The Role of the Endocannabinoid System in Preterm Birth

Thesis submitted for the degree of
Doctor of Philosophy (PhD)
at the University of Leicester

by

Panayoti Bachkangi
MB ChB, DFSRH, MRCOG

Reproductive Sciences Section
Department of Cancer Studies and Molecular Medicine
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Leicester, United Kingdom

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AUTHOR’S DECLARATION

I, Panayoti Bachkangi, state that this thesis represents my own unaided work, except where acknowledged in the text, and has not been submitted previously in consideration for a degree at this or any other university.

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(01/12/2017)
Panayoti Bachkangi
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Abstract

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Panayoti Bachkangi

Preterm labour (PTL) is a serious and costly obstetric complication affecting ~10% of all pregnancies, is difficult to predict and so difficult to prevent. Previous studies have demonstrated that the concentration of the endocannabinoid (eCB), N-arachidonylethanolamine (AEA) increases, in the plasma, whilst levels of its hydrolysing enzyme, fatty acid amide hydrolase (FAAH) decrease in the blood cells of women at risk of PTL.

This project re-examined plasma concentrations of AEA and two other eCBs; N-oleoylethanolamine (OEA) and N-palmitylethanolamine (PEA), in women at high-risk for PTL. Expression studies of the enzymes that regulate eCB concentrations (FAAH and N-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD)), and the receptors to which they bind (cannabinoid receptors 1 and 2 (CB1 and CB2)), were performed on labour affected and unaffected placenta and fetal membranes taken at term and preterm and related to the expression of progesterone receptor (PR) isoforms.

The data showed that a plasma AEA concentration of >1.095 nM had a specificity of 87.1 %, sensitivity of 25.9 %, NPV of 70.2 % and PPV of 61.2 %) to predict PTL. Furthermore, the gestational age at delivery and the number of days remaining in the pregnancy could also be determined by the AEA concentration. Similar to that for AEA, plasma OEA concentration predicted parturition up to eight weeks before it occurred, through a process that involved decreased placental CB1 expression and increased placental CB2 expression, with higher CB2 expression in the FM, especially in prematurity. This was associated with decreased PR expression during PTL in the placenta and choriodecidua, with the PR-C isoform being the dominant isoform involved in pPROM.
In conclusion, plasma AEA predicts PTL in asymptomatic “high-risk” women. The interaction of the endocannabinoid system with PR expression in the placenta and FM, suggest not only its involvement in parturition, but also suggests a novel mechanism for preterm labour.
Acknowledgements

My heartfelt thanks go to Prof. Justin Konje, who offered me the opportunity to embark on this academic journey. He repeatedly helped me with both my academic and clinical careers; he is the man who not only offered me this opportunity in the first place, but helped to save it when it started to unravel. I do not think that any words of gratitude can do him justice, not even if I could spend my entire thesis acknowledging the magnitude of his support. I am eternally grateful to him.

An equal acknowledgement is deserved to Dr. Anthony Taylor. As a supervisor he never stopped teaching and encouraging me, even when he no longer worked at the University of Leicester. He carried on supporting me, while sacrificing his own time, for my successes. He is one of the most helpful tutors I have ever encountered, as he continues to offer assistance generously to every single student in our field. I admit that his input has been pivotal for the success of this project.

Of course, I am grateful to my fellow postgraduate students for offering me an introduction to the practical academic world. They supplied me with essential advice that cannot be found in books or journals. However, a special ‘thank you’ is addressed to Dr. Sarah Melford, who successfully resolved issues within the UHL Department of Research and Development that were so crucial for this success of this project.

Mrs Muna Abbas, our laboratory technician, was always there to provide her support with the laboratory related issues. To her I owe many thanks. I am also deeply obliged to Miss Shashi Rana, who showed a real example of ‘the kindness of strangers’. The moment the Endocannabinoid Research Group started its surreal adjustment, and while I thought my project and doctorate were doomed to failure, she volunteered to help me. Without that support, the study would not have been completed.

In the Leicester Royal Infirmary, the staff and colleagues of the Maternity and the Prematurity Prevention Clinic tried their best to help me with patient recruitment. Hence a big “thank you” is dedicated to them.
One more person I feel grateful to is my good friend and mentor Dr. Jin Li. She offered her help and advice when I was going through a very dark phase of my personal life; a phase that could have threatened my research project and entire career.

Last, but not least, I owe the biggest recognition to the two people who have nothing to do with this project, research, or even medicine: my parents, who with their unconditional love, never stopped believing in me.
To my parents who have always been supporting me.

To my devoted friends, who have been there for me both on the bad and the good days.
Aἰὲν ἀριστεύειν

Ever to Excel

Homer (Iliad 6. 208)
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<tr>
<td>[Ca$$^{2+}$$]</td>
<td>Intracellular calcium ion concentration</td>
</tr>
<tr>
<td>2-AG</td>
<td>2-Arachidonoylglycerol</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl Cyclase</td>
</tr>
<tr>
<td>AEA</td>
<td>Anandamide</td>
</tr>
<tr>
<td>AEA- $$^d_4$$</td>
<td>Tetra-deuterated anandamide</td>
</tr>
<tr>
<td>AEA-$$^d_8$$</td>
<td>Octa-deuterated anandamide</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>BMI</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine 5’-monophosphate</td>
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<tr>
<td>CB</td>
<td>Cannabinoid</td>
</tr>
<tr>
<td>CB1</td>
<td>Cannabinoid receptor type 1</td>
</tr>
<tr>
<td>CB2</td>
<td>Cannabinoid receptor type 2</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CL</td>
<td>Cervical length</td>
</tr>
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<td>CO$$_2$$</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
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<td>Cytochrome P450</td>
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<td>DAB</td>
<td>3, 3’- diaminobenzidine</td>
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<td>DAG</td>
<td>Diacylglycerol</td>
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<td>DAGL</td>
<td>sn-1-DAG lipase</td>
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<td>dH$$_2$$O</td>
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<td>Docosahexaenoic acid</td>
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<td>DNA</td>
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<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
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<td>E2</td>
<td>17β-oestradiol</td>
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<tr>
<td>eCB</td>
<td>Endocannabinoid(s)</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>ECS</td>
<td>Endocannabinoid system</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Endocannabinoid membrane transporter</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
</tr>
<tr>
<td>ER-alpha</td>
<td>Oestrogen receptor alpha/ ER-α</td>
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<tr>
<td>ER-beta</td>
<td>Oestrogen receptor beta/ ER-β</td>
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<td>ERG</td>
<td>Endocannabinoid Research Group</td>
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<td>EMT</td>
<td>Extracellular regulated kinases</td>
</tr>
<tr>
<td>ERK 1/2</td>
<td>Extracellular signal regulated kinase 1/2</td>
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<tr>
<td>FAAH</td>
<td>Fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FM</td>
<td>Fetal membranes</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>Gp-AEA</td>
<td>Glycerophospho-arachidonylethanolamide</td>
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<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
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<td>G protein-coupled receptor 119</td>
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<tr>
<td>GPR55</td>
<td>G protein-coupled receptor 55</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Sulphuric acid</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>Insulin-like growth factor binding protein 1</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
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<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
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<tr>
<td>LPE</td>
<td>Liquid Phase Extraction</td>
</tr>
<tr>
<td>LPI</td>
<td>Lysophosphatidylinositol</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass/charge ratio</td>
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<td>MAGL</td>
<td>Monoacylglycerol lipase</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>NADA</td>
<td>N-arachidonoyl dopamine</td>
</tr>
<tr>
<td>NAE</td>
<td>N-acylethanolamides</td>
</tr>
<tr>
<td>NAPE</td>
<td>N-arachidonoylphosphatidylethanolamine</td>
</tr>
<tr>
<td>NAPE-PLD</td>
<td>N-arachidonoyl phosphatidylethanolamide-phospholipase D</td>
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<td>NAT</td>
<td>N-acyltransacylase</td>
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<td>Normal goat serum</td>
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<td>NPV</td>
<td>Negative predictive value</td>
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<td>OEA</td>
<td>Oleoylethanolamide</td>
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<td>OEA-d$_2$</td>
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<tr>
<td>ofFN</td>
<td>Oncofetal fibronectin</td>
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<td>P4</td>
<td>Progesterone</td>
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<td>p-AEA</td>
<td>Phosphoanandamide</td>
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<td>PAMG-1</td>
<td>Placental alpha macroglobulin-1</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PEA</td>
<td>Palmitoylethanolamide</td>
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<td>Prostaglandin</td>
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<td>Protein kinase A</td>
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<tr>
<td>PLD</td>
<td>Phospholipase D</td>
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<tr>
<td>pPROM</td>
<td>Pre-labour premature rupture of membranes</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive predictive value</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PR-A</td>
<td>Progesterone receptor isoform A</td>
</tr>
<tr>
<td>PR-AB</td>
<td>Progesterone receptor isoforms A and B</td>
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<tr>
<td>PR-B</td>
<td>Progesterone receptor isoform B</td>
</tr>
<tr>
<td>PR-C</td>
<td>Progesterone receptor isoform C</td>
</tr>
<tr>
<td>PR-PAN</td>
<td>All progesterone receptor isoforms</td>
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<td>PTB</td>
<td>Preterm birth</td>
</tr>
<tr>
<td>PTL</td>
<td>Preterm labour</td>
</tr>
<tr>
<td>PTNL</td>
<td>Preterm not in labour</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
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Chapter 1  Preterm labour, the endocannabinoid system and the sex steroid hormones
Chapter 1  Preterm labour, the endocannabinoid system and the sex steroid hormones

1.1  Introduction

1.1.1  Definition and classifications

Preterm birth (PTB) is defined as delivery before 37 completed weeks, or 259 days, of gestation (1) and it constitutes 9.6% of all births worldwide (1) and 7.6% in the UK (2). There are two main classifications based on clinical presentation and/or the gestational age at birth.

With respect to the first classification, there are three main categories (3). The first is medically indicated (iatrogenic), which accounts for 25% (8.7%–35.2%) of preterm births. In these patients, there is absence of Preterm Premature Rupture of fetal Membranes (pPROM) and/or spontaneous labour. The second category includes those patients who have pPROM and represent a further 25% (7.1%–51.2%) of all preterm births. The last category consists of the spontaneous (idiopathic) preterm births and represents 50% of all preterm deliveries (23.2 to 64.1%).

The second classification is based on the gestational age at the time of birth (4, 5). The categories include (a) Extreme Prematurity, where labour occurs before 28 weeks of gestation, representing 5 to 6% of preterm births, (b) Severe Prematurity, where delivery is between 28 and 31 weeks and accounts for 10 to 15% of preterm births, (c) Moderate Prematurity for births between 32 and 33 weeks of gestation and representing 12 to 20% of preterm births, and (d) Near Term for births between 34 and 36 weeks of gestation and representing 60 to 72% of preterm births.

1.1.2  Importance and impacts

Children who are born prematurely are at a higher risk for cerebral, respiratory and cognitive complications. The morbidity associated with PTB can extend to later life with serious physical, psychosocial and economic impacts on the child, family and society. Typically, the earlier the preterm birth, the more severe the associated complications.

Preterm labour (PTL) is responsible for 75% of perinatal mortalities and for more than half of long-term morbidities (4). Premature neonatal deaths, comprise 17% of the annual infant mortality in the USA (6) and 27% globally (7). The impact on the surviving premature new-borns is broad and depends on the body systems involved (8), and commonly include general developmental and growth delay. The respiratory system can be affected by chronic lung disease, respiratory distress syndrome or pulmonary barotraumas (9). Special senses can also be affected in the form of hearing impairment and retinopathy (9). There may also be a patent ductus arteriosus (PDA), necrotising enterocolitis or intraventricular haemorrhage and cerebral palsy (9).
Of course, all these complications require prolonged and recurrent hospitalisation with the corresponding risk of acquiring nosocomial infections. This may then influence the development of mother-infant relationship in the first two years of life (10) and affect the vulnerability of the inter-parental relationship (11). Unfortunately, all these complications have a long-term influence on the health of individuals throughout their lives. Accordingly, there may be physical, psychological and mental consequences, which in turn may lead to impairment in their social performance later in their lives (12).

It has been calculated that in the first 10 years of life, the number of hospital inpatient admissions and costs are 130% and 443%, respectively, higher than for those who are born at term (13). The financial problem is not confined to the health or welfare of the affected individuals and their families, but also affects society in general. For example, the financial burden of prematurity in the USA has been estimated to be 26.2 billion dollars annually (or $51,600 per preterm infant) (14). In the UK, the burden on the National Health Service (NHS) is £939 million per annum (15), whilst in England and Wales, prematurity costs in 2006 were estimated at £2.946 billion, whereas a hypothetical intervention that would delay preterm birth by one week only could possibly save up to one billion pounds (16). Thus, it has been estimated that surviving preterm, very preterm and extremely preterm infants costs £22885 (US $35471), £61781 (US $95760) and £94740 (US $146847) to reach maturity (i.e. the age of 18 years), respectively, with 92% of the costs covering hospital admissions for the preterm individuals (16).

From the maternal point, the economic impact of PTB on the healthcare system starts far before parturition; specifically, as it is reflected in the antenatal admissions of patients to the hospitals with ‘threatened PTB’. One-third of pregnant women, worldwide, are admitted to the hospital, for observation, with ‘threatened PTL/PTB’ (17, 18). Fortunately, among all these, 50 to 80% would reach full term without any further antenatal admissions and deliver at term. (19) Moreover, it is universally appreciated that the implementation of proper diagnostics, protocols and management plans for high-risk women can save unnecessary admissions without jeopardising patient welfare (17, 18, 20).

1.1.3 Aetiology

The precise reason why PTL is initiated remains unknown and in many cases, this is multifactorial. There are a number of risk factors (see Table 1-1), such as a family history of prematurity, infections (both local and systemic), medical, psychological, gynaecological and obstetric conditions and various other factors, such as socioeconomic status, habits and even
paternal factors (21-25). Therefore, PTL prediction is, understandably, very difficult and subsequent prevention even more puzzling.

Table 1-I Selected risk factors for preterm labour

It is obvious that due to the diversity of the aetiological factors the prediction as well the prevention of PTL is very difficult.

<table>
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<th>Aetiological Factor</th>
<th>References</th>
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<td>Family History</td>
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<tr>
<td>Gynaecological</td>
<td>Previous termination of pregnancy</td>
<td>(27-34)</td>
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<td></td>
<td>History of polycystic ovarian syndrome</td>
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<td>Correction of genital (Müllerian) anomalies</td>
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<td>Cervical surgical treatment</td>
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<td></td>
<td>In-vitro fertilisation (IVF) or IVF with intracytoplasmic sperm injection (IVF/ICSI)</td>
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<tr>
<td>Obstetric/ Gestational</td>
<td>Previous PTL</td>
<td>(4, 19, 35-47)</td>
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<td>Mechanical distension of the uterus (e.g. twin pregnancy, polyhydramnions)</td>
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<td></td>
<td>Oligohydramnions</td>
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<td>Antepartum haemorrhage (APH)</td>
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<td>Placental abruption</td>
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<td>Low lying placenta (LLP)</td>
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<td>Pregnancy induced hypertension (PIH)</td>
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<td>Surgery/ General Anaesthesia</td>
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<td>Cervical (1ry cervical</td>
<td>Congenital anomalies of the cervix</td>
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<td>insufficiency)</td>
<td>Cervical trauma or surgery</td>
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<td>Chronic hypertension</td>
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<td>Hypeparathyroidism</td>
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<td></td>
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<tr>
<td>Habits</td>
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<td>Alcohol consumption</td>
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<td>Recreational substances (e.g. Cocaine)</td>
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<td></td>
<td>Coitus</td>
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<td>Obesity</td>
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<td>Low BMI</td>
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<td>Prolonged periods of starvation</td>
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<td>Artificial sweeteners</td>
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<td>Liquorice</td>
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<td>Psychological</td>
<td>Stress</td>
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<td>Depression</td>
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<td>Bipolar disorders</td>
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<td>Infections</td>
<td>Systemic (extra-uterine):</td>
<td>(90-104)</td>
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<tr>
<td></td>
<td>○ Pneumonia</td>
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<td></td>
<td>○ Malaria</td>
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<td>○ UTI (incl. asymptomatic bacteriuria and pyelonephritis)</td>
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<td>○ Periodontal</td>
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<td>Localised (genital-uterine):</td>
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<td>○ Bacterial Vaginosis (BV)</td>
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<td>○ Chlamydia trachomatis</td>
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<td>○ Trichomonas vaginalis</td>
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<td></td>
<td>○ lower educational level</td>
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<td>○ high rate of crime</td>
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<td>○ deprivation</td>
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<td>○ unequal distribution of health services</td>
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<td></td>
<td>Single women</td>
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<td>Paternal factors</td>
<td>IBD</td>
<td>(24, 25, 61, 109)</td>
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<td>Drug user</td>
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<td>Promiscuous lifestyle</td>
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1.1.4 Challenges in managing preterm labour

Previously, clinicians focused on the avoidance of late preterm deliveries with the mistaken belief that knowledge of risk factors and their subsequent amelioration could prevent PTB. Iatrogenic preterm births for example can be prevented if the indications for them were properly understood (110). Unfortunately, this is incorrect since more than 80% of preterm births are considered “inevitable”. A concise explanation for this has been described in an acronym similar to Einstein’s Mass-Energy equivalence: $E = mc^2$, where “E” stands for aetiology of late preterm births and “mc$^2$” stands for multiple complex causes (111).

Despite the complex nature of the pathogenesis of PTL and PTB, most clinical guidelines focus either on separate entities, such as the management of active preterm labour (112-115), the management of individual clinical scenarios such as preterm premature rupture of fetal membranes (pPROM) (116, 117), the use of tocolysis (118, 119), or the antenatal use of corticosteroids for fetal lung maturation (120, 121). Accordingly, the management of confirmed PTL has become a fait accompli, whilst the significance of its early prevention and detection, or even the importance of identifying high risk women, before any symptoms arise, and avoiding unnecessary hospital admissions, is often overlooked. Although this is important, some believe that the management of PTL should be initiated much earlier (i.e. before active labour sets in), because the causes of PTL probably exist pre-conceptually or earlier in gestation and thus may be identified and prevented at these early stages.

The management of PTB, can be divided into primary, secondary and tertiary prevention (122, 123), with most current approaches focussing on tertiary prevention when PTL is imminent. However, what clinical practice needs is to focus on primary prevention, or in other words, accurate and early prediction of PTL.

1.2 Prediction

Predicting PTB early and accurately is the key to early intervention and management. Accurate prediction is likely to prevent unnecessary hospital admissions, and help health professionals to offer care to the patients who actually need it. As a result, different methods of prediction have been adopted.
Chapter 1  Preterm labour, the endocannabinoid system and the sex steroid hormones

1.2.1  Fibronectin

The first bed-side investigation that was found helpful for the prediction of PTL was oncofetal fibronectin (ofFN) (124-131). The American College of Obstetricians and Gynecologists recommend ofFN vaginal swab measurement as the “gold standard” biochemical test for the prediction of preterm delivery in such women (132). This extracellular matrix glycoprotein, which is found in the amniotic fluid, placenta and tissues at the feto-maternal interface, is released through either mechanical or inflammatory mediated damage to fetal membranes before birth.

In women presenting with suspected PTL, meta-analyses and systematic reviews of published studies concluded that ofFN predicts delivery before 34 weeks with a sensitivity of 63% (range 37 to 90%) and a specificity of 86% (79 to 93%) (133). Similar data in ‘high risk’ groups indicated that the risk of delivery before 34 weeks gestation increases 4.48-fold when ofFN concentrations exceeded 50 ng/mL and 9.94-fold when ofFN exceeded 200 ng/mL (134). In multiple pregnancies, ofFN showed promising results with specificity and positive and negative likelihood ratios of 85%, 78%, 3.9, and 0.20 respectively (135). However, contamination with maternal blood, amniotic fluid, urine or semen may result in a false positive test or when the cervix is dilated for 3 cm or more (136) as it may give false positive results (137). Despite its good negative predictive value, its relatively low sensitivity, however, leads to more women being incorrectly diagnosed with PTL.

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

After the discovery of the predictive role of fibronectin, other studies focused on a different chemical component capable of being used as a bed-side diagnostic predictor: insulin-like growth factor binding protein 1 (IGFBP-1) (136-153). Just like ofFN, IGFBP-1 is produced at the maternal-fetal interface and is found in cervico-vaginal secretions, but unlike ofFN it is a decidual product (154) that is also produced by the liver.

Both IGFBP-1 and ofFN have been compared and found to have similar predictability (155) although ofFN was slightly better (156). IGFBP-1 was found to have an advantage as it is believed to be better than ofFN before 34 weeks of gestation (157), plus the fact that it can predict preterm labour in asymptomatic women (158, 159).

Several studies examined the promising predictability of IGFBP-1 tests (136, 143, 144, 146, 160, 161) and found it to have a sensitivity of approximately 75% (69 to 89.5%), a specificity of 89% (87 to 94.1%), a negative predictive value of 91% (88.9 to 92.5%), and a positive predictive value
of 56 to 94.4%. It has been shown to be useful in Prematurity Prevention Antenatal Clinics (PPCs) in identifying women who are not going to deliver, but it is poor at detecting those in need of treatment (159).

Some have suggested that combining the ofFN and IGFBP-1 tests would provide a better prediction, considering that none of these tests offers an absolute answer. The rationale behind this is the fact that each test could offer answers in the areas that are being lacked by the other one. However, such studies have not yet been published. A comparison of the ofFN and IGFBP-1 tests is shown in Table 1-2.

The IGFBP-1 diagnostic kit is cheaper than the ofFN test making it the kit of choice in some Units (162). Unlike the ofFN test, the IGFBP-1 test can be useful in the detection of pPROM (163) because it is undetectable in urine and semen, while its quantities in the amniotic fluid are very high (164).

Both tests have almost equal predictability values, however, studies suggest that IGFBP-1 is better in predicting PTL before 34 weeks (marked with ++), while its negative predictive value (NPV) is slightly better (hence marked with the ≥ symbol). In asymptomatic women ofFN is better than IGFBP-1 in PTL prediction (155, 157, 161, 165-169).
1.2.3 Sonographic cervical length measurement

A cervical length measurement (CL) by trans-vaginal ultrasonography (TVS) has been found to be of high predictive value in high risk pregnant patients (170-176). A long cervix (≥ 30 mm) places the patient in the low risk group, while a cervix of ≤15 mm increases the risk of preterm labour by up to 90% at ≤28 weeks gestation and 50 to 60% at ≤ 32 weeks gestation (170, 171, 175). Routine screening of CL in pregnant women without previous PTB is debatable due to lack of sufficient evidence to support its value as a predictive test in low risk women (177) and consequently has not been adopted in routine clinical settings. In clinical practice, CL measurement is used in combination with ofFN or IGFBP-1, especially when the clinical or sonographic findings indicate it (152, 153, 178).
1.2.4 Other predictors

The tests mentioned above are the most commonly used. However, there are many other predictors available.

A large variety of the studies have been carried out with the aim of predicting PTL and birth using various factors. These predictive factors can be clinical, biochemical or, even, biophysical (179). Table 1-3 summarises the different predictors that have been used. Even though many of the tests show a reliable predictive value when positive, they have a low specificity (179). Some predictors, such as C-reactive protein (CRP), lack specificity and others are not feasible in routine clinical practice, either because they are invasive, like amniocentesis, or are too expensive. Most guidelines focus on specific clinical aspects of PTL, with active labour being their predominant element, while minimal attention is paid to preventable approaches.

Current clinical practice is in need of a practical test that predicts preterm labour early, is both cheap and accurate, and with both good positive and negative predictive values. More than twenty predictive methods have been tested, but the vast majority are either impractical or have limitations. Ideally, a test is needed that not only predicts the risk but also the timing of PTB long before parturition. The answer to such a clinical request maybe present in the endocannabinoid system, since this is a predictor of early miscarriage (180) and a pilot study indicated that a component of the system might be a predictor of delayed labour (181).
Table 1-3: Different types of predictors of preterm birth

<table>
<thead>
<tr>
<th>Predictive Methods*</th>
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<tbody>
<tr>
<td><strong>Microbiology</strong></td>
</tr>
<tr>
<td>Asymptomatic bacteriuria (182)</td>
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<tr>
<td>Bacterial vaginosis (183-185)</td>
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<tr>
<td><strong>Endocrinological</strong></td>
</tr>
<tr>
<td>β-Human chorionic gonadotrophin (β-HCG) (186-189)</td>
</tr>
<tr>
<td>C-reactive protein (CRP) (190-192)</td>
</tr>
<tr>
<td>Glycosylated haemoglobin A1c (HbA1c) (193)</td>
</tr>
<tr>
<td>Oestrogen (Oestriol) (194-196)</td>
</tr>
<tr>
<td>Progesterone (197, 198)</td>
</tr>
<tr>
<td>Oestrogen/Progesterone ratio (199)</td>
</tr>
<tr>
<td>Relaxin (200-202)</td>
</tr>
<tr>
<td>Corticotrophin releasing hormone (CRH) (198, 203, 204)</td>
</tr>
<tr>
<td>Prolactin (189, 205-207)</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
</tr>
<tr>
<td>IL-1 (α and β) (208, 209)</td>
</tr>
<tr>
<td>IL-6 (210-212)</td>
</tr>
<tr>
<td>IL-8 (213-215)</td>
</tr>
<tr>
<td>IL-10 (216, 217)</td>
</tr>
<tr>
<td><strong>Biochemical</strong></td>
</tr>
<tr>
<td>Matrix metalloproteinase-9 (MMP-9) (218, 219)</td>
</tr>
<tr>
<td>Serum α-fetoprotein (MSAFP) (220, 221)</td>
</tr>
<tr>
<td>Alkaline phosphatase (220)</td>
</tr>
<tr>
<td>Plasma fibronectin (222)</td>
</tr>
<tr>
<td><strong>Clinical predictors</strong></td>
</tr>
<tr>
<td>Previous history of preterm birth (223-225)</td>
</tr>
<tr>
<td>Vaginal digital examination (226-234)</td>
</tr>
<tr>
<td>Uterine activity (232, 235-237)</td>
</tr>
<tr>
<td>Mammary stimulation test (238-240)</td>
</tr>
<tr>
<td>Periodontal assessment (241-243)</td>
</tr>
<tr>
<td>Rheobase measurement (244, 245)</td>
</tr>
<tr>
<td><strong>Biophysical</strong></td>
</tr>
<tr>
<td>Absence of fetal breathing movements (246, 247)</td>
</tr>
</tbody>
</table>

*The tests cover every possible clinical, laboratory and biophysical marker. Nonetheless, the limited significance of their predictability makes them all of no much value in the daily clinical practice.

### 1.3 Endocannabinoids and the endocannabinoid system

The endocannabinoid (eCB) system (ECS) consists of endogenous lipids, their metabolising enzymes, and receptors. The lipids are mainly derivatives of arachidonic acid conjugated with ethanolamine or glycerol, while the receptors bind the psychoactive agent delta-9-
tetrahydrocannabinol (Δ⁹-THC) and other active ingredients found in the marijuana plant Cannabis Sativa (C. Sativa (248, 249)). The most commonly studied eCB is arachidonylethanolamide, commonly known as anandamide (AEA) (84). Two other less commonly investigated molecules that are often considered to be eCBs, but of high importance, are N-oleoylethanolamide (OEA) and N-palmitoylethanolamide (PEA), as they both increase plasma AEA concentrations by inhibiting its catabolism and reducing its cellular uptake (250).

1.3.1 Historical perspective

Cannabis has been known to mankind for over three millennia by the Mediterranean and Mesopotamian civilisations; the Indian, Chinese, Tibetan, Arab, and recently North American societies, whilst in China it was cultivated as early as 4000 BC (251). C. Sativa is the most commonly known variety of the Cannabis genus and is known to have more than twenty medicinal uses including analgesic, hypnotic, anticonvulsive, anti-rheumatic and as an appetite promoter (88) and in obstetrics, it was used to facilitate childbirth and stimulate lactation (88). The term “cannabinoids” was originally used to describe a group of C21 terpenophenolic compounds produced from C. Sativa (252). More recently, synthetic cannabinoids and natural cannabinoids (the exocannabinoids) have been discovered and so the cannabinoids derived from C. Sativa have been renamed ‘phytocannabinoids’ (253).

1.3.2 Endocannabinoid system (ECS)

Scientific interest in eCB started in the late1980’s, when a specific G protein-coupled receptor (GPCR) for Δ⁹-THC was identified in porcine brain (83). This receptor was named cannabinoid receptor (CB1). Following that, the first specific ligand for CB1 was identified. Biochemically, this ligand is known as N-arachidonoylethanolamide (AEA) or alternatively and more popularly, anandamide, which is a portmanteau of the Sanskrit word "Ananda”, which means “bliss” (84).

Later, a second eCB receptor (CB2), was cloned and identified in the macrophages of the human spleen; it shares only 44% of the CB1 identity (254), and its main role was originally considered to be that of regulating the immune system and inflammatory response. Surprisingly, when cannabinoids (e.g. Δ⁹-THC) bind to CB2 they inhibit macrophage migration, while a different eCB, 2-arachidonoylglycerol (2-AG) stimulates this migration through the same CB2 (255). This suggests that the actions of CB2 are both ligand-dependent and complex. Following the discovery of CB2, 2-AG was identified (256). Subsequently, several more eCBs have been identified, such as 2-arachidonyl-glyceryl ether (257) (noladin ether), N-arachidonoyl-dopamine (NADA) (258),
and virdhamine (259). Nonetheless, while many different eCBs have been reported, the focus of most pharmacological and biochemical studies has been on AEA and 2-AG.

Following the discovery of the two major cannabinoid ligands and the synthesis of pharmacological agonists and antagonists at the CB1 and CB2 receptors, evidence of the existence of additional receptors appeared in the literature. This was initially suspected because of the cannabinoid-mediated reduction of excitatory neurotransmission in mice lacking CB1 receptors (260) and from studies showing hypotension and mesenteric vasodilation in mice lacking CB1 and CB2 receptors after the administration of “abnormal cannabidiol” (Abn-cbd) (261) or the effective milk ingestion in newborn mice lacking the CB1 receptor (262). Some of these receptors have now been identified and all of them are G-protein coupled receptors (GPCRs) that bind as yet, unidentified ligands and are thus called “orphan receptors” (263). When the ligands are identified, the receptors are renamed “adopted orphans” and, subsequently, three more potential CBs have been identified: GPR55, GPR119 (264) and GPR18 (265).

1.3.3 Signalling in the ECS

When bound by ligands, CB1 and CB2 receptors are activated and initially show mostly inhibitory effects (266). However, further studies have revealed that CB activation is more complex, exhibiting both stimulatory or inhibitory effects (or sometimes both, as in the case of adenylate cyclase) depending on the nature of the tissue under investigation (267). For example, activation of CB1 by AEA shows partial agonistic activity such as the inhibition of adenylate cyclase, inhibitory modulation of Ca^{2+} channels and of inwardly rectifying potassium channels; results that are also consistent with the effects of Δ^9-THC (268). Additionally, AEA inhibits antagonist binding to L-type calcium channels (269) and partial stimulation of the Ca^{2+}-permeable transient receptor potential vanilloid type 1 (TRPV1) receptor (270). By contrast, cannabinoids (endogenous and exogenous) have direct actions on different receptors and channels. Hence, different cannabinoids can stimulate, without the involvement of the cannabinoid receptors, Ca^{2+} ion channels, Na^{+} ion channels, K^{+} ion channels, TRPV1, TWIK-related acid-sensitive K^{+} channel (TASK1) and the serotonin (5-HT3) and other receptors (267, 271, 272).

Studies have also suggested that the eCBs signalling process is even more complex, since eCBs are known to have a role in retrograde signalling in the CNS (273-275). AEA and 2-AG, for example, act as primary retrograde messengers in the hippocampus (273) and moreover, AEA, through retrograde signalling, can inhibit the secretion of different neurotransmitters such as
acetylcholine, serotonin and dopamine, and can have a negative feedback on eCB release too (275).

Of the various natural endocannabinoids, OEA has a strong affinity to the nuclear receptor peroxisome proliferator-activated receptor-alpha (PPAR-alpha) with resulting control on feeding, body weight and lipid metabolism (276). Similarly, PEA targets PPAR-alpha, (277) with little or no affinity to CB1 or CB2 (278), producing an anti-inflammatory and anti-hyperalgesic response (279). Interestingly, recent data suggest that both OEA and PEA, have affinity to the two endocannabinoid orphan GPRs, GPR55 and GPR119 (272).

