridges under a gentle vacuum at a flow rate of approximately 1ml/min. This resulted in the adsorption of AEA within the sample (along with the non-polar impurities) to the reversed phase sorbent particles. The cartridges were then washed with 1ml of 40% aqueous methanol to remove some of the impurities. The adsorbed AEA was then finally eluted from the sorbent particles into 1ml of acetonitrile into sample tubes.

The elute was then dried under a gentle stream of nitrogen, reconstituted in 80µl of acetonitrile and the AEA levels measured using the UPLC-MS/MS method described in section 2.4.

2.7.3 Effect of Extraction Procedure on AEA Levels Measured from Plasma.

To compare the robustness and accuracy of the SPE technique in relation to the normal LPE technique employed for the analysis of AEA in plasma (Habayeb et al., 2004), the AEA levels in pooled depleted plasma (Plasma from multiple donors pooled together, obtained from a blood bank) using both extraction techniques, over a period of 3 days, were assayed.

There was no significant difference in pooled plasma AEA concentration between the two methods (n=15). The mean value observed using either extraction technique was 1.18nM. The intra-day variability and inter-day variability for extraction of AEA using both techniques was less than 5% and approximately 12% respectively using both techniques. The two methods were, therefore, regarded as highly comparable in these respects.

There was a significant difference in the extraction efficiency between the two methods The SPE method demonstrated a 60 ± 3.3% extraction efficiency, whereas the LPE method produced a mean extraction efficiency of only 19 ± 2.7%. Therefore, the SPE method was approximately 3 times more efficient than the LPE in extracting AEA from plasma. The benefit of this was that smaller samples (0.5 ml) could be used to adequately quantify AEA from plasma using the SPE method when compared to the LPE (2 ml) method.

The LOQ for SPE and LPE were 8 and 25fmol/ml plasma, respectively whilst the LOD values were 4 and 18.75fmol/ml respectively (Table 2.4). The time to process the samples were shorter and several samples could be processed at the same time using the SPE method. This was a significant advantage over the LPE method**.**

Table 2.4 Comparison of extraction efficiencies of SPE and LPE methods

Parameter	LPE	SPE
Extraction efficiency (%)	19	60
Intraday variability (%)	2.9	4.0
Interday variability (3 days, %)	12.6	11.6
LOD (pM)	18.75	4
LOQ (pM)	25	8
[AEA] (nM, mean ± SD, n=15)	1.18 ± 0.15	1.18 ± 0.14
Approximate processing time for 10 samples (hr)	3.5	0.4
Amount of plasma required per test (ml)	2	0.5

LOD-Limit of detection; LOQ- Limit of quantification

2.7.4 Validation of SPE Method in Measuring Plasma AEA Concentration in Pregnant Volunteers.

To further demonstrate that the plasma AEA concentrations are comparable irrespective of the extraction technique employed, AEA concentrations were measured in plasma obtained from pregnant volunteers at term. Figure 2.11 demonstrates that for all samples the AEA concentrations were not significantly affected by the extraction method used. The AEA concentrations obtained following SPE relative to concentrations observed following LPE were 100.75 ± 8.67% (n = 56).

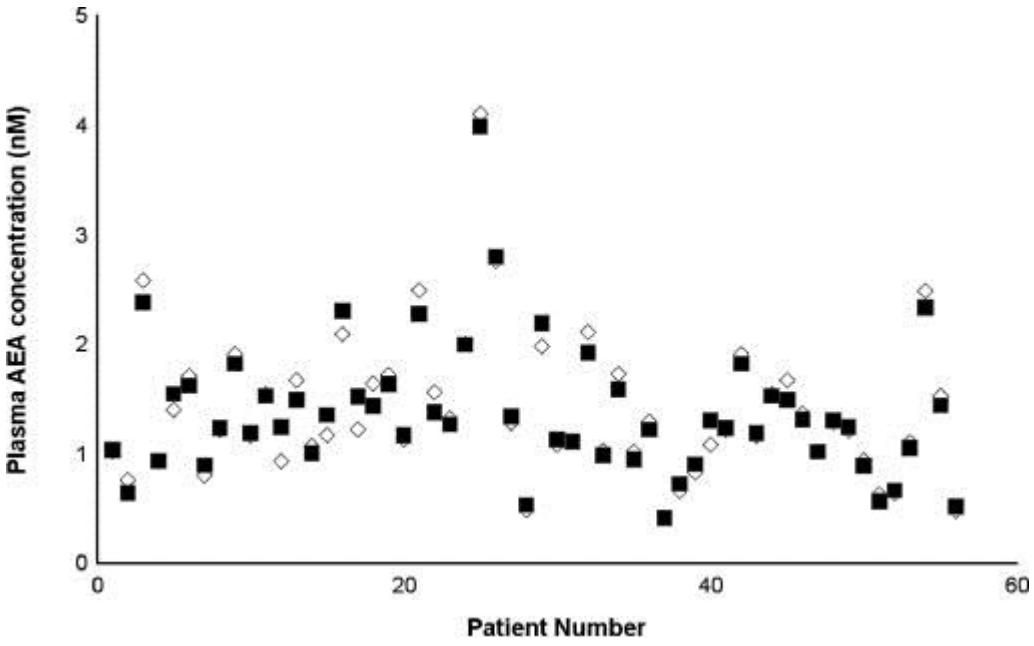


Figure 2.11. **Comparison of Plasma AEA concentrations measured using SPE and LPE technique.** Figure published in Marczylo et al., 2009. AEA was extracted from plasma collected from 56 labouring or non-labouring women at term using SPE (■) and LPE (◇). Results are representative of the mean AEA concentrations following three replicate injections onto the UPLC–MS/MS.

2.8 Discussion:

The combination of UPLC with MS/MS yielded a method for the analysis of AEA at concentrations found in plasma with sensitivity, accuracy, and precision which were a significant improvement on previous methods and which make this method suitable for analysis of many clinical samples.

The MS/MS technique is better than the MS technique employed by our group previously. The MS/MS technique identifies both the parent compound as well as the unique daughter ions into which the parent compounds fragments. This increases the confidence that the compound measured is the analyte of interest, as it is very unlikely that compounds that share the same molecular weight will fragment into similar daughter ions.

The superior efficacy of this methodology, especially the lower LOD and LOQ, means that AEA could be measured in a smaller volume of plasma sample or in other biomatrices with lower concentrations of AEA. The high degree of precision and accuracy means that plasma AEA levels in study populations can be measured accurately and thus provide greater confidence in the interpretation of results.

The reduced run times also allowed multiple samples to be processed over a shorter time period thereby allowing a faster turnover of samples. The increased sample analysis rate also meant that a larger number of replicates could be measured, ensuring increased accuracy of the final measurement. The faster analysis time for this method also represents a financial saving in terms of solvents, labour time, and consumables.

However, a major problem with developing this improved method, was the time taken to optimise each and every component, although the pay-off for all the hard work, was the production of a robust, fairly simple and reproducible method that is transferrable to other users and possibly other biomatrices.

The validation study comparing the levels of plasma AEA concentration at various stages of pregnancy and in labour showed that the plasma AEA levels were similar in the 1st trimester and second trimester. There was a non-significant increase in the level of plasma AEA throughout pregnancy but there was a significant increase in the levels during term labour. In comparison with the studies performed previously (Habayeb et al., 2004), the plasma levels reported here in women in the second and third trimester of pregnancy were higher than those reported. In the previous study, plasma AEA levels significantly decreased from the first trimester and remained low throughout gestation, only rising again at term in women who were not in labour. Examination of the present study indicated that there was no decrease in plasma AEA levels in the second trimester, but there was a gradual non-significant rise in plasma AEA levels throughout gestation. It is not readily apparent why the differences during gestation as seen in the previous study were not observed in the current study.

However, the significant increase in plasma AEA levels with labour confirmed the previous findings in the present study although the percentage increase was not as much. The smaller sample size, in the present study, could be one of the reasons behind these discrepant observations. Nevertheless, the data from the current study are similar to those produced previously and are just as valid as those presented earlier and perhaps more so, because the UPLC-MS/MS method has a greater precision and sensitivity.

The demonstration that short term storage had little or no effect on the absolute concentrations of plasma AEA measurement is encouraging because it allows some flexibility in the procurement and processing of samples for plasma AEA concentration measurements. Whether this is applicable to all biomatrices is subject to further investigations, but so long as any source of the enzymes that either synthesise or degrade AEA are not present in the separated plasma, there should be no reason to suppose that short-term storage of extracted material could not be analysed using this method.

The use of the SPE method, which increased the extraction efficiencies of AEA and AEA-d_8 from plasma, suggests that the combination of improved sensitivity and increased extraction efficiencies

from smaller volumes of biomatrices would assist in the accurate measurement of more difficult to retrieve samples, such as exudates from tissues or small samples from efferent veins. It would also help to measure plasma AEA levels in those patients from whom only a limited amount of blood could be harvested, either due to patient choice or difficulties with collapsing veins.

Nevertheless, despite all of these improvements, it was decided to process all samples for this thesis using the standard LPE technique. This was for 2 main reasons:

(1) Sample collection had already started before the SPE method was validated and therefore it was essential to process all the samples using one particular method, and

(2) This would allow for comparison of the results with those obtained by Habayeb et al. in their seminal work published in 2004.

The demonstration of a quick and robust method for the measurement of plasma AEA in pregnant women meant that plasma AEA levels could then be measured in women in physiological and pathological states of labour including women undergoing induction of labour, women who are at high risk of PTD, women admitted with threatened preterm labour and women admitted with ruptured membranes, all of which will be described in the following Chapters.

Chapter 3

Plasma AEA concentration and induction of labour

3.1 Introduction:

As discussed in Chapter 1, the study by Habayeb et al showed that the plasma levels of AEA in women labouring at term were 3.7 fold higher than the levels in non-labouring women at term (Habayeb et al., 2004). Moreover defective cannabinoid receptor signalling in the pregnant CB1 knockout mouse has been shown to be associated with elevation of CRH and spontaneous onset of PTL (Wang et al., 2008).

Additionally plasma AEA levels have been shown to be reduced by progesterone, mediated through its action on FAAH (Maccarone et al., 2000) and functional progesterone withdrawal is linked to the onset of labour. AEA has also been shown to increase the production of PGs by gestational tissues like chorioamnion (Mitchell and Sato et al., 2008). Additionally, since AEA metabolism by FAAH leads to the production of AA, AEA could be a source of AA which is the principal metabolite from which PGs are produced (Di marzo et al., 2007). It is likely that plasma AEA levels could be elevated during the process of labour and that the endocannabinoid system is involved either in the initiation or progression of labour.

The study by Habayeb et al., was cross-sectional and a better study design will be one in which the women in labouring and non-labouring states act as their own control. An ideal population to study is therefore women undergoing induction of labour where plasma AEA levels are measured longitudinally. The results from such a study might also provide a greater depth of understanding about the role of AEA in the process of labour.

Induction of labour is the stimulation of uterine contractions in an otherwise pregnant quiescent uterus for various maternal and fetal indications. The rates of induction of labour in UK hospitals remained fairly constant, being around 20% between 1995 and 2000 (Mackenzie et al., 2006)

The indications for induction of labour can be subdivided into maternal, fetal, social or a combination of these. The commonest indications are prolonged pregnancy and preeclampsia.

Others include current antepartum haemorrhage, diabetes mellitus, red-cell alloimmunisation, demonstrable placental failure and previous unexplained stillbirth at term (Mackenzie et al., 2006).

Pharmacological and mechanical methods are often used for inducing labour. As a general principle, the simplest inductions of labour are those performed when the cervix is 'ripe' and would have probably preceded a spontaneous onset of labour by a few hours to a day or two, and a mechanical technique alone will suffice. Where the cervix is very unripe, induction of labour is usually accomplished by using a sequential combination of pharmacological agents and a mechanical stimulus. PGs and oxytocics are the most commonly used pharmacological agents while sweeping of the fetal membranes and amniotomy are the common mechanical methods used. Recently various agents including misoprostol, antiprogestogens, relaxin, nitric oxide and DHEA have been investigated as pharmacological agents to induce labour (Mackenzie et al., 2006).

Induction of labour is not without risks. It may fail resulting in caesarean section, or be complicated by uterine hyper stimulation (potentially leading to either fetal distress or hypoxic damage to the fetus or uterine rupture), cord prolapse, chorioamnionitis and amniotic fluid embolism. The risk of instrumental delivery is increased by 1.5 fold and that of caesarean section is increased by 1.8 fold. All these risks are greatest when induction of labour is started when the cervix is unripe (Chamberlain G and Zander L., 1999). This is evident in Figure 3.1 which demonstrates the effect of ripeness of cervix on the outcome of induction of labour.

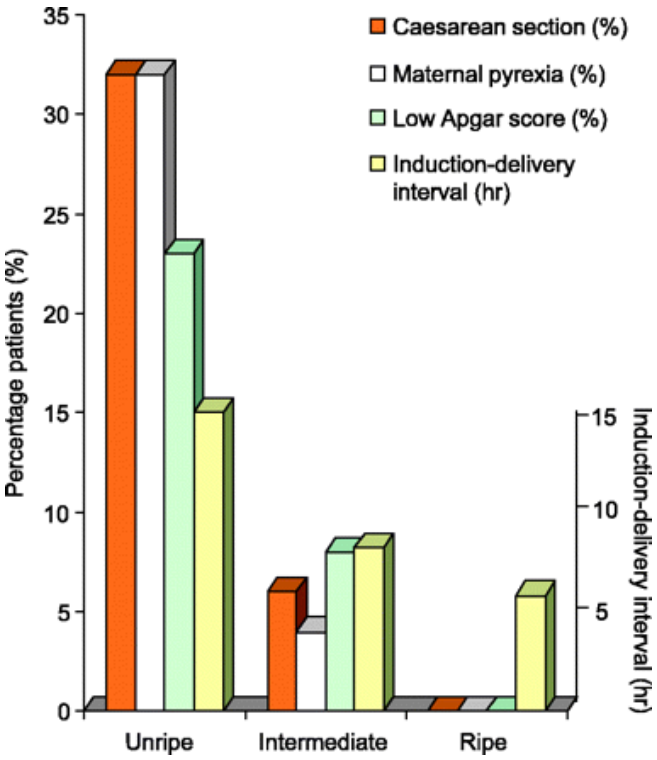


Figure 3.1. Effect Of Cervical Ripeness On the Outcome Of Induction of labour Reproduced from Calder *et al.* 1974.

Since the state of the cervix (cervical ripeness) influences the success of induction of labour clinical tools have been developed to assess the cervical favourability and relate it to the success of induction of labour. These include the Bishop's score, cervical ultrasound, presence of vaginal FFN, and serum nitrite levels. Of these, the semi quantitative assessment of the cervix as described by Bishop is the most frequently used tool (Bishop et al., 1964) and is used routinely to assess the cervix as part of the induction process. The details of the constituents of the Bishop's score are shown in Table 3.1. If the score exceeds 7, the chances of successful delivery are almost equivalent to that following spontaneous labour. On the other hand if the score is less than 4 then the risks of failure of induction are high.

The main aims of the studies in this chapter were therefore to

1. investigate changes in the plasma AEA level in women undergoing induction of labour and
2. to correlate pre-induction plasma AEA levels and the percentage change in plasma AEA levels , Bishop's score and parity with the induction to delivery interval.

Table 3.1. Components Assessed For Bishop's Pelvic Scoring system (Bishop 1964).

Score	**0**	**1**	**2**	**3**
Station of the presenting part (cm)	-3	-2	-1/0	+1
Cervical dilatation (cm)	Closed	1-2	3-4	5+
Cervical effacement	0-39	40-59	60-79	80+
Cervical Consistency	Firm	Medium	Soft	
Position of the cervix in pelvis	Posterior	Mid	Anterior	

3.2 Subjects and Methods:

3.2.1 Subjects

A cohort of 50 women who underwent induction of labour for various obstetric indications at the Leicester Royal Infirmary during the period July 2007- July 2008 were recruited. The inclusion criteria were singleton pregnancy and willingness to provide a blood sample prior to the initiation of induction and again in the active phase of labour. Since each woman acted as her own control it was not necessary to have any exclusion criteria. All the volunteers signed a written informed consent to take part in the study, which was approved and conducted according to the guidelines of the Leicestershire and Rutland Ethics Committee.

Variables obtained from each volunteer included age, BMI, parity, gestational age at recruitment, and the indication for induction of labour. Additional information obtained included the Bishop's score at induction, the duration of labour, the type of analgesia used, the induction to delivery interval and the type of delivery.

Induction of labour was by one of several methods; artificial rupture of fetal membranes (ARM), administration of vaginal PGE_2 pessary, syntocinon infusion or a combination of procedures. Once labour was established different forms of analgesia were offered, these include entonox, epidural or opiates.

3.2.2 Methods:

The plasma levels of AEA were measured using the LPE method as described in Chapter 2.