1.3.4 eCB synthesis and degradation

Among the different types of eCBs, only the biological cycles of AEA and 2-AG have been studied in depth and are considered to be synthesised on demand, depending on the required physiological response and need (280). Synthesis of AEA (and all other eCBs) is thought to occur through a two-step enzymatic process, commonly known as the “transacylation-phosphodiesterase pathway”, which is Ca²⁺-dependant (86, 281, 282). The process (see Figure 1-1) starts from membrane glycerolipids with the transfer of an acyl chain from the sn-1 position of a glycerophospholipid to the amino group of the hydroxyethyl moiety of phosphatidylethanolamine (PE), through the catalysis of Ca²⁺-dependent N-acyltransferase (NAT). This then leads to the formation of N-acylphosphatidylethanolamine (NAPE). Following that, NAPE is hydrolysed by a phosphodiesterase of the phospholipase (PL) D-type known as N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) to N-acylethanolamine (NAE) and phosphatidic acid (283).

More recent studies have identified successful alternative experimental synthesis of AEA in NAPE-PLD (-/-) mice, and suggested the existence of alternative pathways, but without being able to clearly identify them (284, 285).
Figure 1-1: Biosynthesis of anandamide and other N-acyl ethanolamines via the transacylation-phosphodiesterase pathway.
Adapted from Ueda et al. 2013 (286). This is a multistep reaction where NAPE-PLD plays an important role in the synthesis of AEA and other NAEs.

AEA is hydrolysed by a fatty acid amide hydrolase (FAAH) into arachidonic acid (AA) and ethanolamine, while 2-AG is hydrolysed either by FAAH or a monoacylglycerol lipase (MAGL) into AA and glycerol (287-294). In the case of AEA degradation, cyclooxygenase-2 (COX-2), lipoxigenases (LOX) 12 and 15, and cytochrome (Cyt) P450 are all able to metabolise AEA through alternative pathways producing diverse products (see Figure 1-2), some of which are biologically active.
Figure 1-2: A schematic representation of the different AEA hydrolysis pathways.

Adapted from Placzek et al. 2008 (295). AEA is hydrolysed by different enzymes to different components depending on various biological circumstances. Abbreviations: COX-2: Cyclooxygenase-2; PGE2-EA: Prostaglandin endoperoxide ethanolamide intermediates; LOX: lipooxygenases; HPETE-EA: 12-hydroperoxy-5, 8, 10, 14-eicosatetraenoylethanolamide and 15-hydroperoxy-5, 8, 11, 13-eicosatetraenoylethanolamide; Cyt P450: Cytochrome P450; HETE-EA: Hydroxyeicosatetraenoic ethanolamide; EET: Epoxyeicosatrienoic acids; FAAH: Fatty acid amide hydrolase; AA: Arachidonic acid.

ECS signalling is terminated by cellular uptake of the eCB (295) and in the case of AEA and 2-AG, uptake and termination of the signals by FAAH are similar (296). With AEA, the uptake occurs through the actions of a non-cloned membrane transport molecule, the AEA membrane transporter (AMT) (297), while with 2-AG the mechanism of uptake is not clearly identified (298).

A very interesting development was the discovery of the cannabinoid receptor-inactive congeners OEA and PEA, whose activities are similar to that of AEA and 2-AG and also influence their metabolism and function (86). This phenomenon is known as the “entourage effect”, whereby the former congeners are specifically catabolised in preference to the latter. The result is that plasma AEA concentrations increase because this molecule’s catabolism is inhibited, and its cellular uptake reduced (250). It should be noted that inhibitors of FAAH, such as N-arachidonoyl-γ-aminobutyric acid are analgesic and N-arachidonoylglycine (which is not), lead to a rise in the plasma concentrations of all eCBs including OEA and PEA (299, 300).
OEA and PEA, as mentioned earlier, despite their limited affinity to the CB receptors (278), act primarily on the orphan receptors, GPR55 and GPR119 (272), and thus the presence of either N-arachidonoyl-γ-aminobutyric acid or N-arachidonoylglycine might cause activation of these receptors. Thus, OEA and PEA contribute to the effect of the eCB in one of two ways, either through the entourage effect or by direct stimulation of the orphan receptors.

1.3.5 Fatty Acid Amide Hydrolase

Fatty acid amide hydrolase (FAAH) is the most widely studied enzyme that degrades the eCBs to decrease their cannabinomimetic activity, for example by hydrolysing AEA to arachidonic acid and ethanolamine (301) (Figure 1-3); hence, it has previously been called anandamide amidohydrolase or anandamide amidase (302). In addition to its AEA hydrolysis activity, FAAH inhibits 2-AG activity by hydrolysing it to arachidonate (AA) and glycerol (291). This enzyme is abundant in peripheral mononuclear cells, particularly T-lymphocytes (303).

![Figure 1-3: The hydrolysis of AEA by FAAH.](image)

FAAH is the main hydrolysing enzyme of AEA. The outcome of the hydrolysis results in the production of arachidonic acid and ethanolamide. Image adapted from Freund et al. (304).

As indicated by its name, FAAH catabolises different fatty acid amides (FAAs), which are family members of the ECS. Therefore, N-acylethanolamines (305), such as OEA, PEA and oleamide (306), all CB1 agonists (307) that potentiate AEA activity on CB1 (308), are also hydrolysed by FAAH.
1.3.6 eCBs and women’s health

One of the first studies to examine the role of the ECS in women’s health showed that low FAAH protein levels and enzyme activity is associated with a higher risk of miscarriage (309). Subsequent studies showed that plasma AEA levels in humans decline in the luteal phase of the menstrual cycle in women and continue to decrease during the first half of pregnancy to a nadir in the second trimester of pregnancy (310) and then start increasing in the third trimester, reaching the highest levels during active labour (Figure 1-4) (310-312). In addition to these findings, strong evidence indicates a correlation between elevated AEA plasma levels in pregnancy and spontaneous miscarriage (313) supporting the suggestion that high FAAH activities are important during early pregnancy.

Figure 1-4: Changes in plasma AEA during normal pregnancy.
Image adapted from Habayeb et al. 2004 (310).

Plasma levels were assayed using HPLC-MS and recently confirmed in another study using UPLC-MS/MS (311). The figure shows plasma AEA concentrations in women throughout the different trimesters of pregnancy and at term, in both non-labouring and labouring states. The AEA concentrations decrease after the 1st trimester, and remain low during the 2nd and 3rd trimesters. At term, plasma AEA concentrations increase in the non-labouring stage, and reach a maximum during active labour.
1.3.7 ECS and sex steroid hormones

Progesterone is a crucial hormone for the progress of pregnancy. From the moment of conception, serum progesterone levels start rising, and a steady increase in its levels usually represents a healthy pregnancy and better outcome (314-316). A link between progesterone and the ECS has been established where progesterone has been shown to stimulate FAAH activity in human T-lymphocytes (303) with the subsequent downgrading effect on AEA plasma concentrations, but has opposite effects on FAAH activity in the rat uterus where it down regulates its activity (317). Whether progesterone exerts the same control in the human uterus remains unknown.

Conversely, stimulation of CB1 and CB2 receptors affects negatively the luteal secretion of progesterone, with a possible impact on implantation (318). Furthermore, a definitive link between progesterone and ECS activity remains controversial, with data coming from studies of the menstrual cycle, indicating that despite there being a positive correlation between plasma AEA concentrations and serum estradiol, FSH and LH concentrations, there was no such connection between AEA and progesterone concentrations (310, 319). Some investigators (320) have tried to resolve this conflicting information about the interactions between ECS and progesterone by presenting an all-encompassing hypothesis (Figure 1-5) (320).
Figure 1-5: Summary of the major interactions of the ECS with progesterone.
Image redrawn from Gorzalka et al. 2012 (320). The effect of progesterone on FAAH differs according to the tissue under investigation: the uterine FAAH is downregulated while it is upregulated in immune cells.

1.3.8 The placenta, progesterone receptors, ECS and parturition

The human placenta is a target for eCB activity and has a regulatory role in pregnancy outcome, especially in the first trimester where low levels of FAAH and high expression of CB1 are associated to miscarriage (321). Although FAAH and CB1 were initially shown to be present in placental tissue (321, 322), CB2 was not. However, with improved detection tools was subsequently located in the placenta (323). The presence of both CB receptors in the placenta and the possibility that the levels of eCBs in the placenta may be regulated, suggest that endocannabinoids and exocannabinoids may have biological effects on this important organ (324).
In human fetal membranes, the stimulation of CB1 leads to an increase in PGE$_2$ through activation of COX-2 (325). Hence, the ECS has a role in the progression of pregnancy and the timing of labour. Another proof that CB1 may be controlling the gestational length comes from a study on mice with knockout of the CB1 gene: such an effect leads to preterm labour, by altering the oestrogen and progesterone levels, and independently from the prostaglandin production through COX-1 (326). Another study proved the influence of CB1 on the length of pregnancy, in mice, where tocolytic treatment with THC prolonged the pregnancy by acting on the CB1 and through the nitric oxide (NO) pathway (327).

One effect that may result from eCB activation in the placenta is the modification of other signalling pathways. For example, the process of parturition is thought to be initiated when the myometrium, placenta and amniochorion are subjected to a phenomenon known as 'functional progesterone withdrawal', in which each tissue no longer responds to circulating progesterone. One way this event may occur, is through a ‘switching’ from the active progesterone receptor (PR)-B isoform to a less active PR-A isoform (as has been shown in the myometrium at term) or possibly to the more recently discovered isoforms PR-S, PR-C or PR-M (328), shown to be present in amnion (vide infra).

### 1.3.9 Placenta and the progesterone receptors

The two predominant Progesterone receptors (PR) are PR-A (94 kDa) and PR-B (116 kDa) (329); they are both nuclear receptors and encoded by the same gene. (330, 331) In addition to those two there is a PR that acts on the DNA-binding domain (DBD), known as PR-C (60 kDa) (329) which has been suggested to be involved in parturition (328). More receptors include the one binding in the hinge region or D domain, PR-D, those binding in the hormone-binding domain (HBD), PR-E/F (332), and the more recently discovered isoforms PR-S and PR-T (333).
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Figure 1-6: Structure of PR variants.

A, diagram of transcriptional and translational start sites for human PR-A and PR-B. B, domain organization of the human PR-A, -B and -C isoforms. h, hinge region; LDB, ligand binding domain; ID, inhibitory domain. The numbers denote the positions of amino acids for each isoform proteins. AF-1, -2, and -3 are transcription activation domains. (Figure and explanation adapted from Li, X. & O’Malley (334)).

Apart from the nuclear PRs, there is also the PR-M, which is located at the mitochondria with absent DNA-binding domain, (332) while on the cell membrane there are five types of seven-transmembrane progesterone adipo Q receptors (PAQR) identified, so far (mPRs α, β, γ, δ, and ε) (335).
1.3.10 Placenta, progesterone receptors and parturition

The process of parturition is initiated when, in the placenta and amniochorion, there is a 'functional progesterone withdrawal' in the form of switching of PR-B isoform to PR-A or to the more recently discovered isoforms PR-S, PR-C and PR-M (328).
The sites, types and ratios of PR receptor isoforms in pregnancy and parturition have been the subject of much scientific debate. Hence, PR-A/PR-B ratio changes in favour of PR-A in the placental villi of women at term and in labour have been observed, that are different to that of non-labourers, or preterm and post term pregnancies, regardless their labouring state (337). However, others have suggested the exact opposite ratio, favouring PR-B over PR-A, but only in the myometrium (338).

Moving from the predominantly studied receptors, PR-C rather than PR-A was found to be involved with a similar PR-C/PR-B ratio in the myometrium (339). Moreover, PR-C was found largely in the amnion, while PR-A was significantly decreased, at the onset of labour (340). A more recent study proposed equally supportive results about a 60-kDa PR isoform which was found in high levels in the fetal membranes at term (328). As translational sites at the amino acids 289 and 301 express proteins of 64 kDa, (336), i.e. almost equal the length of PR-C, the authors concluded that the abundant protein was either PR-C or a similar sized isoform produced by the AUG codon at amino acid 301 (328).

Still, this proposition is not fully proven and, in addition, the presence of such receptor/protein has not been explored in preterm tissues.

1.3.11 Oestrogen receptor alpha

It is important to highlight a few facts about ER-α and its expression in the human placenta. While there are three main isoforms of ER (alpha, beta and gamma), alpha and beta are the main ones expressed in the placenta (341). It needs to be taken in consideration the fact that previously it was believed that in early pregnancy ER did not exist or that they were hardly expressed (342, 343). Similar doubts existed about the existence of ER-α through pregnancy, until it was shown to exist in healthy placentae (344).

ER alpha and beta placental expressions gradually increase in the first two trimesters and then decrease until parturition, with ER-α expression being ten-fold higher than that of ER-β (341).

The “functional progesterone withdrawal” involves, of course, PR isoforms, but is also regulated by the placental ER-α, which tends to rise in labour (345). Specifically, ER-α remains low in the third trimester until labour advances, where it then increases and is associated with changes in the expressions of PR-A and PR-B, leading to its contribution to the “functional withdrawal” (346). Similarly, an increase of ER-α has been noted in the human myometrium, while ER-β does not
increase in parturition (347). Another role of ER-α in labour augmentation was discovered in murine uteri, where ER-α is the main facilitator of the oestrogenic activity on the expression of oxytocin receptors (348).

Whatever the role of ER-alpha in parturition, the presence of that receptor has not been explored extensively in preterm tissues.

1.3.12 Endocannabinoids and preterm birth

The precise interaction between the endocannabinoid system and preterm birth remains unknown. What is known is that the use of cannabis in pregnancy, and preconceptually, significantly increases the risk of subsequent preterm birth (349), and that FAAH expression is increased by progesterone, which theoretically should prevent preterm delivery in women presenting with PTL (350).

From these observations, it can be concluded that low “functional progesterone activity” is likely to be associated with low FAAH activity and consequently high plasma AEA levels. Indeed, FAAH activity levels in lymphocytes have been suggested to be a possible biomarker for the prediction of early miscarriages (309), because elevated plasma AEA levels are associated with miscarriage. It is thus likely that the higher levels of AEA measured in labour at term are linked to the “functional withdrawal of progesterone” hitherto postulated as one of the possible mechanisms underlying the onset of labour (338). If this is the case, then AEA levels would also be higher in the women at risk of PTL (181) that subsequently deliver prematurely. In this regard, prostaglandins (PGs) could be an important intermediary factor.

Prostaglandins are known to be involved in the initiation of labour, in humans, both at term and prematurely (351), starting with pPROM or SROM, cervical changes and uterine activity to placental separation and uterine involution (352). Prostaglandin synthase (PGHS) activity is evident in the human myometrium and fetal membranes with predominance of PGE₂ in the amnion (352) (Figure 1-6). Raised PG concentrations stimulate the activity of the 11β-hydroxysteroid dehydrogenase enzyme (11β-HSD-1). This enzyme converts cortisone to cortisol, which in turn upregulates the PGHS-2 leading to further rise in the concentrations of PGE₂ and PGF2α (351).
Figure 1-8: Interrelationship between cortisol, PGHS-2, and PGDH.
In human chorion, cortisone is converted to cortisol through the activity of 11β-HSD-1, and the activity of this enzyme is increased locally by PGs. Adapted from Challis et al. 2000 (351)

An interaction between prostaglandins and cannabinoids has been described in different species but with different effect on parturition.

In gravid rats treated with Δ9-THC and AEA serum prostaglandins, PGF 1α and PGF 2α, were found to be significantly lower after treatment, suggesting the possibility that cannabinoids delay the onset of parturition (353). This could be attributed to the possible inhibitory consequence of AEA, due to its eicosanoid nature, in the PG synthesis by competitive effect. As a result to the reduced levels of PG in the murine placenta and uterus there is a delay in the onset of labour (353).

Although this might be true for rodent species, the observation that endogenous cannabinoids, as well as synthetic cannabinoids, affect the production of prostaglandins with a consequent influence on the timing and progression of term and preterm human labour and membrane rupture
differ significantly (325). Cannabinoids increase the production of prostaglandin-E2 (PGE₂) in the human amnion, leading to uterine contractions, and their action on the prostaglandin synthesis in fetal membranes is mediated by CB1 stimulation and COX-2 (325).

As a result, it can be concluded that the ECS interacts with PG synthesis, even though the mechanism(s) and impact on parturition varies between rats and humans due to different PG activity.

One more piece of evidence of an association between the ECS and parturition was discovered when the loss of CB1 receptor expression in rats was shown to induce preterm birth (326). The mechanism is related to a reduction in normal oestrogen and progesterone concentrations, and is independent of PG activity.

Additionally, a pilot study conducted by the endocannabinoid research group at the University of Leicester, showed that plasma AEA levels increase in women at high risk for PTL who subsequently deliver prematurely (p = 0.0406, one-sided Mann-Whitney U test) (181) (Figure 1-7). These data need confirmation, but provide an interesting starting point for the work presented later in this thesis.
Figure 1-9: A pilot study of plasma anandamide (AEA) levels in women at high risk of PTL. Plasma AEA measurements were taken at the time of presentation and the women followed to identify those who subsequently delivered at term (n = 36) or preterm (n = 7). The data are presented as median, IQR and range. The levels of plasma AEA concentrations were significantly (*p = 0.0406) elevated in the women who subsequently delivered preterm (one-sided Mann-Whitney U-test). Nallendran et. al. 2009 (181).

1.3.13 Conclusion

It can thus be concluded that the ECS has a possible role on the length and success of gestation. Plasma AEA depends upon the biological circumstances with high plasma concentrations of AEA being associated with miscarriage and parturition, either at term or prematurely (PTL). This has led to the suggestion that either the plasma concentrations of AEA and other eCBs, or levels of other components of the ECS, could be used as predictors of delivery in women at high risk of PTL or those presenting with symptoms of PTL.
1.4 Hypotheses

On the basis of the preceding literature review and the available evidence, it is therefore hypothesised that:

(1) Plasma concentrations of AEA and possibly other eCBs are raised in women at risk of PTL who end up with premature birth. Moreover, the lymphocytic FAAH levels and activities will be reduced, in these women. Consequently, one or all of the ECS components can be used as biomarkers to predict PTL.

(2) Changes in the expression of eCB enzymes and receptors and of PR isoforms in the fetal membranes and placenta may reflect a possible ECS/progesterone involvement in labour, either term or preterm, and help elucidate a possible mechanism for parturition.

(3) Combining eCB and FAAH values from the previous two hypotheses with clinically used predictive tests (e.g. ofFN and CL) will improve the sensitivity and specificity, PPV and NPV for the prediction of PTL.

1.5 Aims

To test these hypotheses, a series of experiments were designed to:

1. Measure the plasma concentrations of AEA, OEA and PEA, and determine their sensitivity and specificity, PPV and NPV values in predicting PTL.
2. Measure of FAAH activity in peripheral blood lymphocytes to evaluate their value to predicting PTL.
3. Measure plasma concentrations of oestradiol and progesterone and correlate these with eCB and FAAH levels.
4. Quantify of the expression of eCB receptors, CB1 and CB2, the ECS enzymes FAAH and NAPE-PLD, and progesterone receptor isoforms in the placenta and fetal membranes between different groups (labourers, non-labourers, preterm labourers and term labourers) to determine if there is an interaction between these factors during parturition.
Chapter 2  Prediction of preterm birth using plasma endocannabinoid measurements
Chapter 2  Prediction of preterm birth using plasma endocannabinoid measurements

2.1 Introduction

In Chapter 1, the different predictive tests that are clinically available for the prediction of PTL were presented. Although each test has merit, they are imperfect and have the distinct disadvantage of not being predictive of PTL and consequently of PTB, but have some negative predictive value (NPV). For example, both of the commonly used cervico-vaginal swabs (ofFN and IGFBP-1) have a very good NPV but poor sensitivity, resulting in a number of unnecessary hospital admissions and treatments, whilst sonographic cervical length measurements are reliable when there are identified cervical changes, but they require experienced sonographers to make those measurements, and user variation even at a single site, is known to cause false positivity (354). Furthermore, any larger cervical changes that take place usually mean that PTL is imminent. An additional caveat that prevents routine use of these tests for the prediction of PTL is that they are prohibitively expensive (ofFN) or need trained personnel and expensive equipment (e.g. sonographic measurement of cervical length). Consequently, a more reliable method for the prediction of PTL and impending PTB is required. The method that is found, needs to have high sensitivity, with accurate prediction, and be relatively inexpensive. One additional property that would be of benefit would be the ability to predict PTL in asymptomatic women before the signs of labour begin. This would mean that unnecessary admissions and expenses might be avoided, while early prediction will allow prompt management to be planned. The method would also benefit from being relatively non-invasive, such as a blood test that can quantify one or several biomarkers.

This test, which can be used as predictor either on its own or in conjunction with the available predictive tests, currently does not exist, but previous work has suggested that plasma AEA could fulfil this role(355). In a pilot study performed by our research group, plasma AEA concentrations were shown to increase in term pregnancies from the non-labouring to labouring state (312) and that plasma AEA concentrations had already started to increase in the 3rd trimester in preparation for parturition, suggesting that plasma AEA concentrations might increase early before parturition in pregnant women who are at risk for PTB. This was tested in another pilot study (181) and the data demonstrated that plasma AEA concentrations did indeed predict those women who would subsequently go on to deliver preterm. A necessary first step in my studies was to examine if such changes can actually predict PTB. Furthermore, since OEA and PEA may have an “entourage effect” on plasma AEA concentrations, it would seem reasonable to also quantify their plasma concentrations, to determine if these are related to plasma AEA concentrations, and whether combining these measurements would improve the prediction of PTL by these biomarkers.
Additionally, a comparison between the efficacies of the PTL predictability of these eCBs (either alone or in combination) to that of other predictive tests commonly used in current clinical practice (e.g. IGFBP-1 and cervical length measurements) might also be useful. Such an evaluation may highlight a superiority of one test over another or the possibility that combining the results of all tests would provide a better risk assessment.

The measurement of plasma eCB concentrations had already been effectively established and verified in a number of other studies by the endocannabinoid research group (310, 311, 356), so that methodology was used here.

2.2 Methods

2.2.1 Patient Recruitment

The patients who participated in this prospective cohort study were recruited from Leicester Royal Infirmary, either from the Prematurity Prevention Clinic (PPC) or from the Obstetric Wards.

2.2.2 Defining cut-off points for gestational ages

Prematurity includes deliveries from the age of viability (24\textsuperscript{+0} weeks) until 36\textsuperscript{+6} weeks. As a result, corticosteroid treatment is normally administered from 24\textsuperscript{+0} to 34\textsuperscript{+6} gestational weeks (121, 357). However, in some cases where the pregnancy is just before 24 weeks, where viability is considered achievable, corticosteroids are offered to pregnant women. Such decisions are made by senior clinicians and further to discussion with the neonatologists. Women who were pregnant before 24 gestational weeks and to whom steroids were offered were considered eligible for recruitment.

2.2.3 Inclusion Criteria

The criteria for inclusion of this study were as follows:

a) Singleton pregnancies
b) Accurate ultrasound-dated pregnancies (made in the 1\textsuperscript{st} trimester)
c) Gestational age between 24 and 34 weeks
d) Gestational age < 24 weeks, if fetal viability was clinically accepted (defined as being when neonatologists deemed resuscitation of the new-born was probable)
e) The presence of known risk factors, such as previous PTL, late miscarriage or cervical surgery
f) Presenting with symptoms of PTL

g) Agreeing to have an ofFN or IGFBP-1 (Actim Partus®) endocervical swab.

h) Cervical dilatation < 3cm and no effacement (i.e. not in active labour)

### 2.2.4 Exclusion Criteria

The exclusion criteria were:

a) Multiple pregnancies

b) Complicated pregnancies (e.g. uncontrolled diabetes, severe preeclampsia, known fetal anomalies, etc.)

c) Clinical or biochemical evidence or suspicion of chorioamnionitis

d) Suspected placental abruption

### 2.2.5 Recruitment approach

All the patients were recruited at the Leicester Royal Infirmary, which is part of the University Hospitals of Leicester NHS Trust. They were provided with a Patient Information Leaflet (PIL) and Consent form approved by the Leicestershire, Northamptonshire and Rutland Research Ethics Committee. (Reference number: 06/Q2501/48) (See appendix 8-1).

Attendees at the PPC were asymptomatic patients considered to be at high risk for PTL and were approached on the first or second visit to the clinic (in the first trimester or at mid-term, respectively), when a PIL was offered and the study explained. Some of the main reasons for referral to PPC are listed in Table 2-1.
Table 2-1: Criteria for patient referral to the Prematurity Prevention Clinic

<table>
<thead>
<tr>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous spontaneous preterm labour 1 preterm birth at: 14–34 weeks</td>
</tr>
<tr>
<td>Previous pPROM at 14–34 weeks</td>
</tr>
<tr>
<td>Maternal weight, &lt;50 kg</td>
</tr>
<tr>
<td>Maternal BMI, &lt;20</td>
</tr>
<tr>
<td>Previous cervical cerclage</td>
</tr>
<tr>
<td>Uncertainty regarding need for cervical cerclage</td>
</tr>
<tr>
<td>Late booker with cervical cerclage in situ</td>
</tr>
<tr>
<td>Positive screen for Bacterial Vaginosis at 16 weeks of gestation</td>
</tr>
</tbody>
</table>

*Data are taken from Lamont’s paper (358).*

Emergency admissions were recruited either on the labour ward, at the Maternity Assessment Centre, or on the antenatal wards. These patients were admitted with symptoms of PTL (e.g. abdominal pain, uterine tightenings or pPROM), but were assessed not to be in active labour (the cervical os was closed, there was no cervical effacement, etc.).

The CL were measurements were obtained only from women recruited from the PPC. The cut-off point was 25mm, as per the national guideline (357). Any controversial sonographic findings (170-177) outside the guideline regulations were managed according to the discretion of the leading clinician. Fortunately, in our cohort none of the results had controversial interpretation.

### 2.2.6 Sample collection

Venous blood was collected from volunteers from the median cubital vein blood into 9 mL EDTA tubes (Sarstedt Ltd., Leicester, UK) and in to plain 2.7 mL tubes (Sarstedt Ltd., Leicester, UK) prefilled with the acid citrate dextrose (ACD) anticoagulant, for measurement of plasma eCB level and FAAH enzyme activity, respectively.
Blood samples for eCB measurements were placed on ice, transferred to the laboratory and processed within 120 minutes of collection. The tubes were centrifuged at 2500 g at 4°C for 30 minutes, and the plasma transferred into 7mL Kimble scintillation vial (Kinesis, St. Neots, Cambs., UK) before being stored at -80°C for subsequent lipid extraction and eCB measurement.

2.2.7  Sample size calculations

The sample size was determined by a power calculation using the data from a pilot study by Nallendran et al. (181) who showed plasma AEA concentrations to be significantly elevated in women who delivered prematurely. Her ROC analyses indicated that plasma AEA levels above 0.90nM was predictive of preterm birth in a high risk group with a sensitivity of 100% (95% CI 40-100%), specificity of 65% (95% CI 43-84%) and a relative risk (RR) of 2.88 for preterm delivery. However, these data were limited to a relatively small cohort of patients (n = 43) and consequently had insufficient power.

Based on the above information, two categories of volunteers were identified:

(a)  Asymptomatic women:

From this pilot study, women who delivered prematurely had 50% higher plasma AEA concentrations than those who reached full term. At the 5% level of significance, and with a power of 80%, it was calculated that at least 16 patients will be needed for each arm of the study. Moreover, the pilot data showed a PTB labour/term ratio of 1:6.6. Hence, to obtain 16 PTB labourers, 122 participants were required.

(b)  Symptomatic women:

This is based on the fact that of the 6-8% of all pregnancies that present with suspected PTL before 34 weeks, only 50% of these will end up in true PTL, i.e. 3-4% and that for 6000 deliveries per annum at Leicester Royal Infirmary, 360-480 women would present with suspected PTL per annum. If pregnancies with the exclusion criteria (e.g. multiple pregnancies, polyhydramnios, etc.) constituting 25% of those admissions were actually excluded from the selection pool, then there would then be between 248 and 450 eligible pregnancies for recruitment annually.
2.2.8 Improvement of existing tests

To improve the sensitivity of the IGFBP-1 for predicting preterm birth from 63% to 73% at the 5% level of significance and for a power of 80%, a minimum of 232 patients would need to be studied. Previously, our team managed to recruit 141 women over a period of 8 months for an oFN study (359). Consequently, recruiting 250-300 patients (high-risk and normal) over a 36-month period was considered feasible.

2.3 Plasma eCB quantification by UPLC-MS/MS

Quantification of endocannabinoid concentrations (AEA, OEA and PEA) in plasma was performed using Ultra Performance Liquid Chromatography Tandem Mass Spectrometry (UPLC-MS/MS), as described in (310, 311, 356).

2.3.1 Chemicals

HPLC-grade water was from a water purification system (Maxima ELGA, ELGA High Wycombe, UK). Acetonitrile, chloroform, and methanol were purchased from Fisher Scientific (Loughborough, UK) and were all HPLC-grade. Oasis® HLB extraction cartridges used for the solid phase extraction (SPE) were purchased from Waters UK Ltd. (Elstree, Herts., UK).

The endocannabinoids of interest (AEA-d₀, OEA-d₀ and PEA-d₀) together with their different deuterated equivalents (AEA-d₈, OEA-d₂ and PEA-d₄) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Mobile phases were filtered before their use through polytetrafluoroethylene (PTFE) filters (4-7 mm of diameter); 0.2µm pores (Waters UK Ltd.) as previously described (311).

2.3.2 Preparation of internal standards (IS)

The stock for the AEA-d₀ and AEA-d₈ standards were supplied in solvents, which were dried under a gentle stream of N₂ gas and then reconstituted at 5mg/mL and 100µg/mL, respectively, in acetonitrile. OEA-d₀ and PEA-d₀ (supplied as powdered solids) were dissolved in ethanol at 10mg/mL and 2.5mg/mL, respectively. OEA-d₂ and PEA-d₄ were supplied as ethanol stocks at a concentration of 1µg/µL and were stored at -20°C, without any further adjustment.

Prior to the start of measurements, additional dilutions (1:100) were made in ACN, with the d₈ materials used as the internal standards (IS). The IS consisted of 10µl of AEA-d₈ (6.272pmol/µL), 10µl OEA-d₂ (6.272pmol/µL) and 20µl PEA-d₄ (6.272pmol/µL), plus 960µl of ACN, resulting in
final concentrations of 0.06272pmol/µL for AEA-d₈ and OEA-d₃, and twice that for PEA-d₄. The IS mixture was stored in a freezer at -20°C for up to 3 months.

2.3.3 Calibration curves

A calibration curve for each endocannabinoid was generated based on the formula introduced by Giuffrida et al. (360) and which has been previously used successfully previously (310). Eight concentrations of AEA, OEA and PEA in triplicate were used to calibrate the MS/MS. A typical response of the corresponding standards is shown in Figure 2-1.

![Figure 2-1: Calibration curve for AEA.](image)

This example shows the response of the MS/MS to the indicated AEA concentrations (AEA-d₀). The data shown were collated from the first 51 samples that were analysed over 7 different occasions. Linear regression analysis was used for the creation of the calibration curve. In this figure, the SEM is also included as vertical error bars.
Peaks for AEA-\textsubscript{d8}, OEA-\textsubscript{d3}, and PEA-\textsubscript{d4} standards were measured using Masslynx software (Waters Inc., Milford, MA), and concentrations of AEA, OEA and PEA in the standards and samples calculated using the Quanlynx software (Waters Inc.). The measurement of the unknowns was calculated from curves using the equation (361):

\[
\text{Relative response (y)} = \frac{\text{peak area}}{(\text{IS area}/[\text{IS}])}
\]

Where

- **peak area**: peak area of relevant AEA, OEA or PEA
- **IS area**: peak area of internal standard
- **IS**: the concentration of internal standard

### 2.3.4 Extraction and measurement of AEA, OEA and PEA from plasma

Endocannabinoids were extracted from plasma samples previously stored at −80°C. Briefly, samples were thawed on ice, centrifuged at 4°C, for 30 min at 2500 g, to separate any flocculated protein and the supernatants transferred to a fresh Kimble vial for further processing, as described (310, 311, 356). Samples were always processed in duplicate, to eliminate the possibility of discrepancies between results due to faults in the extraction process and 0.5 mL of each plasma sample in the Kimble was initially diluted with 0.5mL of deionized (HPLC-grade) water. To this, 20\(\mu\)L of internal standard was added and vortexed for approximately 10 sec for proper mixing.

Lipids were extracted from the samples with the aid of a Vacmaster vacuum manifold (Biotage, Uppsala, Sweden) using Oasis HLB cartridges (Waters UK Ltd) of 1cc capacity each, attached to the manifold. The vacuum flow-rate was adjusted at 1mL/minute and each Oasis HLB cartridge was initially activated with 1mL 100% methanol and then cleared by 1mL of HPLC-grade water. At this point, 1mL of the premixed sample was added, and any unwanted materials removed by washing the column with 1mL 40% methanol. Finally, the sample was eluted into plastic test tubes (Test Tube PP Push Cap 2.5mL, Sarstedt) by adding 1mL acetonitrile.

Samples were then dried gently at 40°C under a constant stream of \(\text{N}_2\) gas on a Techne® Sample Concentrator and once dried, solubilised with 80\(\mu\)L of acetonitrile to reconstitute the sample. After transfer to a HPLC vial, these were then loaded to the UPLC-MS/MS system (Waters Ltd,
Hertfordshire, UK) and 7 µl measured in triplicate (311). If samples that were not analysed on the same day, HPLC vials were stored for a maximum of 48 hours at -20°C.

### 2.3.5 Ultra-high pressure liquid chromatography – tandem mass spectrometry

The following is an explanation of the ultra-high pressure liquid chromatography – tandem mass spectrometry (UPLC-MS/MS) system and analysis and is adapted from Lam et al. (361).

The UPLC-MS/MS system consisted of an Acquity UPLC system connected to a Quattro Premier Tandem mass spectrometer (Waters Ltd.). The separation system was an Acquity UPLC BEH C₁₈ (2.1 x 50 mm, 1.7µm) column maintained at 40°C. (Figure 2.2 shows a diagram of the UPLC system and the principles of actions of the mass spectrometer).

![Diagram of UPLC-MS/MS system](image-url)

**Figure 2-2: Mass Spectrometry principle and the UPLC-MS/MS equipment.**

Panel A. Schematic diagram of the HPLC system (362).

Panel B. UPLC-Quattro Premier XE Mass Spectrometer System (photo by author).
The two mobile phases were solution A (2 mM ammonium acetate containing 0.1% formic acid and 5% acetonitrile) and solution B (acetonitrile containing 0.1% formic acid). A linear gradient: 0–0.5 min, 20% B; 2.5 min, 100% B; 3.5 min, 20% B then re-equilibrated at 20% B until 4.0 min elapsed. Samples were kept at 4°C throughout the analyses.

Analyte quantification was conducted by the use of tandem electrospray mass spectrometry in positive-ion mode (ES+). The source parameters included: capillary voltage of 1 kV, cone voltage of 21 V, source temperature of 120°C, desolvation temperature of 440°C, cone gas flow of 50L/hour and desolvation gas flow of 800 L/h, as described (311). The optimal MS/MS entry, collision and exit energies were 6, 16 and 2 eV for each precursor [M+H]+ ion. The ions that were generated were monitored in multiple reaction monitoring (MRM) mode. From each eCB molecule, a daughter-ion was produced and measured. The mass transitions were: AEA-d0 (m/z 348.25>61.9), AEA-d8 (m/z 356.25>62.9), OEA-d0 (m/z 326.5>61.9), OEA-d2 (m/z 328.2>61.9), PEA-d0 (m/z 300.5>61.9) and PEA-d4 (m/z 304.2>61.9).

The concentrations of each eCB in plasma were calculated from a comparison to the curves created from the standards injected in each run.

2.3.6 IGFBP-1 assessment

Patients who attended the PPC gave signed informed consent and after obtaining the blood sample, a swab was taken from the anterior fornix of the cervix at speculum examination. In emergency admissions, the swab test was performed as per the routine clinical practice. The results were interpreted as being either positive or negative.

2.3.7 Cervical length measurement

Participants who attended the PPC had a pelvic ultrasound scan performed by an experienced clinician or sonographer, where the measurement of the cervical length was assessed. In some cases of emergency admissions, when the clinical circumstances indicated, a cervical length assessment was performed. The results were interpreted based on the cut-off point of 25mm, indicating that below this length the patients were considered at high risk for PTL.

2.3.8 Measurement of the recruitment to delivery interval

To calculate the recruitment to delivery interval (RTDI), the week of recruitment of each woman was recorded such that if a woman was 29+1 or 29+6, her date would be recorded as week 29. She
was then followed until the week of delivery and a similar strategy employed, e.g. if delivery was 37+1 or 37+6, then the delivery week was recorded as week 37. The RTDI for this example patient would therefore be 37-29 = 14 weeks. Similar data were obtained for all women recruited and used as either the entire cohort, or subsets of the PPC recruited women and those recruited as emergency admissions. Additionally, all data (plasma AEA, OEA and PEA concentrations) were evaluated in this way. To set a baseline control, the eCB concentrations for RTDI values between 0 and 5 weeks were chosen and p-values obtained from an unpaired Student’s t-test between these values and those for the remaining cohort. These were recorded. Next, the RTDI values between 0 and 6 weeks were chosen, and the p-values recorded. This was then repeated until the values for eCB concentrations for RTDI values between 0 and 12 weeks were chosen as the control baseline and tested against the remaining cohorts. The raw p-values were then plotted.

2.3.9 Statistical analysis

Statistical analysis of the data was performed using Microsoft (MS) Office Excel 2010 software (Microsoft, WA, USA) and Prism 6 software (GraphPad, San Diego, CA, USA) with graphical representation of the data of these software packages, as appropriate. D’Agostino & Pearson omnibus normality test were used to determine if data originated from a Gaussian or non-Gaussian distribution. Data that originated from a Gaussian were treated as parametric data and those from non-Gaussian distributions treated as non-parametric data, hence standard deviation (SD) was used for this variable and interquartile range (IQR) for the others.

Mann-Whitney U test for independent samples was used for the analysis of non-Gaussian distributed data and unpaired Student’s t-test for Gaussian data. Statistical significance was accepted when p < 0.05. In addition, ROC analyses were undertaken to determine specificity and sensitivity values and to provide negative (NPV) and positive predictive (PPV) values, using cut-off values presented by the software.

The outliers in data have been included throughout the statistical analyses. The logic behind this decision is that the patients with “outlying” results were recruited as per the clinical inclusion criteria, their samples were analysed the same way as all other samples and hence, there was no clinical or laboratory indication to exclude them from the analyses.
2.4 Results

2.4.1 Patient demographics

At the beginning of the study, patient recruitment was affected by a number of factors that led to a very small number of participants; some patients were apprehensive about having an additional speculum examination, while others had comorbidities in their pregnancy. Patients presenting with pPROM or who had complicated pregnancies (diabetes or gestational hypertension) were initially excluded from this study, but due to the poor recruitment rate a decision was made to include all patients, except those presenting with any of the exclusion criteria (section 2.1.2). Consequently, only 51 patients in total were recruited; 18 from the PPC and 33 from emergency admissions. From the 18 patients recruited from the PPC, only one delivered prematurely. From the patients recruited as emergency admissions, 17 delivered at term and 16 prematurely (Table 2-2). The majority (44 out of 51 (82%)) of the cohort, regardless of whether they were delivered preterm or at term, or were emergency admissions or identified through the PPC, were Caucasian (Table 2-3).

<table>
<thead>
<tr>
<th></th>
<th>Term</th>
<th>Preterm</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPC participants</td>
<td>17</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>Emergency admission participants</td>
<td>17</td>
<td>16</td>
<td>33</td>
</tr>
<tr>
<td>All participants</td>
<td>34</td>
<td>17</td>
<td>51</td>
</tr>
</tbody>
</table>

Volunteers recruited from the PPC had only one patient that delivered prematurely. Participants recruited as emergency admissions divided equally between those that delivered at term and those that delivered prematurely (preterm).
Table 2-3: Ethnic origins of the volunteers

<table>
<thead>
<tr>
<th></th>
<th>African</th>
<th>Asian</th>
<th>Caribbean</th>
<th>Caucasian</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preterm</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>Term</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>30</td>
<td>33</td>
</tr>
<tr>
<td>Totals</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>44</td>
<td>51</td>
</tr>
</tbody>
</table>

*There is limited ethnic diversity and as a result, reduced likelihood of an ethnicity factor being a contributor to delivery timing.

A comparison of the patients’ BMI and ages, showed no significant ($p = 0.98$ for BMI and $p = 0.33$ for age; Mann-Whitney U test) difference between the groups (figure 2.3). The median BMI of the women in the preterm group was 26.80 Kg/m$^2$ (range 20.5 – 29.0) and 24.00 Kg/m$^2$ (range 20.5 - 33.0) for the women in the term group. The median ages were 27.0 years (range 22.5 - 33.0) for the preterm group and 29.0 years (range 22.0 - 31.0) for women that delivered at term. Again, the difference between the two groups was not statistically significant ($p = 0.33$; Mann-Whitney U test).

Figure 2-3: Comparison of BMI and Age among the term and preterm groups.

Data are presented as the median (horizontal line in the boxes), interquartile (horizontal edges of boxes) and ranges (top and bottom ‘whiskers’) for the two patient groups. There were no significant differences for BMI or ages between the term and preterm groups (Mann-Whitney U-test).
2.4.2 Plasma eCB measurements

Normality tests were performed to examine the distribution of the plasma measurements of AEA, OEA and PEA and only the PEA data were found to be normally distributed (D'Agostino & Pearson omnibus normality test; PEA, $p = 0.6$; whilst AEA and OEA, $p<0.0001$). Consequently, analyses of the AEA and OEA data were non-parametric and parametric for the PEA data.

Measurement of plasma AEA concentrations in women recruited from the PPC and delivery suite/wards when combined showed that they were significantly higher in those who delivered prematurely (Figure 2.4), whilst there were no significant differences in OEA and PEA plasma concentrations between the two groups (Figure 2.4).

![Figure 2-4: Plasma AEA, OEA and PEA concentrations in the entire patient cohort.](image)

The concentrations of all 3 eCBs were increased in the women who delivered prematurely, but only the plasma AEA concentrations in the women who delivered prematurely were significantly higher (*$p = 0.0146$; Mann-Whitney U-test) than in those that delivered at term. The AEA and OEA data are plotted as box and whiskers plots, with median, IQR and ranges being shown. PEA data are presented as the mean ± SD, as the data are normally distributed. The number of samples assayed are shown above the bars.
AEA concentrations were significantly (p = 0.0146; Mann-Whitney U test) higher in women who eventually delivered prematurely with a median plasma AEA of 0.50nM (IQR 0.37-0.74) compared to a median plasma AEA of 0.33nM (IQR 0.22-0.55) for those that delivered at term (Figure 2-4).

Plasma OEA concentrations were not significantly different between the preterm and term groups (p = 0.1375), even though the median plasma OEA value at 3.66nM (IQR 2.37 - 4.98) was higher than that found in the preterm group (2.76nM; IQRs 2.14 - 3.93).

There were also no significant differences in plasma PEA concentrations between the groups (Student’s t-test, p = 0.0716) even though the mean values were higher in the preterm group (15.25nM ± 1.27; mean ± SEM) when compared to that of the term group (12.01nM ± 1.10).

AEA measurements in labour were significantly higher (p = 0.015; Mann-Whitney U test) in the group of preterm labourers than in those at term (Figure 2-5).

Figure 2-5: Comparison of AEA concentrations between women in labour (labourers) at term and preterm.
The AEA concentrations are significantly higher in preterm labourers (p = 0.015) with IQRs 0.37 – 0.74nM in PTL vs. 0.22 – 0.55nM at term.)
2.4.3 Sub-analyses of endocannabinoid measurements

The plasma eCB concentrations in patients recruited as emergency admissions were also compared to that of patients recruited from the PPC (Figure 2-5). AEA concentrations were slightly higher (median, IQR) in women who delivered prematurely (0.49nM, IQR49, 0.36-0.75) than at term (0.47nM, 0.29-0.79), but did not reach statistical significance (Mann-Whitney U test; p = 0.56). OEA concentrations were similarly higher in the PTL group (median: 3.21nM, IQR 2.32-5.18) than in the term group (median: 2.87nM, IQR: 2.75-5.65), but the difference was not statistically significant (Mann-Whitney U test; p = 0.81). PEA concentrations were also not statistically significant (Student’s t-test; p = 0.34) between the preterm (Mean ± SEM: 0. ± 0.09) and term group (Mean ± SEM: 0.49nM ± 0.09) and (0.49 ± 0.07), respectively.

Figure 2-6: Comparison of plasma eCBs between PTL and term groups from women recruited as emergencies.

There was no significant difference between the two groups for any of the plasma eCB measurements. (Mann-Whitney U tests; p-values: AEA = 0.56, OEA = 0.81. For PEA: Student’s t-test; p = 0.34).

A similar analysis among samples obtained from PPC was not possible, because only one patient delivered prematurely among the 18 recruited.

2.4.4 Relationship between eCBs in women who delivered at term

A further analysis was undertaken comparing the eCB concentrations according to the time of delivery, in the cohort recruited from the PPC and emergency.

Only women who delivered at term were included in this analysis. An analogous examination of volunteers who delivered prematurely was not possible because only one patient from the PPC
had a PTB. As a result the three eCB comparisons were made only made in the cohort that delivered at term.

In women who delivered at term there was no significant difference in the concentrations of AEA (Mann-Whitney U test; \( p = 0.066 \)) or PEA (Student’s t-test; \( p = 0.46 \)) concentrations. However, with OEA concentrations (Mann-Whitney U; \( p = 0.0147 \)) the difference was significant. (Figure 2.7). So, OEA plasma concentrations have been found to be higher in high-risk women recruited in PPC despite their progression to full term pregnancies. This suggests a difference in the aetiopathology-induced regulation of OEA in pregnant women.

**Figure 2-7: Measurement of plasma AEA, OEA and PEA concentrations in women who delivered at term and were recruited either from the PPC or were emergency admissions.**

The plasma concentrations of OEA, were significantly higher among those who presented as emergencies (*\( p = 0.0147 \); Mann-Whitney U -test). For AEA (\( p = 0.06 \); Mann-Whitney U-test) and PEA (\( p = 0.46 \); Mann-Whitney U-test), despite the higher pattern in the figures, the results for AEA and PEA were not statistically significant.

### 2.4.5 ROC analyses

Having established a difference between plasma AEA concentrations in the entire patient cohort and plasma OEA concentrations in the two groups at term, ROC analyses was used to determine to optimal cut-off value for these two eCBs and the utility of either or both as predictors of PTB.

ROC curves for plasma AEA, OEA and PEA concentrations (see Figures 2-8 and 2-9) were generated using GraphPad Prism software and cut-off points were determined by finding the highest likelihood ratio values. Based on the determined cut-off points, the sensitivity, specificity, PPV and NPV were calculated for each eCB (Table 2-4). Similar statistical values for CL and IGFBP-1 were calculated for comparison (*vide infra*).
AEA had a sensitivity and specificity of 67%, with a PPV of 52% and NPV of 79%. OEA and PEA showed good specificities (85% and 88%) and poor sensitivities (44% and 33%), whereas the NPVs of 74% and 71% respectively. As for the IGFBP-1, the sensitivity and PPV were 44.4% and 25% respectively, while the specificity 61.2% and NPV 79%. In the CL no sensitivity and PPV could be calculated, as no positive findings were discovered, but the specificity was 76% and NPV 89%.

Figure 2-8: ROC curve of plasma AEA for the prediction of PTL.

Area under the curve (AUC): 0.707; p = 0.01. The cut-off points were determined by finding the highest likelihood ratio values with the aid of GraphPad Prism. A plasma concentration of AEA above 0.42nM is suggestive of high risk for preterm birth. The sensitivity and specificity were both 67%, but the NPV was 79% (Table 2-4).
Chapter 2  Prediction of preterm birth using plasma endocannabinoid measurements

Figure 2-9: ROC curves for OEA and PEA for the prediction of PTL.
Cut-off points were identified and sensitivity, specificity, PPV and NPV were calculated (see Table 2-4). The AUC for OEA was 0.606; \( p = 0.22 \). The AUC for PEA was 0.648; \( p = 0.082 \). The results were not statistically significant, but the \( p \)-value of PEA being close to significance advocates that possibly a bigger number of samples could offer promising results.

Table 2-4: Outcome of ROC analyses for cervical length, IGFBP-1 positivity and plasma AEA, OEA or PEA concentrations in the prediction of PTB

<table>
<thead>
<tr>
<th>Cut-off point</th>
<th>CL</th>
<th>IGFBP-1</th>
<th>AEA</th>
<th>OEA</th>
<th>PEA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25mm</td>
<td>+ve/-ve</td>
<td>0.42 nM</td>
<td>4.31 nM</td>
<td>17.50 nM</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>0</td>
<td>44.4</td>
<td>67</td>
<td>44</td>
<td>33</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>76</td>
<td>61.2</td>
<td>67</td>
<td>85</td>
<td>88</td>
</tr>
<tr>
<td>PPV (%)</td>
<td>0</td>
<td>25</td>
<td>52</td>
<td>62</td>
<td>60</td>
</tr>
<tr>
<td>NPV (%)</td>
<td>89</td>
<td>79</td>
<td>79</td>
<td>74</td>
<td>71</td>
</tr>
</tbody>
</table>

The cut off points for the plasma eCB concentrations were determined by the ROC analyses, while for CL it was defined as per the national guidelines (357). IGFBP-1 has two outcomes, either positive (+ve) or negative (-ve). Despite the limited number of volunteers, plasma eCB measurements seem to have better predictability than the current conventional predictive tests (IGFBP-1 or cervical length (CL)).
2.4.6  **Cervical length**

Among the volunteers, 23 had ultrasonic measurement of their cervical length: 19 from the PPC and 4 from the emergency admissions group. The cut-off point for cervical length was set at 25mm (363). Only 2 women proceeded to preterm delivery, one from the PPC group and one from the emergency admissions group and both had cervical lengths > 25mm (Table 2-5), therefore in total 2 pregnancies were delivered prematurely (8.7%). When cervical length measurements were used to generate ROC curve data (figure 2-10 ) they showed a sensitivity of 0%, a specificity of 76%, a PPV of 0%, and a NPV of 89% (Table 2-4).

**Table 2-5: Cervical length**

<table>
<thead>
<tr>
<th>Cervical length</th>
<th>Preterm</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 25 mm</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>More than 25</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>21</td>
</tr>
</tbody>
</table>

No patient with a CL ≤ 25mm delivered prematurely, thus the Sensitivity and PPV for this test are both 0%.

**Figure 2-10: ROC analysis of cervical length in the prediction of PTB.**

The small number of the volunteers in the PTL group and the discrepancy in CL in the term group (as shown in Table 2-5) may have contributed to the stepwise shape of the curve.
2.4.7 IGFBP-1

Cervical IGFBP-1 measurements were performed using Actim Partus® kits (Alere, Stockport, Cheshire, UK) on 40 out of 51 volunteers, 21 of which were emergency admissions and 9 were recruited from the PPC (Table 2-6). The reasons for not obtaining measurements from the other 12 patients are presented in Table 2-7. In many situations, IGFBP-1 was not clinically appropriate (e.g. in pPROM), but the patients agreed to participate in the study. So, only a blood sample was obtained from those patients.

Table 2-6: IGFBP-1 results and pregnancy outcomes in 40 volunteers

<table>
<thead>
<tr>
<th>IGFBP-1</th>
<th>PTB</th>
<th>Term</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>4</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
<td>19</td>
<td>24</td>
</tr>
</tbody>
</table>

The IGFBP-1 was assessed in 9 patients from the PPC and in 31 emergency admissions. As it is notable, whether the results were positive or negative, most of the patients delivered at term, while 19% (5 out of 24) of the patients with a negative test ended up in PTL.

Table 2-7: Reasons for absent data for the IGFBP-1 (Actim® Partus) measurements

<table>
<thead>
<tr>
<th>Reasons for not having IGFBP-1 measurement (Actim Partus®)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not possible</td>
</tr>
<tr>
<td>Declined examination</td>
</tr>
<tr>
<td>In possible labour</td>
</tr>
<tr>
<td>Not appropriate</td>
</tr>
<tr>
<td>pPROM</td>
</tr>
<tr>
<td>Abdominal cervical cerclage in situ</td>
</tr>
<tr>
<td>Bulging membranes</td>
</tr>
</tbody>
</table>
Cervical IGFBP-1 measurements showed a sensitivity of 44.4%, specificity of 61.2%, with a PPV of 25% and NPV of 79% (Table 2-4). Overall, the eCB results showed equal, if not better, PTB predictability in comparison to CL and IGFBP-1.

### 2.4.8 Correlation analyses

Having established that the eCBs were as good as if not better predictors of impending PTB, it was decided to see if the ‘entourage effect’ could explain this by performing correlation analyses. The correlation coefficients were calculated between eCBs on the entire cohort or when eCB based on the timing of delivery (preterm and term), or when the IGFBP-1 results (positive or negative) or when the cervical length measurements were ≤25 or >25mm were performed. Because the CL and IGFBP-1 data were not normally distributed, the strength of the correlations were classified using the nomenclature described Evans (364). These are presented in Table 2-8.

<table>
<thead>
<tr>
<th>Descriptive category</th>
<th>r range</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Very weak”</td>
<td>0.00 to 0.19</td>
</tr>
<tr>
<td>“Weak”</td>
<td>0.20 to 0.39</td>
</tr>
<tr>
<td>“Moderate”</td>
<td>0.40 to 0.59</td>
</tr>
<tr>
<td>“Strong”</td>
<td>0.60 to 0.79</td>
</tr>
<tr>
<td>“Very strong”</td>
<td>0.80 to 1.00</td>
</tr>
</tbody>
</table>
There was a positive correlation between all eCBs (see Table 2-9). The most notable correlation was between AEA and OEA, followed by AEA and PEA and lastly OEA and PEA. Emergency admissions who delivered prematurely had similar correlations, while correlations between AEA and PEA for those who delivered at term showed the highest r-value \( r = 0.76, p = 0.001 \). A similar sub-analysis from PPC samples was not possible, as it was 1 of the 18 patients delivered at preterm. Moreover, the correlations between the eCB concentrations of term and preterm groups were neither strong nor significant.

During the analyses, calculations were performed separately among women who delivered at term and prematurely. This was because our Leicester expert group agrees with the theory that the initiation of labour at term is different than that of preterm (351, 365-369).
Table 2-9: Correlations between eCBs in all samples, all PTB vs. Term labourers and PTB vs. Term labourers among emergency volunteers

<table>
<thead>
<tr>
<th></th>
<th>Correlation (r)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ALL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA vs. OEA</td>
<td>0.69</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>AEA vs. PEA</td>
<td>0.66</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>OEA vs. PEA</td>
<td>0.64</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>PTB</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA vs. OEA</td>
<td>0.70</td>
<td>0.001</td>
</tr>
<tr>
<td>AEA vs. PEA</td>
<td>0.65</td>
<td>0.003</td>
</tr>
<tr>
<td>OEA vs. PEA</td>
<td>0.62</td>
<td>0.005</td>
</tr>
<tr>
<td><strong>Term</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA vs. OEA</td>
<td>0.64</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>AEA vs. PEA</td>
<td>0.62</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>OEA vs. PEA</td>
<td>0.58</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

In the upper part of the table, the correlations included the entire cohort of samples (both those recruited from PPC and emergencies). The comparisons were performed in the entire 51 samples (“ALL”), in women who delivered prematurely (“PTB”) and in those who delivered at term (“Term”). In the lower part of the table similar comparisons were performed only the samples obtained from the emergencies. A comparison in patients from PPC was not possible as only 1 patient delivered prematurely. Detailed explanation of these values is presented in the discussion.
A different analysis of the correlations between eCBs based on the results of IGFBP-1 showed similar findings. There was a positive correlation between eCBs in all patients who had negative IGFBP-1 test (Table 2-10). This was found in both analyses of the whole lot of patients and the PPC participants. However, the eCB correlation in patients who tested positive showed either poor or statistically insignificant correlations (e.g. in the PPC group, with positive IGFBP-1 test, all three eCB correlations were of $p = 0.44$ – Table 2-10).

Similarly, eCB values from emergency volunteers with negative IGFBP-1 had a poor correlation. In opposition, emergencies with positive IGFBP-1 showed positive correlation in eCBs in a similar order as before (AEA-OEA, then AEA-PEA and OEA-PEA).
Table 2-10: Correlations among eCBs based on whether an IGFBP-1 result was positive or negative

<table>
<thead>
<tr>
<th>Correlations for cervical IGFBP-1 results</th>
<th>Correlation ($r$)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All samples</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA OEA</td>
<td>0.7233</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>OEA PEA</td>
<td>0.7352</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>AEA PEA</td>
<td>0.5781</td>
<td>0.0039</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA OEA</td>
<td>0.549</td>
<td>0.0244</td>
</tr>
<tr>
<td>OEA PEA</td>
<td>0.6176</td>
<td>0.0096</td>
</tr>
<tr>
<td>AEA PEA</td>
<td>0.527</td>
<td>0.0318</td>
</tr>
<tr>
<td><strong>PPC samples</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA OEA</td>
<td>0.6608</td>
<td>0.0028</td>
</tr>
<tr>
<td>OEA PEA</td>
<td>0.645</td>
<td>0.0038</td>
</tr>
<tr>
<td>AEA PEA</td>
<td>0.636</td>
<td>0.0045</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA OEA</td>
<td>-0.3571</td>
<td>0.4444</td>
</tr>
<tr>
<td>OEA PEA</td>
<td>0.3571</td>
<td>0.4444</td>
</tr>
<tr>
<td>AEA PEA</td>
<td>0.3571</td>
<td>0.4444</td>
</tr>
<tr>
<td><strong>Emergency samples</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA OEA</td>
<td>0.6084</td>
<td>0.0399</td>
</tr>
<tr>
<td>OEA PEA</td>
<td>0.6783</td>
<td>0.0185</td>
</tr>
<tr>
<td>AEA PEA</td>
<td>0.5594</td>
<td>0.0628</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA OEA</td>
<td>0.8909</td>
<td>0.0011</td>
</tr>
<tr>
<td>OEA PEA</td>
<td>0.8061</td>
<td>0.0072</td>
</tr>
<tr>
<td>AEA PEA</td>
<td>0.7818</td>
<td>0.0105</td>
</tr>
</tbody>
</table>

With the exception of the positive results in PPC patients, which show no statistical significance, eCBs retain a positive correlation in all analyses. This suggests that the “entourage effect” is in action during labour as well as during the quiescent phases.

There was no correlation between CLs and eCBs (Table 2-11) and likewise, none between individual eCBs and CLs, having divided the lengths into two groups with 25 mm being the cut-off point ($\leq 25$ and $>25$ mm). Interestingly, when the CL $\leq 25$ mm, there was a negative correlation with PEA ($r = -0.8$), this was not statistically significant ($p = 0.133$). A further analysis of the correlation between eCBs based on the CL ($\leq$ or $>25$ mm), revealed a positive linear relationship between AEA and OEA, and a very strong positive relationship between AEA and PEA and OEA...
and PEA when CL ≤25mm. Unfortunately, only the AEA and OEA correlation was significant. A similar analysis in the groups whose CL was > 25mm, showed a significant but moderate positive relationship among AEA and OEA (Spearman, $r = 0.6$, $p = 0.001$) and between OEA and PEA (Spearman, $r = 0.6$, $p = 0.004$).

**Table 2-11: Correlations among eCBs based on the CL measurements**

<table>
<thead>
<tr>
<th>Correlations for Cervical lengths</th>
<th>Correlation ($r$)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CL to eCBs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All CL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA</td>
<td>0.113</td>
<td>0.605</td>
</tr>
<tr>
<td>OEA</td>
<td>0.006</td>
<td>0.975</td>
</tr>
<tr>
<td>PEA</td>
<td>-0.076</td>
<td>0.729</td>
</tr>
<tr>
<td><strong>CL ≤25 mm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA</td>
<td>-0.5</td>
<td>0.45</td>
</tr>
<tr>
<td>OEA</td>
<td>-0.5</td>
<td>0.45</td>
</tr>
<tr>
<td>PEA</td>
<td>-0.8</td>
<td>0.133</td>
</tr>
<tr>
<td><strong>CL &gt; 25 mm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA</td>
<td>0.319</td>
<td>0.196</td>
</tr>
<tr>
<td>OEA</td>
<td>0.041</td>
<td>0.870</td>
</tr>
<tr>
<td>PEA</td>
<td>0.043</td>
<td>0.864</td>
</tr>
<tr>
<td><strong>eCBs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL ≤25 mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA OEA</td>
<td>1</td>
<td>0.016</td>
</tr>
<tr>
<td>OEA PEA</td>
<td>0.9</td>
<td>0.083</td>
</tr>
<tr>
<td>AEA PEA</td>
<td>0.9</td>
<td>0.083</td>
</tr>
<tr>
<td>CL &gt; 25 mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA OEA</td>
<td>0.687</td>
<td>0.001</td>
</tr>
<tr>
<td>OEA PEA</td>
<td>0.636</td>
<td>0.004</td>
</tr>
<tr>
<td>AEA PEA</td>
<td>0.613</td>
<td>0.006</td>
</tr>
</tbody>
</table>

In the upper part of Table 2-11 (marked as “CL to eCBs”) the correlations examine the eCB concentrations against the different cervical lengths. As it is shown, none of the correlations were significant.

In the bottom part of the table (marked as “eCBs”) the correlations were between the eCB concentrations in women with different CLs. It is obvious that there is a predominant positive correlation among all three eCBs. The non-significant correlations still suggest that the high p values were most likely due to the small number of samples.
2.4.9 Recruitment to delivery intervals (RTDI)

The next step in the investigation of the potential of eCBs as biomarkers of PTB has to examine the relationship between the interval from sampling to delivery (recruitment to delivery interval: RTDI), which was calculated in as the number of weeks from the time of recruitment and sampling to the time of delivery.

The comparisons for AEA and OEA were done by Mann-Whitney U test, as not all of the data were equally distributed, while for PEA the tests were conducted by Student’s t test. The analyses were performed repeatedly where every time the same AEA data was used but they were compared according to different weekly RTDIs (Tables 2-11 and 2-12). This carried on up to the highest possible comparison between women who delivered within 14 weeks of RTDI and those who delivered later than 14 weeks since recruitment. Comparisons for RTDI of 4 and 13 weeks was not possible as there was no available data under this RTDI. In the PCC cohort, the number of women who delivered before 9 and beyond 12 weeks was very small, making such a comparison impossible (Table 2-11 and Figure 2-18).

The different p values were recorded and plotted. Afterwards, the same sequence of analyses was performed for OEA and PEA. The analyses were performed independently for all samples, samples obtained from only the PPC (Table 2-11) and only from emergency admissions (Table 2-12).

Plasma AEA (p = 0.03) and OEA (p = 0.016) were significantly higher (using Mann-Whitney U test) in those who had a RTDI of 9 weeks. Similarly, patients with RTDI of 10 weeks showed a significant difference in these two endocannabinoids (AEA p = 0.03 and OEA p = 0.01). (Table 2-11 and Figure 2.17). Analysis of the results of the 19 patients from the PPC, also showed significant changes in OEA concentrations (p < 0.001) in 10-week intervals (Table 2-11 and Figure 2.17).

From this analysis, it was concluded that women who delivered within 10 weeks of their recruitment had AEA and OEA concentrations significantly higher than women who delivered after 10 weeks of recruitment.

Similar analyses on the samples from emergency admissions alone did not show any significant results (Table 2-12). In this cohort, there was no data available for RTDI of 4 weeks and as a result such a comparison was not performed.
Chapter 2  Prediction of preterm birth using plasma endocannabinoid measurements

Figure 2-11: Graph showing the p values of RTDI of each eCB in all 51 patients. AEA and OEA reach concurrent significant differences in RTDIs of 9-10 weeks. (AEA and OEA analyses were performed with Mann-Whitney U test).