3.2.3 Statistical Analyses:

Power analysis of published AEA data (Lam et al., 2008) with $\alpha = 0.05$ and $\beta = 0.8$ indicated that the minimum number of subjects required in the study group that would allow a significant change in plasma AEA concentration to be observed was 29. In order to totally avoid a type II error it was decided to recruit 50 subjects. (A type 11 error is failure to reject a false null hypothesis).

Variables that were normally distributed are presented as means ± SD and data that did not follow a Gaussian distribution are expressed as medians and inter quartile range. The plasma AEA concentrations before and after induction of labour were compared using two-tailed Student's paired t-test. A P value of <0.05 was considered statistically significant. The statistical software used was Graphpad Instat version 3, (GraphPad Software, San Diego, CA, www.graphpad.com). Correlations and general linear model analyses were performed with the SAS software package (Version 9.1, SAS Institute, Cary, NC, USA). Spearman's rho was used to assess correlations between induction-to-delivery interval, Bishop's score and percentage change in plasma AEA levels. All associations were pre-assessed for linearity using scatter plots. A fixed- effects multiple regression model was used to determine the variable that significantly predicted the natural log-transformed induction-to-delivery interval. The variables that were analysed included Bishop's score, parity, pre-induction plasma AEA level and percentage change in plasma AEA level. A backward stepwise model was used to eliminate non significant variables. The residual variables from the final model were then assessed for normality and the association between these residuals and predicted values were also assessed. The effect of method of induction, based on two categories; those treated with prostaglandins and those treated with other methods, was examined as a possible effector on plasma AEA levels using Mann-Whitney U test.

3.3 Results:

3.3.1 Subject:

Out of the 50 women who were recruited into the study 20 were primigravidas and 30 were multiparas. The basic characteristics of the volunteers who participated in the study are shown in Table 3.2. The median age of the women was 28 (18-40) and the median gestational age of the women was 40 weeks but all women were at a gestational age > 36 weeks. The median bishop's score was 4 (0-10). The median time interval from sampling to delivery was 2 hours and 12 minutes (9 minutes - 14 hours and 30 minutes). The indications for induction of labour are shown in Table 3.3 and the various methods of induction used are shown in Table 3.4. Most of the women were induced for postdatism (16/50) (32%). The next commonest indications were preeclampsia (7/50) (14%) and SROM (6/50) (12%). Most of the women had a combination of all 3 methods of induction including prostin, ARM and syntocinon (13/50) (26%).

Table 3.2. Basic Demographic Characteristics Of Women In The Study Group

	Median (IQR)	Range	Mean (SD)
Age (yr)	28	18-40	
BMI (wt/ht^2)	24	18-36	
Gestational age (wk)	40	36-42	
Bishop's score	4 (3-6)	0-10	
Induction-to-Delivery interval (hrs)	13hrs and 8min (10hr-24hr)	4hr and 7 minutes-47hrs and 39 minutes	
Sample-to-delivery interval (hrs)	2hrs and 12 min (1hr-4hr)	9min-14hrs and 30 minutes	
Pre-induction AEA (nM)			1.20 (0.6)
Post-induction AEA (nM)			1.82 (0.9)
Percentage change in AEA level			61.4% (59.6)
Ln (IDI)			2.7 (0.61)

AEA=anandamide; IQR=Inter quartile range; Ln (IDI)=natural log of the predicted induction-to-delivery interval.

Table 3.3. The Indications For The Induction Of Labour

Indications	Number of women
Postdatism	16
Reduced fetal movements	2
Preeclampsia	7
Symphysis pubis dysfunction	2
Diabetes	4
Spontaneous rupture of membranes	6
Intra uterine growth retardation	4
Twins	1
Polyhydramnios	2
Maternal request	2
Baby with congenital heart malformation	1
Multiple indications	2
Previous precipitate labour	1

Table 3.4. The methods of induction

Method of induction	Number of women
Prostin[1] + ARM[2]	**9**
Prostin +ARM+ Syntocinon	**13**
Prostin +Syntocinon	**8**
Prostin	**7**
ARM	**3**
Syntocinon	**1**
ARM +Syntocinon	**9**

Prostin=Prostaglandin vaginal pessary; ARM=Artificial rupture of membranes

During the active phase of labour, thirty-one women had epidural analgesia for pain relief, 17 used entonox (combination of nitrous oxide and oxygen), 1 had intravenous fentanyl "patient controlled analgesia" and 1 woman did not require any pain relief.

3.3.2 Plasma AEA concentrations:

The mean ± SD plasma AEA levels increased from a pre-induction value of 1.20 ± (0.57) nM to an active labour value of 1.82 ± (0.87) nM, an average increase of 0.62nM ($P<0.0001$; Student's paired t test; Figure 3.2.) The percentage of change in plasma AEA levels did not vary significantly between women induced for different indications. The method of induction had no effect on pre-induction plasma AEA levels ($P=0.17$), post-induction plasma AEA levels ($P=0.78$) or the percentage change in plasma AEA levels ($P=0.76$; Mann-Whitney U Test).

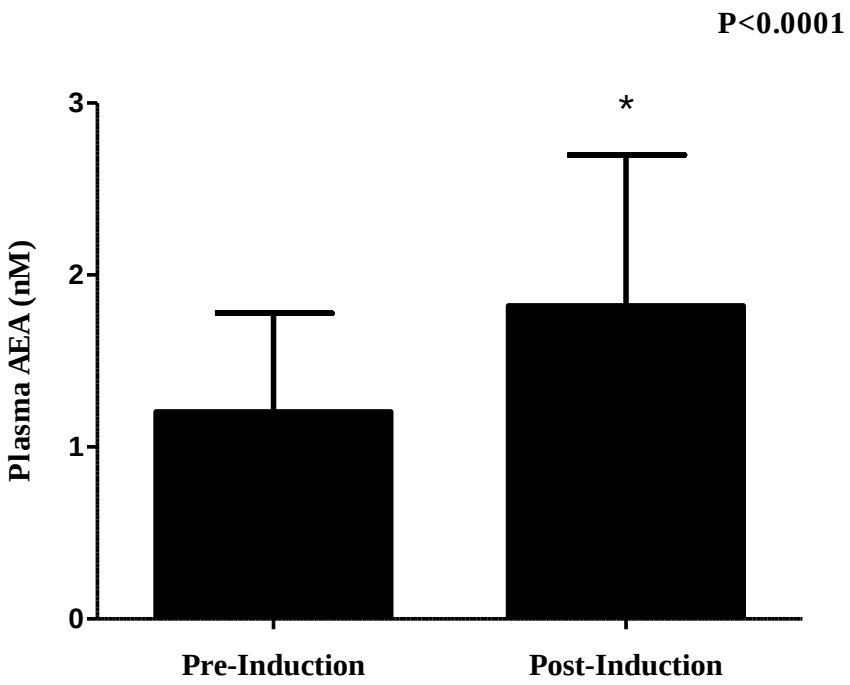


Figure 3.2. Comparison of plasma AEA levels in women pre-induction and post-induction. Plasma AEA pre-induction (1.20 ± 0.57).nM; Plasma AEA post-induction (1.82 ± 0.87).nM; Student's paired "t" test; n=50

There was a significant negative correlation between the induction-to-delivery interval and the Bishop's score (Spearman's r=-0.45; P=0.0015; univariate model). There was no-significant correlation between pre-induction plasma AEA levels and Bishop's score (r=-0.1; P=0.51); and between pre-induction plasma AEA levels and induction-to-delivery interval (r=0.19; P=0.1833). There was a statistically significant negative correlation between the percentage increase in plasma AEA levels during the transition from the non-labouring to the labouring phase and the induction-to-delivery interval (Spearman's r=-0.32; P=0.02). There was also a significant negative correlation between plasma AEA levels during the active phase of labour and the interval between the active phase of labour and delivery (Spearman's r=0.36; P=0.025).

A main effects general linear model (which produced normally distributed residuals) indicated that only the Bishop's score was associated with the induction-to-delivery interval (P=0.0125) (Table 3.5). There was no statistically significant association between induction-to-delivery interval and pre-induction AEA levels (P=0.083) and parity (P=0.0715).

However, a model, which included all significant interactions, demonstrated a significant association between the induction-to-delivery interval and parity and pre-induction plasma AEA levels (P=0.0448) and also between Bishops score and pre-induction plasma AEA levels (P=0.0084) (Table 3.6). A sub-analysis of this interaction demonstrated that there was a more positive association between pre-induction plasma AEA levels and the induction-to-delivery interval for nulliparous than for parous women (Figure 3.3). The regression estimates were based on a fitted model. For primigravidas the slope was 16.16 x pre-induction AEA concentration (r^2=0.293; P=0.017; n=20) and for the multigravidas the slope was 5.28 x pre-induction AEA concentration (r^2=0.0998; P=0.103; n=28).

Table 3.5. Relationship Between Bishop's Score, Pre -induction Plasma AEA Level, Gravidity and Induction-To-Delivery Interval.

Variable	F	Regression Coefficient	P
Bishops Score	6.78	-1.51	0.0125 *
Pre_AEA	3.14	-3.81	0.0833
Gravidity	3.41	4.94	0.0715

A main effect general linear model.*Significant at 5% level

Table 3.6. The Association Between Bishop's Score, Pre-induction Plasma AEA Level, Gravidity and Induction-to-delivery Interval After Adding Interactions

Variable	F	Regression coefficient	P
Bishop's Score	6.52	-1.43	0.0143*
Pre-induction AEA level	7.64	14.9	0.0084*
Gravidity	1.54	8.93	0.2210
Pre-induction AEA level X Gravidity	4.27	12.75	0.0448*

A general linear model where the interactions between all independent variables on induction-to-delivery interval are taken into account.*Significant at 5% level

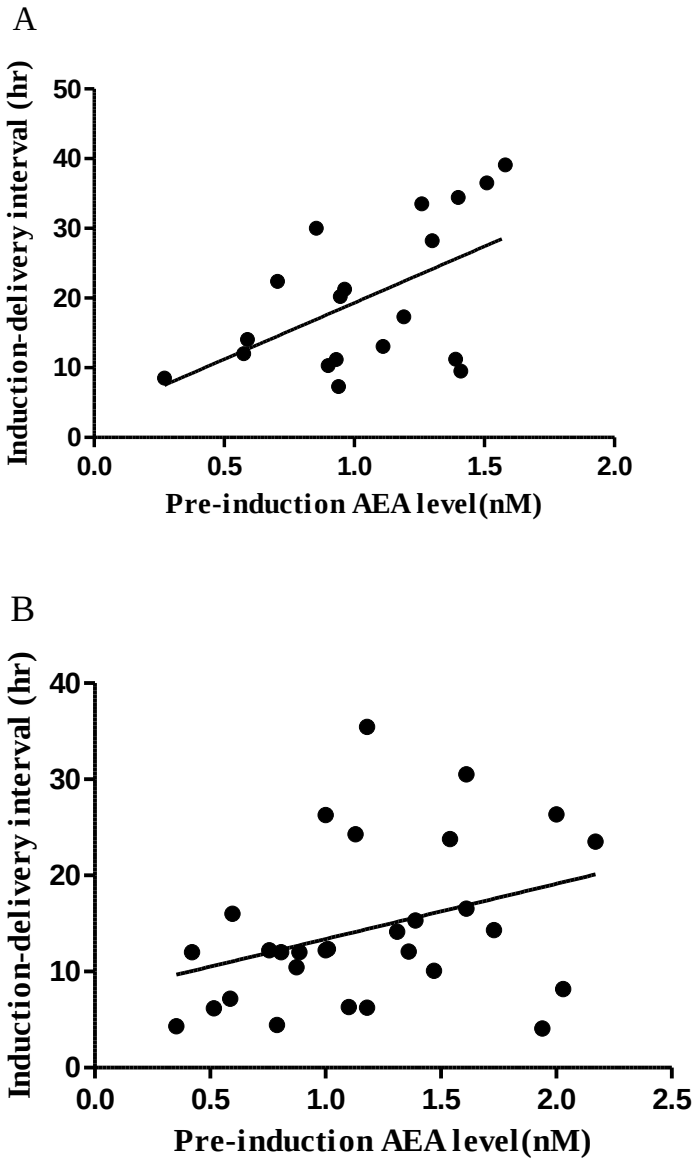


Figure 3.3. The effect of gravidity on the correlation between the induction-to delivery interval and pre-induction plasma AEA concentrations. Plasma AEA concentrations determined in A are for nulliparous women and in B are for multiparous women. For nulliparas the slope is 16.16 x pre-induction plasma AEA concentration (r^2=0.293; P=0.017; n=20) and for parous women the slope is 5.28 x pre-induction plasma AEA concentration (r^2=0.0998; P=0.103; n=28).

A fixed effect multiple linear model was applied to investigate the variables that best predicted induction-to-delivery interval. The variables included Bishops score, percentage change in plasma AEA levels, gravidity and pre-induction AEA levels. The induction-to-delivery interval was positively skewed, so it was log transformed to normality for analysis (Shapiro-Wilk's test; P=0.35 post transformation). Non-significant predictors were removed from the model one at a time in an iterative backward selection procedure until only two significant predictors remained. The first variable removed from the model was pre-induction AEA (P=0.36) and the last variable removed was gravidity (P=0.07). In the final model (Table 3.7) both Bishop's score (Figure 3.4) and percentage change in plasma AEA levels (Figure 3.5) remained the most significant predictor of induction-to-delivery interval. The residuals from the final model were normally distributed and displayed no obvious association with the predicted values.

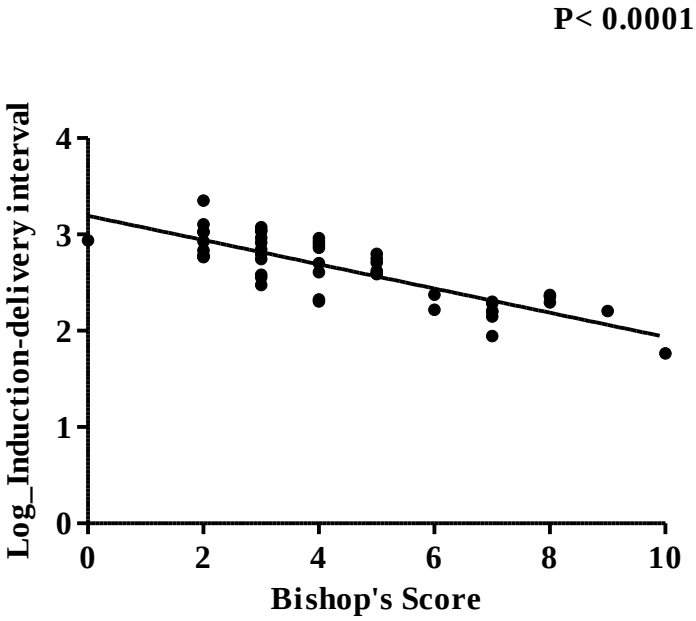


Figure 3.4. Prediction of induction-to-delivery interval for Bishop's score with percentage change in plasma AEA concentration as a covariate. The graph depicts the relationship between the predicted natural log value of induction-to-delivery interval and Bishop's score (slope=-0.13; r^2=0.6744; P<0.0001; n=48). The r^2 for the full model is 0.30.

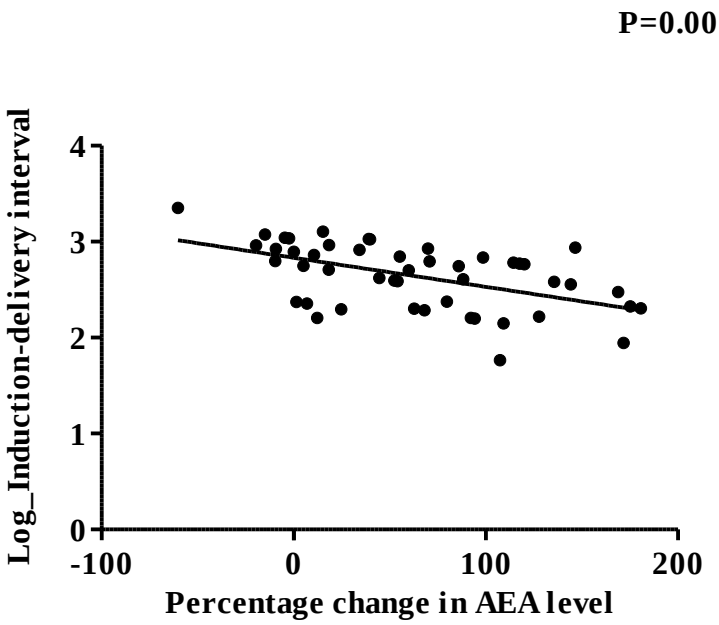


Figure 3.5. Graph Demonstrating Regression Of Predicted Natural Log Value Of Induction-to-delivery Interval From Percentage Change In Plasma AEA Level.
Slope of the graph is -0.003; r^2=0.282; P=0.0001; n=48). The r^2 for the full model is 0.30.

Table 3.7. Multiple Regression coefficients obtained from fixed effects multiple regression model of natural log of induction-to-delivery interval predicted from Bishop's score and percentage change in plasma AEA level.