Figure 2-12: Graph showing the p values of RTDI of each eCB in PPC patients. AEA and OEA were analysed with non-parametric test. The results are similar to the preliminary RTDI findings in PPC patients, with the curves maintaining the same pattern, and suggesting that RTDI of 10 weeks could be of predictive value.
Table 2-12: RTDI analysis of the 3 eCBs in all patients and PPC patients

<table>
<thead>
<tr>
<th>Weeks</th>
<th>p values for all patients (n = 51)</th>
<th>p values for PCC patients (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RTDI Predictability*</td>
<td></td>
</tr>
<tr>
<td>&lt;1</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
<td>0.6</td>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
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<td>0.2</td>
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<tr>
<td>5</td>
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<td>0.08</td>
</tr>
<tr>
<td>6</td>
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</tr>
<tr>
<td>8</td>
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</tr>
<tr>
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<td>0.03</td>
</tr>
<tr>
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</tr>
<tr>
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<td>0.2</td>
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<tr>
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<tr>
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<td>0.12</td>
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<tr>
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<tr>
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<td>0.9</td>
</tr>
<tr>
<td>12</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*The table shows the different p values obtained after multiple analyses (Mann-Whitney U test for AEA and OEA, and Student’s t-test for PEA) according to the different RTDIs. The multiple individual analyses of all the samples (n = 51) and those from the PPC (n = 19) point out that the ECS may have a predictive RTDI around to 10 weeks.
A similar analysis was performed for patients who were recruited from 24 to 30 weeks only, despite the limited number in this cohort. There was significant difference in the RTDI for all three eCBs at 10 weeks, in the entire cohort, while among the emergencies only OEA showed significant difference at 8 weeks (Table 2-13). Analyses among samples from patients from PPC did not show any significance. However, the PPC cohort did not show any significant results at RTDI of 10 and 11 weeks.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>AEA</th>
<th>OEA</th>
<th>PEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>0.47</td>
<td>0.63</td>
<td>0.94</td>
</tr>
<tr>
<td>1</td>
<td>0.27</td>
<td>0.73</td>
<td>0.89</td>
</tr>
<tr>
<td>2</td>
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<td>0.65</td>
<td>0.89</td>
</tr>
<tr>
<td>3</td>
<td>0.57</td>
<td>0.42</td>
<td>0.85</td>
</tr>
<tr>
<td>5</td>
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<td>0.67</td>
<td>0.48</td>
</tr>
<tr>
<td>6</td>
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<td>0.72</td>
<td>0.60</td>
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<tr>
<td>7</td>
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<td>0.81</td>
<td>0.26</td>
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<tr>
<td>8</td>
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<td>0.97</td>
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<tr>
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<td>0.89</td>
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<tr>
<td>10</td>
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<td>0.84</td>
<td>0.62</td>
</tr>
<tr>
<td>11</td>
<td>0.44</td>
<td>0.69</td>
<td>0.70</td>
</tr>
<tr>
<td>12</td>
<td>0.57</td>
<td>0.41</td>
<td>0.84</td>
</tr>
</tbody>
</table>

*Unlike the analyses from the entire cohort and PPC patients, the emergencies alone did not show any significant results. This could indicate that the plasma eCBs are of more value in the RTDI predictability in quiescent patients.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>AEA</th>
<th>OEA</th>
<th>PEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>0.03</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
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<td>0.01</td>
<td>0.03</td>
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<tr>
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<td>0.21</td>
<td>0.14</td>
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<tr>
<td>8</td>
<td>0.07</td>
<td>0.003</td>
<td>0.10</td>
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<tr>
<td>9</td>
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<td>0.09</td>
<td>0.95</td>
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<tr>
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<td>0.30</td>
<td>0.59</td>
<td>0.15</td>
</tr>
<tr>
<td>11</td>
<td>0.36</td>
<td>0.76</td>
<td>0.23</td>
</tr>
</tbody>
</table>

*There is a consistent significance in the RTDI at 10 weeks in all patients, while in the emergency group OEA (p = 0.0003) remains a promising predictor for a RTDI of 8 weeks.
2.5 Discussion

The results from the studies presented in this chapter point towards the possibility of predicting PTB with some certainty long before the event. The key observation that supports the primary hypothesis of this chapter, that plasma AEA concentrations would be significantly higher in women at risk of PTB, is supported by the data presented.

That plasma AEA concentrations had a diagnostic value similar to or even better than that of IGFBP-1 (Table 2-4) or cervical length, suggesting that this measurement may have a future benefit for the prevention of PTB. Furthermore, plasma AEA and OEA concentrations, at appropriate cut-off values (as determined by ROC analyses), had the potential to predict parturition over 2 months in advance of the actual event. Interestingly, the results from women who were recruited before 30 gestational weeks showed similar probabilities of delivery (RTDI values) for all 3 eCBs, suggesting that sampling of blood and subsequent measurement of the 3 eCBs at around the time of fetal viability (24 weeks of completed gestation) might provide a fairly accurate prediction of the actual week of delivery. There are, however, several limitations to this study that need to be addressed before a definitive conclusion can be made. These relate to the actual timing of recruitment, the patient demographics and the limited study design (see section 2.6 ‘Limitations of the study’).

2.5.1 Plasma eCB concentrations in prematurity

Although the plasma AEA concentrations were significantly higher in both symptomatic and asymptomatic women prior to delivery, making it a good predictor of PTB, a finding that agrees with the suggested hypothesis by the pilot study authors, and that second trimester plasma AEA measurements are better predictors of PTB than cervical IGFBP-1 or cervical length measurements, the prematurity rate did not seem to be similarly associated with plasma OEA and PEA measurements, despite their concentrations being higher in women who subsequently delivered prematurely. The reason for the lack of effect could be due to the small numbers of women recruited (n = 51), and the mixed presentation of those women. It appears that pregnant women did not want to take part in clinical trials, because of fears that doing so may damage the pregnancy or cause damage to the fetus. Accordingly, it was not possible to recruit the numbers anticipated at the beginning of the study and only 51 instead of the 212 required were recruited. The actual number required for sufficient power, was 16 women who would deliver prematurely and 196 women who would go on to deliver at term. Although the first (important) number was
achieved, a larger number that subsequently went to term before delivery would probably provide stronger evidence that plasma eCBs can be used as a reliable test for prediction of PTB.

The analysis of the plasma eCB concentrations from the patients who were recruited only as emergencies, showed no significant difference between the PTB and term delivery groups (Figure 2-6). These patients were admitted with symptoms of either PTL or other clinical reason that can simulate symptoms of labour (e.g. UTI, strong Braxton-Hicks contractions, GI symptoms, musculoskeletal pains, etc.) so, eCB concentrations are anticipated to be high even though this might be for different reasons, with the most prevalent and common one being the fact that they often presented with painful symptoms. At this point it is appreciated the value of a predictive test that can offer reliable answer in asymptomatic women.

Unfortunately, a similar analysis among PPC patients was not possible as only one delivered prematurely. Hopefully, with a larger number of volunteers such analyses will be possible.

A comparison of the plasma eCB concentrations between the PPC and the emergency admissions cohort, who subsequently delivered at term, showed significant differences in their plasma AEA and OEA concentrations, but not their PEA concentrations. To the researcher’s knowledge, this is the first time that the plasma OEA and PEA concentrations have been assessed in such clinical groups. As eCBs are thought to be released on demand and not stored, asymptomatic patients in PPC, who have no other risk factors or health issues and delivered at term, had no obvious medical/biochemical reason for elevated plasma eCB concentrations at the time of sampling. However, the emergency admissions group, may have had numerous medical/biochemical reasons related to their admission, such as pain associated with their actual condition that was not even related to PTB, or in some cases, this was actually due to their predilection towards preterm birth. This idea needs to be investigated more, but because the causes of emergency admission are varied (as described earlier), then a much larger patient recruitment strategy would need to be employed.

The analysis of the RTDI for the entire cohort indicated a significant relationship between RTDI and AEA and OEA plasma concentrations. For AEA, a high plasma measurement (> 2nM) was predictive of a delivery within 7-10 weeks (RTDI 7-10 weeks), whilst that for elevated OEA (>2.3nM) was for delivery within 9-11 weeks. These are potentially exciting data, and regardless of when the blood sample was taken, when the RTDI was either 9 or 10 weeks, the concentrations of both plasma AEA and OEA were significantly related to the prediction of birth at that point. The same RTDI (either 9 or 10 weeks) for PEA was almost as good with a slightly poorer
probability for prediction of birth \( (p = 0.08) \), which with a larger cohort might improve. These findings are very promising as they show possible predictability of PTB of up to 10 weeks in advance, and could allow prematurity prediction that has eluded perinatologists for decades (370, 371).

An analysis of the samples from the emergency admissions group alone did not reveal any significant relationship between plasma eCBs and RTDI. By contrast, the samples from the PPC group alone showed a significant difference in OEA at a 10 and 11-week RTDI, suggesting that plasma OEA may be a good predictor of preterm delivery in this group and that a subtle difference in the aetiopathology of parturition in the PPC group and the emergency admissions group exists. These data also indicate that a conservative 8-week period and a more consistent 10-week period can be provided for the preparation of primary prevention (e.g. administration of corticosteroids or \textit{in utero} transfer), with plasma OEA concentrations in particular, seemingly a good predictor of the RTDI for asymptomatic women. Considering that patient recruitment took place between the age of viability and 34 gestational weeks, a clinically relevant predictive test could be performed at 24 weeks (the age of viability) of pregnancy that would be sufficient for clinical management of pregnancies that were shown to have elevated plasma OEA concentrations. Having said that, it is possible, that these results could have been affected by an artefact in the study design. As a result, a further study, is indicated where plasma eCB concentrations are measured in the same type of volunteers but at earlier gestational ages such as at the twentieth gestational week.

By contrast, data on the use of IGFBP-1 in the cohort were almost exactly as shown in published studies, with their respective NPVs being close to that published (133, 136, 143, 144, 146, 160, 161). It is known that oFN has limited value in predicting PTB in asymptomatic high-risk women but has a good NPV for symptomatic women (155, 157, 161, 165-169). IGFBP-1 is also a poor predictor of PTB but has a good NPV (136, 143, 144, 146, 160, 161). Moreover, both tests can only predict PTB up to 2 weeks in advance, while researchers suggest of results of oFN reaching a 3-week predictability (with specificity of 96.1% and NPV of 98.6%) (372). Recently, a new test has been introduced for the prediction of PTL. PartoSure\textsuperscript{TM} [placental alpha macroglobulin-1 (PAMG-1)] is a new swab test with cited predictive values within seven days being as follows: sensitivity 90%, specificity 93.8%, PPV of 78.3%, and NPV of 97.4% (373) and that it is superior to oFN and CL assessments (374). Moreover, it has been tested from 20\textsuperscript{th} to 36\textsuperscript{th} gestational weeks, something that allows a big spectrum of gestational ages. However, a positive PAMG-1 test predicts labour within 7 days and a negative result suggests that delivery is improbable in the
following 14 days (373, 374). Consequently, this test can be a very good tool for symptomatic women (i.e. secondary prevention), but when it comes to its long-term input and primary prevention it does not exceed the two weeks.

In this study, though, the data suggest that a simple blood test, that measures the plasma concentrations of the eCBs, in asymptomatic patients, may allow the prediction of birth as early as 2 months in advance of the event.

### 2.1.1 Testing the entourage effect

Because concentrations of the eCBs are closely linked through the ‘entourage effect’ where one eCB might be ‘sacrificed’ by the actions of FAAH, then an analysis of the relationships between the 3 eCBs could possibly reveal such an effect. There was a strong positive correlation between all three eCBs in the cohorts, with the strongest being between AEA and OEA, then AEA and PEA with the weakest being between OEA and PEA.

A sub-analysis of the correlates that focussed solely on the plasma concentrations obtained from the emergency admission patients, showed similar strong correlations between eCBs in both those that delivered at term and those that delivered prematurely. In general, the positive correlation between the three eCBs, as well as the rise in concentrations, may provide further evidence of the concept of the “entourage” effect in the female reproductive system. OEA and PEA are known to boost the effect of AEA in the nervous, intestinal, and vascular tissues (375, 376) and in the female reproductive system (250, 377).

Following this, it was important to examine the eCB correlation depending of the results of the other predictors: IGFBP-1 and CL. This could elude a mechanism by which eCBs respond in certain circumstances, like, for example, when there is cervical shortening.

The correlations between eCBs based on the Actim Partus® results (Table 2-9) was significant in all patients. While the patients who had positive IGFBP-1, in the PPC showed a positive correlation between all eCBs, in women with negative IGFBP-1 the correlations were weak and insignificant. In emergency admissions with negative IGFBP-1 the correlations between AEA and OEA and OEA and PEA were significant, while the correlation between AEA was PEA was insignificant. On the other hand, patients with positive IGFBP-1, showed significant correlation between their eCB.
The significant correlations between all eCBs in all the negatively tested women for IGFBP-1 (PPC and emergencies) corresponded to the fact that the cervical swabs have a very high NPV. As a result, in patients with low risk of PTB (i.e. patients who had negative swab test) an accord between the eCB is expected as there is no indication for their excessive production and release. In other words, in quiescent states eCBs concentration would be lower, as proven, and their correlation similar.

The correlations between the 3 eCBs in asymptomatic women (from PPC) with positive IGFBP-1 were not high, as there is no evidence of the cervical swab use in asymptomatic women. On the other hand, in symptomatic women (i.e. emergencies) with positive IGFBP-1, the strong eCB correlation is possibly due to the fact that the same reason that “triggered” the IGFBP-1 could be behind the corresponding correlation among eCB.

Finally, CL showed no significant correlations when the lengths were compared to the eCBs concentrations. However, in women with a CL of ≤25mm the correlation between AEA-OEA had the highest significant strength. The rest of the correlations in this group of women even though were very strong they were statistically insignificant. Women with a CL of > 25mm had a strong and significant correlation among all three eCBs. Understandably, a short CL increases dramatically the risk of PTB (170-176) and as a result our eCB measurements, a strong correlation is expected. In the group of CL > 25mm, all correlations were strong, which means in women who are asymptomatic and with a strongly negative test (NPV 89%) an accordance is expected also among eCBs. The effect of the ECS on the cervix and the induction of cervical ripening is not clear, as the studies in this field are limited (250). There is evidence, though, suggesting the involvement of CB1 in the cervix, through AEA stimulation, leading to ripening, given the fact that such an interaction leads to uterine muscle relaxation and cervical ripening (378, 379).

Another interesting point arising from the data was that women with a cervical length of <25 mm delivered at term. The literature suggests that about 11% of these women should have delivered prematurely. None did in this study and that was probably because these women were under close clinical care having all been in the PPC cohort, and so receiving the appropriate management that allowed them to consequently deliver at term. It is also possible, though, that the number of participants was too small.
Chapter 2  Prediction of preterm birth using plasma endocannabinoid measurements

2.6 Limitations of the study

The first limitation was related to the number of participants. The number of patients who refused the participation was relatively high. At some point it had reached a stage where only one in every six approached patients would consent for participation. The main reason was the refusal of a speculum examination. Women were not keen for an “unnecessary” examination, while a lot of those who had experienced a previous PTL were apprehensive of the examination. Other patients refused due to cultural reasons or the fear of becoming “Guinea pigs”. A considerable number of patients showed indifference simply because this study would not “offer answers” or “predictions” for their on-going pregnancy. There were patients from ethnic minorities who refused participation due to language barrier or religious reasons, or both.

It is important to mention that in many circumstances an introduction about the study was offered, voluntarily, to patients by some hospital staff, before even the researcher meets the patients. Depending on the quality of that initial communication, the patients agreed or refused to participate. Of course, all these reasons mentioned are recognised, both in the general population and minority groups (380-382) and the patients’ wishes were respected.

Another challenge with this study was the fact that there was only one person who had to recruit, collect the samples and run them in the laboratory. Given the fact that the samples had to remain on ice, no more than two patients were possible to be recruited at any time. This part is described again with more detail in chapter 7.

Although the data presented in this Chapter support a role for the measurement of plasma eCBs in the prediction of PTB, the study was limited by the small number of patients being recruited. Additionally, a very important group were excluded from study: women with multiple fetal pregnancies. These pregnancies were excluded because the source of the eCBs in pregnancy is currently unknown. If they are produced by the placenta or fetal membranes, then multi-fetal pregnancies would possibly increase the plasma eCB concentrations, i.e. two placentae, twice the volume of plasma eCB. This would ‘fit’ with the current data, but might make the analysis that much more difficult. In order to find a definitive conclusion for the prediction of PTB from a single blood sample taken between 24 and 30 weeks gestation, and a larger number of samples will be required. A power analysis run by the current data and a similar analysis that was performed on the data from the pilot study, suggested 212 samples would be required for a definitive conclusion.
From a research perspective, the actual role of plasma eCBs have in PTB and parturition requires further examination, but these molecules probably have roles in all tissues of the uterus, such as the myometrium, the decidua, chorion, amnion and even the placenta, since these eCBs have been found in all of these tissue (356).

One more limitation is the definition of the cut-off points for gestational ages when it comes to the IGFBP-1 test. The cervical predictive tests of ofFN or IGFBP-1 are reliable in their prediction up to 34-35 weeks (156, 157, 161), with no studies examining their efficacy beyond this gestation, as there is no clinical (realistic) indication to use them. Logically, recruiting patients who had exceeded the 34th week of gestation, but were still less than 37 weeks pregnant, fell in a grey area.

However, corticosteroid treatment is normally administered at any point up to 34 weeks (121) and the cervical predictive tests of ofFN or IGFBP-1 are reliable in their prediction up to 34-35 weeks (156, 157, 161), with no studies examining their efficacy beyond this gestation, as there is no clinical (realistic) indication to use them.

Among the volunteers and their subgroups (women who delivered at term or prematurely and participants from the PPC or were emergencies), there was no significant difference in ages and BMI. The vast majority of the cohort were unintentionally Caucasians. Patients of other ethnicities, and from socially deprived backgrounds, who often were at high risk for PTB (21, 23, 106, 383) refused participation. Considering that the reasons for refusal are essential to be mentioned in any study (384), and even though the PIL offered the freedom to candidates not to disclose their reasons for refusal, the reasons in all cases were obvious and discussed in the section covering the limitations. It can be argued that, as the majority of the patients are Caucasians the study should be considered flawed. However, taking into consideration that the patients were of the same ethnic origin, with no effect of the age and BMI (Figure 2-3) on their plasma eCBs, and without any notable medical conditions, that means that the cohort obtained was of similar qualities and with expected “cleaner” results.

Only one patient had PTL from the PPC group (n = 18) and 17 volunteers from the emergency group (n = 33). This difference on its own implies the significant role of PPC in managing and preventing PTL.
2.7 Conclusions

The data presented in this Chapter provides evidence that eCBs can predict PTB in asymptomatic, as well as symptomatic, patients at high-risk of PTL. The preliminary RTDI data suggest a 10-week prenatal prediction for parturition, something that requires further extensive studying.

Although the main limitation of this study was that recruitment was limited and more volunteers would be needed to provide a convincing argument that plasma AEA (at least) can be used to predict PTB, the results of RTDI obtained from women recruited before 30 gestational weeks (Table 2-15) offer some additional data to support the original hypothesis that a single blood test taken in the second/third trimester can predict whether a woman will deliver prematurely or not. The added benefit that these molecules can interact with specific receptors that are measureable within pregnant tissues (379), suggest they have an intimate role in parturition. This idea will be examined in the following chapters.
Chapter 3  FAAH, oestradiol and progesterone measurements
3.1 Introduction

In Chapter 1 the hydrolysing effect of FAAH on AEA (301), as well as on OEA and PEA, the so-called ‘entourage effect’ (305) was described. Moreover, it was highlighted how plasma FAAH has been used to predict miscarriage (309) and its expression is affected by progesterone (303, 317). Therefore, plasma levels of FAAH and eCBs are considered to be inversely related.

Since plasma eCB levels change significantly in women at high risk for PTL, it is necessary to study the lymphocytic activities of FAAH, to see if FAAH activities could account for these. If found, then it could be used as an individual testing parameter or in combination with the eCB values for the prediction of PTB.

Because the concentrations of the eCBs (especially AEA) increase in women who are at high-risk for PTL (Chapter 2), and other data suggest that serum oestradiol and progesterone alter significantly in women transitioning from the quiescent state to the process of active labour (385), then it seemed prudent to determine if a correlation between plasma eCB concentrations and female sex hormones and if a relationship with those parameters with the FAAH enzyme activities exists.

Previous studies that have focussed on the evaluation of eCB levels in pregnancy and their changes with the different gestational ages or obstetric complications, have revealed that they are controlled by hormones, primarily oestradiol and progesterone (314-316). This is clinically relevant because progesterone caproate is used successfully in the preventative management of PTL (386-389). Furthermore, several connections between the ECS and progesterone have been demonstrated (Chapter 1).

Of relevance to this chapter, is data indicating that when either CB1 or CB2 are stimulated in the ovary, in sheep, luteal progesterone secretion is perturbed (318), and that there appears to be a lack of correlation between plasma AEA and progesterone concentrations through the menstrual cycle in humans (310, 319). With the observations that there is an apparent discrepancy between how progesterone controls lymphocytic and uterine FAAH levels and activities (303, 317) and that oestradiol inhibits FAAH production and activity in endothelial cells and the uterus (320, 390), with a resulting increase in AEA levels, then the possible relationship between FAAH activity and sex steroid hormone concentrations needs attention in the at risk PTL population.
3.2 Methods

3.2.1 Patient recruitment and sample storage

The samples for FAAH quantification were collected from the same patients who were recruited for the eCB study (Chapter 2). A blood sample was collected in plain 2.7 mL tubes (Sarstedt Ltd., Leicester, UK) prefilled with the acid citrate dextrose (ACD) anticoagulant. Once blood samples were obtained, they were placed in ice. Then, they were transferred to the laboratory where they were stored at −80 °C until the time of their analysis. When all samples had been collated, they were shipped to Mauro Maccarrone’s laboratory in Rome (Italy), where FAAH activities were measured (described in section 3.2.3).

Samples for hormonal quantification were taken at the same time and plasma divided into two parts, one the analysis of plasma eCB concentrations (Chapter 2) and one for the measurement of oestradiol (E2) and progesterone (P4). In both cases, samples were stored at -80°C until analysed.

3.2.2 Sample collection

Of the 51 samples collected for eCB measurement, only 43 additional samples were collected for FAAH analysis. However, for the hormonal quantification only 36 samples were used on the basis of the following:

a. Analysis could be performed in duplicate
b. For each sample there was a relevant sample available for FAAH analysis, for future comparison and correlation of results
c. There was sufficient sample for re-analysis, if indicated.

Blood samples for FAAH quantification were collected and kept in prefilled tubes with ACD. ACD is an ideal anticoagulant as it preserves blood pH at 6.5 and as a result it prevents platelet aggregation (391, 392) and it needs to be kept at 4 °C. The volume of ACD required in each tube should cover the ratio of 1:10 of the blood sample (based on the protocol of Maccarrone et al. (309)). Hence, the volume of 0.3 mL of ACD was added to each empty tube, and all tubes were stored at 4 °C until recruitment time.
3.2.3 FAAH activity measurement

The methodology for the quantification of FAAH in human blood have been optimised and in use for several years (309, 393). The experiments were carried in a different laboratory, by the group that has originally optimised the methodology of FAAH quantification.

The preparation of the lymphocyte membranes took place by the ice-cold hypotonic lysis method and through the pelleting of resultant membranes at 11000 x g at 4°C for 20 mins. The suspension of the membranes was at 1 mg/mL concentration. Then, 50 μg of the membrane protein was incubated at 37°C for 15 minutes with 10μM AEA-ethanolamine-1-[^3H] (60 Ci/mmol; NEN DuPont de Nemours, Wilmington, DE, USA). At that stage the process was halted to add 800 μl of ice-cold chloroform/methanol (1:2 v/v) and the new mixture was vortexed thoroughly. Then, a further addition of 240 μl of chloroform and 240 μl of dH2O was followed by vortex mixing. The mixture was incubated at room temperature for 10 minutes and afterwards it was centrifuged for 5 minutes at 3000 x g. The supernatant aqueous layer was discarded while the lower layer was dried by further centrifugation through a DNA mini speedvac at 100 mbar for 30 min at 30°C. The dissolving of the dried lipids occurred by the use of 50 μL of methanol. At that stage the lipid levels were measured by reverse phase HPLC. The activity of FAAH was extracted as pmol of the resulting[^3H]-AA per min per mg of protein.

HPLC was performed on a Nelson 1022 Plus chromatograph prepared with a series 200 LC pump, an LC295 UV/VIS detector, and a Canberra Packard flow scintillation analyser (500 TR series) (Perkin-Elmer). The relevant separations were performed on a C18 (5 μm, 3.0 mm x 150 mm) column (Waters, Milford, MA, USA) with a mobile phase of methanol-water-acetic acid (85:15:0.1, v/v/v) at a flow rate of 0.8 mL/min. The solvent was then mixed with UltimaFlo M liquid scintillation cocktail (Perkin-Elmer) at a 2:1 (v/v) ratio post column.

The peak areas of the quantified[^3H]-AEA verified the linearity as well as the sensitivity of the counting, while the detection threshold (signal-to-noise ratio > 4) was 0.5 fmol[^3H]-AEA (0.12 nCi). The resulting[^3H]-AEA and[^3H]-AA were calculated from the corresponding peak areas. Moreover, the peak identities were assessed by UV detection of cold (unlabelled) standards at 204 nm.
3.2.4 ELISA

The ELISA kits for the quantitative measurement of plasma oestradiol (E2-EASIA kit) and progesterone (PROG-EASIA kit) concentrations were obtained from DIAsource ImmunoAssays S.A. (Brabant Wallon, Belgium), and consisted of a 96-well microtiter antibody-coated plate and all the chemicals required performing the assays.

3.2.5 Distribution of samples on the plates

For both E2 and P4 measurements, samples of human plasma were assayed in duplicate (Figure 3-1).

Figure 3-1: ELISA plate showing the distribution of samples for E2 (a.) and P4 (b.) quantification.

The first 12 wells (A1 to F2) were Calibrators (pg/mL). Wells G1 and G2 contained a positive control of known concentration (supplied in the kit). Samples 1 to 36 (H1 to C12) in duplicate and the last 10 wells (D11 to H12) were duplicates of randomly chosen 5 undiluted samples (9, 4, 5, 21 and 22) that would provide an idea on how high the actual values can be and if they are measurable in that undiluted state.
Both E2 and P4 are known to produce exceedingly high values in pregnancy (394) especially in the third trimester (see Appendix 8.4 and 8.5), therefore the samples were diluted with assay buffer (details are described in corresponding description of the assay procedure) to bring them in the usable range of the kits.

### 3.2.6 E2 ELISA assay

The first step was to prepare the calibrators by adding 4 mL of deionised water (dH₂O) to the zero calibrator, while 0.5 mL of dH₂O was added to each of the other calibrators (43, 72, 117, 298 and 994 pg/mL respectively), followed by mixing by gentle inversion. Meanwhile, the positive control was created by adding 0.5 mL of dH₂O to the control vial followed by mixing by gentle inversion to create a concentration of 178 ± 54 pg/mL and the HRP-estradiol conjugate was prepared by adding 0.1 mL of the concentrated HRP-estradiol solution to the ready-to-use ‘conjugate buffer’.

Consideration was taken that the maximum stability of the mixture at room temperature was 4 hours, and hence, delays were avoided. The ‘Wash Buffer’ was created by mixing 2 mL of concentrated ‘washing solution’ with 400 mL of dH₂O and the ‘substrate solution’ prepared by mixing 0.2 mL of the chromogen (TMB - tetramethylbenzidine) to the ready-to-use ‘substrate buffer’ (H₂O₂ in acetate/citrate buffer). As maximum stability the substrate buffer was 15 minutes at room temperature, it was prepared just before use.

### 3.2.7 ELISA procedure

Plasma samples were allowed to thaw for 2 hours and then vortex mixed to ensure a homogenous solution. Each sample (3 μL of plasma) was then diluted with 117 μL of ‘assay buffer’ to provide a 1:40 diluted sample. This was stored on ice whilst other dilutions were made. The next step was to pipette 50 μL of each calibrator, positive control and sample into the appropriate wells of the microtiter plate, as shown in Figure 3-1; the time between distribution of the first calibrator and the last sample was less than 40 minutes. At this point, 50 μL of estradiol-HRP-conjugate was dispensed in each well using a multichannel pipette followed swiftly by 50 μL of anti-estradiol antibodies. The plate was incubated for 3 hours at room temperature on a horizontal orbital shaker set at 700 ± 100 RPM.

The contents on the plate were removed rapidly and replaced with 0.4 mL of washing solution per well and this step repeated 4 times before 200 μL of the freshly TMA solution was dispensed in each well. The plate was incubated for 30 min at room temperature, avoiding direct sunlight, on a horizontal orbital shaker set at 700 ± 100 RPM. Finally, the reaction was stopped by adding...
50μL of stopping reagent (1M H₂SO₄) and the absorbance at 450nm with a reference reading at 620nm, was read within 1 hour, on a ‘Labsystems Multiskan Ascent ELISA plate reader’.

### 3.2.8 P4 ELISA assay

The steps for the production of calibrators, positive control and samples were performed in exactly the same manner as for the E2 ELISA assay, except that the P4 calibrators were 0.20, 0.75, 2.00, 7.50 and 20.00 ng/mL respectively, the concentration of the positive control was 1.88 ± 0.90 ng/mL, the samples were diluted 1 in 4.8-fold (i.e.25 μL of sample plus 95 μL of assay buffer) and the volume of progesterone-HRP-conjugate (200 μL instead of 50 μL).

### 3.2.9 Calculations of results

Having determined the mean absorbance for each sample, the net absorbance was calculated by deducting the value at 620 nm from 450 nm. Then a calculation was performed for each calibrator and sample, to evaluate the per cent bound (B/Bo x 100% = OD (calibrator or sample)/ OD (zero calibrator) x 100%) and a standard curve was calculated and plotted based on the Reciprocal (B₀/B). Standard curves for E2 and P4 were also obtained through log, anti-log, and B/B₀. However, as the reciprocal of B₀/B offered the best value for the coefficient of determination (r²), it was used for the analyses (Figures 3-3 and 3-4).

![E2 standard curve](image)

**Figure 3-2: Standard curve for E2.**

The plotted results show the accuracy of the experimental performance. Data are plotted as the individual data points and are not shown where they overlap.
Figure 3-3: Standard curve for P4.

As with the E2, the experimental methodology was followed accurately, resulting in accurate findings.

The mean absorbance was calculated in duplicate for the samples and the concentrations of plasma E2 and P4 calculated from the respective regression equations. The obvious outliers (neat samples) were rejected from further analysis. For the E2 ELISA measurements, the concentrations were multiplied by 40 (dilution factor 120/3 = 40) and for P4 by 4.8 (dilution factor 120/25 = 4.8).

Subsequently, values of plasma E2, P4 concentrations and the E2/P4 ratio were analysed for possible correlations between each other E2 and P4 with the plasma measurements of the three endocannabinoids (Chapter 2). These data were then used to determine if a relationship between the sex steroids and, the timing of delivery (PTB or term) or the site of admission (PPC vs. Emergency) exists. The software employed for the analysis and graph acquisition was MS Office Excel 2010 (Microsoft, WA, USA) and Prism 6 (GraphPad, San Diego, CA, USA). The strength of these correlations was interpreted (as in Chapter 2) by the same guide (364) where the values, positive or negative, were classified as follows: “very weak” 0.00-0.19, “weak” 0.20-0.39, “moderate” 0.40-0.59, “strong” 0.60-0.79 and “very strong” 0.80-1.0.
3.3 Results

3.3.1 FAAH measurements

There was no significant statistical difference (p = 0.2911) in the serum FAAH values between women who delivered prematurely (mean± SEM: 110.3 ± 6.072) and at term (96.57 ± 8.801) (Figure 3-5). The means, SEM, and figures may be favouring the PTL group with higher values, but such difference was not backed statistically.

![Peripheral lymphocytic FAAH activity](image)

**Figure 3-4: Comparison of plasma FAAH levels between women who delivered prematurely and at term.**

The figure shows the means and SEM for each group [PTL (n = 15): 110.3 ± 6.072 vs. Term (n = 28): 96.57 ± 8.801]. There was no significant difference between the two groups (p = 0.29).