	Slope (95% CI)	P value	r^2
Bishop's score	-0.13(-0.2, -0.06)	0.003	0.30
Percentage change in plasma AEA level	-0.003(-0.006 , -0.0008)	0.01	

There was no significant difference in the percentage change in plasma AEA concentration and the type of analgesia used in labour. Fifteen women used entonox (A mixture of oxygen and nitrogen) and twenty seven women had epidural for analgesia. The percentage change in plasma AEA level among entonox users 72.45 ± 13.442.was not significantly different compared to that in epidural users 50.84 ± 10.52 (P=0.21).

p=ns

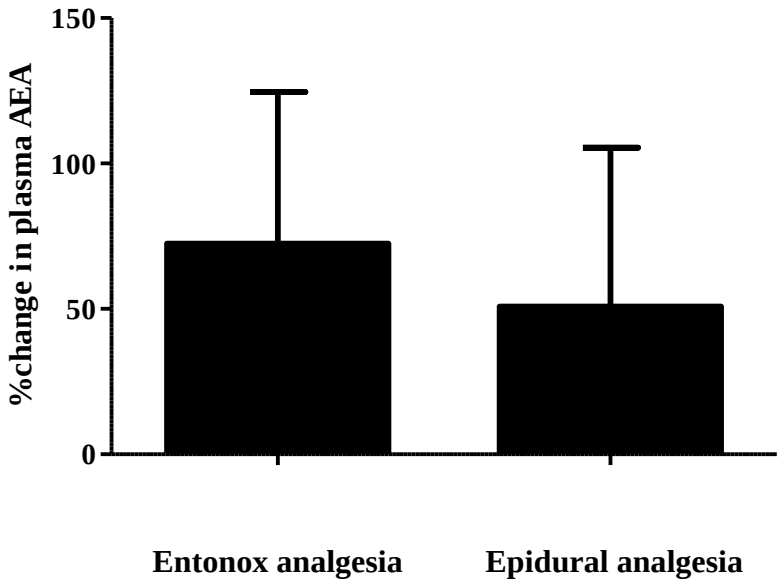


Figure 3.8. Comparison of the effect of different types of analgesia on percentage change in plasma AEA levels during the transition from the non-labouring to labouring phase. There was no significant difference in the percentage change in plasma AEA concentration in women having different modes of analgesia Number of women using entonox=15; Number of women using epidural=27.

3.4: Discussion:

In this study the plasma AEA levels in women after the induction of labour rose 1.5-fold. This increase provides an additional evidence to suggest that AEA may be associated with the process of labour either in its initiation or progression.

This 1.5 fold rise was considerably lower than the 3.7-fold rise from term non- labouring to term labouring reported by Habayeb et al., (2004). The differences could be due to the fact that in this study labour was induced, whereas the previous study included a mixture of women who had spontaneous and induced labours. The previous study was performed using a cross-sectional design, whereas this was a longitudinal study with women being followed through the process of induction, with samples obtained from the non-labouring and labouring states in each woman. The better insight into causality and control for non observed heterogeneity that longitudinal studies provide suggests that the present data should be more accurate.

Despite these findings, there was still a clear and significant increase in plasma AEA levels during labour, whether spontaneous or induced and therefore, it is highly likely that AEA has an effect in the process of labour. As receptors for AEA (CB1 and CB2) and FAAH have been demonstrated in human term myometrium (Dennedy et al., 2004) placenta and gestational membranes (Park et al., 2003), AEA may be involved in the mechanisms initiating and/or maintaining labour. AEA has been shown to be synthesised in the uterus (Paria et al., 2001) and has been found in amniotic fluid (Schuel et al.,2002). It has also been shown to exert a relaxing effect on pregnant myometrium in an *in vitro* study and this action was mediated through CB1 receptors (Dennedy et al., 2004). However, direct evidence for a contractile effect on the human myometrium is currently lacking.

The difference in the strength of association between the pre-induction plasma AEA and induction-to-delivery interval amongst nulliparous and multiparous women is probably due to the difference in the basic underlying mechanism of labour which could explain the general difference in the labour progression between the two groups that is usually observed clinically.

The significant inverse correlation between the induction-to-delivery interval and Bishop's score suggests that this cohort of women followed the accepted trend whereby a high Bishop's score predicts a short induction-to-delivery interval.

The negative correlation between the percentage change in plasma AEA levels and induction-to-delivery interval and post-induction plasma AEA levels and interval from sampling to delivery suggest that it is most likely not the absolute levels of the AEA but the rate of change in the levels that are involved in the initiation/ progression of labour by stimulating uterine contractions supporting the proposition that AEA has a direct effect on the myometrium (Dennedy et al., 2004). AEA mainly acts through CB1 and CB2 receptors and causes a reduction in AC levels leading to a decrease in cAMP levels (Petrocellis et al., 2004). A decrease in cAMP levels in myometrium leads to myometrial contraction (Sanborn et al., 2001).

An alternative explanation of why plasma AEA levels increase during labour could be related to the suggestion of Schmid et al (1997) that AEA acts as a precursor of AA required for the production of PGs, because AEA degradation by FAAH converts it to AA and ethanolamine Alternatively, the increased AEA levels could independently stimulate the production of PGs by gestational tissues, as PGs are essential for the initiation and progression of labour (Mitchell et al.,2008). Of course, both mechanisms could be working in concert.

Recent evidence from the mouse model showing that defective CB1 signalling can lead to preterm birth (Wang et al., 2008) suggests that defective cannabinoid signalling may play a role in human preterm parturition. The finding of a shorter duration of labour and faster parturition associated with a higher percentage rise in plasma AEA levels suggests that AEA signalling is intimately involved in human parturition and so AEA may be involved in the cascade of events that leads to delivery, but how, is not exactly clear. One suggestion is that AEA acts differentially on the upper and lower uterine segments; enhancing uterotonic signalling in the fundus while facilitating relaxation and cervical ripening in the isthmus leading to a shorter labour duration. Clarification of the exact role of AEA in human parturition is therefore essential, and if shown to have a

modulatory effect on the myometrium, then CB receptor antagonists that block the action of AEA could be investigated as potential inhibitors of preterm labour.

3.5 Conclusion:

As plasma AEA concentrations increase during the transition from the non-labouring to labouring state it was hypothesised that plasma AEA levels in women with threatened PTL and women who are at high risk of delivering preterm might predict women who will deliver preterm. Therefore in the next studies plasma AEA levels were measured in women at high risk of delivering preterm (Chapter 4) and in women presenting with threatened preterm labour (Chapter 5).

Chapter 4

Prediction of PTD among high risk pregnant women

4.1 Introduction:

In chapter 3, it was shown that in women undergoing induction of labour plasma AEA levels increased 1.5-fold during the transition from the non-labouring to the labouring phase. This pattern of change was similar to that previously reported from a cross-sectional study of term non-labouring and labouring women (Habayeb et al., 2004). This suggests that plasma AEA levels could be used as a biomarker for labour. The plasma AEA levels in women at risk of PTB who subsequently proceed to deliver preterm could be elevated and could be used to predict the women who are really at risk.

PTB, defined as delivery between 24 and 37 completed weeks of gestation, contributes to approximately 70% of neonatal mortality and 75% of the neonatal morbidity in industrialized nations (Wen et al., 2004). In order to reduce the rate of preterm deliveries it is important to accurately predict those who are really at risk of delivering preterm so that appropriate interventions can be directed at them.

Women who are considered to be at high risk of delivering preterm include those who have experienced previous spontaneous PTB; women who have had previous mid trimester miscarriages; recurrent first trimester miscarriages; women with uterine abnormalities and those who have undergone large loop excision of the transformation zone (LLETZ) or cone biopsy (Chandiramani and Shennan et al., 2006). However, in a large group of women who deliver preterm there are no identifiable risk factors (Iams et al., 2001)

Women with a prior spontaneous PTD have a 2.5 fold increased risk of PTD in the current pregnancy in comparison to women who have never experienced PTBs (21.7% vs. 8.8%; $P<0.001$) (Mercer et al., 1999) or a recurrence risk of 15-50% depending on the number and gestational age of previous deliveries (Goldenberg et al., 2008). The risk is also inversely related to the gestational age of the previous PTB (Goldenberg et al., 2003). The mechanism for the recurrence is unclear but

persistent or recurrent intrauterine infection is considered to be one of the main contributory factors for recurrent spontaneous PTB (Goldenberg et al., 2008).

The risk of PTD following previous miscarriage is much less when compared to that following previous PTB. The RR associated with previous miscarriage is 1.57 (Berkowitz and Papiernik.,1993). The risk does not increase until after two or three spontaneous miscarriages. The risk is much higher following a previous second trimester miscarriage when compared to a first trimester miscarriage as the causes of late miscarriages are not uncommonly related to those of PTB.

The rate of PTB among women with congenital uterine abnormalities is between 25-47% (Raga et al., 1997). Some of the studies have shown that the risk of PTD is increased in women who have undergone LLETZ, for example Jakobsson et al (2009) showed that the risk is increased threefold in women who have undergone LLETZ and was fivefold higher in women who had undergone repeat procedures when compared to the background risk. However, some studies have not reproduced similar risks (Shanbhag et al., 2009).

In these high risk women, screening for signs and symptoms of PTD offers opportunities for early intervention. Such interventions include the early administration of steroids and tocolytics and/or transfer to appropriate units with neonatal intensive care facilities. One such predictor of PTB is cervical length as described in the introduction chapter (section 1.2.7).

Spontaneous PTB is usually preceded by a shortening and dilatation of the cervix (Berghella and Berghella, 2005). The normal cervical length at gestational age between 14 and 24 weeks varies between 25 and 50mm, whilst between 24-30 weeks the cervical lengths are maintained at 25mm and thereafter there is a physiological shortening of the cervix (Iams et al, 1996). There is no apparent difference in the cervical length between primigravidas and multiparous women (Berghella et al., 2003).

Cervical length has therefore been used for screening asymptomatic high risk women to identify those likely to deliver preterm (Andrews et al., 2000; Guzman et al., 2001). Cervical length measurement is routinely made by transvaginal ultrasound (Figure 4.1) and has been found to be safe and acceptable to patients (Owen et al., 2003). The specificity and sensitivity of this method depends on the gestational age at screening and the background risk of the population screened (Berghella and Berghella, 2005). The consensus view from these predictive studies is that the earlier the shortening of the cervix occurs during pregnancy and the greater the degree of the shortening, the greater the risk of PTD.

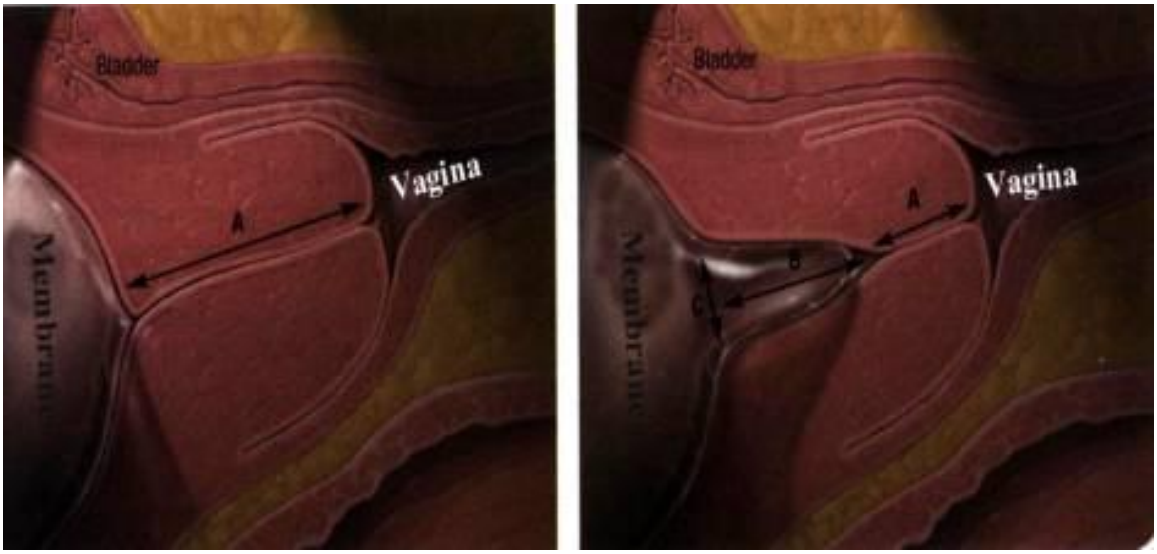


Figure 4. 1. Transvaginal ultrasonography of closed normal cervix (left) and of a short cervix with significant funnelling (right) From: www.fetalultrasound.com

Furthermore, in a prospective study of predictors of spontaneous PTB in women with prior PTB, the probability of delivering preterm was 15% compared with 3% in woman without a prior PTB (Iams et al., 1998). A positive FFN test, a cervical length <25mm or a combination of both factors further increases the probability of a PTB in a woman with prior PTB to 49%, 31% and 64%, respectively whereas the same tests in women without prior PTB increased the probability to only 13%, 8% and 25%, respectively (Iams et al., 1998). Thus, screening and identification of women who are really at risk should allow the initiation of risk-specific treatment and the potential to produce more risk-specific interventions than are currently available.

On the base of this information, it was hypothesised that among high-risk women, plasma AEA levels would be elevated in those who are truly at risk of PTD. The aims of the study in this chapter were to

1. measure plasma AEA levels in women at high risk of delivering preterm.
2. investigate whether plasma AEA levels can predict which of them would really deliver preterm.
3. compare the accuracy of plasma AEA levels with that of cervical length in predicting PTD and also
4. to examine whether there were any correlation between cervical length and plasma AEA levels.

4.2 Subjects and Methodology:

4.2.1 Subjects:

Women attending the Preterm Prevention Clinic at the Leicester Royal Infirmary, who were considered ‘high risk’ for PTD due to various risk factors such as previous spontaneous PTD, cervical surgery, >3 first trimester or 1 second trimester miscarriage or uterine morphological abnormality were consented and recruited into the study. Exclusion criteria were multiple pregnancies, pregnancies complicated by medical conditions such as diabetes mellitus and hypertension, or pregnancies with placenta praevia. Volunteers had their cervical length measured

using transvaginal ultrasound as described in the methods section 4.2.2.2 (page 154) as part of their antenatal care and in addition gave a blood sample between 20 and 30 weeks of gestation for the measurement of plasma AEA. The outcome of the pregnancy was monitored through the EUROKING database system of the Leicester Royal Infirmary Hospital. The gestational age at delivery, the interval from blood sampling for AEA measurement to delivery, and the cervical length measurement at the gestation when plasma AEA levels were measured were all noted. Other variables recorded were maternal age and BMI.

Power analysis of the published AEA data (Lam et al., 2008) with α=0.05 and β=0.8 indicated that the minimum number of subjects required in the group that would allow a significant change in plasma AEA concentration to be observed was 29. In order to avoid a typeII error it was decided to recruit 50 subjects.

4.2.2 Methods:

4.2.2.1 Measurement of Plasma AEA Concentrations

Plasma AEA levels were measured from 4ml of blood using the LPE method described in detail in chapter 2.

4.2.2.2 Measurement of Cervical Length:

Each at risk woman had cervical length measurements performed serially as part of their PTD prevention programme. The first measurement was taken at the 16th week of gestation and the frequency of subsequent measurements depended on the number of high risk factors, the measured cervical length and the gestational age at which the previous pregnancies were delivered

Before a cervical length measurement was made, the volunteer's bladder was emptied. The measurement was made with a transvaginal real-time ultrasound probe placed in the anterior fornix of the vagina. The appropriate sagittal view of the cervix was identified by the location of the

triangular area of echo density at the external os and by the V-shaped notch at the internal os with a faint line of echo density or echolucency between the two. To ensure that a satisfactory image was obtained, a detailed image was first observed, and then the probe was withdrawn until a blurred image was observed, the probe was then repositioned to restore the original image, avoiding undue pressure on the cervix, which is known to artificially increase its length (Berghella et al., 2003). The cervical length was measured three times with each examination being performed over 3 minutes to observe dynamic cervical changes. The shortest measurement of the 3 was taken as the final reading.

4.2.2.3 Statistical analysis:

Data analysis was performed using GraphPad Instat version 3, (GraphPad Software, San Diego, CA, www.graphpad.com). Descriptive statistics were calculated for baseline demographics and obstetric characteristics (mean and SD for continuous variables and numbers and percentages for categorical variables). Comparison of gaussian distributed, continuous data was by Student's unpaired t-test and that of non-gaussian distributed data using the Mann Whitney U Test. Categorical data were compared using chi-square test.

4.3 Results.

Of the 50 volunteers recruited, 8 had undergone a cervical cerclage either abdominally or vaginally and were therefore excluded from further analysis. The risk factors for PTD present in the volunteers are shown in Table 4.1. Twenty four of the 42(57%) volunteers had undergone cervical surgery, the commonest risk factor. The second commonest risk factor was previous PTD 16/42(38%). The demographics and the obstetric characteristics of these are shown in Table 4.2. There was no difference in any of the confounding factors like ethnicity, parity, weight and smoking status.

Table 4.1. Risk Factors For Recurrent PTD

Indications	Number of patients
Previous PTD	16
LLETZ	22
Cone Biopsy	2
>3 1st trimester miscarriages	1
Uterine abnormality	1

Table 4.2. Demographics And Obstetric History Of The Study Population

Variable	Delivered Preterm	Delivered Term	Difference
Maternal age (mean±SD)	29.57 ± 2.1	29.72 ± 6.8	ns
Ethnicity n (%)			
White	6/7 (83)	29/33 (87)	ns
Asian/African	1/7 (17)	4/33 (12)	ns
BMI (mean±SD)	20.67 ± 1.4	24.5 ± 0.80	ns
Parity n (%)			
Multi	5/7 (71)	17/33 (51)	ns
Primi	2/7 (28)	16/33 (48)	ns
Smoking status			
Smokers n (%)	2 (28)	7 (21)	ns
Non-smokers n (%)	5 (71)	26 (78)	ns

Of the 42 volunteers, 7 delivered preterm (16%). The mean AEA levels in these women were 1.01 ± 0.70nM. It was not significantly different from that in the 35 women delivered at term (0.72 ± 0.39nM) (Figure 4.2). The variation in plasma AEA levels in those that delivered preterm was significantly larger than that in the term group.

A subgroup analysis was made in which the plasma AEA levels of the women who delivered preterm at various intervals from the time of sampling of plasma were compared to that of women who delivered at term (1 week; 2 week; 3 week; 4 week; 5 week; 6 week and >6 week). One of the volunteers delivered within 2 weeks of sampling and 2 of them delivered within 3 weeks. Due to the sample size being small it was not possible to compare the plasma AEA levels in these groups with those delivering at term. However the plasma AEA level (1.65 ± 0.62nM) of women who delivered preterm (within 6 weeks of sampling) were compared with the levels of those who delivered at term (0.72 ± 0.40nM) and were found to be significantly different (P=0.01; Mann Whitney U test; Figure 4.3).

A ROC (Figure 4.4) was constructed to test the ability of plasma AEA level to predict PTD in a high risk population. A plasma AEA level of 1.110nM predicted the risk of preterm delivery at < 37 weeks gestation with a sensitivity of 66.6% and a specificity of 81.8%. The area under the curve (AUC) for this study was 0.909.

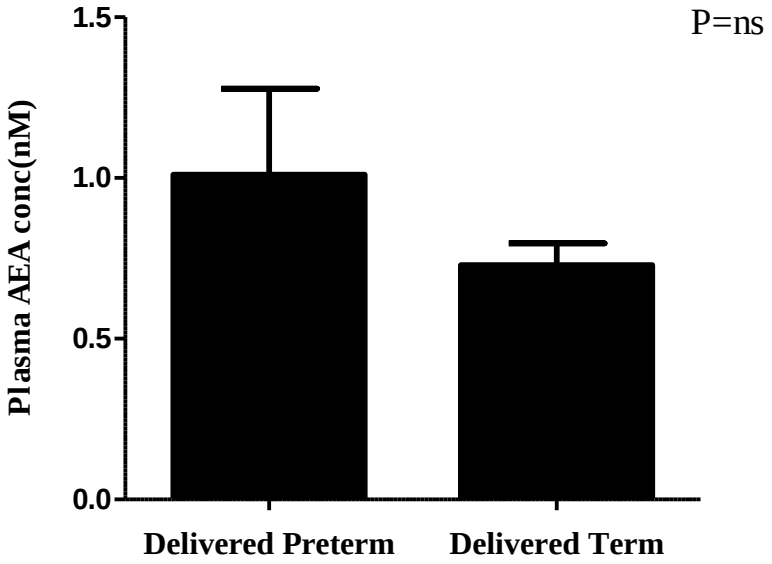


Figure 4.2. Comparison of plasma AEA levels between women who delivered preterm and term. The plasma AEA levels in women who delivered preterm (n=7) was 1.01 ± 0.07nM and 0.72 ± 0.39nM in those who delivered at term (n=31). Although plasma AEA levels were slightly higher in those women that delivered preterm, the difference was not significant (P>0.05; Student's t-test).

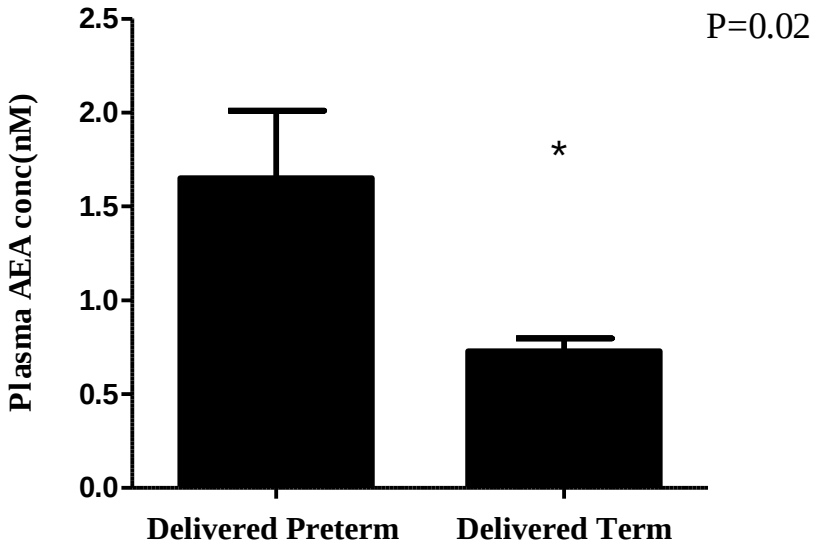


Figure 4.3. Comparison of plasma AEA levels of women who delivered preterm within 6 weeks of sampling of blood and those who delivered at term. The plasma AEA levels of women who delivered preterm within 6 weeks of sampling (n=3) were significantly different from those of women who delivered at term (n=33); (P=0.02; Mann Whitney U test).

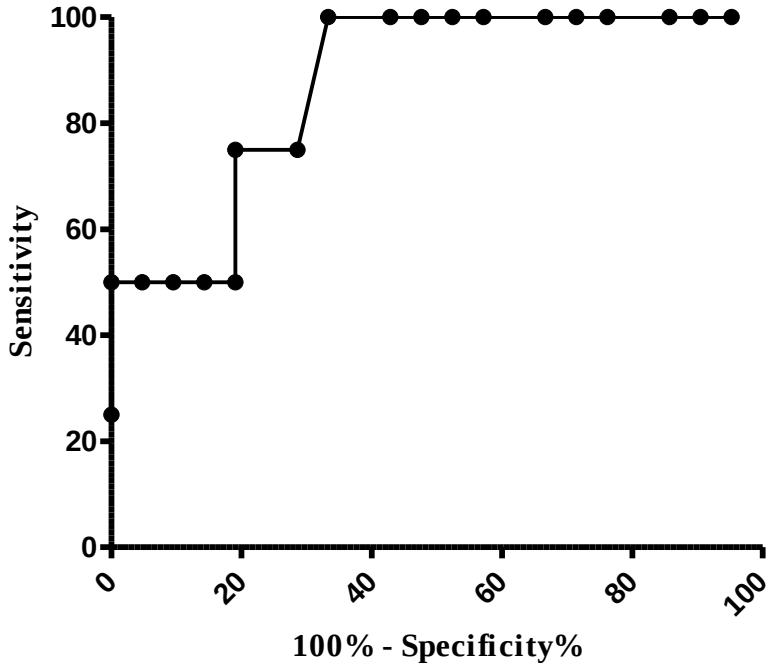


Figure 4.4. **Receiver Operating Curve of Plasma AEA level in predicting PTD within 6 weeks of sampling.** A plasma AEA level of 1.11nM predicted PTD with a sensitivity of 66.6% and specificity of 81.8%. The positive likelihood ratio was 3.67. AUC=0.909 (95%CI 0.74-1.0); P=0.02.

The cervical length measurements were found to follow a Gaussian distribution (Kolmogorov-Smirnov test) with the mean cervical length being 31.8mm. Twelve of the 42 volunteers (28%) had a cervical length ≤25mm. Of the 12 women with cervical a length ≤25mm, 3 (25%) delivered preterm. The cervical length of women who delivered preterm (28.7 ± 13.6mm) was not significantly different from that of those who delivered at term (33.7 ± 11.9mm) as shown in Figure 4.5.

A ROC of the cervical length data is shown in Figure 4.6. The sensitivity, and specificity of a cervical length <25mm in predicting PTD were 42.9% and 71% respectively. The likelihood ratio of PTD with a cervical length of <25mm was 1.5. The AUC for this study was 0.575. By contrast, a cervical length of <15mm, gave a sensitivity and specificity of 28.6% and 93.6%, respectively and the likelihood ratio of delivering preterm increased to 4.4.

The cervical length of the women who had previous PTD (Excluding those of women who had cervical surgery) was found to be significantly inversely correlated to plasma AEA levels (r^2=0.36; n= 16). The relationship between cervical length and plasma AEA levels was defined by the equation

Y=MX+C where,

Y (Cervical length)= Cervical length (Y)=-12.04 ± 4.2 (AEA-X) + 3.56. as shown in Figure 4.7

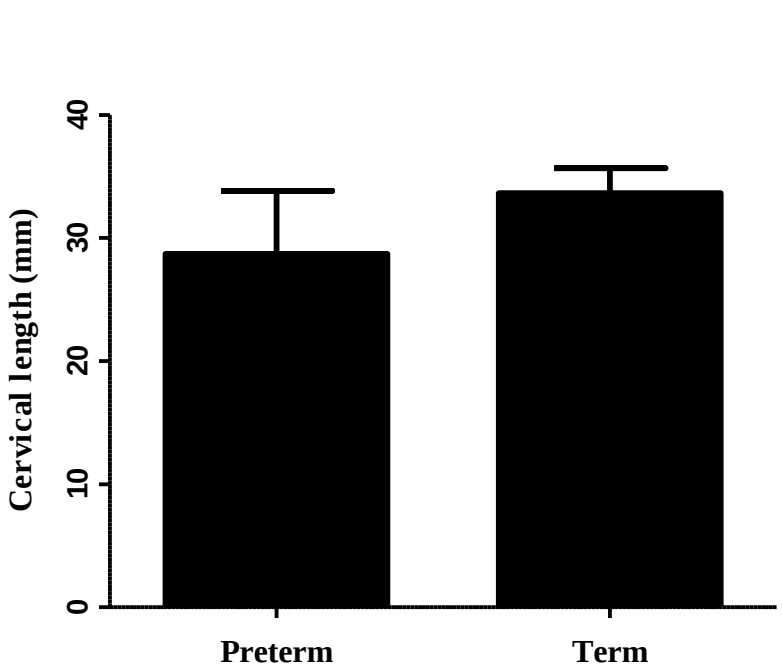


Figure 4.5. Comparison of cervical length in women who delivered preterm and term. The mean cervical length of women who delivered preterm (n=7) and those who delivered at term (n=35) was not significantly different (P>0.05; Student's t test).

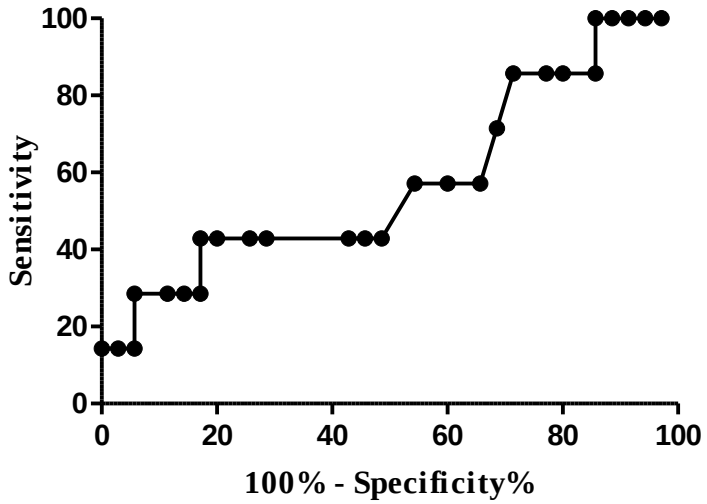


Figure 4.6. **Receiver Operating Curve of cervical length in predicting PTD.** A cervical length of <25mm predicted a risk of PTD with a sensitivity of 42.9% and a specificity of 71%. The positive likelihood ratio was 1.5. A cervical length <15mm predicted a risk of PTD with a sensitivity of 28.6 % and specificity of 93.6%. AUC=0.59 (95% CI-0.32-0.85); P=0.46.

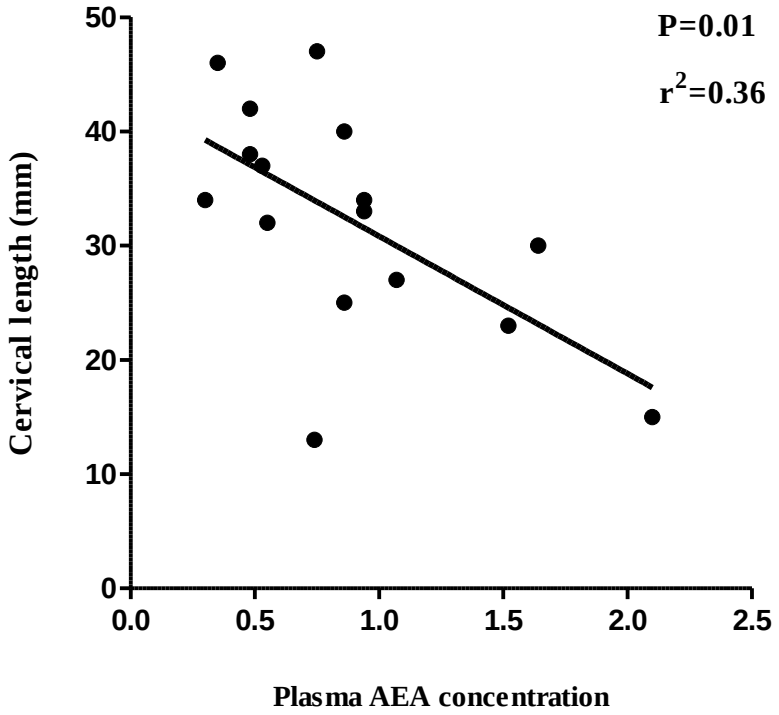


Figure 4.7. The relationship between plasma AEA level and cervical length. Pearson correlation coefficient=0.36; n=16; Cervical length (Y)=-12.04 ± 4.2 AEA (X) + 3.56.

4.4 Discussion:

In this chapter plasma AEA levels in a cohort of pregnant women at high risk of recurrent PTD were measured and there was a significant difference in women who delivered preterm (1.65 ± 0.62nM) within 6 weeks of sampling when compared to that of women who delivered at term (0.72 ± 0.39nM) (P=0.02). There was however no significant difference in plasma AEA levels between all women who delivered preterm (1.01 ± 0.70nM) and those who delivered at term (0.72 ± 0.39nM) (P>0.05).

The reason for this difference could be that plasma AEA levels rise only a few weeks before the onset of PTL and delivery and therefore the levels in women whose blood was sampled remotely from delivery (i.e. week 20 who delivered in week 30) would not have been expected to be significantly different from that in those who delivered at term. This is also supported by the findings of Habayeb et al that plasma AEA levels in normal pregnancy remained low during the second and third trimester and only rose at term prior to the onset of labour (Habayeb et al., 2004).

Using a plasma AEA level of 1.11nM, the sensitivity of the assay in predicting PTD at less than 37 weeks was 66.6% and the specificity was 81.8% with a positive likelihood ratio was 1.7. The AUC for this study was 0.909.

Cervical length and funnelling have been investigated in a number of studies as predictors of PTD in high risk women and has been found to be accurate (Berghella et al., 2003). The mean cervical length in this cohort of pregnant women who delivered preterm was 28.7 ± 13.6mm. Using a 25mm cut off, the sensitivity of the test was 42.9% and specificity was 71% with a positive likelihood ratio of 1.5. Using a cut off of 15mm, the sensitivity was 28.6%, specificity was 93.6% and the positive likelihood ratio was 4.4. Although the results are poor, they are comparable to those of Andrews et al (2000) whose sample size was also small as in this cohort.

The positive predictive value of the cervical length test was only 27%, but similar to those of others (Guzman et al., 1998, Berghella et al 1999).This could be due to a larger proportion of women in this study who had undergone the LLETZ procedure (52%). It has been previously noted that cervical length in women with prior cone biopsy/ LLETZ is comparatively shorter than women who have never undergone this procedure (Fischer et al., 2009). Even though there is anatomical reduction in cervical length the mean gestational length at delivery was not significantly different from those without prior cervical surgery, though a higher proportion of women delivered preterm.
This is the limitation of the study. The cohort in the study consists of heterogeneous population of women who had previous PTD (a cohort with very high risk of recurrent PTD) and women who had undergone cervical surgery (cohort with very mild risk). Moreover, the pathophysiological mechanism of initiation of PTL might vary in both these conditions and AEA might not be involved equally in the preterm parturition pathway in both conditions. The results have to be interpreted with caution and it is important that this study is carried out in a homogenous group of women.