Similarly, insignificant correlations of FAAH to all 3 eCB and sex hormones (table 3-1). The correlations of FAAH to eCBs were generally very weak, with r values fluctuating from 0.06 for PEA, to 0.11 for AEA, to 0.15 for OEA. Still, the p values were between 0.45 and 0.66. Likewise, the FAAH correlation to E2 and P4 were both very weak (r = 0.048 and 0.057, respectively) with insignificant p values (p = 0.78 and 0.74). Not surprisingly, the correlation of FAAH to E2/P4 had a negative extremely weak value of -0.00953 and p value of 0.958.
Table 3-1: The Spearman correlations ($r$) of plasma FAAH concentrations versus the eCB concentrations (AEA, OEA and PEA), and female sex hormones (P4, E2, and E2/P4 ratio)

<table>
<thead>
<tr>
<th></th>
<th>Spearman r</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEA</td>
<td>0.11</td>
<td>0.45</td>
</tr>
<tr>
<td>OEA</td>
<td>0.15</td>
<td>0.31</td>
</tr>
<tr>
<td>PEA</td>
<td>0.06</td>
<td>0.66</td>
</tr>
<tr>
<td>P4</td>
<td>0.057</td>
<td>0.74</td>
</tr>
<tr>
<td>E2</td>
<td>0.048</td>
<td>0.78</td>
</tr>
<tr>
<td>E2/P4</td>
<td>-0.009</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Figure 3-5: Correlation of blood FAAH activity to plasma concentrations of the three eCBs.

The FAAH activity did not show any possible correspondence to the concentrations of any of the three eCB. As shown in table 3-1, not only did the tests (Spearman’s) show tremendously poor correlation, but also the p values were far from any statistical significance.

Finally, having followed the same example of analysis of the RTDI predictability of eCBs (chapter 2), a similar analysis was performed to assess the effect that FAAH activity might have on RTDI. None of the RTDIs were significantly related (Table 3-2), as the lowest p values were
0.2722 (at 5 weeks) and 0.407 (at 11 weeks). The p values were plotted in a graph to assess a possible pattern, such as the elliptical patterns obtained with eCB measurements (Chapter 2). However, no such pattern was observed.

**Table 3-2: The p values for the different RTDI, in weeks, for plasma FAAH levels**

<table>
<thead>
<tr>
<th>RTDI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 weeks</td>
<td>0.2722</td>
</tr>
<tr>
<td>6 weeks</td>
<td>0.8851</td>
</tr>
<tr>
<td>7 weeks</td>
<td>0.8052</td>
</tr>
<tr>
<td>8 weeks</td>
<td>0.8314</td>
</tr>
<tr>
<td>9 weeks</td>
<td>0.5779</td>
</tr>
<tr>
<td>10 weeks</td>
<td>0.6219</td>
</tr>
<tr>
<td>11 weeks</td>
<td>0.407</td>
</tr>
</tbody>
</table>

* It is obvious that there is no significant RTDI, and as a result has no promising predictive value of labour.

**Figure 3-6: A graphic presentation (plotting) of the p values for the different RTDI.**

Unlike in the case of eCBs, where all RTDI showed a u-shaped pattern reaching significant p values, in this curve such an array is not applicable.
3.3.2 E2 and P4 measurements

The data in table 3-1 shows the plasma concentrations of E2 and P4 in the 36 samples. The data show no significant difference in plasma E2 concentrations among the three groups (p = 0.6279, Kruskal-Wallis test). Similarly, plasma P4 concentrations were not significantly different among the three groups (p = 0.8318, Kruskal-Wallis test). Additionally, there was no statistical significance among the E2/P4 ratio groups (p = 0.2055)

Figure 3-7: Comparison of the concentrations of P4 and E2 among the different groups of patients.

The groups consisted of emergency admissions (Em) who delivered at term (T) or prematurely (PT), and patients recruited from the prematurity prevention clinic (PPC) who delivered at term. The P4 concentrations did not show significant difference among the three groups (data are presented as the mean ± SEM; ANOVA test, p = 0.62). The E2 concentrations in the term emergency group (Em T) appear to be lower than the preterm emergency group (Em PT), the difference did not reach significance (data presented as box and whisker - Kruskal-Wallis test, p = 0.18). This could be due to the wider range of IQR in the preterm group (Em PT: 64.3 – 116.7, Em T: 70.3 – 94.92).
Figure 3-8: Examples of individual comparisons of P4 concentrations in different groups of patients.

The data is shown in box and whiskers and analysed by Mann-Whitney test due to unequal distribution. The P4 in the emergency groups showed no difference between those who delivered prematurely from those who delivered at term (p = 0.82). Likewise, P4 concentrations showed no significant difference among women who delivered at term whether those were recruited as emergencies or PPC (p = 0.53).

Figure 3-9: Examples of individual comparisons of E2 concentrations in different groups of patients.

As the data were not equally distributed, non-parametric tests were performed and the data is presented as box and whiskers. While there was no statistical significance among the emergency groups (Em PT vs Em T: Mann-Whitney test, p = 0.49), the patients from PPC who delivered at term showed higher concentrations than the emergency admissions who, similarly, delivered at term (Mann-Whitney test, p = 0.050).
Figure 3-10: Examples of comparison of the E2/P4 ratio among the different groups.
There was no significant difference between the three groups (Kruskal-Wallis test, p = 0.1687).
The data shown as box and whiskers. Despite the obvious difference between the values of term
recruitees from PPC and the emergencies, there was no statistical significance (the data are
equally distributed and presented in a graph with mean ± SEM; Unpaired t-test, p = 0.063).

3.3.3 Correlation analyses

Tables 3-2 to 3-5 and Figures 3-12 and 3-13 show that no significant correlations (Spearman)
were observed between P4 and E2, or between P4, E2 or E2/P4 and any of the eCBs. Moreover,
after dividing the samples into groups according to the site of recruitment and the timing of
delivery, no significant correlations were observed.

Figure 3-11: Correlation of serum P4 concentrations to E2 concentrations.
As it is visually obvious there was very weak positive correlation between the two hormones, that
was not statistically significant (n = 36. Spearman’s test; r = 0.176 and p = 0.3).
Table 3-3: Correlation co-efficients of all 36 samples: oestradiol (E2) to progesterone (P4), progesterone to each eCB and oestradiol to each eCB

<table>
<thead>
<tr>
<th>Correlation</th>
<th>r</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4 vs E2</td>
<td>0.097</td>
<td>0.5</td>
</tr>
<tr>
<td>P4 vs AEA</td>
<td>0.045</td>
<td>0.9</td>
</tr>
<tr>
<td>P4 vs OEA</td>
<td>0.174</td>
<td>0.3</td>
</tr>
<tr>
<td>P4 vs PEA</td>
<td>-0.220</td>
<td>0.7</td>
</tr>
<tr>
<td>E2 vs AEA</td>
<td>-0.247</td>
<td>0.3</td>
</tr>
<tr>
<td>E2 vs OEA</td>
<td>-0.326</td>
<td>0.2</td>
</tr>
<tr>
<td>E2 vs PEA</td>
<td>-0.295</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Table 3-4: Correlation co-efficients for E2 and P4 to each eCB among patients recruited from the PPC (C) or as emergencies (E)

<table>
<thead>
<tr>
<th>Correlation</th>
<th>r</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C P4 AEA</td>
<td>-0.02</td>
<td>0.9</td>
</tr>
<tr>
<td>C P4 OEA</td>
<td>0.50</td>
<td>0.09</td>
</tr>
<tr>
<td>C P4 PEA</td>
<td>0.07</td>
<td>0.8</td>
</tr>
<tr>
<td>C E2 AEA</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>C E2 OEA</td>
<td>-0.21</td>
<td>0.4</td>
</tr>
<tr>
<td>C E2 PEA</td>
<td>-0.13</td>
<td>0.6</td>
</tr>
<tr>
<td>E P4 AEA</td>
<td>-0.011</td>
<td>0.2</td>
</tr>
<tr>
<td>E P4 OEA</td>
<td>0.164</td>
<td>0.3</td>
</tr>
<tr>
<td>E P4 PEA</td>
<td>-0.226</td>
<td>0.06</td>
</tr>
<tr>
<td>E E2 AEA</td>
<td>-0.236</td>
<td>0.03</td>
</tr>
<tr>
<td>E E2 OEA</td>
<td>-0.310</td>
<td>0.06</td>
</tr>
<tr>
<td>E E2 PEA</td>
<td>-0.311</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Figure 3-12: Correlation co-efficients for E2 and P4 to each eCB among patients recruited from the PPC.

As described in Table 3-4 there was no relevant correlation of any type among E2 or P4 and any of the three eCBs among patient recruited from the PPC. It can be concluded that an interaction among each hormone and the eCBs in unlike during the quiescent phase of the uterus.
Table 3-5: Correlation between each hormone to each eCB according to the timing of delivery

<table>
<thead>
<tr>
<th>Correlations</th>
<th>r</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTB P4 vs AEA</td>
<td>0.03</td>
<td>0.4</td>
</tr>
<tr>
<td>PTB P4 vs OEA</td>
<td>0.25</td>
<td>0.1</td>
</tr>
<tr>
<td>PTB P4 vs PEA</td>
<td>-0.11</td>
<td>0.6</td>
</tr>
<tr>
<td>PTB E2 vs AEA</td>
<td>-0.27</td>
<td>0.1</td>
</tr>
<tr>
<td>PTB E2 vs OEA</td>
<td>-0.30</td>
<td>0.2</td>
</tr>
<tr>
<td>PTB E2 vs PEA</td>
<td>-0.24</td>
<td>0.1</td>
</tr>
<tr>
<td>Term P4 vs AEA</td>
<td>-0.06</td>
<td>0.4</td>
</tr>
<tr>
<td>Term P4 vs OEA</td>
<td>-0.09</td>
<td>0.3</td>
</tr>
<tr>
<td>Term P4 vs PEA</td>
<td>-0.40</td>
<td>0.1</td>
</tr>
<tr>
<td>Term E2 vs AEA</td>
<td>-0.25</td>
<td>0.3</td>
</tr>
<tr>
<td>Term E2 vs OEA</td>
<td>-0.43</td>
<td>0.1</td>
</tr>
<tr>
<td>Term E2 vs PEA</td>
<td>-0.38</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Table 3-6: Correlation of the E2/P4 to each eCB according to the timing of delivery

<table>
<thead>
<tr>
<th>Correlations</th>
<th>r</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTB Ratio vs AEA</td>
<td>-0.27</td>
<td>0.3</td>
</tr>
<tr>
<td>PTB Ratio vs OEA</td>
<td>-0.39</td>
<td>0.1</td>
</tr>
<tr>
<td>PTB Ratio vs PEA</td>
<td>-0.16</td>
<td>0.5</td>
</tr>
<tr>
<td>Term Ratio vs AEA</td>
<td>-0.19</td>
<td>0.3</td>
</tr>
<tr>
<td>Term Ratio vs OEA</td>
<td>-0.39</td>
<td>0.07</td>
</tr>
<tr>
<td>Term Ratio vs PEA</td>
<td>-0.10</td>
<td>0.6</td>
</tr>
</tbody>
</table>
3.4 Discussion

The main conclusion from the findings of the plasma FAAH concentrations is that they do not have an active role in the prediction of preterm birth. Unlike in early pregnancy, where plasma FAAH concentrations increase in cases of miscarriage and can be used as a predictor or even a diagnostic tool, in both preterm and term labour, it seems that the ECS works differently. Even in the RTDI prediction, lymphocytic FAAH activities do not seem to have any impact on the outcome predictability. Of course, it is possible that a larger sample size may have offered different conclusions, although it is unlikely that the results would have been significantly different favouring the “FAAH vs. AEA” disproportion for two main reasons:

(1) FAAH concentrations are “paradoxically” slightly higher in the PTL group, suggesting that a slightly lower plasma AEA measurement should have been obtained if the original hypothesis that plasma AEA concentrations are dependent upon lymphocytic FAAH activities. Taking into consideration that FAAH is the main, but not the only, hydrolysing enzyme of AEA, there is always a possibility that a different mechanism of interaction takes place between those two elements of the ECS when it comes to labour, either that is at term or prematurely.

(2) As the p value lies far from any promising significance ($p = 0.29$), it can be hypothesised that plasma FAAH acts in a different mechanism or, even, that it has no direct role in labour. One more possible theory could be that the FAAH input in labour lies in the expression of its tissue receptors rather its plasma activity. In other words, as progesterone receptors have different isoforms that switch from one type to another according to the uterine activity or quiescence (329-336), without the requirement of a change in the plasma levels of progesterone, the same could be the case with FAAH. That is to say, that labour can have an effect on the expression of its receptors, not counting solely on the plasma activity. This, on the other hand, is something that will be examined in chapters 4 and 5.

A power analysis showed that 67 patients need to be in the PTL group in order to obtain significant results in the results of FAAH plasma activity. One more important point is the fact that FAAH activity in early pregnancy was found high in the lymphocytes as it was examined in these cells in particular. Lymphocytes are known to decrease in pregnancy in the first two trimesters as well as in the early post-natal period, with the only time to rise in concentrations being the third trimester (395). One explanation to the changes of FAAH activity could be the change in the number of lymphocytes and consequently the change in FAAH activity.
On the other hand, it is possible that FAAH activity and eCB regulation, in general, originate from different tissues in addition to the lymphocytes. For example, the fetal membranes, in pregnancy, reach full growth, with a further subdivision to three different tissues: the decidua, the chorion, and the amnion. Considering that the chorio-decidual unit can be one of the chief controller of the mechanism of labour (345, 396, 397), it would be worth examining if the placenta has any control on the labouring process.

In the case of the sex steroid hormones, things should be seen from a different angle. In a healthy pregnancy, both hormones keep rising in their plasma concentrations until the time of labour. At delivery the progesterone concentrations fall, while oestrogen remains unaffected. Adding to that, in parturition there is a switch between the isoforms of the PR receptors, creating a functional progesterone withdrawal (335). The current predominant theory suggests a PR-B isoform being responsible for the uterine quiescence as well as for the suppression of the oestrogen receptor ER. Once the PR-B changes to PR-A, the predominant isoform in labour, there is a functional inhibition of the progesterone hormone and equally a functional increase in oestrogen, resulting into labour (394).

Having found a connection between eCB and PTL, and knowing the role of FAAH, in controlling the concentration of plasma eCBs, it was important to examine the hormonal levels of the recruits and to establish if any association between them and that of the plasma eCB levels exists.

The data indicated that no significant difference existed between plasma E2, P4 or E2/P4 ratios for those women that delivered at term or prematurely. These data were unexpected and could have been due to errors in the E2 and P4 measurements, but this seems unlikely since, all the hormonal values were compatible with those of reported gestational E2 and P4 plasma levels (see tables 1 and 2 in Appendices 8-4 and 8-5, respectively).
One other hypothesis, that P4 or E2 controls the levels of the eCBs does not appear to be supported by the data. Despite the attempts to analyse correlation coefficients from every single different level possible no significant correlation was detected between the hormones and any of the eCBs. The correlations between E2 and P4, and between E2 or P4 and each eCB, from all samples (Table 3-4 – Figure 3-12) were very weak to weak, with mixed positive and negative associations, leading to the conclusion that there is no significant correlation between sex steroid hormones and eCBs in this type of pregnant woman.

Similarly, weak and insignificant findings for the comparison of each hormone to each eCB among patients recruited in the PPC (Figure 3-13), as well as the Emergency samples (Table 3-5) were obtained. The only noticeable fact is that most of the E2 correlations to the eCBs were negative, while most of the P4 positive. Among women who delivered prematurely, there was still, very weak to weak correlation between sex hormones and eCBs, with negative association between E2 and eCB (Table 3-6). Similarly, in term labourers there were weak negative correlations between each hormone and eCB (Table 3-6).

Then, and to be thorough, a different approach of analysis was adopted: instead of examining the correlation between E2 and P4 and those of the eCB, the E2/P4 ratio was calculated for each sample and further correlations were calculated. In addition to all that, the analysis of the “r” of E2/P4 ratio and each eCB in women who delivered at term and prematurely, individually, showed weak negative correlation (Table 3-7).

From these observations, it appears that the changes in the plasma eCB levels in the “at risk” pregnant woman are independent of E2 and P4. Both P4 and E2 have a control on the eCB levels, but as indicated earlier, it is usually tissue-related. A clearer assessment will be obtained once the values for the FAAH and NAPE-PLD are available; from that we can identify if there is any significant correlation between the hormones and the enzyme levels and activities. Nonetheless, the P4 findings are in accord with the “functional withdrawal” theory of progesterone and parturition, where the levels of P4 are high but not recognised by the pro-pregnancy receptor-signalining pathways. In addition, most studies that examined the correlation of P4 and the ECS have not focussed on one of the main tissues where parturition is initiated: the placenta and
amnion-chorion, where oestradiol and progesterone act through specific receptors that are positioned in close proximity to the cannabinoid receptors.

3.5 Conclusions

The plasma FAAH concentrations do not seem to have a predictive role in the case of PTL. The findings suggested the control of eCB, during parturition, is possibly through other tissues rather than peripheral lymphocytes. The female sex steroid hormones E2 and P4 did not show any significant correlation to the eCBs or FAAH values at any gestational stage or state as has been shown in early pregnancy. A comparison between these findings and the expression of ECS and hormonal receptors in the placenta-amnion-chorion will enrich the image of the mechanism of PTL and the possible role of ECS in its initiation.
Chapter 4  Expression of the ECS and progesterone receptors in the placenta
4.1 Introduction

It has been established that CB1 and CB2 receptor expression and function are involved in the control of gestational length and PTB, at least in laboratory animals (325-327). It was therefore important to examine possible changes in the expression of the ECS in those tissues that may be principally involved in parturition, such as the placenta and fetal membranes. The other important tissue to examine is the human myometrium at term, but because there are ethical and clinical reasons for the maintenance of the intact myometrium, an examination of the possible changes in CB1 and CB2 receptor expression in myometrial tissue could not be achieved (398). Consequently, this chapter focuses on the expression of the receptors and enzymes of the ECS, and potential factors in their modulation within the placenta, whilst Chapter 5 addresses these factors in the fetal membranes.

In Chapter 2, it was established that plasma concentrations of the eCBs changed significantly, and at an early stage in women who were at high risk for PTL. However, these changes were not related to changes in serum progesterone concentrations (Chapter 3), suggesting that the theory of “functional withdrawal” rather than quantitative withdrawal of progesterone during human parturition (337, 399) is probably responsible, whereby the dominant PR isoform (PR-B) is either no longer active, or its expression changes at the fetal-maternal interface, resulting in reduced progesterone action. One part of this theory, is that the less active PR-A isoform predominates late in pregnancy or is the initiator of parturition (337). Some have proposed that a non-active PR-C isoform may have a role to play (328).

Variations in CB1 and CB2 receptor expression have been associated with changes in the enzymes FAAH and NAPE-PLD expression in murine uterine and gestational tissues (400), suggesting that any changes in the expression could also be important here, especially, as their expression in human and murine blood (lymphocytes and endothelial cells) are predicted to be controlled by oestradiol (E2) and progesterone (P4) (309, 401). If that were the only controlling influence on plasma eCB concentrations, then there should have been an inverse relationship between the concentrations of the eCBs and FAAH expression in peripheral blood lymphocytes (PBL) of the human cohort studied in (Chapter 3), but that was not demonstrated, suggesting that FAAH expression in PBLs may not be the control point for plasma eCB concentrations in this population. This raises the questions: where do the eCBs in blood come from and how are they regulated? It is possible that they come from the placenta, since the cannabinoid receptors and the eCB
modulating enzymes have been shown to be expressed in the placenta in early pregnancy and their modulation associated with miscarriage (309, 321), although similar data for the placenta at term, or preterm are missing. Therefore, this Chapter will examine the possibility that the placenta at term and preterm differentially regulates plasma eCB concentrations.

### 4.1.1 Placenta and the ECS

It has been suggested that the human placenta is the target of eCB activity and has a regulatory role in the outcome of the pregnancy (321). The discovery of the presence of CBRs in the placenta was made when it was shown that cannabinoids and marijuana targeted the placenta and delayed the clearance of serotonin (402). Further studies confirmed the presence of FAAH, CB1 (321, 322) and CB2 (323) in the placenta, where the presence of CBRs and eCBs levels are likely to be directly related to the biological effect of the cannabinoids as in other reproductive organs (324).

In human fetal membranes, the stimulation of CB1 leads to increase in PGE₂ through activation of COX-2 (325). Hence, the ECS has a role in the progression of pregnancy and the timing of labour. More proof that CB1 may be controlling the length of gestation comes from a study on CB1 knockout mice which through increased oestrogen and progesterone levels and prostaglandin production through COX-1 (326), have increased preterm labour rates. Another study demonstrated the influence of CB1 on the length of pregnancy, in mice, where tocolytic treatment with Tetrahydrocannabinol prolonged the pregnancy by acting on the CB1 receptor and through the nitric oxide (NO) pathway (327).

### 4.1.2 Placenta, progesterone and oestrogen receptors

In Chapter 1, there is a detailed description about the link between serum progesterone concentrations and pregnancy success (314-316) and its link to FAAH activity (303) and the ECS in general (317). Also discussed are the roles of PR and ER-alpha in pregnancy and parturition (328-333, 335, 341-348).

### 4.1.3 Summary

The aims of the studies in this Chapter were therefore to examine the expression of CB1, CB2, FAAH and NAPE-PLD in preterm and term placenta, and to determine if the expression levels of these ECS components are affected by labour. This may unravel a connection of these expressions to the measured plasma eCB concentrations. Additionally, because E2 and P4 are known factors that affect the ECS expression in other parts of the body (303, 310, 317, 319, 320, 390), and their
 serum levels did not significantly change in pregnancy duration or parturition (Chapter 3), even though they should have (303), then there may be changes in the receptors for these sex steroid hormones that are related to the ECS component expression. Thus, a relationship between changes in oestrogen (ER) or the progesterone receptor (PR) isoforms in the placenta might be related to the expression of the four ECS components listed above.

4.2 Materials and Methods

4.2.1 Patient groups

The samples for this study were taken from four groups of patients: (1) term and in labour (called ‘term labourers; TL); (2) term and not in labour (called ‘term non-labourers; TNL); (3) preterm and in labour (called preterm labourers; PTL); and (4) preterm and not in labour (called preterm non-labourers; PTNL).

A power analysis of similar groups (328) showed that 6 samples are sufficient for each group to show a 40% change in expression with $\alpha = 0.05$ and $\beta = 0.80$. However, previous experience has been that insufficient or incomplete samples (e.g. chorion without amnion in fetal membrane samples or calcification of the placental cotyledons) sometimes occurs and so a decision to collect a minimum of 10 samples for each group was made.

4.2.2 Definition of patients in each group

The term labourers were low-risk women, at or beyond 37 weeks of gestation who had vaginal birth. Term non-labourers were also low-risk women, at or beyond 37 weeks of gestation but having elective Caesarean section due to previous Caesarean section. Preterm labourers were women, less than 37 weeks of gestation who presented with suspected PTL and had vaginal deliveries, and preterm non-labourers, were women of < 37 weeks of gestation who had Caesarean section, either for fetal reasons (e.g. severe IUGR) or maternal obstetric complications (e.g. severe preeclampsia) but were not in active or suspected labour. As it is not common practice to perform elective Caesarean sections prematurely, all the volunteers in this group had unavoidable fetal or maternal indications for early delivery.

In each case, the placenta was collected from the delivery suite and transported on ice to the research laboratories where a large cotyledon was selected and dissected free under sterile conditions. The piece was then divided into three parts:
1. One for Immunohistochemistry (IHC)

2. One for RNA extraction

3. One for Protein quantification (although this sample was never used)

The details of the tissue collection and processing are presented in the following section.

4.2.3 Tissue collection

The human placental samples were collected from patients at the Leicester Royal Infirmary. Samples measuring 1 cm x 1 cm x 0.5 cm was placed into a tissue cassette and fixed chemically in Formalin (10% formaldehyde in normal saline) for 48 hours, with the formalin changed at 24 hours. Samples were then processed (dehydrated) and then embedded in paraffin wax. After allowing to air-dry at 37°C for 1 week, the blocks were stored at room temperature, until samples from all 4 groups had been collected. At this point, tissue was sliced with a microtome and 4 μm-thick sections mounted on silanised microscope slides and re-dried at 37°C for 7 days.

Additionally, a portion of placenta weighing approximately 100 mg was placed in a sterile polypropylene tube, and the tissue snap-frozen in liquid nitrogen for a few minutes. The frozen tissue was then placed in -80°C for future biochemical analyses.

4.2.4 RNA preparation

The preparation of RNA was based on a previously optimised method (403). Briefly, RNA extraction was performed using TRIZOL reagent (Invitrogen, Paisley, UK) as per the manufacturer’s instructions; 100 mg of placenta was defrosted and homogenised in TRIZOL (1 mL) using a pre-chilled UltraTurrex® blade. Homogenisation was completed using 20 repeated strokes of a pre-chilled tight-fitting-glass Dounce® homogeniser. The homogenate was either stored at -80°C overnight or subjected to the next step by being transferred to microfuge tubes followed by the addition of 200 μl of chloroform. The tubes were shaken for 15 secs vigorously and allowed to stand for 3 minutes at room temperature and then centrifuged in Sanyo Hawk 15/05 microfuge (Fisher Scientific, Loughborough, UK) for 10 minutes at 7,000 rpm at 4°C. The supernatant was transferred to a new 1.5 mL-microfuge tube and 500 μl of isopropanol added. The new mixture was vortex mixed briefly then left to stand for 10 minutes at room temperature. A further centrifugation at 13,000 rpm for 10 minutes at 4°C was used to pellet the RNA. The supernatant was discarded, and the pellet re-suspended with 75% ethanol in
Chapter 4 Expression of the ECS and progesterone receptors in the placenta

diethylpyrocarbonate (DEPC)-treated de-ionised water, followed by vortex mixing. Samples were re-centrifuged in the microfuge for 5 minutes at 7,000 rpm at 4°C, the supernatant removed and the inside of the top of the tube wiped with a paper tissue to remove any residual ethanol and water. The pellet was left to air-dry by placing the tubes upside down for 10 minutes at room temperature. The pellet was then re-suspended in 100µl DEPC treated water and the tubes placed in the thermal cycler for 5 minutes at 56°C to aid pellet solvation. The RNA solution was briefly collected by centrifugation and was either stored at -80°C or immediately quantified.

4.2.5 RNA quantification

The samples frozen for RNA quantification were thawed quickly in a thermal cycler at 65°C for 2 minutes then placed on ice for 5 minutes. RNA was quantified by diluting the thermal sample 1:12 in DEPC-treated water and the mixture (60 µl) vortex mixed. Absorbance at 260nm with reference to protein contamination at 280nm and phenol contamination at 230nm were read using a UV Ultrospec 3000 spectrometer with DEPC-treated water used as a “zero” reference. For each sample, the absorbance readings at 230, 260 and 280 nm, the absorbance ratios 260/230 and 280/280, and the actual concentration (µg/mL) were measured.

4.2.6 RNA agarose gel electrophoresis

Agarose gel electrophoresis was used as a visual method to quickly easily and accurately check that the RNA obtained was pure and intact. Briefly, 1 µg of total RNA was measured into a microfuge tube and brought to the convenient volume of 10 µL by adding RNase-free water. Two volumes of sample buffer were added (20 µL), the mixture vortex mixed, heated at 65°C for 5 minutes and the left to cool to room temperature. Then, 2 µL of gel loading buffer and 2 µL of ethidium bromide (EtBr) (1 mg/mL) were added. The mix (30 µL) was loaded onto a standard agarose gel. The ready-load size marker standards (Invitrogen, Paisley, UK) needed only the addition of 2 µL of EtBr per 20 µl and were also loaded (5 µL per lane).

The gel was made by mixing low melting agarose (1 g/100 mL) in 1x TAE buffer, and by dissolving with heat for 1 minute in a microwave at 800 Watts until the solution boiled. Once the gel was fully dissolved, it was allowed to cool to ~50°C and the solution poured into a tray with well-formed combs and left to set. The gel tray was placed in a prefilled tank containing 1x TAE to a level that just covered its surface. The equipment was constructed so that the gel ran at 5 Volts/cm (120 Volts/hour) for approximately 1.5 hours (i.e. until the bromophenol blue band
reached half-way through the gel’s length). The gel was placed on the ultraviolet (UV) transilluminator and the 28S, 18S, and 5S RNS species were identified from a photograph.

### 4.2.7 DNAse 1 treatment of RNA extract

For each sample that was thawed slowly on ice from -80°C, the volume was brought to 100 µl by adding DEPC-treated water. Then, in order, the following materials were added: 15µl of 10× DNAse reaction buffer, 10 µl of RNAse-free DNAse 1, 3.15 µl of RNAse inhibitor (RNasin, Promega, Southampton) and 21.85 µl of DEPC-treated water. After vortex mixing, samples were incubated at 37°C for an hour. An equal volume (150 µL) of acidic phenol:chloroform:iso-amyl alcohol mixture (50:24:1) was added and the mixture thoroughly combined by vortex mixing on high power for one minute. The phases were separated by a centrifugation at 11,000 rpm for 2 minutes at 4°C. The top aqueous layer was carefully transferred in a new 1.5 mL microfuge tube and an equivalent volume of chloroform:iso-amyl alcohol mixture (24:1) was added. The mixture was vortex mixed again at high power for 1 minute and then centrifuged at 11,000 rpm for 2 minutes at 4°C. Again, the aqueous top layer was transferred to a new tube where 100 µl of iso-propanol was added, and the mixture vortex mixed for 5 seconds and placed at -20°C for 1 hour. The DNA-free RNA precipitate was collected by centrifugation at 11,000 rpm for 5 minutes at 4°C. Then, the pellet was washed with 1 mL of 75% ethanol (25% DEPC-treated water) by vortex mixing at high power for 20 seconds and re-centrifugation at 11,000 rpm for 5 min at 4°C. The ethanol was allowed to evaporate by inverting the tubes on a paper towel for 7 minutes, and the excess ethanol removed by wiping carefully the inside the tube with a paper tissue. The RNA was re-dissolved in 100 µL of DEPC-treated water by heating at 56°C for 5 minutes and stored at -80°C.

### 4.2.8 Reverse transcription with AMV-RT

The RNA samples were thawed on ice and two sets of microfuge tubes, corresponding to the sample type, were labelled +RT and -RT. Two sets of mastermixes were prepared, as shown in table 4-1, providing 9.62 µl of mastermix for each RNA sample. In the appropriately labelled tubes, 1 µg of RNA was added and the volumes brought to 15.38 µl by adding DEPC-treated water. Then 9.62 µl of mastermix was added. The mixture was vortex mixed gently and centrifuged at 13,000 rpm for 10 seconds to collect the reactants to the bottom of the tube before being transferred to the thermal cycler, where they were incubated at 42°C for one hour followed
by a 5 minutes denaturation of the enzymes at 95°C. Samples were either analysed immediately or stored at 4°C.