The clear inverse correlation between plasma AEA levels and cervical length and the fact that the correlation persisted even after excluding women who had cervical surgery. suggests that there could be a direct causal relationship between AEA levels and cervical function. CB1, CB2 and TRPV1 receptors have all been identified in cervical cancer cells (Contassot et al., 2004) and AEA has been measured in cervical cancer cells. Unpublished data from our group demonstrated CB1, CB2 and FAAH in the normal cervix suggesting that the cervix may be a target of AEA. CB1, CB2 receptors and FAAH protein as well as the mRNA have been demonstrated in term fetal membranes and myometrium. Recently, the levels of AEA were quantified in normal human fetal membranes and placenta (Marczylo et al., 2010). Arachidonic acid, which is the principal metabolite from which prostaglandins are produced, could arise from AEA as hydrolysis of AEA by FAAH leads to the production of AA and ethanolamine (Di Marzo and Petrosino, 2007). AEA has been reported to increase PG synthesis by gestational tissues such as the chorioamnion through

its action on CB1 receptors (Mitchell et al., 2008). It is therefore possible that AEA causes cervical effacement indirectly by increasing PG synthesis.

In conclusion this study has shown that plasma AEA levels were significantly different in a subgroup of women who delivered preterm among a cohort of high risk patients. However this study was limited by a small sample size and heterogenous study population and therefore further larger studies in a homogenous group of women will be needed to confirm these findings.

Chapter 5

Prediction of PTD among women with threatened PTL

5.1 Introduction:

In chapter 4, it was shown that plasma AEA levels in women at high risk of delivering preterm, due to a significant past obstetric history or having undergone cervical surgery, were significantly higher in women who delivered preterm within 6 weeks of obtaining the sample when compared to women who subsequently delivered at term.

These interesting observations were extended to studies (presented in this chapter) to women presenting with threatened PTL to investigate whether plasma AEA levels could be used to identify those who will deliver preterm (i.e. true preterm labour).

PTL is defined as the onset of regular uterine contractions associated with progressive cervical change between viability and 37 completed weeks of gestation (Chatterjee et al., 2005). A significant number of women presenting with abdominal pain do not deliver preterm. Only a small proportion, (8–24%) of those who present with symptoms will go on to deliver prematurely (Hamilton et.al., 2010). However, clinically it is difficult prospectively to separate those who will eventually deliver preterm (i.e. presenting in true preterm labour) from those who progress to term. This is largely due to lack of an accurate test to diagnose true preterm labour.

The diagnosis of PTL is usually made clinically based on the presence of regular uterine contractions (3 within one 10 minute period) associated with cervical change, as assessed on vaginal examination. Many women presenting with symptoms suggestive of PTL, however even if combined with cervical effacement and dilation, do not progress to PTD. So a clinical diagnosis of PTL is often unreliable. This is because each component of the diagnosis - uterine contractions and cervical changes, is subject to variation, and each of the parameters involved in the diagnosis of labour is highly subjective and can therefore lead to wide inter- or intra-observer differences. Also in some women the symptoms of PTL can be insidious, non-specific and vague. They include menstrual like cramps, backache,

increased vaginal discharge, uterine contractions, or a general feeling of 'being unwell' (Mussad et al., 2005).

Clinically, an association has been established between the occurrence of two or more contractions in 30 min at 28 weeks gestation and subsequent risk of PTB (Iams et al., 2002). However, the positive predictive value of a contraction rate of over four per hour at 27–28 weeks pregnancy for PTD at less than 35 weeks of gestation is low (23%), and the sensitivity is also poor (28%) (Iams et al., 2002). When several parameters are compared using a ROC analyses, the performance of uterine contractions frequency was found to be lower than that of clinical evaluation of the cervix (Iams et al., 2002). Uterine contractions occurring in the case of an unripe cervix are less likely to result in PTB.

Cervical assessments are commonly performed by digital/speculum examination or by ultrasound visualisation of the cervix. Digital examination, though an essential part of clinical diagnosis of PTL is subjective and is associated with wide inter- and intra-observer variability. Some studies have shown that ultrasound assessment is better at delineating the presence of a ripened cervix, when compared to digital examination (Herbst et al., 2006). For example, in one study, a cervical length of <15 mm in women admitted with PTL was associated with a 50% risk of delivering within 7 days (Norman et al., 2007). Only 1% of women with cervical length >16mm delivered within 7 days. Thus, ultrasound cervical length measurement has a good negative predictive value. However, the test is invasive and needs to be performed by trained personnel, often out of hours and immediately after such women are admitted. In addition, the positive predictive value of cervical assessment remains low, except for those with a very short cervical length <10mm. Even with a cervical length of 10 mm or less, the PTB rate within 7 days of testing was less than 50% (Tsoi et al., 2005). Thus, the poor association between clinical symptoms and signs and the likelihood of delivering preterm means that a large number of women still receive unnecessary treatments which are expensive. In addition, this also causes significant problems for trials of potential treatments for the prevention of PTL.

Since only 8-24% of women admitted with threatened PTL deliver preterm (Hamilton et al., 2010), a better means of identifying women with true threatened PTL going on to deliver is needed to prevent women unnecessarily receiving steroids, tocolysis and possibly being transferred to other centres away from their community, all of which represent a significant financial cost both to the family and NHS. Identification of the women who are at true risk of PTD in those presenting with features of threatened PTL will therefore enable the targeting of appropriate resources with potential benefits to the patients and the NHS whilst avoiding unnecessary interventions in women who are not at risk.

In recent years, FFN testing has been used to identify these high risk women (Leitich et al., 1999). FFN, as discussed in Chapter 1 is a glycoprotein which is released into the vaginal secretions when there is disruption of the chorio-decidual membrane. FFN has been detected in cervicovaginal secretions between 21 and 37 weeks of gestation in only 3% to 4% of uncomplicated pregnancies delivered at term (Lockwood et al., 1991). The presence of FFN at this period of gestation is associated with a high risk of delivering preterm and its usefulness in the prediction of PTD has been extensively tested in women with threatened PTL (Honest et al., 2002).

Historically, FFN tests have been of two different types; (1) a quantitative lab-based assay, which gives absolute values, with 50ng/ml being the accepted upper limit of normal and (2) a rapid qualitative bedside test based on the same antibody, but only giving either a positive or negative result (McParland et al., 2004).

Four published studies have examined the reliability of bedside testing with FFN and its correlation with subsequent PTD in women with symptoms of PTL (Parker et al., 1995; Senden et al., 1996; Benattar et al., 1997 and Owen et al., 1997). All four studies used the ROM-Plus (Adeza Biomedical, Sunnyvale, Calif) vertical-flow immunoassay method (spot test with control ring) to detect the levels of FFN. Subsequently, the bedside test has been refined and has evolved into the Full-Term® test marketed by Cytyc, who acquired Adeza Biomedical in 2001. The prototype FFN membrane

immunoassay (Adeza Biomedical) dipstick test is easier to perform and more economical than the ROM-Plus kit. This has been evaluated in one study (Coleman et al., 1998).

In a systematic review of symptomatic women, the mean sensitivity and specificity for the FFN testing in predicting delivery within seven days of the test were 77% (95% CI 67-88%) and 87% (95% CI 84-91%) respectively; FFN was more predictive as a short term marker than as a long term marker for PTB. The mean sensitivities for delivery within 14 days and 21 days of the test and before 34 weeks of gestation were 74%, 70% and 63% respectively, with corresponding mean specificity rates of 87%, 90% and 86% (Leitich et al., 1999).

The clinical performance of the FFN test in symptomatic women is derived from pre-test and post-test probabilities. From a median pre-test probability of 3% for a delivery within 7-10 days of the test, the positive and negative post-test probabilities were 14.4% and 0.8% respectively (Honest et al., 2002).

The negative predictive value of the test for delivery within 7 days in symptomatic women was approximately 99% (Honest et al., 2004), making this the most useful aspect of the test. This has immense cost-saving implications, as management can be targeted to the correct group of women. Evidence for this came from a Canadian study where the availability of the FFN test reduced antenatal admissions from 24 to 12%, with no difference in the PTBs (8.6 versus 7.8%) (Abenhaim et al., 2005).

However, the main disadvantage of the FFN test is that the sensitivity and the positive predictive value of the test are low, meaning that a large number of women will need interventions; hence the test is not very useful for interventional studies. Another limitation is that it cannot be performed in women who have had sexual intercourse within the previous 24 hours of testing (Lukes et al., 1997) and in women with significant vaginal bleeding (Feinberg et al., 1994). Furthermore, the test is costly and the test kit needs to be refrigerated prior to analysis.

In order to overcome the disadvantages of this test, a number of other potential markers have been tested, but to date no other tests have proven in multiple clinical trials to be significantly better than the FFN test. Therefore, there is a need to test novel markers for the prediction of PTL to establish whether they will have better sensitivity and specificity compared to the gold standard FFN test. Plasma AEA is one such biomarker, as the levels have been shown to be elevated just prior to the onset of spontaneous labour (Habayeb et al., 2004).

In this chapter, plasma AEA levels were quantified in women presenting with threatened PTL to evaluate its accuracy in predicting those who truly delivered preterm. The aims of the studies presented in this chapter were, therefore, to determine:

1. if there was a significant difference in the plasma AEA levels between women who delivered preterm and those who delivered at term.

and,

2. whether plasma AEA levels could accurately predict true PTD and to compare the efficacy of plasma AEA in predicting true PTL with that of the gold standard, the FFN test.

5.2 Subjects and Methodology:

5.2.1 Sample size:

From the literature, 6-8% of pregnancies before 34 week of gestation will present with suspected PTL but only 3-45% will proceed to deliver (i.e. are true PTL). From a hospital population of 5500 deliveries therefore, 550-733 women will present with suspected PTL over a 20 month period. Approximately 75% of these will have multiple pregnancies, polyhydramnios or other exclusion criteria. When these are excluded, there will be a minimum of 412 women over the 20 month period of the study from whom to recruit the cohort for the study.

5.2.2 Power calculation:

To improve the sensitivity of the gold standard for predicting true PTL from 63% to 73% at the 5% level of significance and for a power of 80% a minimum of 232 patients was calculated to be required for the cohort.

5.2.3 Subjects:

These were women who attended the delivery suite at Leicester Royal Infirmary with symptoms of threatened PTL between 24 and 37 weeks of gestation. Those who agreed to part take in this ethically approved study signed a written informed consent. The exclusion criteria include multiple pregnancies, pregnancies complicated by medical conditions (such as diabetes mellitus and hypertension, or pregnancies with placenta praevia), women who had ruptured membranes, chorioamnionitis, presented with bleeding, or who were in advanced labour (dilated cervix > 4 cm).

A blood sample was collected at the time of admission for measurement of plasma AEA levels. Additionally, for those who consented and had not had sexual intercourse in the 24 hours prior to admission a vaginal FFN test was performed.

The outcome of the pregnancy was monitored through the EUROKING database system of the Leicester Royal Infirmary and the gestational age at delivery was recorded. The time interval from sampling of blood for AEA measurement to delivery was also noted, whilst other variables such as maternal age, BMI, ethnicity, presence of other risk factors like smoking, or previous preterm delivery were recorded.

5.2.4 Methods:

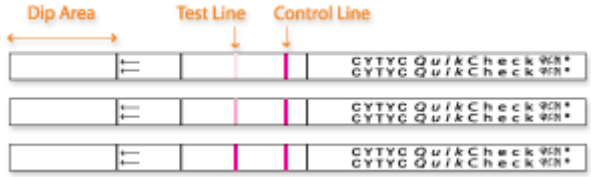
5.2.4.1 Measurement of plasma AEA concentrations

Plasma levels of AEA were measured from 4ml of blood after extraction using the LPE and measured using a UPLC-MS/MS method as described in detail in Chapter 2.

5.2.2.2 Measurement of FFN

This was measured using FFN membrane immunoassay test kits purchased from Cytyc UK Ltd., Crawley, West Sussex. To perform the FFN test, women were placed in the lithotomy position and a sterile speculum lubricated with a very small amount of KY lubricating jelly (Johnson & Johnson, New Brunswick, NJ) was inserted into the vagina to expose the cervix. A sterile polyethylene terephthalate fiber (Dacron) swab was then placed onto the posterior fornix where it was gently rotated for ≥10 seconds, in accordance with the manufacturer's instructions. The swab was inserted into a separate tube containing extraction buffer and mixed vigorously in the buffer for 10 to 15 seconds. The swab was then removed and the lower end of the dipstick inserted into the same buffer for 10-15sesconds. The results were interpreted after 5 minutes. A positive result was indicated by the presence of 2 lines, whereas a negative result was indicated by the presence of a single control line. The additional line that indicated a positive result could vary from faint to dark. An example of these possible results is shown in Figure 5.1.

- A **positive** (+) patient result will appear as two lines: a test line and a control line. The presence of a very light test line should be interpreted as a positive result.



- A **negative** (-) patient result will appear as one distinct line: a control line.

- The absence of a distinct control line should be interpreted as an invalid result.

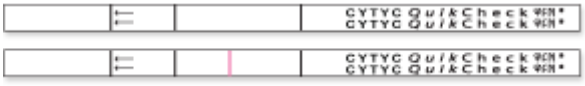


Figure 5.1. Interpretation of the FFN test results.

The result of the FFN test was made available to the clinicians for the management of the women. All the women who were considered at significant risk of delivering preterm were given steroids (two doses of dexamethasone or betamethasone by injection) at the discretion of the managing clinician. Women who needed transfer out of the hospital received atosiban, an oxytocin antagonist, which is used to stop uterine contractions. Any woman who had evidence of urinary tract infection was treated with appropriate antibiotics.

5.2.4.3 Statistical analysis

Data analysis was performed using GraphPad Instat version 3, (GraphPad Software, San Diego, CA, www.graphpad.com). Descriptive statistics were calculated for baseline demographics and obstetric characteristics (mean and SD for continuous variables and numbers and percentages for categorical variables). Comparison of Gaussian distributed, continuous data was made using Student's unpaired t-test and that of non-Gaussian distributed data was made using Mann-Whitney U-Test. Categorical data were compared using χ^2-test.

5.3 Results:

A total of 135 women were originally recruited into the study, but only 100 fulfilled the criteria for inclusion into the study. Table 5.1 shows the reason for exclusion.

Fifteen out of the 100 women delivered preterm giving a rate of 15% for spontaneous PTD; 2 other women had an emergency Caesarean section (in one woman for fetal distress and labour after previous 4 Caesarean sections in the other). If the indicated PTD is included, the PTD rate was 17% in the study population.

Table 5.1. Reasons for exclusion of women from study.

Reason for Exclusion	Number
Preterm premature rupture of membranes	9
Outcome not known	15
Advanced cervical dilatation	2
Twins	3
Placenta praevia	1
Significant bleeding	1
Gestational Diabetes Mellitus	2
Benign intracranial hypertension	1
Lost specimen	2

The demographic characteristics of the women who delivered at term and preterm are shown in Table 5.2. There were no differences in any of the demographic variables for both groups.

The mean plasma AEA levels in the women who delivered preterm (0.65 ± 0.42nM) were not significantly different from that of women who delivered at term (0.71 ± 0.40nM) (Figure 5.2).

The plasma AEA levels of women (n=7) who delivered within one week of presentation (0.73 ± 0.21nM) were also not significantly higher than those in women who delivered at term (0.71 ± 0.40nM) (P=0.19). These data are shown in Figure 5.3.

A ROC analysis of the plasma AEA levels showed that at a cut-off level of 1.036nM, the specificity of the test in predicting PT within 7 days of testing was 82.35% (95%CI 56.57-96.20%) but the sensitivity at this level was only 22.89% (95%CI 14.38-33.42%). The positive predictive value was 21% and the negative predictive value was 16%. The area under the ROC curve was 0.55. This is shown in Figure 5.4.

Table 5.2. Demographics and Obstetric History of the Study Population.