**Table 4-1: RT reaction mastermixes**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>+RT</th>
<th>-RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x AMV buffer</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>RNasin</td>
<td>0.62 µl</td>
<td>0.62 µl</td>
</tr>
<tr>
<td>Anchored oligo (dT)23 primer</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>10mM dNTPs</td>
<td>2.5 µl</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>AMV-RT</td>
<td>0.5 µl</td>
<td>-</td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>-</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Total Volume</td>
<td>9.62 µl</td>
<td>9.62 µl</td>
</tr>
</tbody>
</table>

**4.2.9 Standard PCR reaction**

The cDNA taken from -80°C were thawed slowly on ice, vortex mixed and briefly collected by centrifugation before use in PCR. A mastermix was prepared in sufficient volume for n+1 samples. The mastermix prepared for each sample/tube contained: 42 µl of sterile deionised H₂O, 5 µl of 5x AJ buffer, 1 µl of each 5’ and 3’ primers (10 pmol/µl) (see table 4-2), and 2 µl of dilute Taq polymerase, making a total volume of 51 µl. To this, 1 µl of cDNA or –RT control was added and centrifuged at 13,000 rpm for 1 minute. Tubes were placed in the thermal cycler and run with different programmes according to the gene of interest (Tables 4-3 and 4-4) for 40 cycles.
### Table 4-2: Primer sequences used in qPCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Expected size (bp)</th>
<th>Annealing Temp (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER_alphaF</td>
<td>GAGACATGAGAGCTGCCAAC</td>
<td>381</td>
<td>60</td>
</tr>
<tr>
<td>ER_alphaR</td>
<td>CCAAGAGCAAGTTAGGAGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAAHF</td>
<td>GGGCGTCAGCTACACTATGC</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>FAAHR</td>
<td>ATCAGTCGCTCCACCTCCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAPE-PLDF</td>
<td>AAGAGATAGGAAAAAGATTTGGACCTT</td>
<td>99</td>
<td>60</td>
</tr>
<tr>
<td>NAPE-PLDR</td>
<td>CTGGGTCTACATGCTGGTATTTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR-A_PR-BF</td>
<td>GAATTCTATTGGAACGCCCACCTG</td>
<td>396</td>
<td>60</td>
</tr>
<tr>
<td>PR-A_PR-BR</td>
<td>CTGCAGGTCTACCCGCCCCTATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR-BF</td>
<td>CCTGAAGTTTGCGCCATACCT</td>
<td>197</td>
<td>60</td>
</tr>
<tr>
<td>PR-BR</td>
<td>AGCAGTCCGCTGCTTTTCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRF</td>
<td>AGGAGTCTGCAAGCTTCAA</td>
<td>284</td>
<td>60</td>
</tr>
<tr>
<td>PRR</td>
<td>CTGCAGGGACTGGATAATGTATTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB1F</td>
<td>CTTCCCACAGAAATTCCC</td>
<td>853</td>
<td>62.4</td>
</tr>
<tr>
<td>CB1R</td>
<td>TACCTCCCATCCCTCAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB2F</td>
<td>GGGCGTCAGCTACACTATGC</td>
<td>851</td>
<td>62.4</td>
</tr>
<tr>
<td>CB2R</td>
<td>ATCTCGGGGCTTCTTCTTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDHF</td>
<td>AGAACATCATCCCTGCCTC</td>
<td>347</td>
<td>60</td>
</tr>
<tr>
<td>GAPDHR</td>
<td>GCCAAATTCGTTGTCAATACC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4-3: Thermal cycler conditions for ER-α, PR-B, PR-AB and PR (all isoforms)

<table>
<thead>
<tr>
<th>Step</th>
<th>Cycles</th>
<th>ER-α</th>
<th>PR-B, PR-AB and PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>1</td>
<td>94°C degrees, 10 min</td>
<td>95°C degrees, 10 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td></td>
<td>94°C degrees, 30 sec</td>
<td>94°C degrees, 1 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>10</td>
<td>60°C degrees, 30 sec</td>
<td>60°C degrees, 1 min</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>68°C degrees, 1 min</td>
<td>72°C degrees, 1 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td></td>
<td>94°C degrees, 30 sec</td>
<td>94°C degrees, 1 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>30</td>
<td>60°C degrees, 30 sec</td>
<td>60°C degrees, 1 min</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>68°C degrees, 1 min 5 sec</td>
<td>72°C degrees, 1 min 5 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>1</td>
<td>68°C degrees, 5 min</td>
<td>72°C degrees, 5 min</td>
</tr>
</tbody>
</table>

The light and darker blue shading are to indicate the change in PCR conditions, with the second set having an extension time that increased by 5 second per cycle.

Table 4-4: Thermal cycler conditions for CB1, CB2, GAPDH, and FAAH

<table>
<thead>
<tr>
<th>Step</th>
<th>Cycles</th>
<th>CB1, CB2 and GAPDH</th>
<th>FAAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>1</td>
<td>95°C degrees, 10 min</td>
<td>95°C degrees, 5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td></td>
<td>95°C degrees, 1 min</td>
<td>95°C degrees, 45 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>35</td>
<td>60°C degrees, 45 sec</td>
<td>55°C degrees, 45 sec</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>72°C degrees, 1 min</td>
<td>72°C degrees, 1 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>1</td>
<td>72°C degrees, 10 min</td>
<td>72°C degrees, 10 min</td>
</tr>
</tbody>
</table>
4.3 Immunohistochemistry

4.3.1 Samples

Stored 4 μm-thick sections mounted on silanised microscope slides and dried at 37°C for 7 days were collated from the 4 patient groups. Each was labelled and run in a single IHC run, to minimise inter-assay variability. Since normal endometrium also contains all the antigens being tested, two samples of proliferative endometrium were run, one as a positive and the other as negative control in each experiment. The methodology had previously been developed within the Department and needed only minor modification.

4.3.2 Materials

Table 4-5 lists the source of reagents used in the IHC studies.
Table 4-5: Source and Materials used for the immunohistochemistry (IHC) experiments

<table>
<thead>
<tr>
<th>Company</th>
<th>Materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma Aldrich Ltd</td>
<td>CB1 (Rabbit Polyclonal Anti-Cannabinoid Receptor 1 antibody; catalogue number)</td>
</tr>
<tr>
<td></td>
<td>CB2 (Rabbit Polyclonal Anti-Cannabinoid Receptor 2 antibody; catalogue number)</td>
</tr>
<tr>
<td></td>
<td>NAPE-PLD(Rabbit Polyclonal Anti-NAPE-PLD antibody; catalogue number)</td>
</tr>
<tr>
<td></td>
<td>Bovine serum albumin Fraction V</td>
</tr>
<tr>
<td></td>
<td>Tween</td>
</tr>
<tr>
<td></td>
<td>Trizma Base</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
</tr>
<tr>
<td></td>
<td>MgCl$_2$</td>
</tr>
<tr>
<td></td>
<td>Citric Acid</td>
</tr>
<tr>
<td></td>
<td>Mounting medium DPX</td>
</tr>
<tr>
<td></td>
<td>Mayer’s Haematoxylin</td>
</tr>
<tr>
<td>Dako Denmark A/S</td>
<td>Normal goat serum (NGS)</td>
</tr>
<tr>
<td></td>
<td>Normal rabbit serum (NRS)</td>
</tr>
<tr>
<td></td>
<td>Biotinylated Rabbit anti-Mouse antibody</td>
</tr>
<tr>
<td></td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td></td>
<td>Biotinylated Goat anti-Rabbit Antibody</td>
</tr>
<tr>
<td>Alpha Diagnostics Intl.</td>
<td>FAAH antibody (Rabbit Polyclonal Anti-FAAH)</td>
</tr>
<tr>
<td>Leica Microsystems Ltd</td>
<td>PR Mouse Monoclonal antibody (NCL-PGR)</td>
</tr>
<tr>
<td>Vector Laboratories Ltd</td>
<td>Avidin/Biotin Blocking Kit</td>
</tr>
<tr>
<td></td>
<td>ABC Elite (Horseradish-peroxidase conjugate)</td>
</tr>
<tr>
<td>Abcam plc</td>
<td>3,3’-diaminobenzadine (DAB)</td>
</tr>
<tr>
<td>Fischer Scientific Ltd</td>
<td>Hydrogen peroxide (H$_2$O$_2$)</td>
</tr>
</tbody>
</table>

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4.3.3 CB1 and CB2 immunohistochemistry

The methodology of immunohistochemistry using CB1 and CB2 antibodies were essentially identical, the only difference being the antibody concentrations and negative controls used. For all IHC methods the first steps were as detailed below.

4.3.3.1 General IHC methodology

Slides were de-waxed in xylene for 3 minutes, 3 times, followed by re-hydration through 99% alcohol (Industrial Methylated Spirits (IMS)) for 3 minutes twice and then 95% IMS once before being place in distilled water for another 3 min. Antigen retrieval was achieved by microwaving the slides in 10mM citric acid buffer (pH 6.0) for 10 minutes at 700 Watts and then being allowed to cool for 20 minutes before being washed in tap water for 5 minutes. Slides were then incubated in 6% hydrogen peroxide (H2O2) for 10 mins to suppress endogenous peroxidase activity, followed by a wash in running tap water for 5 minutes. Non-specific binding sites were then blocked by placing the slides in Tris-buffered saline (pH 7.4) containing 10% (w/v) bovine serum albumin (TBA), for 5 minutes. Next, 100 µl of Avidin Blocking solution was added to each slide for 15 minutes in a humid chamber. That was prepared by adding 4 drops of avidin solution per 1 mL of Blocking Solution (10% (v/v) of normal goat serum (NGS) in TBA). The slides were then washed in TBS for 5 minutes. At this point, 100 µl of Biotin Blocking solution was added to each slide for 15 minutes in a humid chamber. That was prepared by adding 4 drops of biotin solution per 1 mL of Blocking Solution. The excess liquid was drained from each slide and 100 µl of primary antibody was added to all slides except the negative controls. Slides were incubated in a humid chamber at 4 °C for 18 hours (overnight).

The CB1 primary antibody was prepared at a concentration of 1:500 in Blocking Solution. For the negative controls, Rabbit IgG was used, diluted in blocking solution, at the same concentration as the primary antibody.

The CB2 primary antibody was prepared at a concentration of 1:150 in Blocking Solution. For the negative controls, Rabbit IgG was used, diluted in blocking solution, at the same concentration as the primary antibody.
The following day, the slides were washed in TBS for 30 minutes and then 100 µl of the secondary antibody were added to each slide and were left in a humid chamber for 30 minutes at room temperature. The secondary antibody was biotinylated goat anti-rabbit antibodies, diluted 1 in 400 of TBS. The slides were washed in TBA for 20 minutes and 100 µl of ABC Elite solution added to each slide and incubated at room temperature for 30 minutes. The solution was prepared at least 30 minutes beforehand, by adding 1 drop of A and 1 drop of B to 2.5 mL of TBS, and then vortexed for a few seconds. Slides were then washed in TBA for 20 minutes and 100 µl of DAB added and slides incubated in room temperature for 5 minutes. DAB was prepared by adding 1 drop (30 µl) of chromogen to 50 drops (1500 µl) of substrate and mixed by inversion. The slides were then washed in distilled water for 5 minutes and counterstained in Mayer’s haematoxylin for 2 minutes, washed in running tap water for 5 minutes, dehydrated in 95% and 99% IMS, cleared twice in xylene and a coverslip mounted use XAM mountant and allowed to air-dry for 24 hours before being analysed.

### 4.3.4 FAAH immunohistochemistry

Immunohistochemistry for FAAH was performed in a similar way to the CB1/2 IHC with a few differences.

1. There was no antigen retrieval step.
2. The FAAH primary antibody was prepared at a concentration of 1:2000 in the same Blocking Solution as for the CB1/2 protocol.
3. For the negative controls rabbit serum was used, diluted in blocking solution at the same concentration as the primary antibody.
4. On day 2, in the first TBA wash (for 30 min) the slides with Anti-FAAH antibody were washed in a different box from those with the rabbit serum. These were recombined for the remaining part of the protocol.
4.3.5 NAPE-PLD immunohistochemistry

The NAPE-PLD protocol, followed the principles of the CB1/2 protocol but some further differences.

1. The slides were de-waxed in xylene for 5 minutes 3 times as in other protocols but then rehydration was performed in 100% ethanol for 10 minutes, followed by successive 5-minute incubations in 95%, 90%, 80%, 70%, and 50% ethanol and then distilled water for another 5 min.
2. The slides were then incubated in 3% Hydrogen Peroxide \( \text{H}_2\text{O}_2 \) for 15 mins to suppress endogenous peroxidase activity.
3. All washes were in TBS-Tween20.
4. The NAPE-PLD primary antibody was prepared at a concentration of 1:50 in Blocking Solution.
5. For the negative controls Rabbit IgG was used, diluted in blocking solution to the same concentration as the primary antibody. The slides were then incubated in a humid chamber at room temperature for 18 hours (overnight).
6. The following day, the slides were washed in TBS-Tween20 for 5 minutes 3 times.
7. The 2nd secondary antibody (biotuinylated goad anti-rabbit, diluted 1:400) was added to each slide, diluted 1 in 400 TBS.
8. Slides were dehydrated through different concentrations of ethanol rather than through IMS.

4.3.6 Progesterone receptor isoforms

The IHC procedure for the PR isoforms was similar to that for CB1/2, but with the following differences.

1. The washing buffer was PBS-Tween20.
2. The blocking solution was made by dispersing 10% NRS in TBS.
3. The PR primary antibody was prepared at a concentration of 1:40 in Blocking Solution.
4. For the negative controls, mouse IgG was used, diluted in blocking solution, at the same concentration as the primary antibody.
5. The secondary antibody added, was biotinylated goat anti-mouse, and diluted 1 in 400 with PBS.
4.4 Data interpretation and statistical analyses

The PCR results were analysed by the relative quantification method described by Livak and Schmittgen (404). Briefly, the level of each gene’s transcript was corrected for the Ct value obtained for the housekeeping gene GAPDH. The arithmetic mean of the ΔΔCt value was calculated and then used to determine the relative gene expression value for a particular sample. This was then used to calculate the levels of the corrected gene expression for the different groups and the individual values used in correlation analyses.

To calculate the amounts of the different PR isoforms, the corrected Ct values for the A-isofom were subtracted from the B+A isofom to provide the relative levels of the B-isofom. The amount of C-isofom was calculated by subtracting the corrected Ct values of the B+A isofom from the corrected Ct values of all the PR isoforms (which are called PAN-PR). This was because PCR conditions for all PR-isoforms and the housekeeping gene were identical (Tables 4-3 and 4-4).

4.4.1 Image capture and immunohistomorphometric analysis

Photomicroscopy images were taken on an Axioplan transmission microscope (Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire) equipped with a Sony DXC-151P analogue camera (Sony Inc., Tokyo, Japan) connected to a computer, running Axiovision image capture and processing software (Axiovision version 4.4; Carl Zeiss Ltd.). All images were captured at 200x magnification and analysed using image analysis software (ImageScope version 10.2.2.2319; Aperio Technologies, Inc., Vista, CA) as previously described (399, 405, 406). Immunoreactivity (unbiased histoscore; H-score) was assessed semi-quantitatively by assigning scores as 0 (no staining), 100 (weak staining), 200 (moderate staining) and 300 (strong staining) as determined by the software algorithm.

For each slide, the tissue was examined to gain a general overview of the staining pattern, then 10 representative images were obtained from different representative areas. For each antibody, the images were captured in one continuous single session; hence technical bias or changes in the microscope or analysis settings were avoided. The capturing and analysis of the images took place with the operator blinded to the samples.

4.4.2 Statistical analyses

The ratiometric nature of the qRT-PCR data makes the data non-Gaussian, therefore they are reported as median and interquartile ranges. Differences in the 4 groups were determined using
Krukal-Wallis one-way ANOVA, with Dunn’s multiple comparisons test. Normality of the histoscore data was examined using three tests: D’Agostino-Pearson omnibus normality test, Shapiro-Wilk normality test and Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillie for P value within PRISM version 6.00. The IHC data were not consistently normally distributed and as a result Mann-Whitney U-test was used for all the two-group comparisons, Kruskal-Wallis one-way ANOVA with Dunn’s multiple comparisons test for multi-group analyses, and Spearman’s test for defining correlations. Statistical significance was accepted for p< 0.05. The control group in all the studies were term non-labour (TNL).

4.5 Results

4.5.1 CB1 transcript levels

There were significant statistical differences in CB1 transcript levels between the groups (Figure 4-1) (Kruskal-Wallis; p = 0.0015). CB1 transcript levels in the TNL group (median: 1.39, IQR: 0.75 – 1.65) were significantly higher than in the PTL (median: 0.02, IQR: 0.02-0.03) (p = 0.01) and PTNL (median: 0.06, IQR: 0.02 – 0.14) (p = 0.02) groups. A similar significant difference was found between TL (median: 0.5, IQR: 0.25 – 2.49) and PTL (p = 0.004), as well as between TNL and PTNL (p = 0.004) groups.
4.5.2 CB1 immunohistochemistry

The staining of CB1 immunoreactivity was confined mainly to the cytotrophoblast and syncytiotrophoblast layers of the placental villi (Figure 4-2). Histomorphometric analysis of these data (Figure 4-3) revealed there was no statistically significant difference between the median H-score for the TNL (median: 100.9, IQR = 51.4-128.1) and TL (median: 77.1, IQR = 65.8-87.2; p = 0.69) groups. Similarly, the median CB1 H-score for the PTL group (median: 74.8; IQR = 65.1-98.1) was not statistically significantly different (p = 0.81) from median for the PTNL group (median: 90.1; IQR = 46.4-121.1). What was significant was that in both the labouring groups the median CB1 H-scores were lower than those in the non-labouring group. This suggests that
protein levels may be affected by labour, but not as much as would significantly affect tissue function.

Figure 4-2: Representative photomicrographs of CB1 immunoreactivity in the 4 patient groups.
There is reduced CB1 expression in both labour-affected placentae groups, in accord with the higher immunohistomorphometric H-scores. Images are representative of 6 samples per group, 10 images per sample and were taken at 200x magnification.
Figure 4-3: Histomorphometric analysis of CB1 immunoreactivity in the placenta.

Data are the H-scores for the 4 patient groups and are presented as the median and IQR for 6 samples in each group. Kruskal-Wallis one-way ANOVA showed that all groups were not statistically significantly different (n.s.) from each other.

4.5.3 CB2 transcript levels

There was a difference in the levels of CB2 transcripts among the groups (Figure 4-4) but this was not statistically different (p = 0.11). The data in the PTL group (median: 0.11, IQR: 0.06-69.08) seem to be of higher values, in comparison to the other three ones, but the median remained low and at the same levels of the others. As a result, the individual comparisons of PTL group to TL (median: 0.48, IQR: 0.33-2.23) (p = >0.9), PTNL (median: 0.07, IQR: 0.06-0.14) (p = 0.2), and TNL (median: 1.9, IQR: 0.43-3.62) (p = >0.9) were all insignificant.
Chapter 4  Expression of the ECS and progesterone receptors in the placenta

4.5.4 CB2 immunohistochemistry

The staining of CB2 immunoreactivity was confined mainly to the cytotrophoblast and syncytiotrophoblast layers of the placental villi (Figure 4-5) as it was for the immunostaining of CB1 (Figure 4-3). Histomorphometric analysis of these data (Figure 4-6) revealed there was no significant difference between the median H-score for the TNL (median: 195.4, IQR = 174.2-206.3) and TL (median: 192.8, IQR = 166.6-196.3) groups (p = 0.87). Similarly, the median CB2 H-score for the PTL group (median: 194.2, IQR = 180.6-202.1) was not statistically significantly different (p = 0.40) from median for the PTNL group (median: 172.6, IQR = 147.6-192.7). What was significant was that the median in the PTL group the CB2 H-score was higher than the median in the PTNL group, mirroring the changes in the transcript levels. This suggests that both CB2 protein and transcript levels are differentially affected by prematurity and the presence of labour, with CB2 expression increasing the closer the patient is to term or parturition.
### CB2 expression in the Placenta

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**Figure 4-5:** Representative photomicrographs of CB2 immunoreactivity in the 4 patient groups.

Note the reduced CB2 expression in both non-labour groups when compared to the CB1 immunoreactivity shown in Figure 4-2. Images are representative of 6 samples per group, 10 images per sample and were taken at 200x magnification.
Figure 4-6: Histomorphometric analysis of CB2 immunoreactivity in the placenta.
Data are the H-scores for the 4 patient groups and are presented as the median and IQR for 6 samples in each group. The medians in the TL and PTNL groups were lower compared to the TNL control group and the median expression of CB2 immunoreactivity in the PTL group when compared to the PTNL group. Despite this, Kruskal-Wallis one-way ANOVA showed that all groups were not statistically significantly different (n.s) from each other.

### 4.5.5 FAAH transcript levels

There were differences in the FAAH transcript levels between the groups (Figure 4-7), especially between labourers and non-labourers, in both term and preterm groups, but the statistical results were not significant (Kruskal-Wallis test; \( p = 0.6 \)). All Dunn’s multiple comparisons were not statistically significant either (all \( p \) values were > 0.99). [TNL (median: 0.66, IQR: 0.35-0.83), TL (median: 0.28, IQR: 0.17-4.3), PTNL (median: 0.24, IQR: 0.08-0.35), and PTL (median: 0.16, IQR: 0.08-1.08)].
Figure 4-7: FAAH transcripts in the placenta.
The data show higher transcript levels in the labouring groups both at term and preterm. However, none of the comparisons (Kruskal-Wallis one-way ANOVA or Dunn’s multiple comparisons) showed a statistical significance. Kruskal-Wallis one-way ANOVA showed that all groups were not statistically significantly different (n.s) from each other. The FAAH: GAPDH refers to the ratio of FAAH transcripts to GAPDH transcripts.

4.5.6 FAAH immunohistochemistry

The staining of FAAH immunoreactivity was confined mainly to the cytotrophoblast and syncytiotrophoblast layers of the placental villi (Figure 4-8) as it was for the immunostaining of CB1 (Figure 4-4) and CB2 (Figure 4-6). Histomorphometric analysis of these data (Figure 4-9) revealed there was no statistically significant difference between the median H-score for the TNL (median: 239.8, IQR = 212.7-257.8) and TL (median: 239.3, IQR = 231.3-252.4) groups (p = 0.78). Similarly, the median FAAH H-score for the PTL group (median: 235.1, IQR = 222.2-246.6) was not statistically different (p = 0.58) from median for the PTNL group (median: 239.0, IQR = 237.3-242.6).
Figure 4-8: Representative photomicrographs of FAAH immunoreactivity in the 4 patient groups.
Images are representative of 6 samples per group, 10 images per sample and were taken at 200x magnification.

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Figure 4-9: Histomorphometric analysis of FAAH immunoreactivity in the placenta.
Data are the H-scores for the 4 patient groups and are presented as the median and IQR for 6 samples in each group. Kruskal-Wallis one-way ANOVA showed that all groups were not statistically significantly different (n.s) from each other.

4.5.7 NAPE-PLD transcript levels

There were no significant differences in the NAPE-PLD transcript levels between the groups (Figure 4-10) (Kruskal-Wallis test; \( p = 0.39 \)). Like with FAAH transcripts, all individual comparisons were not significant (\( p >0.99 \)). [TL (median: 0.33, IQR: 0.02-47.86), TNL (median: 4.81, IQR: 0.08-11.01), PTL (median: 0.18, IQR: 0.09-1.76), and PTNL (median: 4.9, IQR: 0.22-60.27) (\( p = 0.7 \))].
Figure 4-10: NAPE-PLD transcripts in the placenta.

The transcript levels of PTL and PTNL, with no significant difference was not significant difference in the medians (Mann-Whitney U; p = 0.13). Kruskal-Wallis one-way ANOVA showed that all groups were not statistically significantly different (n.s) from each other. The NAPE-PLD: GAPDH refers to the ratio of NAPE-PLD transcripts to GAPDH transcripts

4.5.8 NAPE-PLD immunohistochemistry

The staining of NAPE-PLD immunoreactivity was very weak when compared to that of other ECS components and confined mainly to the syncytiotrophoblast layer of the placental villi of the PTL group and to the villus body of all the other groups (Figure 4-11).

Histomorphometric analysis of these data (Figure 4-12) revealed no statistically significant difference between the median H-score for the TNL (median: 5.8, IQR = 3.0-15.5) and TL (median: 10.7, IQR = 4.5-15.5) groups (p = 0.54). Similarly, the median NAPE-PLD H-score for the PTL group (median: 0.52.1, IQR = 0.09-3.16) was significantly different (p = 0.016) from that in the PTNL group (median: 9.7, IQR = 6.0-18.8).
## NAPE-PLD expression in the Placenta

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**Figure 4-11:** Representative photomicrographs of NAPE-PLD immunoreactivity in the 4 patient groups.

There is lack of NAPE-PLD immunoreactivity in all patient groups and a generally ‘cleaner’ staining in the PTL group, suggestive of a lower NAPE-PLD protein level in PTL. There is also increased amount of staining in the villus core of all the other groups. Images are representative of 6 samples per group, 10 images per sample and were taken at 200x magnification.
Figure 4-12: Histomorphometric analysis of NAPE-PLD immunoreactivity in the placenta.

Data are the H-scores for the 4 patient groups and are presented as the median and IQR for 6 samples in each group. Note the statistically significant (*p<0.05) lower median in the PTL when compared the PTNL group. Kruskal-Wallis one-way ANOVA showed that all other groups were not statistically significantly different (n.s) from each other.

4.5.9 PR transcript levels

The PR transcripts were analysed at two levels (see section 4.4): (1) in qRT-PCR for the PR-B isoform alone, as a combination of the A and B isoforms and a primer set that that encompassed all PR isoforms (PR-PAN). The actual transcripts assessed were thus PR-PAN, PR-AB and PR-B. At the second level, (2) the corrected Ct values of the other PR isoforms were obtained through subtracting the values of the particular PR isoform from PR-PAN or PR-AB. In this way, the PR-A transcripts were obtained by subtracting the level of PR-B from PR-AB, while PR-C values were obtained by subtracting the level of PR-AB from PR-PAN. These data will now be reported one at a time.
4.5.9.1 All PR isoforms (PR-PAN)

No statistically significant differences were observed among the four groups (p = 0.49) for the levels of PR-PAN (all PR isoforms). There were higher values noted in the non-labouring states, both at preterm and term, but all individual comparisons showed p values of >0.99 (Figure 4-13). [TNL (median: 1.15, IQR: 0.55-1.76), TL (median: 1.11, IQR: 0.57-1.66), PTNL (median: 0.54, IQR: 0.21-1.63), and PTL (median: 0.35, IQR: 0.2-0.8)].

Figure 4-13: PR (PR-PAN) transcripts in the placenta.

The data show lower but not statistically significant transcripts during labouring states. Kruskal-Wallis one-way ANOVA showed that all groups were not statistically significantly different (n.s) from each other. The PR: GAPDH refers to the ratio of PR transcripts to GAPDH transcripts.

PR staining in the placenta was widespread and not confined solely to the cytotrophoblast and syncytiotrophoblast layers, but also to the body of the villi and capillaries (Figure 4-14). There was a clear increase in staining intensity in the PTNL groups that was variable within the group but consistently higher that either of the term groups or the PTL group.
## PR-PAN expression in the Placenta

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**Figure 4-14: Representative photomicrographs PR-PAN immunoreactivity in the 4 patient groups.**

There is an increased PR-PAN immunoreactivity in the PTNL patient group and generally ‘cleaner’ staining in the PTL group. Images are representative of 6 samples per group, 10 images per sample and were taken at 200x magnification. Histomorphometric analyses of the median PR staining pattern (Figure 4-15) confirmed that PR-PAN expression was significantly ($p = 0.049$) elevated in the PTNL group (median: 147.9, IQR = 119.4-184.3) when compared to the TNL control group (median: 60.3, IQR = 35.3-109.9), whilst the level in the TL group (median: 67.0, IQR = 37.6-123.6) was unaffected when compared to that of the TNL group ($p \geq 0.999$). The expression of PR-PAN in the PTNL group was significantly ($p = 0.034$) higher than in the PTL group (median: 52.7, IQR = 20.9-108.8).
Figure 4-15: Histomorphometric analysis of PR-PAN immunoreactivity in the placenta.

Data are the H-scores for the 4 patient groups and are presented as the median and IQR for 6 samples in each group. Note the statistically significant (** p<0.05) lower median in the PTL when compared the PTNL group, something noticed also in the transcript levels but without statistical significance. Kruskal-Wallis one-way ANOVA showed that all other groups were not statistically significantly different (n.s) from each other, although the PTNL group was significantly higher than the TNL control group.

Determination of the amounts of PR-A and PR-C isoform levels using immunohistochemistry were exceedingly difficult. Therefore, examination of the levels of these two isoforms and that of PR-B were performed using qRT-PCR alone (as described in Section 4.4).

4.5.10 PR-A transcript levels

There was no significant difference (p = 0.854) in the amount of PR-A transcripts in the groups (Figure 4-16). Even all the individual comparisons were not statistically significant (p = >0.99 for all comparisons). [TNL (median: -0.27, IQR: -1.10-1.15), TL (median: -0.67, IQR: -0.81- -0.19), PTNL (median: -0.6, IQR: -0.98- 0.28), and PTL (median: -0.59, IQR: -0.95- -0.02)] (Figure 4-16).
Figure 4-16: PR-A transcripts in the placenta.

The graph shows all medians with no significant differences among all four groups. Kruskal-Wallis one-way ANOVA showed that all other groups were not statistically significantly different (n.s) from each other. The PR-A: GAPDH refers to the ratio of PR-A transcripts to GAPDH transcripts.

4.5.11 PR-AB transcript levels

Like PR-A, the PR-AB transcript did not show any statistical significance neither among the four groups (p = 0.7) nor in the individual comparisons (p > 0.99 in all comparisons). [TNL (median: 0.79, IQR: 0.57-3.09), TL (median: 0.57, IQR: 0.5-0.9), PTNL (median: 0.39, IQR: 0.16-0.66), and PTL (median: 0.44, IQR: 0.17-0.81)] (Figure 4-17).
Figure 4-17: PR-AB transcripts in the placenta.
The graph shows all medians with no significant differences among all four groups. Kruskal-Wallis one-way ANOVA showed that all other groups were not statistically significantly different (n.s) from each other. The PR-AB: GAPDH refers to the ratio of PR-AB transcripts to GAPDH transcripts.

4.5.12 PR-B transcript levels

There was no significant difference (p = 0.6) in the amount of PR-B transcripts between the groups (Figure 4-18). Again, none the individual comparisons showed any significance (p = >0.99 in all comparisons). [TNL (median: 1.13, IQR: 0.42-2.33), TL (median: 0.6, IQR: 0.51-0.88), PTNL (median: 0.67, IQR: 0.62-0.71), and PTL (median: 0.60, IQR: 0.10-0.95)] (Figure 4-18).
Figure 4-18: PR-B transcripts in the placenta.
The results were not statistically significant (n.s.). The PR-B: GAPDH refers to the ratio of PR-B transcripts to GAPDH transcripts.

4.5.13 PR-C transcript levels

The PR-C transcript levels were similar to those of the other isoforms; showing no significant difference among all four groups (p = 0.9) or in individual comparisons (p = >0.99). [TNL (median: 0.11, IQR: -1.74-2.28), TL (median: 0.12, IQR: -0.99 – 1.91), PTNL (median: 0.04, IQR: -1.30 – 1.79), and PTL (median: -0.24, IQR: -1.90 – 0.74)] (Figure 4-19).
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Figure 4-19: PR-C transcripts in the placenta.
The graph shows the median, IQR and ranges for PR-C transcript levels in each group after correction for the levels of the housekeeping gene, GAPDH and after the transcript levels for the A and B isoforms had been subtracted from the PR-PAN transcript levels. Although graphically there appears to be lower PR-C transcript levels in the placentae of the PTL group when compared to the PTNL group, this difference was not statistically significant (n.s.); the lower PR-C expression in the TL group was also not statistically significantly different from the TNL median (Kruskal-Wallis one-way ANOVA; n = 6).

4.5.14 ER-alpha transcript levels

There was no significant difference (p = 0.95) in the amount of ER-α transcripts found among the groups (Figure 4-19), nor in any individual comparisons (p > 0.99 in all comparisons). [TNL (median: 0.76, IQR: 0.34 - 3.33), TL (median: 0.53, IQR: 0.27 - 0.79), PTNL (median: 0.41, IQR: 0.23- 0.43), and PTL (median: 0.35, IQR: 0.30 – 0.82)] (Figure 4-20).
Figure 4-20: ER-alpha transcripts in the placenta.

The results have not shown any statistical significance. The ER-alpha: GAPDH refers to the ratio of ER-alpha transcripts to GAPDH transcripts.
4.5.15 Correlation analysis

4.5.15.1 Transcript and protein

The correlations between the different proteins and transcripts were performed individually for each group of interest (full data available in appendix 8.7).

An analysis of the correlation of the H-score against transcript values for each protein examined by IHC was performed. This correlation aimed to examine the consistency or changes in the protein values from the transcript level to the protein expression.

All correlations were performed by Spearman’s test and were not statistically significant. The correlations for CB1 ($r = 0.1$, $p = 0.5$), FAAH ($r = 0.018$, $p = 0.93$) and NAPE-PLD ($r = 0.2$, $p = 0.24$) were neither strong nor significant. Similarly, insignificant were the correlations for CB2 ($r = 0.3$, $p = 0.07$) and PR ($r = -0.3$, $p = 0.09$) (Figures 4-21 to 4-23).