Variable	Delivered Preterm n=17	Delivered Term n=83	P value
Maternal Age (years) (mean ± SD)	25.41 ± 5.4	27.48 ± 5.5	ns
White n (%)	16 (94)	71 (85)	ns
Asian or African descent n (%)	1 (6)	12 (15)	ns
BMI (Kg/m^2) (mean ± SD)	24.69 ± 5.7	24.84 ± 5.0	ns
Multiparous n (%)	9 (52)	53 (63)	ns
Primiparous n (%)	8 (48)	30 (37)	ns
Smokers n (%)	4 (25)	32 (39)	ns
Gestational age at Presentation (Median)	28	31	P=0.007

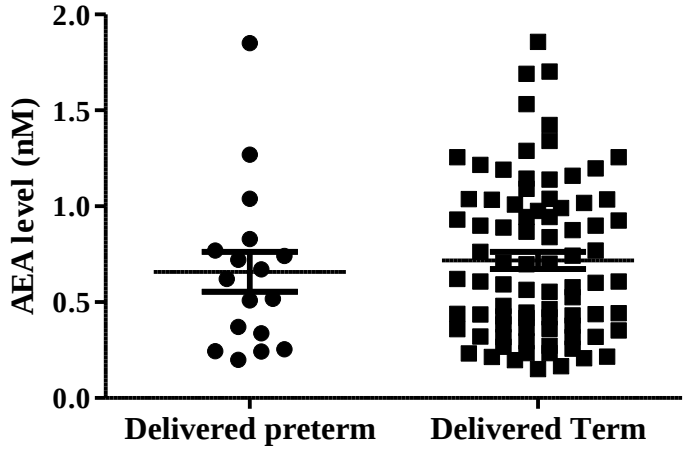


Figure 5.2. Comparison of plasma AEA levels between women who delivered preterm and term. The plasma AEA levels in women who delivered preterm (n=17) and women who delivered at term (n=83) are shown as individual data points along with the mean (long horizontal bar) ± SD (shorter horizontal bars). The plasma AEA levels was not significantly different between the two groups (P>0.05; Student's *t*-test).

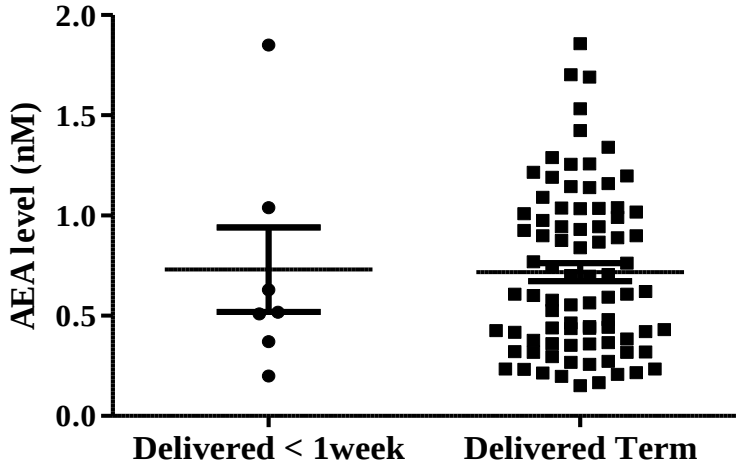


Figure 5.3. Comparison of plasma AEA levels between women who delivered preterm within 1 week of presentation and those who delivered at term.

The plasma AEA levels in women who delivered preterm (n=7) and women who delivered at term (n=83) are shown as individual data points along with the mean (long horizontal bar) ± SD (shorter horizontal bars). The plasma AEA levels were not significantly different between the two groups (P>0.05; Student's *t*-test).

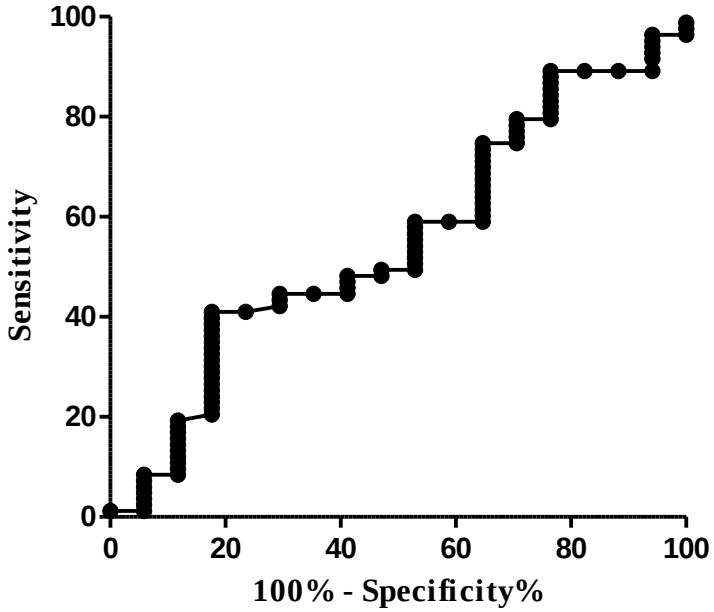


Figure 5.4. Receiver Operating Curve of plasma AEA levels in predicting PTD. A plasma AEA level of 1.036nM predicted the risk of PTD with sensitivity of 22.89% and specificity of 82.35%. The positive likelihood ratio was 1.30. AUC=0.65(95% CI 0.49-0.82); P=0.07.

There was a significant correlation between plasma AEA levels and the time interval between sampling and delivery suggesting an association between the two parameters (Pearson's correlation r=-0.25; P=0.01). There was a negative correlation between plasma AEA concentration and sampling to delivery interval. The slope of the regression curve was -17.85 x AEA concentration (r^2=0.06). This is illustrated in Figure 5.5.

The FFN test was performed in only 78 of the 100 women. The reasons for not performing the test in the remaining 22 women included the patient's refusal of the test (n=16), having had intercourse in the last 24 hours (n=4) and/or the presence of bleeding (n=2).

The FFN test was performed in 11/17 (64%) women who delivered preterm and 67/83 (80%) who delivered at term. The FFN test was positive in 4/11 (36%) women who delivered preterm and 9/67 (13%) who delivered at term.

There were no statistically significant differences in maternal age, parity, or gestational age at the time of sampling in those who were FFN positive and those who were negative (Table 5.3**).** However, there was a statistically significant difference in the length of gestation at delivery with a longer gestation if the FFN test was negative (Table 5.3). However, the proportion of women who delivered preterm was not significantly different in the two groups, although it was close to the statistical cut-off of P<0.05 (P=0.07).

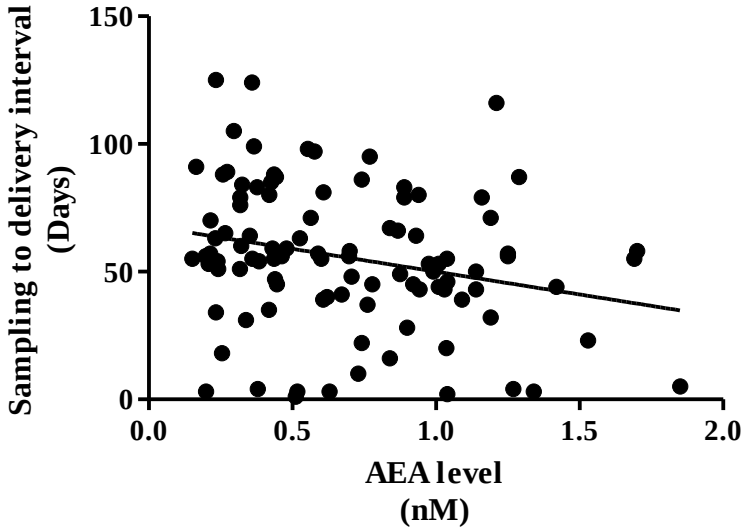


Figure 5.5. Regression of sampling to delivery interval from plasma AEA concentration. Regression coefficient is -17.85 ± 6.91 (r^2=0.06).

Table 5.3. Comparison between FFN positive and negative cases among patients sampled between 24 and 37 weeks of gestation

Variable	FFN positive n=13	FFN negative n=65	Significance
Maternal Age (years) (mean ± SD)	27.69 ± 6.6	27.62 ± 5.6	ns
Gestation at sampling (weeks) (mean ± SD)	29 ± 3.2	30 ± 2.6	ns
Gestation at delivery (weeks) (mean ± SD)	36.3 ± 6.5	38.6 ± 1.8	P=0.01
Multiparous n (%)	10 (76)	43/65	ns
Primiparous n (%)	3 (23)	22/65 (33)	ns
PTB rate	4 (30)	7/65 (10)	P=0.07

The sensitivity of predicting PTD within 7 days of testing with the FFN test was 30% (95%CI 6-65%); specificity was 85 % (95%CI 74-92%); positive predictive value was 23% (95%CI 5-53%) and the negative predictive value was 89% (95%CI 79-95%). These data are shown in Table 5.4. The relative risk of delivering within 7 days of a positive FFN test was 2.14 (95% CI 0.63-7.2).

The sensitivity of predicting PTD at <37 weeks of gestation was 36% (95%CI 10-69%); specificity was 86% (95%CI 76-93%); positive predictive value was 30% (95%CI 9-61%) and the negative predictive value was 89% (95%CI 79-95%). These data are shown in Table 5.5. The relative risk of delivering within 7 days of testing positive with a FFN test was 2.85 (95% CI 0.97- 8.3).

The mean plasma AEA level (0.83 ± 0.43nM) in the 13 women who had a positive FFN test was significantly (P=0.04; Student's two tailed t-test) higher than the mean level in women who tested negative (0.61 ± 0.35nM). These data are shown graphically in Figure 5.6.

Table 5.4. FFN test results in cervicovaginal secretions sampled at 24-37 weeks gestation, as a predictor of birth within 7 days of testing.

FFN Test Result	Delivery <7days	Delivery >7days	Total
Positive	3	10	13
Negative	7	58	65
Total	10	68	78

Table 5.5: FFN test results in cervico-vaginal secretions sampled at 24-37 weeks gestation as a predictor of birth at <37 weeks of gestation.

FFN Test Result	Delivered < 37 weeks	Delivered >37 weeks	Total
Positive	4	9	13
Negative	7	58	65
Total	11	67	78

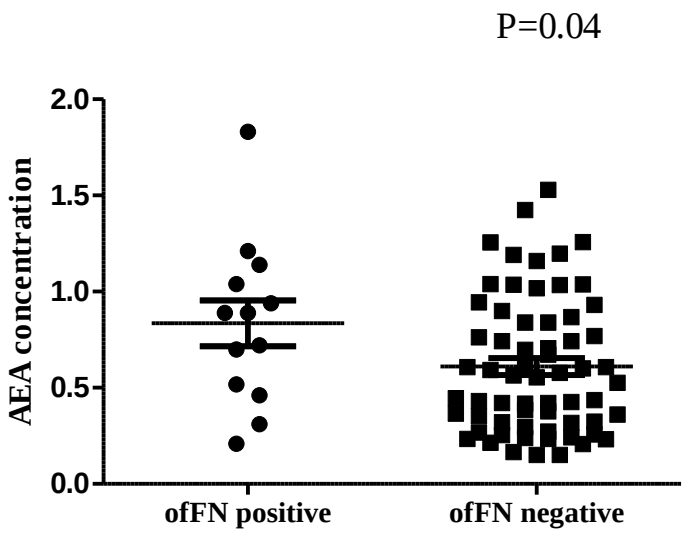


Figure 5.6. Comparison of plasma AEA levels between women who tested positive and negative for FFN. The data are presented are shown as individual data points along with the mean (long horizontal bar) ± SD (shorter horizontal bars). The plasma AEA level in one woman who had a positive FFN test was very high when compared to rest of the women in the group.

5.4 Discussion:

In this chapter, plasma AEA levels were measured in a cohort of pregnant women admitted with symptoms of threatened PTL. This is a high risk group with an estimated PTD rate of 20-25%. The PTD rate in this cohort was 17% which is consistent with that quoted in other studies (Hamilton et al., 2010; Honest et al., 2003). Since most of these women do not deliver preterm, it is vital that those with true PTL are identified for subsequent tailored management.

With regards to possible confounding factors, there was no difference between those who delivered preterm and those who delivered at term with regards to ethnicity, age, parity, smoking status or BMI, all factors which have been associated with PTB (Goffinet , 2005). One woman in the preterm group and two women in the term group had a history of previous preterm deliveries, but this was also not significant. The lack of a statistical association with these well documented risk factors may be related to the small sample size of this study.

The FFN test, the current gold standard test for identifying those who will deliver preterm in women presenting with threatened preterm labour was used for comparison. The FFN test was performed in only 78 of the 100 women.

Out of the 78 women in whom the FFN test was performed, the test was positive in 13 of them. The test produced a sensitivity in predicting PTD within 7 days of testing of 30% (95%CI 6-65%); a specificity of 85 % (95% CI 74-92%); a positive predictive value of 23% (95%CI 5-53%) and a negative predictive value of 89% (95% CI 79-95%). These values are different from those reported by Malak et al. in Leicester (Malak et al., 1996) who obtained sensitivity, specificity, positive and negative predictive values of 80%, 90.2%, 44.4% and 97.9%, respectively in predicting delivery within 7 days of the test. The differences between the two studies may be as a result of the type of test performed. Previously, an ELISA method was used to quantify the FFN levels and the treating clinicians were blinded to the results of the test whereas in the present cohort, FFN membrane

immunoassay test was used and the clinicians were not blinded to the test result. A systematic review by Honest (Honest et al., 2006), reported that there was significant heterogeneity in the results among the various studies using the FFN test. This could not be explained by a difference in the risk status of the groups tested, type of recruitment, the presence or absence of digital examination before testing, sexual intercourse within 24 hours preceding testing, bleeding before testing, methods of testing, serial testing, blinding of the test result, study design, publication language or the quality of studies.

In a meta-analysis done by Leitich et al.,(1999) the overall sensitivity and specificity in predicting PTD within 7 days in symptomatic women was 89% (95% CI 80-97%) and 86% (95% CI-81-91%), respectively. However, the latest meta-analysis performed by Sanchez-Ramos et al. (2009) also showed that the accuracy of the FFN test in predicting PTD within 7 days of testing among symptomatic woman was limited in its scope with a sensitivity and specificity of 76.1% (CI 95% 69.1-81.9) and 81.9% (95% CI 78.9-84.5), respectively. The results presented in this Chapter are similar to those of the latest meta-analysis and the FFN test efficacy appears to be less effective when compared with other previous studies.

The false positive rate of 69% (9/13) in the present study was relatively high. The release of FFN is thought to be both from the disrupted chorio-decidual interface and from the zone of altered structure within the fetal membranes at the rupture site (Malak et al., 1996). The disrupted chorio-decidual interface could be due to decidual membrane activation occurring as a part of a common terminal pathway in preterm and term labour. This activation could result in further membrane degradation, and promote uterine contractions resulting in labour and delivery. However, the activation process may be reversible in its early stages or may be controlled by “unidentified pregnancy maintenance mechanisms" explaining some of the false positive results (Malak et al., 1996). It is also known that FDC-6 (the monoclonal antibody used to detect FFN) binds specifically to plasma fibronectin isoforms. Contamination of cervico-vaginal samples with blood may occur from cervical friability, abrasion with a speculum or swab. But that possibility is doubtful in the study presented in this

Chapter, because all samples were carefully obtained and the one sample with blood contamination was excluded from the analysis (Table 5.1).

The rate of false negative result in this study was 7/65 (10%). False negative results may be due to sampling error or sampling during the early stage of decidual/membrane activation process before the chorio-decidual separation. The sampling error was minimised, so it can be concluded that decidual/membrane activation must have occurred or the FFN test may have been inadvertently misinterpreted.

It is well known that the FFN bedside test is associated with difficulty in interpretation of test results. In the study by Coleman et al.,(Coleman et al. 1996), the assessment of three FFN test strips was evaluated for 36 clinicians and the inter-observer variation calculated. Eighty-nine percent (32/36) agreed with the clearly positive strip result and one hundred percent agreed with the negative test strip result. For the faintly positive result, 53% (19/36) classified it as positive and 47% (17/36) classified it as negative (Coleman et al., 1998). Thus, some of the false positives and negatives in this series could be attributed to these faint positive results. However, 89% of women who tested negative did not deliver within the next 7 days of sampling. The negative predictive value was thus much lower when compared to the 97.9% reported in the study by Malak (1996).

The plasma AEA concentrations were not significantly different in women who delivered preterm (65 ± 0.42nM) when compared to those in women who delivered at term (0.71 ± 0.40nM). They were also not significantly different in women who delivered within 7 days of testing and those who delivered after 7 days of testing. The sensitivity, specificity, positive predictive and the negative predictive values using a plasma AEA level of 1.036nM in predicting PTD within 7 days of testing were 22.89%, 82.35%, 21% and 16% respectively. The ability of AEA in predicting PTD is not accurate when compared to FFN.

The plasma AEA levels in the initial validation study in the second and third trimesters were 0.90 ± 0.08nM and 1.14 ± 0.11nM respectively (Chapter 3). The women in this series were in the second and third trimesters. The plasma AEA level in the high risk group was 1.01 ± 0.70nM among women who delivered preterm and 0.73 ± 0.40 in women who delivered at term. There was no significant difference in the plasma AEA levels in those who delivered at term in this series and the series on high risk women. However, it is interesting to note that the women in this study who had a positive FFN test had a significantly higher concentration of plasma AEA (0.83 ± 0.43nM) when compared to those who were FFN negative (0.61 ± 0.35nM). These observations suggest that there is a relationship between the two factors since both have been associated with subsequent delivery. Whether they have the same biological origin is uncertain and perhaps needs further investigation.

AEA has been quantified in human biological fluids including amniotic fluid, follicular fluid and oviductal fluid and many gestational tissues such as placenta and fetal membranes (Taylor et al., 2010). There is, however, a paucity of information about the role of AEA in later pregnancy and parturition. The fact that marijuana users were at high risk of preterm labour, stillbirth, fetal growth restriction and placental abruption (Zuckerman et al., 1989) suggest that AEA may indeed play a role in late pregnancy and labour. Also, the demonstration that CB1 and CB2 receptors are expressed in placental and decidual tissues throughout gestation (Park et al., 2003; Heliwell et al., 2004; Habayeb et al., 2008), support the conclusion that endocannabinoids are involved in parturition. Moreover, plasma AEA levels in the rat are increased during two critical stages in pregnancy; on day 10 when there is regression of the placenta and decidua and on day 19 (Fonseca et al., 2009), when there is activation of the myometrium in preparation for parturition on day 21. Furthermore, the CB_1 knockout mouse delivers its pups prematurely (Wang et al., 2008), suggesting that the endocannabinoid system has a key, but not yet fully understood, role in the parturition process.

5.5. Conclusion:

There is some evidence that plasma AEA levels are elevated during labour (Habayeb et al., 2004; Lam et al., 2008) and in women who are at high risk of delivering preterm due to their significant past obstetric history or to having undergone cervical surgery (Chapter 4). However, the levels of AEA in women admitted to the delivery suite with threatened PTL who subsequently delivered preterm or at term were not different. The lack of a difference could conceivably be due either to a small sample size or that the pathology that initiated the labour in this group of women did not influence the plasma AEA levels. Furthermore, the pathophysiological mechanisms involved in PTL could likely be different from the term labouring process.

Nevertheless, this study has shown that there was a significant correlation between plasma AEA levels and positivity of FFN test, suggesting a relationship between fetal membrane integrity and the plasma AEA regulation.

These interesting observations need to be further investigated in studies with larger numbers to critically appraise the potential value of plasma AEA levels.

Chapter 6

General Discussion

Introduction:

The aim of this thesis was to investigate the role played by the endocannabinoid, AEA in labour. This lipid has been shown to play a role in pregnancy there has been little research on its role in labour. Previous research had already established that, chronic administration of AEA in rats leads to prolongation of pregnancy and stillbirth (Wenger et al., 1997). This was associated with a reduction in the levels of serum PGs. Such manipulations in humans are unethical, but data concerning the use of exogenous cannabinoids, such as marijuana and hashish demonstrated that they have a significant effect on pregnancy. For example, marijuana smoking is associated with an increased risk of delivering preterm (Gibson et al., 1983). Furthermore, AEA has been shown to be synthesised in the uterus (Paria et al., 2001) and has been found in AF (Schuel et al., 2002). The study by Habayeb et al.,(2004) showed that there was an elevation in plasma AEA levels with the onset of labour. All of these studies suggested that there could be a possible role for the endocannabinoid system in labour. The aim of the thesis was to explore this relationship further.

The original hypotheses that were explored were:

1. plasma AEA levels are involved in the process of labour and that this is mediated through an increase in the level of plasma AEA.
2. plasma AEA levels are elevated in women who deliver preterm in comparison with women who deliver at term, in women presenting with threatened PTL and in asymptomatic women who were high risk of delivering preterm.

The initial step in the process of investigation was to develop a robust method for measuring AEA in human plasma. Although AEA had previously been measured in the plasma there were several shortcomings in the methods used, such as a relatively long run time, poor limits of detection and wide variations in the levels measured.

The LPE method as described in Chapter 2 allowed a more rapid throughput of clinical samples and had better limits of detection and quantification (Lam et al., 2008). However, even with the liquid phase the extraction method became the rate limiting step. So the SPE method, which was equally efficient in the extraction process and also required a shorter processing time, was developed. It took

3.5 hours to process 10 samples with the LPE method whereas processing of a similar number of samples took only 40 minutes with the SPE method. The other advantage with the SPE method is that a larger number of samples can be processed at the same time (Marczylo et al., 2009).

Samples had to be processed as soon as they were collected, which took nearly 2 hours. This meant valuable time that could be used for recruiting patients for the research studies was lost. In order to overcome this, plasma AEA levels were analyzed in 10 stored and fresh plasma samples. The results showed that there were no significant differences in plasma AEA levels, however, in order to ensure that there was uniformity in the way the samples were processed immediately and the,SPE method was not used in these studies. However, this method has the potential to be made use of in future research, and has been adopted by the research group (Marczylo et al., 2010).

Using LPE method (Lam et al., 2008), changes in the plasma AEA levels during pregnancy were compared with the levels found using the method previously used by Habyeb et al., (2004). The levels of plasma AEA remained low during pregnancy until term and then increased significantly in labour, corresponding with previously reported data. The rise in plasma AEA in labour again reconfirmed that there may be a possible role for plasma AEA in labour. The above two studies were, however, cross-sectional studies, and thus could be subjected to the increased inter-patient variability inherent in such studies.

In order to eliminate this bias, the study was undertaken in a cohort of women who underwent induction of labour where each woman acted as her own control (Chapter 3). There was a statistically significant 1.5-fold increase in plasma AEA levels with labour ($P<0.0001$). As functional progesterone withdrawal is considered to be linked to the onset of labour it could be deduced from this that the changes in the plasma AEA level during labour could be hormonally mediated as plasma AEA levels have been shown to be reduced by progesterone, mediated through its action on FAAH activity in the peripheral lymphocyte (Maccarone et al., 2000). This elevation in plasma AEA levels could also be initiated to increase the production of PGs by gestational tissues, as suggested by Mitchell and Sato, (2008). All gestational tissues; myometrium, fetal membranes, placenta decidua and cervix are known

to express cannabinoid receptors (Park et al., 2003; Dennedy et al., 2004; unpublished observations from our group). Additionally, since AEA metabolism by FAAH leads to the production of AA, AEA could be a source of AA, which is the principal metabolite from which prostaglandins are produced (Di Marzo et al., 2007). This may be important because PGs are essential for the initiation and progression of labour (Mitchell et al., 2008).

There was a statistically significant negative correlation between the percentage increase in plasma AEA levels during the transition from the non-labouring to the labouring phase and the induction-to-delivery interval (Spearman's r=-0.32; P=0.02). There was also a significant negative correlation between plasma AEA levels during the active phase of labour and duration between the active phase of labour and delivery (Spearman's r=0.36; P=0.025). These data suggest that AEA could have an uterotonic action on the myometrium. However, the study by Dennedy et al., (2004), showed that AEA had a relaxing effect on pregnant myometrium in an *in vitro* study and this action was mediated through CB1 receptors. However, it is also known that AEA acts through CB1 and CB2 receptors and can cause a reduction in AC activity leading to a decrease in intracellular cAMP levels (Petrocellis et al., 2004). A decrease in cAMP levels in the myometrium leads to myometrial contraction (Sanborn et al., 2001). Thus, it is possible that AEA has a different effect on the myometrium depending on the stage of pregnancy and or local concentrations. Alternatively, AEA could have a different effect on the myometrium in the upper and the lower segments, as specimens collected in the study by Dennedy et al., (2004) were from the lower uterine segment and all women had elective caesarean section.

Having established that plasma AEA levels increased with active labour it was hypothesised that among high-risk women, plasma AEA levels would be elevated in those who are truly at risk of preterm delivery. The aims of the next parts of this study were, (1) to measure plasma AEA levels in a cohort of high risk women and to investigate whether plasma AEA levels can predict which of them would truly deliver preterm and (2) furthermore to compare the accuracy of plasma AEA levels with that of cervical length in predicting PTD.

This cohort of women were at high risk of delivering preterm, for example, women with a prior spontaneous preterm delivery who have a 2.5-fold increased risk of PTD in the current pregnancy in comparison to women who have never experienced preterm births (Mercer et al., 1999) and women who had undergone the LLETZ procedure with a 3-fold increased risk (Jakobsson et al., 2009). In these high risk women, screening for signs and symptoms of PTD offers opportunities for early intervention.

Cervical length has therefore, been used for screening asymptomatic high risk women to identify those likely to deliver preterm (Andrews et al., 2000; Guzman et al., 2001). The consensus view from these predictive studies is that the earlier the shortening of the cervix occurs during pregnancy and the greater the degree of the shortening, the greater is the risk of PTD.

The study showed that plasma AEA levels in women who delivered within 6 weeks of sampling were significantly elevated when compared with the levels in those who delivered at term (P=0.01). The reason for this difference could be that plasma AEA levels rise only a few weeks before the onset of PTL and delivery and therefore the levels in women whose blood was sampled remotely from delivery (i.e.at week 20 who then delivered in week 30) would not have been expected to be significantly different from those who delivered at term. This is also supported by the findings of Habayeb et al., (2004), who showed that plasma AEA levels in normal pregnancy remain low during the second and third trimesters and only rose at term prior to the onset of labour.

From this study, I found that a plasma AEA level of 1.11nM predicted the risk of PTD with a sensitivity of 28.6% and a specificity of 82.9%. The positive likelihood ratio was 1.7 and the area under curve for the predictive value using the plasma AEA level was 0.598. The sensitivity, and specificity of a cervical length <25mm in predicting PTD were 42.86% and 70.97% respectively. The likelihood ratio of PTD with a cervical length of <25mm was 1.5. By contrast, a cervical length of <15mm, gave a sensitivity and specificity of 28.6% and 93.6%, respectively and the likelihood ratio of delivering preterm increased to 4.4. The area under the curve for the predictive value using cervical length of 25mm was 0.575

This study also showed that the cervical length was also found to be significantly inversely correlated to plasma AEA levels (r=-0.35; P=0.02). The clear inverse correlation between plasma AEA levels and cervical length suggests that there could be a direct causal relationship between AEA levels and cervical function. CB1, CB2 and TRPV1 receptors have all been identified in cervical cancer cells (Contassot et al., 2004) and AEA has also been measured in cervical cancer cells. Unpublished data from our group demonstrated CB1, CB2 and FAAH in the normal cervix suggesting that the cervix may be a target for AEA. Recently, the levels of AEA were quantified in normal human fetal membranes and placenta (Marczylo et al., 2010). As already discussed, AA, which is the principal metabolite from which prostaglandins are produced, could arise from AEA (Di Marzo and Petrosino, 2007) and AEA has been reported to increase PG synthesis by gestational tissues such as the amniochorion through its action on CB1 receptors (Mitchell et al., 2008). It is therefore possible that AEA causes cervical effacement indirectly by increasing PG synthesis.

The next group of women who are also at significant risk of delivering preterm are those who present with threatened PTL. These group of women pose a particular diagnostic challenge to clinicians as only a small proportion, (8–24%) of those who present with symptoms will go on to deliver prematurely (Hamilton et.al., 2010), and, clinically, it is difficult prospectively to separate those who will eventually deliver preterm (i.e. presenting in true preterm labour) from those who progress to term. This is largely due to lack of an accurate test to diagnose true PTL. Digital examination of the cervix, although an essential part of clinical diagnosis of PTL, is subjective and is associated with wide inter- and intra-observer variability. Ultrasound assessment of cervical length is a better test at delineating the presence of a ripened cervix, when compared to digital examination (Herbst et al., 2006). For example, in one study, a cervical length of <15 mm in women admitted with PTL was associated with a 50% risk of delivering within 7 days (Norman et al., 2007). Only 1% of women with cervical length >16mm delivered within 7 days. Thus, ultrasound cervical length measurement has a good negative predictive value. However, the test is invasive and needs to be performed by trained personnel, often out of hours and immediately after such a woman is admitted.

A test with a good positive predictive value and a good negative predictive value is needed to identify women with threatened PTL, who subsequently go on to deliver preterm, to prevent women unnecessarily receiving steroids, tocolysis and possible being transferred to other centres away from their community. In this regard, testing women presenting with threatened PTL with a FFN test changed the median pre-test probability of delivering within 7-10 days of the test from 3% to a positive and negative post-test probabilities of 14.4% and 0.8%, respectively (Honest et al., 2002). The negative predictive value of the test for delivery within 7 days in symptomatic women was approximately 99% (Honest et al., 2002). This is the most useful aspect of the test as the management can be targeted to the correct group of women. This helps saving cost as evidenced by a Canadian study which showed that the availability of the FFN test reduced antenatal admissions from 24 to 12%, with no difference in the PTBs (8.6 versus 7.8%) (Abenhaim et al., 2005). However, the main disadvantage of the FFN test is that the sensitivity and the positive predictive values of the test are low, meaning that a large number of women will need interventions; another limitation is that it cannot be performed in women who have had sexual intercourse within the previous 24 hours of testing. So it seemed sensible to measure the plasma AEA levels in women presenting with threatened PTL to evaluate its accuracy in predicting those who truly delivered preterm and compare the efficacy of this test in predicting true PTL, with that of the gold standard fFN test.

The data indicated that this cohort of women had a PTD rate of 17%, which is consistent with that quoted in other studies (Hamilton et al., 2010; Honest et al., 2003), but the mean plasma AEA levels in the women who delivered preterm were not significantly different from that of women who delivered at term. The plasma AEA levels of women (n=7) who delivered within one week of presentation were also not significantly higher than those in women who delivered at term (P=0.19).

Taking a plasma AEA level of 1.036nM as a cut-off level, the specificity of the test in predicting PTD within 7 days of testing was 82.35% but the sensitivity at this level was only 22.89% The positive predictive value was 21% and the negative predictive value was 16%. The area under the ROC curve was 0.55, whilst the sensitivity of predicting PTD within 7 days of testing with the FFN test was 30%,

specificity was 85%, positive predictive value was 23% and the negative predictive value was 89% The RR of delivering within 7 days of a positive FFN test was 2.14.

Nevertheless, the mean plasma AEA level in the women who had a positive FFN test was significantly (P=0.04) higher than the mean level in women who tested negative. These observations suggest that there is a relationship between the two factors since both have been associated with subsequent delivery, but that the measurement of plasma AEA is not a good test for predicting PTD. This is particularly difficult to understand, when one considers the recent evidence showing that the CB_1 knockout mouse delivers its pups prematurely (Wang et al., 2008). The authors suggested that a defect in the endocannabinoid system exists in the mouse that leads to elevated AEA levels resulting in PTB. They, however, did not measure the plasma AEA levels in the pregnant knockout mice, so it is not possible to make that link. This suggests that further studies are needed to increase the understanding in this area.

Conclusions

Prior to the start of the thesis, it was known that the plasma AEA levels were elevated with the onset of labour (Habayeb et al., 2004). CB1 and CB2 receptors are expressed in placental and decidual tissues throughout gestation (Park et al., 2003; Heliwell et al., 2004; Habayeb et al., 2008) and AA acid, which is the principal metabolite from which PGs are produced, could arise from AEA, as hydrolysis of AEA by FAAH leads to the production of AA and ethanolamine (Di Marzo and Petrosino, 2007). AEA has been reported to increase PG synthesis by gestational tissues such as amniochorion through its action on CB1 receptors (Mitchell et al., 2008). AEA mainly acts through CB1 and CB2 receptors and causes a reduction in AC activity leading to a decrease in cAMP levels (Petrocellis etal., 2004). A decrease in cAMP levels in the myometrium leads to myometrial contraction (Sanborn et al., 2001). It has also been shown to exert a relaxing effect on pregnant myometrium in an *in vitro* study and this action was mediated through CB1 receptors (Dennedy et al., 2004).

The data presented in the thesis add to this existing knowledge by providing:

a. a much improved method of measuring plasma AEA than previously described.
b. an alternative form of AEA extraction; the SPE technique which is equally effective but less time consuming.
c. plasma could be stored for a day prior to extraction without any evidence of decrease in concentration of AEA.
d. that plasma AEA levels become elevated in active labour in women after undergoing induction of labour. This is a much stronger evidence when compared to a previous study (Habayeb et al., 2004) confirming a possible association of plasma AEA levels with labour.
e. that higher the percentage of elevation in plasma AEA levels, the shorter the time duration from sampling (active phase) to delivery.
f. that plasma AEA levels are elevated in women who are at high risk of PTD and who deliver within 6 weeks of sampling when compared to those who deliver at term.
g. that plasma AEA levels had a significant negative correlation with cervical length suggesting that AEA could have an effect on the cervix. This could be either by a direct action of AEA on cervix (unpublished report suggest the presence of CB1 and CB2 receptors in the cervix) or indirectly by acting as a source of AA.
h. that plasma AEA levels were not elevated in women who delivered preterm amongst a cohort of women who presented with threatened PTL. However, this could be the effect of small sample size. The plasma AEA levels were higher in women who had a positive FFN test suggesting that there may be a relationship between these two factors, the nature of which is unknown.

Therefore, although the described here generate new observations, there remain several unanswered questions regarding the involvement of AEA in parturition. These could be addressed through the following studies:

a. Increasing the sample size in the work of both Chapters 4 and 5.
b. Including cervical length measurements in those presenting with threatened PTL.
c. Measuring the levels of PGs as well as those of AEA in women who are not induced with PGs and relating these.

d. Measuring AEA levels in the various gestational tissues before and after the onset of labour.
e. Immunohistochemistry studies of the changes in the expression of CB1, CB2 receptors, FAAH levels (and new members of the endocannabinoid system discovered since I started this research) in the various gestational tissues before and after the onset of labour.
f. Manipulating the endocannabinoid system with AEA/AEA antagonist to see if labour could be induced or arrested. This could be done in experimental animals, such as laboratory rats, mice or rabbits.