Figure 4-21: Correlations of transcripts to H-Scores of CB1 and CB2

None of the correlations were statistically significant.
Figure 4-22: Correlations of transcripts to IHC in FAAH and NAPE-PLD

None of the correlations were statistically significant.

Figure 4-23: Correlations of transcripts to IHC in PR.

This was not statistically significant.

4.5.15.2 Protein (IHC)

In the TNL group, the CB1 and CB2 showed a strong and significant correlation ($r = 0.7$, $p = 0.03$) and in PTNL group there was a strong positive correlation between PR-PAN and FAAH ($r = 0.8$, $p = 0.01$) (Figure 4-24)
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Figure 4-24: Correlations in IHC

There was a very strong correlation between CB1 and CB2 in the TNL group (Figure 4-24 a.) and a positive correlation between FAAH and PR in the PTNL group (Figure 4-24 b.).

4.5.15.3  PCR

At the transcript level there were some interesting correlations in all the groups (full data available in the appendix 8.6).

In the TL, there was a strong positive correlation between CB1 and PR-A (r = -0.8, p = 0.03) but not between ER-alpha and PR-C (r = 0.9, p = 0.07) (Figure 4-25).

Figure 4-25: Correlations of TL groups in PCR results

As was in the TNL group there was a positive correlation again between CB1 and PR-A (r = 0.9, p = 0.017) as well as between CB1 and FAAH (r = 0.9, p = 0.033) and ER-alpha (r = 0.9, p =
0.033) respectively. There was also a positive correlation between CB2 and PR-B ($r = 0.92, \ p = 0.02$) (Figure 4-26).

![Figure 4-26: Correlations of PCR results in the TNL group.](image)

CB1 showed statistically significant correlation with FAAH, PR-A and ER-alpha, whereas CB2 to PR-B similar positive correlation.

In the PTNL group there was a positive correlation between NAPE-PLD and both PR-B ($r = 0.8$ and $p = 0.033$) and ER-alpha ($r = 0.77, \ p = 0.017$) (Figure 4-27).

![Figure 4-27: Correlations of PTNL in PCR results.](image)

PR-B shows a positive correlation to ER-alpha and NAPE-PLD

In the PTL group the only statistically significant correlation was between PR and PR-B ($r = 0.71, \ p = 0.017$) (Figure 4-28).
Figure 4-28: Correlations of PTL in PCR results for PR and PR-B.
The correlation is very strong and statistically significant ($r = 0.71$, $p = 0.017$).
4.6 Discussion

The analysis of the placental tissues in the four different stages (TL, TNL, PTL, and PTNL) offered some interesting results that helped in the explanation of the labour process in both term and preterm stages.

The placental examination in the groups revealed interesting results at two levels. Firstly, it showed some significant changes that could possibly explain the changes in the plasma levels of the eCBs and sex hormones. From a different perspective, most results, even the non-significant ones, showed interesting patterns in the changes of the different transcripts and proteins.

CB1 transcripts remained low in the preterm phase with an increase at term. This suggests a high response of the receptors to the plasma eCBs. The difference between labouring and non-labouring state at term, even though not statistically significant, still indicates a change during parturition. CB1 changes with IHC were not statistically significant but showed a pattern of reduction of expression during labouring states. The transcript results did not follow those of IHC, but this could be explained by the fact that different techniques were used in the experiments.

Another way to explain the discrepancies is the fact that in humans, unlike rodents, two isoforms of CB1 exist (a and b) (407, 408). As the isoforms do not originate from the same gene, it is not surprising that our transcript levels showed statistically significant differences, while in IHC, where no isoform-specific antibodies are used, a pattern of change from non-labouring to labouring states is suggested.

There is a possibility that our sample-size for the placental study is small and as a result possible statistically significant results were not achieved. Then again, such a size was previously confirmed adequate through statistically significant tests (328). Even more recent studies in our group showed that a sample size of n = 3 per group could offer statistically significant results (409). Therefore, accepting the non-statistically significant results uncritically can lead to an erroneous conclusion and as a result to that to type II statistical error.

The CB2 transcripts were exceptionally high in the PTL group, but due to low medians there was no statistical significance in the results. Nonetheless, the pattern clearly showed very low CB2 transcripts at every gestational age and state except the PTL group. In the protein expression, the CB2 medians remained similar without any statistical significance. But there was a similar pattern of rise from the PTNL to PTL group, even though not as visually impressive as in the transcripts.
Considering that CB2, like CB1, has two isoforms (410), it is difficult to establish if the difference in pattern between transcripts and IHC had been “neutralised” due to the non-specificity of the latter’s antibodies. The antibodies used in IHC were non-specific for any isoform while the transcript primers could have detected one isoform in favour of another.

The increase in placental CB2 transcript and expression in preterm samples, suggests CB2 activation could be important in the aetiopathogenesis of PTB (323). These data indicate that the placental endocannabinoid system may be linked to the development or progression of PTB and that downstream events associated with altered CB expression in the placenta need further investigation. An interesting finding was the dramatic rise in CB2 transcript in PTL samples with a meek change in the actual expression examined by IHC. CBs seem to be programmed for a specific role in PTL but in interfering factor alters this plan.

What was interesting was the median FAAH H-score. It was remarkably constant throughout the 4 groups, with only the PTL group showing any change that might be physiologically or clinically significant, because the small loss of placental FAAH expression, might be linked to the increased plasma AEA concentrations noted in this group of patients (Chapter 2). The H-score data do not mirror the changes in the transcript levels. This suggests that there is a disconnection between FAAH protein production and transcript levels in the placenta.

On the other hand, the FAAH transcripts were, possibly, the most intriguing ones as they showed higher values in both labouring states, either prematurely or at term. Irrespective of the statistical significance, FAAH transcripts rose from the non-labouring to the labouring state. Such a finding agrees with the plasma AEA changes from the stage of non-labouring to parturition.

The NAPE-PLD results from IHC are dissimilar to the transcript levels and do not seem to correlate. The small loss of placental NAPE-PLD expression is consistent with the increased plasma AEA concentrations noted in this group of patients (Chapter 2). The H-score data do not mirror the changes in the transcript levels. This suggests that there is a disconnection between NAPE-PLD protein production and transcript levels in the placenta of women in preterm labour.

With NAPE-PLD transcripts the main noticeable pattern was that of the drop from non-labouring to labouring state in prematurity, even though it was not statistically significant. A similar fall in NAPE-PLD expression in PTL was found by IHC. The patterns in prematurity were identical in both transcripts and IHC, but that could be expected, especially as NAPE-PLD has only one isoform in mammals (411).
Knowing that NAPE-PLD is involved in AEA production, this last change, at term, may explain the established rise of plasma AEA from non-labouring to labouring state (312). At preterm, changes can still be connected to the reverse alterations of FAAH transcripts. Moreover, the increased plasma AEA concentrations of women in labour or at risk of PTB who subsequently deliver prematurely, can be explained by the alterations in NAPE-PLD expression and reductions in CB1 receptor expression.

As for PR, the IHC results prove that in the preterm non-labouring the expression is high, possibly aiming to keep the uterus in a quiescent state. Of course, things change during labour, especially in PTL. Unfortunately, in this study it was not established, which type of PR is the one that dominates in the labouring state.

The PR-PAN transcripts showed an interesting pattern of reduction from the non-labouring states in both term and preterm stages. This finding, even though it is not statistically significant, agrees with the “functional withdrawal” theory.

The PR-AB transcripts showed a pattern similar to that of PR-PAN. PR-B transcripts followed the pattern of ER-alpha, while PR-C transcripts did not show any significant changes, suggesting a possibly minor role in labour.

The lack of significant changes in the examined isoforms, despite the changes in PR-PAN (both transcripts and IHC) may be explained by the role of different isoforms that were not studied in this project. It could also be possible to explain the lack of pattern or significance due to the small number of the samples. In addition to all these, one more simple explanation may clarify the discrepancies between all transcript and IHC results. With transcripts, the tissues were grinded up and the analyses included the entire tissue. On the other hand, with IHC the analyses were more specific and the “positive” results were specific to the histological structures of interest.

It is known that PRs do regulate ERs, and ER-alpha was discovered to increase in PTL, but probably is controlled by different isoforms from the ones studied. The same conclusion can be drawn when examining the correlation of PR and ER-alpha, as it was not significant. Still, despite the absence of statistical significance, the change of transcript values follows the pattern of ER-alpha changes that happen in pregnancy: the expression keeps increasing during pregnancy (from PTNL to TNL) and then fall during parturition (TL) (341).
Regardless of the results, the examination of ER-alpha in this study was performed only to ensure the presence of ER in the placental tissues in all different stages of labour and gestation, especially as no similar studies have examined the ER in the placental tissue in all those four groups before.

As for the statistically insignificant results, there is no doubt that p values are an important indicator of the statistical significance. Having said that, it is important to be aware of the actual ‘significance’ of the p values, rather than accepting them non-critically.

The findings from the correlations in IHC did not match those of the transcripts. So, in IHC a positive correlation between CB1 and CB2 at TNL was found, whereas in PTNL, FAAH and PR-PAN were positively correlated. In transcripts, some correlations were more notable than others. CB1 was positively correlated to PR-A at term, whether in labour or not. In the PTNL group NAPE-PLD was positively correlated to the most active PR isoform, PR-B.

The study had two main limitations. The first was the small sample size. But as explained earlier, this number – and even a smaller one – had been proven sufficient to conduct the study.

The other limitation was the difficulty in obtaining samples from the myometrium. Ideally similar analyses would be necessary from myometrial samples, in these four different states, as this could offer more accurate and objective explanation of the parturition process. On the other hand, such a comparison in myometrial tissue cannot be achieved. It is always possible to obtain myometrial samples during caesarean sections with relative safety. But, obtaining a sample from a quiescent uterus would compromise the safety of the pregnancy. Adding to that, obtaining a myometrial biopsy after a vaginal delivery is quite challenging. It requires skilled personnel to obtain the biopsy exactly from the placental bed and even with that there are still risks, like uterine injury, obtaining a biopsy outside the placental bed site, haemorrhage, infection, inadequate sampling and failure (398).

Regarding the p values and their actual significance in the results, a quick scoping of importance needs to be taken into consideration. Since the concept of p value was introduced by Laplace in the 18th century (412) and then properly described by Pearson over 120 years later (413), controversies about its misinterpretation have prevailed. On the other hand, it was Fischer who specified p ‘significance’ being less than 0.05, in 1925, and since then this value has been universally accepted (414). Since then, a p value of less than 0.05 has become a conventional cut-off point for statistical significance.
It is not the purpose nor place to doubt any previous mathematical and statistical research. The aim of this brief introduction is to highlight that the cut-off point of the statistical significance can sometimes be a grey area. A 0.05 or 5% chance of “error” or “luck” can be considered a small percentage sometimes or very high in other circumstances.

Nevertheless, there are a lot of significant findings suggesting an active role of the ECS in parturition and especially in prematurity. Nonetheless, as the placenta in not the only tissue in the feto-maternal system that plays a role in labour, fetal membranes were considered as well. Consequently, the same study and analyses were directed further to fetal membranes, and these are presented in the next chapter.

4.7 Conclusion

The placenta plays a role in controlling the ECS and PTL. CB1 remains significantly low in PTL, unlike CB2 which rises dramatically. FAAH receptors rise in labouring states while NAPE-PLD receptors change from high expression in preterm non-labouring state to very low expression during PTL. PR -PAN expressions tend to be higher in quiescent premature uteri but once PTL starts their expressions decreases. The PR isoforms examined did not show a significant role, except PR-B which followed the pattern of PR-PAN. ER-alpha was shown to exist in the placental tissues at term and preterm, and as in any healthy placenta kept increasing with the advancement of gestational age (TNL).
Chapter 5  The endocannabinoid system and sex steroid hormone receptor expression in human fetal membranes


5.1 Introduction

The mechanism by which preterm premature rupture of membranes (pPROM) occurs is unclear but in a significant number of cases results in PTB (415, 416). Plasma AEA concentrations have been shown to increase in women at high-risk for PTB, suggesting that the cannabinoid receptors (CB1 and CB2) and the regulatory enzymes (NAPE-PLD and FAAH) the endocannabinoid system (ECS) could be involved in rupture (325). Although transcripts for CB1 and FAAH have previously been identified in the human fetal membranes (322, 417), changes in the expression of these proteins in human fetal membranes (FM) at term or preterm have not yet been examined.

The fetal membranes are considered to be part of the placenta and because they arise from the same part of the blastocyst, considered to be part of the same tissue (418). However, since FM have a different structure and function to the cotyledons of the placenta, they are usually examined independently. Although several studies have shown the presence of the ECS in the placenta/FM, these studies were only observational. They showed the presence of the ECS in first trimester (321, 323, 406, 417, 419) or term FM (406, 420, 421) but did not examine all 3 layers of fetal membranes (see table 5-1). As a result, most findings were qualitative, while any quantitative suggestions (420) were confined to the samples from term FM. Furthermore, the expression of CB2, which has been shown to have a putative role in preterm labour (422), has not been studied in human FMs beyond the first trimester (Table 5-1).

In preceding chapters, the significance of progesterone in the continuation of pregnancy until term and its role in preventing preterm labour were discussed. In the fetal membranes, progesterone is known to inhibit apoptosis with a subsequent prevention of pPROM and preterm labour (423), and that during term labour the expression of PR falls dramatically in the fetal membranes (340). The actual biochemical role of the FM in the labour process in women remains poorly understood primarily because of the heterogeneous nature of the tissue (amnion, chorion and decidua) and the diversity of the cell types that each of these layers contain (424). Even though the decidua originates from the maternal endometrium, unlike the chorion and amnion that originate from fetal tissues (425), it is still considered to be a part of the FM. Consequently, when the FM is studied, it should be considered as three different tissues collaborating in the maintenance of pregnancy, with a putative role in the initiation of parturition. Hence, once the amniotic layer is separated from the chorion and decidua, the FM becomes weaker and prone to rupture, with subsequent initiation of labour (426).
The expression of progesterone receptor isoforms (PRs) in the FM have not been studied extensively and all studies have previously focused on full term patients. It is known that serum progesterone, when acting on the fetal membranes, stimulates the release of prostaglandins, which subsequently leads to parturition (352, 427). More specifically, in the amnion, the main source of PGE\textsubscript{2} and other PG precursors and enzymes, PR-C seems to be the dominant PR isoform (340). The two commonly studied isoforms, PR-A and PR-B, are also present in the amnion and their ratio changes during parturition (428-430). Although PR expression in the chorion has been reported to be non-existent (340, 429), other techniques, such as western blotting and PCR have recently demonstrated the presence of both PR-A and PR-B isoforms in the chorion, albeit at a very low level (428, 430).

In the decidua, five isoforms have been identified: PR-A, PR-B, PR-C, and two smaller isoforms (45 and 36 kDa) (328, 340, 429). While PR-B seems to be dominant during quiescence, the PR-B to PR-A ratio changes during labour favouring the latter isofrom, with PR-A being to dominant isoform during labour, in the amnion and decidua (430). Nevertheless, all agree that expression of both isoforms are decreased (340, 428, 429). Furthermore, our group reported the presence of PR-C in the FM at term and confirmed its presence in all three layers with predominance in the amnion and chorion (328).

Similarly, there is a scarcity of studies on ER alpha and beta isoform expression in the FM, with ER-alpha involved in cellular proliferation and ER-beta in cellular maturation (431). The paucity of studies examining ER in FM has been primarily due to technical difficulties, as ER gets desaturated during the processing of the tissues and as a result it is lost (431). Despite these challenges, both ER alpha and beta have been reported in the FM (431-433), with ER-beta strongly expressed in the amnion at term, rather than in the decidual cells (431), with ER-alpha appearing towards term in the decidua and in the amnion (432).

Because ER-alpha expression in the placenta was examined in Chapter 4, and because there is limited knowledge regarding its expression in prematurity in FM, it was considered essential to examine its expression here too.
5.2 The ECS in fetal membranes

In order to study the ECS in FM, an appreciation of the tissue histology is required. In this section, I will briefly review the histology of the FM will be briefly discussed.

5.2.1 Amnion

The amnion is the innermost layer of the FM. It is an avascular layer and although macroscopically it seems very thin, it is in fact composed of five distinct layers (Figure 5-1)(434). The uppermost layer is formed of a single layer of non-ciliated cuboidal epithelial cells, which is in immediate contact with the amniotic fluid at its apical surface. The basolateral surface abuts the basement membrane, which forms the strongest part of the amniotic membrane (424). These two layers rest on a stratum of fibroblasts that lies superior to a layer of fibres that criss-cross in a random pattern with very few cells (434). The amnion produces cytokines, prostaglandins, interleukins and other chemotactic factors that influence pregnancy maintenance and parturition (424, 435-438).

5.2.2 Chorion

The chorion is composed of three layers: the reticular layer, which is in contact with the amnion, a basement membrane, and a cytotrophoblastic layer, which comes into close contact with the maternal decidua (434). The reticular layer is formed by Hofbäuer cells and fibroblasts entrenched in a fibrous web (424, 434). The basement membrane is a dense layer formed of connective tissue (424) and the cytotrophoblastic layer is composed of polygonal cells (439). The cells of the chorion are closely adherent near the amnion, but less so proximal to the decidua, where wide intercellular spaces due to the presence of villi are often seen (434).

5.2.3 Decidua

The decidua is the maternal part of the fetal membranes. It is formed of a thick layer of differentiated fibroblastic cells from the endometrial stroma (434). In pregnancy, it is subdivided into three parts: decidua basalis, decidua capsularis and decidua parietalis (440), with each involved in the production of several hormones, cytokines and growth factors that differ to those produced by the amnion and chorion. The decidua has receptors for oestrogen, progesterone and other hormones. Its role is important from the time of implantation of the conceptus to the growth and development of the placenta, and is critical for the establishment and maintenance of early pregnancy (440).
Figure 5-1: Diagrammatic representation of the histological layers of the FM.

The amnion consists of five layers and the chorion of three. The decidua shows vacuolations towards the placental surface that become less visible at the areas close to the chorion. Image by Uchide et al. (441).
Chapter 5 The endocannabinoid system and sex steroid hormone receptor expression in human fetal membranes

Table 5-1: A summary of studies examining different ECS components in the placenta/trophoblast in the first trimester and FM at term

<table>
<thead>
<tr>
<th>Protein</th>
<th>First trimester</th>
<th>Term</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Decidua</td>
<td>Trophoblast</td>
</tr>
<tr>
<td>NAPE-PLD</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>FAAH</td>
<td>+</td>
<td>+</td>
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<td>CB1</td>
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</tr>
<tr>
<td>CB2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NAPE-PLD</td>
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<td>FAAH</td>
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<td>CB1</td>
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<td>+</td>
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<tr>
<td>CB2</td>
<td>-</td>
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</tr>
</tbody>
</table>

Cells marked “+” indicate the expression of the examined protein and those with a “−” indicate either the lack of studies or negative results. Most studies have focused on the qualitative expression of the proteins, while no studies have examined all the three layers of the FM.

CB2 has not been the focus of any study in term placentae or FMs. The quantitative expression of the ECS in preterm placentae and FMs has not been studied previously. The table has been modified from Chan et al. (442).

In this chapter, the focus will be on the presence of the ECS (as was performed in the previous chapter on the placenta) with transcript and protein expression in all three layers of FM assessed. Similarly, to interrogate the potential for the actions of oestrogens and progesterone in these changes in ECS components, the expression of PR isoforms and the presence of ER-alpha will also be studied.
5.3 Materials and methods

To study the levels of transcripts and proteins in fetal membranes, the same techniques used to study the ECS and the PR isoforms, together with ER alpha in Chapter 4 (see section 4.2) are used here.

5.3.1 Tissue handling

The fetal membranes were obtained from the same patients recruited for placental tissues study detailed in Chapter 4. All FM samples were processed simultaneously with the placenta tissues for both later immunohistochemical (IHC) and PCR experiments.

A 2 cm by 2 cm fetal membrane was obtained, by laying the membranes onto a sterile white tray. This allowed clear viewing of all 3 layers of decidua, chorion and amnion. A sterile scalpel was used to cut the square of tissue and the tissue rolled so that the amnion was internal. This was done to prevent the amnion from being detached during the fixation and processing steps for IHC. Two adjacent pieces were taken: one for IHC, was fixed as described in Chapter 4 (section 4.2.1) and the second was snap-frozen in liquid nitrogen as described in Chapter 4 (section 4.2.1).

Further treatment of the samples for IHC and PCR were exactly as described in Chapter 4.

5.3.2 Histomorphometric and statistical analyses

Histomorphometric analysis of the FM samples differed from that of the placenta in that each tissue layer (amnion, chorion and decidua) was analysed individually for each of the target antigens. As a result, each layer was dealt with as an individual tissue in the analysis. In order for a comparison between PCR (which was all tissue layers) and the IHC data (which was individual tissue layers), the H-scores for the individual tissue layers were summed before the comparison was made.

The statistical analyses performed depended on the distribution of data. For equally distributed data ANOVA tests were performed for the comparison of the groups and unpaired Student’s t-test for paired comparisons. For non-normally distributed data, Kruskal-Wallis was used to compare group data, whilst Mann-Whitney U-test was used to compare paired data. All correlations were performed using Spearman correlation analysis.
Chapter 5 The endocannabinoid system and sex steroid hormone receptor expression in human fetal membranes

5.4 Results

To present all of the data generated in this study in this Chapter would be a very onerous task, hence, each gene and its product are described in turn as they were in the previous chapter and all of the various possible correlations are presented in appendices 8.6 to 8.9; only significant correlations are thus presented later in the Chapter. For the H-score studies, the data are divided into the 3 tissues of the fetal membranes; amnion, chorion and decidua. Each section has the same structure as the previous Chapter, with transcript levels for the entire fetal membranes reported before the H-scores for each of the target antigens.

5.4.1 CB1 transcript levels in FM

There was no statistical difference in the levels of CB1 transcripts among the four groups (Kruskal-Wallis; p = 0.5) (Figure 5-2). At term, both TNL (median: 1.36, IQR: 0.51-2.63) and TL groups (median: 1.23, IQR: 0.67-1.41) showed lower transcript levels than either of the preterm groups [PTNL (median: 4.08, IQR: 1.97-6.02) and PTL (median: 6.65, IQR: 0.53-12.67)]. Paired comparisons did not show any statistical significance [Mann-Whitney U-test: TNL and TL (p = 0.8), TNL and PTNL (p = 0.2), TNL and PTL (p = 0.8), and PTNL and PTL (p = 0.66)].

![Figure 5-2: PCR transcripts of CB1 in FM.](image)

There was no statistical differences in the groups. Image shows box and whisker plots, with median, IQR range values shown. The CB1: GAPDH refers to the ratio of CB1 transcripts to GAPDH transcripts.
5.4.2 CB1 immunoreactivity in FM

CB1 immunoreactivity was found in the amnion, chorion and in the decidua (Figure 5-3). In preterm samples, there appeared to be increased expression in all three layers with the highest expression present in the amnion and chorion (Figure 5.3).

<table>
<thead>
<tr>
<th></th>
<th>CB1 expression in the FM</th>
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<tbody>
<tr>
<td></td>
<td>Non-Labour</td>
<td>Labour</td>
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<tr>
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<td><img src="image2" alt="Image" /></td>
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<tr>
<td>Preterm</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
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</tbody>
</table>

**Figure 5-3: Photomicrographs showing CB1 immunostaining for Term non-labour; Term labour; Preterm non-labour; and Preterm labour FM samples.**

The letters A, C and D correspond to amnion, chorion, and decidua accordingly. CB1 expression is higher in the labouring states, with increased expression discernible in the amnion and chorion. The images are representative of 6 samples in each group.
5.4.3 CB1 H-scores

5.4.3.1 Amnion

There were no statistically significant differences in the expression of CB1 (ANOVA, \( p = 0.27 \)) in the amnion (Figure 5-4). Paired comparisons, using unpaired Student’s t-tests, showed no significance in all comparisons to TNL (115.9 ± 14.02) [TL (126.5 ± 6.42) (\( p = 0.5 \)), PTNL (114 ± 9.44) (\( p = 0.9 \)), and PTL (137.8 ± 4.07) (\( p = 0.16 \))]. By contrast, CB1 expression was significantly increased from PTNL to PTL (\( p = 0.04 \)).

![CB1 IHC in Amnion](image)

**Figure 5-4: H-score values for CB1 immunohistochemistry in the amnion.**
Data are presented as the mean ± SEM for 6 samples in each of the four patient groups. There was no statistical significance in comparisons of the four groups (ANOVA, \( p = 0.270 \)), but in prematurity the expression increases significantly with labour (Student’s unpaired t test, *p = 0.04*).

5.4.3.2 Chorion

CB1 expression in the chorion was similar in all four groups (ANOVA, \( p = 0.61 \)) (Figure 5-5). None of the paired comparisons to TNL (136.1 ± 4.66) were significant [Student’s unpaired t test;
TNL vs. TL (145 \(\pm\) 4.95) (\(p = 0.21\)), TNL vs. PTNL (132.4 \(\pm\) 10.04) (\(p = 0.74\)), and TNL vs. PTL (140.9 \(\pm\) 7.17) (\(p = 0.58\)). Similarly, the comparison of PTNL to PTL group was non-significant (\(p = 0.5\)).

![Figure 5-5: H-score for CB1 immunohistochemistry in the chorion.](image)

There are no significant differences (mean \(\pm\) SEM) in the four groups (ANOVA, \(p = 0.61\)).

### 5.4.3.3 Decidua

There was no statistically significant difference between the four groups (ANOVA, \(p = 0.69\)) nor between all paired comparisons of TNL (115.3 \(\pm\) 5.25), with TL (126.9 \(\pm\) 6.55) \(p = 0.19\), PTNL (123.2 \(\pm\) 5.62), (\(p = 0.32\)), with PTL (124.6 \(\pm\) 10.48) (\(p = 0.44\)) (Figure 5-6).
Figure 5-6: H-scores for CB1 immunohistochemistry in the decidua.
No significant differences (mean ± SEM) were present in the four groups (ANOVA, p = 0.69).

5.4.4 CB2 transcript levels in FM

The CB2 transcripts in the FM showed more interesting results, as there were significant differences between the groups (Kruskal-Wallis; p = 0.0019) (Figure 5-7). Like with CB1, the transcripts were lower at term, but there was no significant difference between TNL (median: 1.51, IQR: 0.90-2.23) and TL (median: 0.33, IQR: 0.22-0.52) (Mann-Whitney U-test, p = 0.0519). In the preterm groups PTNL (median: 8.78, IQR: 7.70-12.08) and PTL (Median: 107.03, IQR: 56.04-1585.53) showed significant difference (Mann-Whitney U-test, p = 0.031). There was no difference among the non-labouring groups PTNL and TNL (p = 0.082). Women at term and in labour (TL) had much lower levels of CB2 transcripts than the PTL group (p = 0.016). The maximum difference was between TNL and PTL (p = 0.009).
Figure 5-7: PCR transcript levels of CB2 in FM.
The transcripts were significantly different among the four groups (Kruskal-Wallis; p = 0.0019). PTL shows the highest levels of transcripts in comparison to every group (Mann-Whitney U-test; compared to PTNL p = 0.031, TL p = 0.015, TNL p = 0.009). The CB2: GAPDH refers to the ratio of CB2 transcripts to GAPDH transcripts

5.4.5 CB2 immunoreactivity in FM

CB2 immunoreactivity was found in the amnion, chorion and in the decidua (Figure 5-8). The lowest CB2 immunoreactivity was observed in the preterm non-labouring samples, and there appeared to be increased expression in all three layers in the labour affected tissues, with the highest expression changes occurring in the amnion epithelial cells and in the decidual stromal cells (Figure 5.8).
### CB2 expression in the FM

<table>
<thead>
<tr>
<th></th>
<th>Non-Labour</th>
<th>Labour</th>
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<tbody>
<tr>
<td><strong>Term</strong></td>
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<td><img src="image" alt="D" /></td>
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<tr>
<td><strong>Preterm</strong></td>
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<td><img src="image" alt="A" /></td>
<td><img src="image" alt="C" /></td>
<td><img src="image" alt="A" /></td>
</tr>
</tbody>
</table>

**Figure 5-8:** Photomicrographs showing CB2 immunostaining for Term non-labour; Term labour; Preterm non-labour; and Preterm labour FM.

The letters A, C and D correspond to amnion, chorion, and decidua respectively. There were significant differences in CB2 expression in the amniotic and chorionic layers, but not in the decidua.
5.4.6 CB2 H-scores

5.4.6.1 Amnion

The comparison in all four groups showed statistical significance (Kruskal-Wallis, \( p = 0.0095 \)) (Figures 5-9). No significance change was found between TNL (median: 238.9, IQR: 233.8-242) and TL (median: 225.8, IQR: 210.3-242.5) (Mann-Whitney U-test, \( p = 0.4 \)) nor between TNL and PTL (median: 248.2, IQR: 245-251.4) (\( p = 0.13 \)) or PTNL (median: 218.5, IQR: 167.7-228.9) and PTL (\( p = 0.07 \)). The CB2 expression was found to be statistically lower in prematurity in the non-labouring groups [TNL and PTNL (\( p = 0.019 \)].

![CB2 IHC in Amnion](image)

**Figure 5-9: H-score values for CB2 immunohistochemistry in the amnion.**

Significant difference in CB2 expression (Kruskal-Wallis, \( p = 0.009 \)) and between TNL and PTNL (Unpaired Student’s t test, \( p = 0.01 \)).

5.4.6.2 Chorion

Like with CB1, none of the CB2 comparisons showed a significant difference in the chorion (Kruskal-Wallis, \( p = 0.28 \)) (Figure 5-10). Comparing the TNL (median: 240.6, IQR: 225-256.1) to TL (median: 227.2, IQR: 213.9-228.8) (Mann-Whitney U-test, \( p = 0.39 \)), or PTNL (median:
211, IQR: 196.7-242.4) (p = 0.12), or PTL (median: 244.4, IQR: 236.6-252.1) (p = 0.85) revealed no significant results, as did the comparison of PTNL to PTL (p = 0.42).

![Figure 5-10: H-score values for CB2 immunohistochemistry in the chorion.](image)

The expression did not show statistical significance (Kruskal-Wallis, p = 0.28).

### 5.4.6.3 Decidua

In the decidua, no difference was observed in CB2 expression (Kruskal-Wallis, p = 0.61) (Figure 5-11). There were no statistically significant differences in the comparison (Mann-Whitney U test) of TNL (median: 250.7, IQR: 227.1-254.2) to TL (median: 240.9, IQR: 229.1-259.1) (p = 0.99), PTNL (median: 241.4, IQR: 225.3-249.8) (p = 0.24), and PTL (median: 250.5, IQR: 248-253.1) (p = 0.99). A comparison of PTNL to PTL was not significant (p = 0.28).
Figure 5-11: H-score values for CB2 immunohistochemistry in the decidua.
No significant differences were observed among the four groups (Kruskal-Wallis \( p = 0.61 \)). Figure shows box and whiskers for median, IQR and ranges.

5.4.7 FAAH transcript levels in FM

The FAAH transcripts showed significant difference between the groups (Kruskal-Wallis; \( p = 0.038 \)) (Figure 5-12). TNL (median: 1.42, IQR: 0.31-4.19) and TL (median: 1.90, IQR: 0.52-3.18) did not show significant difference (Mann-Whitney U-test; \( p = 0.9317 \)). Similarly, no significant difference was shown between PTNL (median: 22.68, IQR: 9.40-49.29) and PTL (median: 2.98, IQR: 0.22-11.15) (\( p = 0.056 \)) and between TNL and PTL (\( p = 0.9 \)). There was, however, a significant difference between TNL and the very high transcripts of PTNL (\( p = 0.017 \)).
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5.4.8 FAAH immunoreactivity in FM

Immunoreactivity for FAAH was particularly strong in the chorion of the TNL (control) group (Figure 5-13). At term, the amount of FAAH immunoreactivity appeared to decrease in FM biopsies that had been affected by labour. By contrast, the exact opposite appeared to be the case in the preterm groups, with increased expression in those FM affected by labour (Figure 5-13). Histomorphometric analyses of these data (Figures 5-14 to 5-16) demonstrated that the immunohistochemical staining for FAAH to be variable and heterogeneous in the 4 patient groups.