Appendix1: Publication arising from the thesis

1. Ultra Performance Liquid Chromatography Tandem mass Spectrometry Method for the Measurement of Anandamide in Human plasma.

Analytical Biochemistry, Volume 380, Issue 2, September 2008, Pages 195-201.

Patricia M.W.Lam, Timothy H. Marczylo, Mona El-Talatini, Mark Finney, Vijaianitha Nallendran, Anthony H. Taylor, Justin C. Konje

2. A solid phase method for the extraction and measurement of anandamide from multiple human biomatrices.

Analytical Biochemistry, Volume 384, Issue 1, January 2009, Pages 106-113.

Timothy H. Marczylo, Patricia M.W. Lam, Vijaianitha Nallendran, Anthony H. Taylor, Justin C. Konje.

3. **The plasma levels of the endocannabinoid, anandamide, increase with the induction of labour.**

British Journal of Obstetrics and Gynaecology, Volume117, Issue 7, June 2010, Pages 863-869.

Nallendran.V, Lam.P, Marczylo.T, Bankart.M, Taylor.A, Taylor.D, Konje.J

Appendix 2: Presentation arising from the thesis

Poster Presentations :

1. Role of anandamide in labour.

Annual Scientific Meeting of the Society for Gynaecologic Investigations, San Diego USA, March 2008.

2 Prediction of preterm labour among asymptomatic high risk patients using plasma anandamide levels.

Annual Scientific Meeting of the Society for Gynaecologic Investigations, Glasgow UK, March 2009.

University of Leicester

ROLE OF ANANDAMIDE IN LABOUR

Vijaianitha Nallendran, Anthony H. Taylor, David J. Taylor, Justin C. Konje

Endocannabinoid Research Group, Reproductive Sciences Section, Department of Cancer Studies & Molecular Medicine, University of Leicester, Leicester, United Kingdom

Introduction

- The precise regulatory mechanisms associated with human labour remain unresolved, despite the significant progress in the field of reproductive medicine at the cellular and molecular level.
- A better understanding of the contractile process within myometrial smooth muscle cells may help us to determine the pathogenesis of abnormal labour, leading to prevention and novel treatment strategies.
- Recent research has demonstrated that the levels of the endocannabinoid, anandamide (AEA), are elevated in labouring women[1].
- *In vitro* studies have shown that anandamide has a relaxing effect on oxytocin-stimulated myometrial strips, possibly by inhibiting the production of intracellular cyclic AMP[2].
- *In vitro* studies have demonstrated that anandamide inhibits PGE_2-stimulated cAMP production by primary myometrial smooth muscle cells suggesting that AEA may improve the response of the myometrial cell to stimulation by oxytocin and other uterotonics.
- The role of anandamide in labour remains controversial.

Aim

To confirm that plasma anandamide levels are elevated during labour and to further research into the relationship between labour and anandamide.

Subjects & Methods

Forty women who were admitted to the Leicester Royal Infirmary for induction of labour were recruited. Nine were primigravida and 31 were multiparous (Table 1). In all cases, blood samples were collected both prior to induction and when the women had entered the active phase of labour. Induction was made using standard procedures (Table 1) and the Bishop Score made.

Table 1: Characteristics of the women entered into the study

	Mean	Range/Nº
Gestational age	40.2	37- 42
Gravidity		
Primigravida		9
Multiparous		31
Body mass index	24.5	18-36
Bishop Score	4	0-8
Induction Type		
Artificial Rupture Of Membranes (ARM)		3
ARM + syntocinon		6
Prostin		8
Prostin + ARM		8
Prostin + syntocinon		7
Prostin + ARM + syntocinon		8
Pain Relief		
Entonox		13
Epidural		26
Fentanyl patient controlled analgesia		1

Blood samples were centrifuged at 12,000 x g for 30 min and plasma separated within 2 hours of collection. The plasma was either processed immediately or stored at -20 c for 24 hours.

AEA was extracted from 2mL of plasma following addition of 1.25pmol/mL internal standard (octo-deuterated AEA) by protein precipitation with 2mL of acetone. The supernatant volume was reduced by a half by evaporation under nitrogen gas. Lipids were extracted into an equal volume of chloroform:methanol (2:1) and the aqueous phase discarded. The lipid extract was evaporated to dryness under nitrogen gas and the residue reconstituted in 80µL of acetonitrile. The AEA contents were then determined by UPLC-MS/MS.

Results

The mean plasma anandamide concentration was significantly different between labouring and non-labouring patients.

Figure1: Comparision of AEA level in non-labour and labour

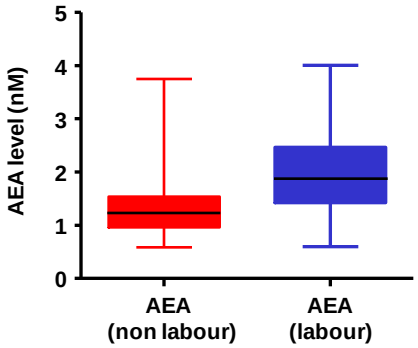
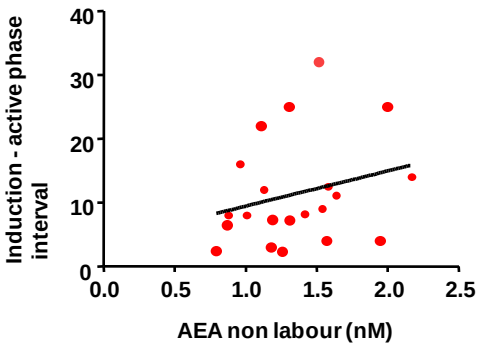


Figure 2: Relationship between AEA non labour and Induction to active phase interval



Discussion

The data confirm that plasma AEA levels do indeed increase during labour, but the lack of a strong correlation between plasma AEA levels and the induction-labour phase interval suggest that AEA is not the cause of labour, but a consequence of it. Since AEA acts on 'pain' pathways, it could be raised in response to pain during labour, although this needs to be established through further research.

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Prediction of Preterm Labour Among Asymptomatic High Risk Patients Using Plasma Anandamide Levels

Vijaianitha Nallendran, Patricia M.W. Lam, Timothy H. Marczylo, Anthony H. Taylor, Penny C. McParland, Stephen C. Bell, David J. Taylor and Justin C. Konje.
Endocannabinoid Research Group. Reproductive Sciences Section, Department of Cancer Studies & Molecular Medicine, University of Leicester, Leicester, UK.

IgG Control CB1 receptor CB2 receptor Serum control FAAH IgG Control NAPE-PLD

Introduction

Previous studies have shown that plasma anandamide (AEA) levels rise during labour[1] and CB1 receptor inactivity is related to an increased preterm delivery risk in mice[2].

These studies suggest that AEA may be involved in the initiation or progression of labour and that plasma AEA levels will be elevated in women who deliver preterm.

Aims

This prospective observational study aimed to determine the effectiveness of using plasma AEA concentrations in predicting preterm birth among high risk pregnant women.

Methods

Forty three pregnant women at high risk for preterm delivery (Table 1) either due to a previous history of preterm delivery resulting from spontaneous preterm labour or preterm premature rupture of membranes or second trimester miscarriage or 3 consecutive 1st trimester miscarriages or having undergone cervical surgical procedure (either LLETZ or cone biopsy) were recruited between 16 and 30 weeks of gestation from the preterm prevention clinic at Leicester Royal Infirmary.

All women consented to take part in the study. Out of the 43 patients 5 underwent cervical cerclage either abdominally or vaginally and therefore excluded from further analysis.

In all patients blood was collected at the clinic and transported to the research laboratory on ice. Blood samples were centrifuged at 12,000g for 30 min and plasma separated within 2 hours of collection. The plasma was either processed immediately or stored at -20°C for 24 hours.

To extracted AEA, 1.25 pmol/mL internal standard (octo-deuterated AEA) was added to 2mL of plasma and plasma proteins precipitated with 2mL of ice-cold acetone and centrifugation. The supernatant volume was reduced by a half by evaporation under nitrogen gas.

Lipids were extracted into an equal volume of chloroform:methanol (2:1) and the aqueous phase discarded. The lipid extract was then evaporated to dryness under nitrogen gas and the residue reconstituted in 80µL of acetonitrile. The AEA concentration was then determined by UPLC-MS/MS[3].

Table 1. Clinical Indications for the High Risk Prematurity Patients

Indications	Number of patients
Previous preterm delivery	10
Large Loop Excision of Transformation Zone	14
Cone Biopsy	1
Second trimester miscarriage	1
Uterine abnormality	1

Results

The pregnancy outcomes are available for 27 out of 38 patients in the study. 4 out of 27 (14%) delivered preterm (<37 weeks of gestation). The median plasma AEA level of women delivering preterm was 1.50nM (IQR 0.98-2.1nM) and was significantly higher than the plasma AEA level 0.74nM (IQR 0.30-0.64nM) of women delivering at term (P=0.02;Mann -Whitney U test; Fig1).

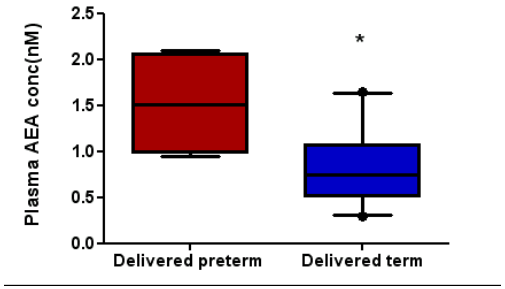

Figure 1. Comparison of the plasma anandamide (AEA) concentrations of women that went on to deliver at term and in those that delivered preterm. The data are presented as the median, interquartile ranges and the 95% centiles. The dots beyond the 95% centiles indicate two patients that are considered to be outliers, but are included here because one was above and one below the limits. *P=0.02, Mann-Whitney U-test.

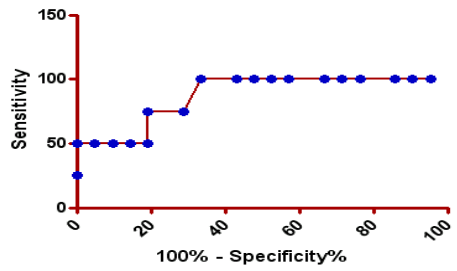

Figure 2. ROC analysis of these data revealed that at a plasma AEA concentration of 0.90nM the sensitivity for predicting PTD was 100% (95% CI 40-100%), specificity was 65% (43-84%) and a likelihood ratio was 2.88. The positive (PPV) and negative predictive values (NPV) were 30% and 100%, respectively.

Conclusion

Our preliminary data demonstrates that plasma AEA concentration among high-risk asymptomatic patients appears to be a valuable negative predictor of preterm delivery. We suggest that further studies are required, comparing plasma AEA levels to the currently used fetal fibronectin testing.

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1. .

Appendix 3: Ethics Documentation, Patient information leaflet Data collection sheet and consent forms

Patient name, address, DOB (or ID label)

Patient Identification Number for this trial: _____

CONSENT FORM

Title of Project:
The role of endogenous cannabinoids (cannabis like compounds produced in the human body in reproduction
Name of Researcher / Principal Investigator:

Please initial box

1. I confirm that I have read and understand the information sheet dated 28th December 2005 version 3 for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that I may withdraw my consent to my tissue being used at any time without justifying my decision and without affecting my normal care and medical management. ☐

4. I agree to donate the tissue samples as detailed below and allow their use in medical research as described in the Patient Information Leaflet. ☐

5. I understand that the tissue is a gift and that I will not benefit from any intellectual property that results from the use of the tissue. ☐

6. I understand that tissue samples will not be used to undertake any genetic tests whose results may have adverse consequences on my or my families insurance or employment. ☐

7. **I agree / do not agree** to my tissue samples being used to undertake genetic research as described in the patient information leaflet **(Delete as applicable)** ☐

8. I understand that sections of any of my medical notes may be looked at by responsible individuals from the research team, or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records ☐

9. I understand that tissue samples and associated clinical data may be transferred to commercial / non-commercial research partners of the University Hospitals of Leicester NHS Trust but that the information will be anonymised prior to transfer. ☐

10. The samples which I hereby consent to donate are (enter the samples here)…….. ☐
……..
…….:

11. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Parent/guardian/ legal representative (if applicable)	Date	Signature
Researcher	Date	Signature

Original for researcher/site file/CRF
copy for patient, copy for hospital notes

PATIENT INFORMATION

NAME

SERIAL NUMBER

TIME AND DATE OF COLLECTION

ID NUMBER

DOB

AGE

ETHNICITY

SOCIAL HISTORY

OCCUPATION

SMOKER

LMP

EDD

WEIGHT

ANTENATAL HISTORY

STAGE OF GESTATION

SYMPTOMS

CONTRACTIONS

URINARY SYMPTOMS

BOWEL SYMPTOMS

EXAMINATION FINDINGS

MEMBRANES

CERVIX DILATATION

DIAGNOSIS

EXCLUSION CRITERIA

MULTIPLE PREGNANCY

MEDICAL COMPLICATIONS
a.Diabetes
b.Preeclampsia
c.IUGR
d.Polyhydramnios

PRELABOUR RUPTURE OF MEMBRANES

SUSPECTED PLACENTAL ABRUPTION

OUTCOME

DELIVERED

GESTATIONAL AGE

DATE AND TIME

University Hospitals of Leicester NHS
NHS Trust

DIRECTORATE OF RESEARCH AND DEVELOPMENT

Director: Professor D Rowbotham
Assistant Director: John Hampton
Co-ordinator: N Turner
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27 June 2007

Professor J.C. Konje
Department of Obstetrics and Gynaecology
Robert Kilpatrick Clinical Sciences Building
Leicester Royal Infirmary
LE2 7LX

Dear Professor Konje

10038 **ID: The Role of Endogenous Cannabinoids (cannabis-like compounds produced in the human body) in Reproduction**

LREC Ref: 06/Q2501/49 **MREC Ref:**

Sponsor UHL NHS Trust
Funder BUPA

Please note that Trust Indemnity ceases on 28/02/2011

Thank you for the proposed amendment (listed below) to this project. I can confirm that this amendment only requires Trust Approval. The REC does not consider this to be a "substantial amendment" therefore the amendment does not require Ethical Review by the Committee. Please continue to submit all protocol amendments to the R&D Office and we will obtain appropriate approval on your behalf.

Amendment form dated 13.5.07 to add Dr Nallendran, Dr El-talatini and Dr Janine Elson to the list of researchers for the study. CVs and GCP certificates were included.

I can therefore now re-confirm the full approval of this project on behalf of the University Hospitals of Leicester NHS Trust.

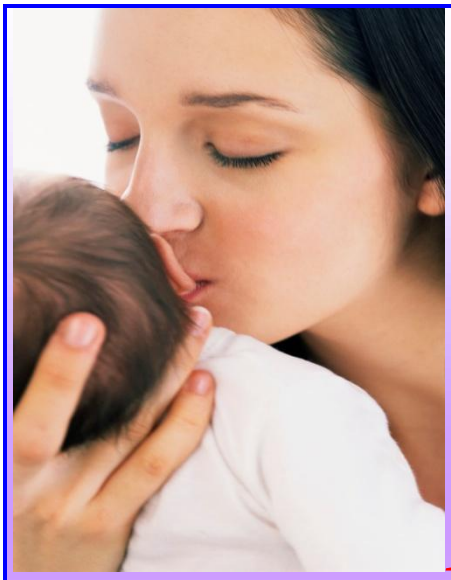
This approval means that you are fully authorised to proceed with the project, using all the resources which you have declared in your original notification form (and subsequent amendments).

The project continues to be covered by Trust Indemnity, except for those aspects already covered by external indemnity (e.g. ABPI in the case of most drug studies).

We will be requesting annual and final reports on the progress of this project, both on behalf of the Trust and on behalf of the Ethical Committee.

If you want to extend the study's end date you will have to submit an annual report available through the R&D website which will be forwarded to the relevant Ethics Committee. This allows you to continue working on the study under the previous arrangements covered by Trust Indemnity. Please note ethics approval is only granted until the proposed end date as reflected in A3 of the COREC form. You are no longer indemnified beyond this date unless you have submitted the annual report form detailing this extension.

Please make sure if you or other researchers have an honorary contract with the Trust that this stays within date whilst working on the research study.



RESEARCH ON PRETERM LABOUR AND ENDOCANNABINOIDS

Dear Colleagues,

We are investigating the value of a naturally occurring endocannabinoid called anandamide in predicting true preterm labour. For this we need blood, saliva and urine of women between 24 and 34 completed weeks of gestation presenting with abdominal pain.

I would be very grateful if you could inform me whenever a patient presents with abdominal pain(suspected labour or not).

Could these patients have their normal fibronectin swab test please as this will avoid me doing a double speculum examination.

Thank you very much for your help.

You can contact on

Mobile: 07886191 896

Phone: 0116 2525899

Thank you

Anitha Nallendran

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