Figure 5-12: PCR transcripts of FAAH in FM

There was significant difference in the transcript levels in the four groups (Kruskal-Wallis; \( p = 0.038 \)) and between PTNL and TNL (Mann-Whitney U-test; \( p = 0.017 \)). The FAAH: GAPDH refers to the ratio of FAAH transcripts to GAPDH transcripts.
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<table>
<thead>
<tr>
<th>FAAH expression in the FM</th>
<th>Non-Labour</th>
<th>Labour</th>
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<tbody>
<tr>
<td>Term</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>Preterm</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 5-13: Photomicrographs showing FAAH immunostaining for Term non-labour; Term labour; Preterm non-labour; and Preterm labour FM.
The letters A, C and D correspond to amnion, chorion, and decidua accordingly. No significant differences were noted in any of the three layers. There was, however, a pattern in the chorio-decidual interface, as the expression seemed more prominent in PTL and lower in PTNL. The expression then becomes more prominent in TNL.

5.4.9  FAAH H-scores

5.4.9.1  Amnion

FAAH expression showed no significant differences in all four groups (Kruskal-Wallis, \( p = 0.37 \)) nor in any of the paired comparisons [Mann-Whitney U-test; TNL (median: 244, IQR: 231.6-257.7) and TL (median: 249.7, IQR: 240.6-257.8) \( p = 0.53 \), TNL and PTNL (median: 243.4,
IQR: 228.7-249.7 (p = 0.69), TNL and PTL (median: 239.2, IQR: 211.7-248.8) (p = 0.48), and PTNL and PTL (p = 0.58) (Figure 5-14).

![FAAH IHC in Amnion](image)

**Figure 5-14: H-score values for FAAH immunohistochemistry in the amnion.**
No significant changes were demonstrated in FAAH expression in the amnion.

### 5.4.9.2 Chorion

The FAAH expression in the chorion was variable, but this was not statistically significant (Figure 5-15) (Kruskal-Wallis, p = 0.34). No significant difference was found between TNL (median: 260, IQR: 234.4-268.4) and TL (median: 247.9, IQR: 241.8-257.6) (Mann-Whitney U-test, p = 0.48), or between TNL and the other two groups [PTNL (median: 236.2, IQR: 227.1-251.2) (p = 0.06) and PTL (median: 258.4, IQR: 205.8-268) (p = 0.8)]. PTNL to PTL comparison was insignificant (p = 0.39).
**Figure 5-15:** H-score values for FAAH immunohistochemistry in the chorion.

The comparison was not statistically significant (Kruskal-Wallis, $p = 0.34$).

### 5.4.9.3 Decidua

FAAH expression was lower in the PTL group (median: 244.8, IQR: 207- 247.9), but this was not statistically significant in the four groups (Kruskal-Wallis, $p = 0.9$) (Figure 5-16). Even among paired comparisons, TNL (median: 239.3, IQR: 228.7-228.7) and TL (median: 234.9, IQR: 228.9-251.7) showed no significant difference (Mann-Whitney U-test, $p = 0.9$) as there was no difference between TNL and PTNL (median: 237.7, IQR: 226.9- 244.5) ($p = 0.69$) and TNL and PTL ($p = 0.8$). Despite the differences in the graph (Figure 5-16) between PTNL and PTL, this was not statistically significant ($p = 0.58$).
Figure 5-16: H-score values for FAAH immunohistochemistry in the decidua.
The comparison was not statistically significant (Kruskal-Wallis, \( p = 0.9 \))

### 5.4.10 NAPE-PLD transcript levels in FM

NAPE-PLD transcripts did show significant difference when compared in the comparison of four groups (Kruskal-Wallis \( p = 0.13 \)) (Figure 5-17). The increase in transcripts from TNL (median: 1.19, IQR: 0.20-2.69) and TL (median: 6.06, IQR: 0.07-64.85) did not statistical significance (Mann-Whitney U-test, \( p = 0.7922 \)). Neither significant were the comparisons of TNL to PTNL (median: 0.11, IQR: 0.08-3.36) \( (p = 0.06) \) or to PTL (median: 1.79, IQR: 0.98-12.80) \( (p = 0.55) \). The difference between PTNL and PTL, on the other hand, showed significant difference \( (p = 0.02) \).
Figure 5-17: PCR transcripts of NAPE-PLD in FM.
The only significant difference in transcript levels was between PTNL and PTL (Mann-Whitney U-test, p = 0.02). A similar comparison at term TNL and TL did not statistical significance (Mann-Whitney U-test, p = 0.7). The NAPE-PLD: GAPDH refers to the ratio of NAPE-PLD transcripts to GAPDH transcripts

5.4.11 NAPE-PLD immunoreactivity in FM

Immunohistochemical staining for NAPE-PLD was weak when compared to the staining obtained with CB1, CB2 or FAAH. The strongest staining was observed in the TNL group and was visible in the amnion, chorion, and decidual layers (Figure 5-18). Strong staining was seen in the amnion of FM at term and appeared to diminish to undetectable levels in FM affected by labour (Figure 5-18). By contrast, staining seemed to be strongest in the chorion of FM taken from preterm women who were not in labour (PTNL) and such staining was greatly diminished by the actions of labour, not only in the chorion, but also in the amnion epithelial cells (Figure 5-18).
### NAPE-PLD expression in the FM

<table>
<thead>
<tr>
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<th>Non-Labour</th>
<th>Labour</th>
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<tbody>
<tr>
<td>Term</td>
<td><img src="image1" alt="Term Non-labour" /></td>
<td><img src="image2" alt="Term Labour" /></td>
</tr>
<tr>
<td>Preterm</td>
<td><img src="image3" alt="Preterm Non-labour" /></td>
<td><img src="image4" alt="Preterm Labour" /></td>
</tr>
</tbody>
</table>

**Figure 5-18:** Photomicrographs showing NAPE-PLD immunostaining for Term non-labour; Term labour; Preterm non-labour; and Preterm labour FM.

The letters A, C and D correspond to amnion, chorion, and decidua, respectively. NAPE-PLD expression in both labouring groups (TL and PTL) was lower in comparison to their corresponding labouring control groups.

#### 5.4.12 NAPE-PLD H-scores

**5.4.12.1 Amnion**

NAPE-PLD expression in the amniotic membranes showed variations, but none of the statistical tests was significant [Kruskal-Wallis ($p = 0.27$); Mann-Whitney U-test TNL (median: 22.82, IQR: 4.618-151.4) and TL (median: 48.05, IQR: 23.01-127.1) ($p = 0.69$), TNL and PTL]
(median: 3.028, IQR: 1.488-63.07) (p = 0.39), TNL and PTL (median: 15.61, IQR: 0.5516-44.46) (p = 0.48), and PTNL and PTL (p 0.8) (Figure 5-19).

Figure 5-19: H-score values for NAPE-PLD immunohistochemistry in the amnion.
The data were not statistically significantly different.

5.4.12.2 Chorion

None of the statistical analyses of NAPE-PLD expression showed significance (Kruskal-Wallis, p = 0.94) (Figures 5-20). All comparisons (Mann-Whitney U-test) of TNL (median: 47.47, IQR: 9.265-98.3) to the other groups were not significant [TL (median: 32.89, IQR: 15.03-86.82) (p = 0.8), PTNL (median: 41.74, IQR: 3.345-119.1) (p = 0.9), and PTL (median: 24.55, IQR: 9.398-68.1), (p = 0.69)]. Again, despite the visual difference between PTNL and PTL, there was no significant difference (p = 0.93).


Figure 5-20: H-score values for NAPE-PLD immunohistochemistry in the chorion.
The comparisons were not statistically significant.

5.4.12.3 Decidua

NAPE-PLD expression was similar in all groups (Figure 5-21) (Kruskal-Wallis, p = 0.99) (Figures 5- and 5-19). Mann-Whitney tests between TNL (median: 76.52, IQR: 81.75-136.1) and TL (median: 80.74, IQR: 24.81-116.5) (p = 0.99), TNL and PTNL (median: 93.3, IQR: 4.251-149.2) (p = 0.93), and TNL and PTL (median: 81.75, IQR: 20.92-135.1) (p = 0.99) showed no significant difference. The comparison of PTNL to PTL was not significant (p = 0.99).
5.4.13 PR transcript levels in FM

PR-PAN transcripts did not show any significant difference in all the four groups of patients (Kruskal-Wallis; p = 0.6022) (Figure 5-22). A comparison of TNL (median: 0.62, IQR: 0.36-1.24) to TL (median: 0.69, IQR: 0.35-1.87) (Mann-Whitney U-test; p = 0.66), PTNL (median: 1.23, IQR: 0.74-2.27) (p = 0.24), and PTL (median: 2.63, IQR: 0.24-8.80) (p = 0.42). Even a comparison of PTNL and PTL did not show significant differences (p = 0.99).
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Figure 5-22: PCR transcripts of PR-PAN in FM.
No statistical significance for comparisons between the four groups (Kruskal-Wallis; $p = 0.6$). The PR: GAPDH refers to the ratio of PR transcripts to GAPDH transcripts.

5.4.14  PR immunoreactivity in FM

Immunohistochemical staining for all PR isoforms using an antibody that detects all major PR isoforms (PR-PAN) indicated the presence of PR protein in all three layers of the FM (Figure 5-23). The intensity of staining was fairly constant across all 4 groups of patients, but there appeared to be a small decrease in staining intensity in the PTL group when compared to all other groups. This observation was confirmed by histomorphometric (H-score) analysis of the 3 tissue types (Figures 5-24 to 5-26).
Figure 5-23: Photomicrographs showing CB2 immunostaining for Term non-labour; Term labour; Preterm non-labour; and Preterm labour FM.

The letters A, C and D correspond to the amnion, chorion, and decidua respectively. The expression of PR using this PR-PAN antibody was fairly constant across all patient groups, with a slight decrease in staining in the PTL group, and with ‘patchy’ and variable staining in the amnion.

5.4.15 PR H-scores

5.4.15.1 Amnion

PR expression appeared to be lower in both labouring groups but none of the comparisons was statistically significant (Figure 5-24). Kruskal-Wallis (p = 0.13), Mann-Whitney U-test; TNL
(median: 262.4, IQR: 252.5-271.8) and TL (median: 268.2, IQR: 215-272.9) (p = 0.9), TNL and PTNL (median: 260.7, IQR: 240.5-269.7) (p = 0.69), TNL and PTL (median: 242, IQR: 87.37-255.2) (0.065), and PTNL and PTL (p = 0.18]) (Figure 5-24).

Figure 5-24: H-score values for PR immunohistochemistry in the amnion.
The comparisons were not statistically significant. Two comparisons [TNL and PTL (p = 0.065) and TL and PTL (p = 0.065)].

5.4.15.2 Chorion

PR expression did not show significant differences within the four groups (Kruskal-Wallis, p = 0.1) (Figures 5-25). All paired comparisons to TNL (median: 247.1, IQR: 211.4-258.3) were not statistically significant (Mann-Whitney U-test; TL (median: 235.9, IQR: 222.7-248.6) (p = 0.69), PTNL (median: 251.6, IQR: 240.5-254.2) (p = 0.69), and PTL (median: 207.5, IQR: 149.8-243.1) (p = 0.132)]. There was, however, a significant difference between PTNL and PTL (p = 0.026).
Figure 5-25: **H-score values for PR immunohistochemistry in the chorion.**

The PR expression showed statistical significance in all four groups (Kruskal-Wallis, \( p = 0.1 \)). Same significance was shown when comparing PTNL to PTL (Mann-Whitney U-test, \( p = 0.026 \)).

### 5.4.15.3 Decidua

The expression of PR did not show statistical significance within group comparisons (Figures 5-26) (Kruskal-Wallis, \( p = 0.1 \)). Paired comparisons by Mann-Whitney U-test of TNL (median: 233.4, IQR: 207.4-239.7) to TL (median: 227, IQR: 205.9 -235.7) \( (p = 0.589) \), to PTNL (median: 238.9, IQR: 222.6-243.4) \( (p = 0.394) \), or PTL (median: 208.6, IQR: 118.7-228.7) \( (p = 0.132) \) showed no statistically significant difference. The comparison of PTNL to PTL was significantly different \( (p = 0.041) \).
Figure 5-26: H-score values for PR immunohistochemistry in the decidua.

The comparisons between groups did not reveal statistical significance (Kruskal-Wallis, $p = 0.1$).

### 5.4.16 PR isoforms

Due to time constraints, it was not possible to complete the intended IHC studies on the different receptor isoforms for PR. The data in this section are therefore confined to transcript levels only. Figure 5-22 has therefore been redrawn as Figure 5-27 for comparison purposes.

#### 5.4.16.1 PR-PAN

PR-PAN transcripts did not show any significant difference within all the four group (Kruskal-Wallis; $p = 0.6022$) (Figure 5-27). A comparison of TNL (median: 0.62, IQR: 0.36-1.24) with TL (median: 0.69, IQR: 0.35-1.87) (Mann-Whitney U-test; $p = 0.66$), to PTNL (median: 1.23, IQR: 0.74-2.27) ($p = 0.24$), or PTL (median: 2.63, IQR: 0.24-8.80) ($p = 0.42$). Even a comparison of PTNL and PTL did not show significant changes ($p = 0.99$).
Figure 5-27: Comparison of PCR transcripts for the PR isoforms in FM.

There was no statistical significance (n.s.) in the comparison of all four PR isoform analyses across any of the patient groups; for PR-PAN (Mann-Whitney U-test; $p = 0.99$); PR-AB combined ($p = 0.79$); PR-A alone ($p = 0.69$); PR-B alone ($p = 0.6$); or PR-C alone ($p = 0.13$).
5.4.16.2 PR-AB

PR-AB transcript levels for the 4 patient groups (Figure 5-27), were similar to those for PR-PAN (Kruskal-Wallis; p = 0.16). Both TNL (median: 0.86, IQR: 0.66-1.62) and TL (median: 0.83, IQR: 0.52-1.28) were low and were not significantly different (Mann-Whitney U-test; p = 0.39). The transcripts increased a lot from PTNL (median: 2.07, IQR: 1.66-2.10) to PTL (median: 2.40, IQR: 1.14-4.45), but despite the obvious difference it was not significant (p = 0.79). Comparisons of TNL transcripts with those of PTNL (p = 0.17) and PTL (p = 0.24) were not significant either.

5.4.16.3 PR-A, PR-B and PR-C isoforms

PR-A transcripts were higher in the membranes from labouring states, but none of the comparisons were statistically significant (Figure 5-27) (Kruskal-Wallis; p = 0.07). TNL (median: -0.02, IQR: -0.87-0.97) and TL (median: 0.61, IQR: -0.23-1.16) transcripts were not statistically different (Mann-Whitney U-test; p = 0.48), neither was the comparison of PTNL (median: 1.42, IQR: 1.09-5.91) and PTL (median: 1.79, IQR: 0.54-8.41) (p = 0.69). In addition to that, paired comparisons of TNL to PTNL (p = 0.08) and PTL (p = 0.08) were not significant.

PR-B transcripts (Figure 5-27) showed a similar pattern to that of PR-AB, but without any significant comparisons (Kruskal-Wallis; p = 0.26). TNL (median: 1.35, IQR: 0.69-1.79) and TL (median: 0.88, IQR: 0.38-1.53) were not significantly different (Mann-Whitney U-test; p = 0.5). PTNL (median: 1.77, IQR: 1.00-1.79) and PTL (Median: 2.17, IQR: 1.46-7.10) were also not different (p = 0.6). The TNL comparisons to PTNL (p = 0.3) and PTL (p = 0.3) were not found to be significant.

PR-C transcript (Figure 5-27) showed a similar pattern to PR-A, where transcript were high in labouring states, but there was no significant difference among the four groups (Kruskal-Wallis; p = 0.15). TNL (median: 0.00, IQR: -0.37-19.15) were not significantly different from neither TL (median: 0.57, IQR: -0.19-1.86) (Mann-Whitney U-test; p = 0.24), nor PTNL (median: -0.59, IQR: -4.46-0.23) (p = 0.24), or PTL (median: 0.58, IQR: -0.48-9.07) (p = 0.42). PTNL and PTL transcripts were not statistically different (p = 0.13).
5.4.17 ER alpha transcript levels in FM

There were no statistically differences in ER-alpha transcripts in analyses between groups (Kruskal-Wallis; p = 0.166) (Figure 5-28). For the TNL (median: 1.01, IQR: 0.68-1.62) versus the TL group (median: 0.85, IQR: 0.33-1.10) there were no differences (Mann-Whitney U-test, p = 0.48). TNL when compared to PTNL (median: 1.27, IQR: 0.93-5.72) or PTL (median: 4.37, IQR: 3.90-6.38) were also non-significant (p values 0.80 and 0.12, respectively). There was a remarkably higher level of ERα transcripts in PTNL compared to PTL, but this difference was not statistically significant (p = 0.19).

![PCR transcripts of ER-alpha in FM.](image)

Figure 5-28: PCR transcripts of ER-alpha in FM.

No significant differences were observed in the transcript levels in the four patient groups (Kruskal-Wallis; p = 0.166). The PTL and PTNL showed similar patterns to those of PRs, with PTL transcript levels being higher, but that increase did not reach statistical significance (Mann-Whitney U-test, p = 0.19). Image shows box and whiskers, medians and IQR values. The ER-alpha: GAPDH refers to the ratio of ER-alpha transcripts to GAPDH transcripts.
5.4.18 Correlation analyses

In this section, an attempt was made to relate the observed differences for the ECS and sex steroid hormone receptors, to determine if there are any significant relationships between the levels of protein or levels of gene transcript. The entire data set are presented in appendix 8-4.

5.4.18.1 IHC vs PCR

In order to make a valid comparison between the H-scores and transcript levels, the values from the three tissues from the immunohistomorphic analyses of each sample were added together and as a result, a common value was obtained for each FM sample. All the IHC values (protein expression) were then summed up and examined for a possible correlation to all the PCR values (transcripts) for all five proteins: CB1, CB2, FAAH, NAPE-PLD, and PR (PR-PAN).

None of the correlations was statistically significant [CB1 \( r = -0.03, p = 0.91 \); CB2 \( r = -0.09, p = 0.75 \); FAAH \( r = 0.28, p = 0.22 \); NAPE-PLD \( r = -0.16, p = 0.51 \); PR \( r = 0.15, p = 0.5 \); Spearman correlation analysis] hence these data are not shown.

5.4.18.2 IHC

There were only two statistically significant correlations shown with IHC. Within the TNL group, CB1 and CB2 protein expression (Figure 5-29) showed a strong negative correlation (Spearman \( r = -0.943, p = 0.017 \)) and in the PTNL there was a direct positive correlation between FAAH and PR expression (\( r = 0.987, p = 0.003 \)) (Figure 5-29). These data suggest that whatever regulates CB2 expression inversely regulates CB1 expression at the end of normal pregnancy and that the activation of PR in the preterm group was not in labour directly influences the expression of FAAH.
Figure 5-29: Correlations in IHC in the fetal membranes.
The only significant H-score correlations were between CB1 and CB2 in the TNL group and between PR and FAAH in the PTNL group.

5.4.18.3 PCR

5.4.18.3.1 Term non-labouring (TNL) group

In the TNL group, CB1 showed very strong positive correlations to both PR ($r = 0.99$, $p = 0.017$) (Figure 5-30) and ER-alpha ($r = 0.88$, $p = 0.03$) (Figure 5-30).

Figure 5-30: Correlations in PCR in the fetal membranes of the TNL group.
5.4.18.3.2 Term labouring (TL) group

CB1 showed positive correlation to PR-A ($r = 0.88, p = 0.03$) in the TL group (Figure 5-31), while a similar correlation between PR and PR-C ($r = 0.88, p = 0.03$) (Figure 5-31) was shown in the same groups.

![Figure 5-31: Correlations in PCR in the fetal membranes of the TN group.](image)

5.4.18.3.3 Preterm non-labouring (PTNL) group

In the PTNL group, only PR was found to be significantly correlated to CB1 ($r = 0.999, p = 0.003$) (Figure 5-32).

![Figure 5-32: Correlation in PCR in the fetal membranes of the PTNL group.](image)
5.4.18.3.4 Preterm in labour (PTL) group

In the PTL group, PR showed strong positive correlation to both of its isoforms PR-B \( (r = 0.998, p = 0.003) \) and PR-C \( (r = 0.997, p = 0.03) \) (Figure 5-33). PR-B was also positively correlated to CB1 \( (r = 0.999, p = 0.017) \) and PR-C was similarly positively correlated to the levels of PR-B transcripts \( (r = 0.998, p = 0.033) \) (Figure 5-33).

![Correlation PCR in PTL FM PR-PAN vs. PR-B and PR-C](image1)

![Correlation PCR in PTL FM PR-B vs CB1 and PR-C](image2)

Figure 5-33: Correlations in PCR in the fetal membranes of the PTL group.

5.5 Discussion

The results here offered many interesting answers to several questions regarding the involvement of the ECS and sex hormone receptors in the process of parturition. Certainly, the first important finding from both IHC and PCR studies was the confirmation of the presence of all the examined components of the ECS, as well as the presence of transcripts for ERα and PR receptors in FM in all four patient groups. Not many studies have managed to show the existence of both eCB receptors and enzymes in FM and to the best of my knowledge, no studies have been undertaken to demonstrate the presence of CB2 in human FM whether at term or preterm.

The amniotic layer of the FM seems to have a pivotal association with various components of the ECS, while the chorion and decidua are significantly associated with changes in the expression of PR. Another interesting finding was that of similar patterns of expression (whether statistically significant or not), with all five antibodies in the chorion and decidua. These observations, so far, agree with the common concept that the amnion is an independent tissue, rather than just being ‘a
layer’ in the FM (426, 443). Embryologically, the chorion and amnion are directly related (444), but mechanically the chorion and decidua rupture first as the amnion is much stronger than the other two layers (426, 445-447). Additionally, the chorion and decidua appear to remain functionally connected at the ‘chorio-decidual interface’, where any deviation from its normal development or inflammatory changes can lead to the loss of pregnancy or preterm birth (448-451).

Amniotic CB1 expression was higher in membranes from both labouring states, with a major change during prematurity. CB1 transcripts were higher in membranes the non-labouring groups, with those in preterm being much higher than in those at term. The increase in CB1 expression in FM at labour coincides with the rise in the plasma AEA in labouring women. These data point to a general involvement of the ECS in labour, and a more specific role in preterm labour. By contrast, CB1 expression in the placenta had the exact opposite pattern – low in labouring states as shown in the previous chapter. Since rupture of the FM is a major factor in the initiation of PTL (40% of preterm deliveries (117)) it can be concluded that CB1, along with AEA, probably play an active part in parturition. The unchanged expressions of CB1 in the chorio-decidual interface suggest that regardless of the exact mechanism, CB1 is involved in the process of labour, and this effect is confined to the amniotic layer.

CB2 protein expression was similar to that of CB1; it increased in the FM of the preterm groups, as its expression in the PTL group was higher than in the PTNL patients. These results were confirmed at the transcript level, with PTL values being significantly higher than in any of the other groups. The higher expression of CB2 at the chorio-decidual interface in both states, even though insignificant, suggest a role of this receptor isoform in labour. Since the findings of CB2 in the FM agree with the changes in the placenta, two conclusions can indeed be confidently drawn: firstly, CB2 is likely to have a greater role than CB1 in parturition, and especially in premature labour, and secondly that low CB2 expression is essential for quiescent pregnancy progression.

FAAH protein expression in the amnion did not indicate any specific role for this enzyme in this layer. Nonetheless, FAAH transcripts confirmed significant changes in the groups, with higher values in FM from both labouring groups, especially those in PTNL. In the chorio-decidual interface, FAAH expression was higher in FM during preterm labour (PTL), but not in the non-labouring state at term (TL). By FAAH remaining higher in the non-labouring stages possibly, it explains the equilibrium of the ECS during quiescence, as hydrolysis of the eCBs takes place no uterine contractions are initiated. The higher FAAH expression in the chorio-decidual interface
in the PTL group has many explanations. Despite high plasma AEA in PTL, it is possible that its hydrolysis does not depend on FAAH, but on alternative possible mechanisms (287-294) (Figure 2-1). Moreover, as it has been indicated that before progesterone stimulates FAAH activity in human T-lymphocytes (303) and in labour, plasma progesterone concentrations increase, a similar increase is expected in FAAH.

All the FM from PTL patients had higher FAAH expression, but it is unlikely that all of them had gone through the same mechanism. It is still unclear the type of parturition in which the ECS is involved at most. Not surprisingly, FAAH was higher in FM from PTL but this was not necessarily statistically significant.

The observations for NAPE-PLD on face value do not make sense. NAPE-PLD protein expression was lower in FM from labouring states, especially in the preterm groups. The exact reverse patterns were found at the transcript level; transcripts were higher in FM from labouring states, with significant difference in the PTL group. The expression of NAPE-PLD protein in the chorio-decidua, however, was lower in PTL compared to PTNL groups. Regardless of the statistical results, these patterns make perfect sense. During labour, the transcripts are higher, especially in PTL, as NAPE-PLD is the primary enzyme involved in AEA biosynthesis. The lower NAPE-PLD expression in labour could be due to its consumption, as more AEA is produced in labour, resulting to the depletion of NAPE-PLD. The changes in NAPE-PLD expression (together with those for CB1, CB2, and FAAH), support the serological and placental changes that were presented in previous chapters.

The difference in the magnitude of the NAPE-PLD patterns between term and preterm groups was similar to that found in the placenta (Chapter 4) supporting the previous proposition that the ECS is actively involved in parturition, but on the other hand, its role in PTL may differ from that at term.

PR expression, even though non-significant in the amnion, was lower in FM from the labouring groups, and more in the preterm group. These results agree with the significantly low PR expression in PTL, compared to PTNL, in both the chorion and decidua. By contrast, at the transcript level, the pattern was the opposite, with PR-PAN transcripts being higher in the FM from the labouring groups.

The PR expressions in all the three layers is explained by the already known natural changes of PR expression and different isoforms in labour. Understandably, the patterns follow the same
patterns of IHC and PCR in the placenta; meaning the transcripts and expressions change in every group in the FM are same as those changes in the placenta in the corresponding group. Similar to the PR-PAN transcript pattern, all of the PR-AB, PR-A, PR-B, and PR-C results showed a similar arrangement. There was a tendency for the distribution of most isoforms to be low in all groups, except from the PTL group where all transcripts were distinctively higher. Interestingly, with the PR-C results, there was an impression that the difference between PTNL and PTL groups was larger, as in the non-labourers where PR-C was even lower than in the term groups. These data suggest that PR-C is possibly the dominant isoform linked to preterm labour or, more precisely, to pPROM.

The ER-alpha transcripts showed identical pattern to PR-PAN, with an enormous, yet not statistically significant, difference between PTNL and PTL, favouring the latter. IHC was not performed, as explained earlier, as the examination of ER-alpha was not the primary aim of the study and as stated earlier there was not enough time to conduct the experiments.

Despite the lack of statistical significance with the transcript and immunohistochemical results for ER-alpha, the findings are indeed interesting. ER-alpha is certainly present in preterm birth and this has not been reported extensively before. Another interesting finding is that the changes, were noted only in the preterm group. If ER-beta is the main contributor in TL, as the literature suggests, then there is a possibility that this role is not also important in preterm labour. Such a hypothesis would need, ideally, a completely new study examining all the ERs at both transcript and protein levels, in the four groups included in this study.

Studying the correlations of the transcripts and IHC stain in the groups showed a possible role for CB1. In the IHC results, CB1 had a negative correlation with CB2 in the quiescent state at term. This can be explained by the fact that AEA acts mainly on CB2 in the peripheral tissues (452) and is in accord with the CB2 contribution on FM. In the transcript levels, CB1 showed a strong positive correlation with PR-PAN or other PR isoforms in all four groups. In the TNL group, the correlation between CB1 and ER-alpha was strongly positive. The lack of CB2 contribution in these findings, despite its substantial changes, is possibly explained by the fact that not all isoforms had been studied in these experiments.

Another interesting finding, from the correlations was that of the PRs with the components of the ECS. In the PTNL group, PR and FAAH had a strong positive correlation, something that explains the ‘protective’ mechanism against PTL. At the transcript level, apart from the abovementioned correlations between PR-PAN and CB1, PR-PAN showed a persistent positive correlation with
PR-C in both labouring states and to PR-B in the PTL group only. This finding agrees with the previous suggestion of a PR-C role in FM in labour.

Of course, there are several limitations to this study. First of all, the number of samples was small, as discussed in the previous chapter, and possibly the results could have been “tighter” with a larger sample size. Possibly with a larger number of samples it would have been possible to achieve statistical significance for the difference in the amniotic PR expression between PTL and TNL groups ($p = 0.065$). Having said that, there have been many results that “almost” reached statistical significance. However, the power of statistical analyses is not going to be discussed again in this chapter. The other point of limitation is that, like in the placental experiments, not all isoforms, in either the eCB components or hormonal receptors, had been taken into consideration. Probably IHC analysis of the CB2, PR and ER isoforms the ones that could have answered a lot of the questions about the mechanism of labour, especially in prematurity.

It has been reported in the literature studies demonstrating discrepancies between transcript levels and protein expressions. In some of the studies, the transcripts are much higher than the protein expressions, while in others the opposite is true. RNA silencing could be one of the mechanisms contributing in these inconsistent findings. MicroRNAs (miRNA) may be involved in the degradation of the mRNA of the protein in our studies, leading to lower than expected expression. Considering the numerous reasons and mechanisms that can lead to labour, more research is needed to study this hypothesis extensively. Likewise, more research will be necessary if the discrepancy between transcripts and proteins is thought to be because of a feedback loop system or any other mechanism that leads to the loss of the proteolytic activity in the examined tissues.

5.6 Conclusions

The ECS in FM probably plays a significant role in parturition. The amnion acts independently from the chorio-decidual interface, but all three layers play an important role both in quiescence and in labour. CB2 seems to be the key component in labour and there is a strong connection between CB1 and PR expression. PR-C and ER-alpha are two sex steroid hormone receptor isoforms that unexpectedly seem to play a role in PTL and therefore need to be studied further. Last, but not the least, all the observed differences between FM taken from women who had been in labour and those that had not, showed a consistently different pattern at term from the pattern observed preterm. This could suggest that the mechanisms involving the ECS and sex hormone receptors in labour at term differ profoundly from those that occur in preterm birth.
Chapter 6  Re-analysis of plasma anandamide for detection of preterm birth, the gestational age at delivery and the recruitment-to-delivery-interval and the possible contribution of other variables
Chapter 6   Re-analysis of plasma anandamide for detection of preterm birth, the gestational age at delivery and the recruitment-to-delivery-interval and the possible contribution of other variables

6.1 Introduction

In Chapter 2, I showed that plasma AEA concentrations increased significantly and early enough in women at ‘high-risk’ of delivering preterm prior to labour and that its measurement could be used as a predictive test for PTL. By contrast, plasma concentrations of OEA and PEA did not show a statistical difference, despite plasma OEA concentrations increasing in women who delivered prematurely. Likewise, cervical length (CL) measurements were not different between the two groups (PTL and TL versus PTNL and TNL), nor were the endocervical IGFBP-1 tests, despite several literature sources indicating that CL and IGFBP-1 measurements are fairly good negative predictors of PTL. The conclusion from the studies presented in Chapter 2, were that the lack of statistical significance was probably due to the limited number of patients recruited into the study. Furthermore, blood FAAH activities, on their own, showed no predictive value. Again, this could have been due to the limited number of samples studied or that peripheral FAAH activity plays no role in regulating plasma AEA concentrations in women at risk of PTL.

When the plasma concentrations of all 3 eCBs were compared, it was shown that AEA may predict the timing of labour (through RTDI) over two months before parturition, regardless of the gestational age at parturition. In fact, all 3 eCBs had similar patterns of significance in both symptomatic and asymptomatic women, even in those women who were recruited before 30 weeks of gestation, suggesting that not only may these 3 eCBs be involved in the timing of labour, but that may be combined into a new predictive test for both PTL and term labour. The recruitment to delivery interval (RTDI) was used as this closely resembles what might occur in a clinical setting. These data also suggested that this interesting observation required further in-depth study, because it suggested that the biology of the eCBs is somehow related to parturition. One way of doing this, was to combine all the data available and re-examine the various components and determine which component (if any) contributes to a better prediction of PTL. But this is quite a complex process, when one considers all of the parameters that could contribute to PTL in the patient cohort.
Consequently, it was necessary to:

a. Increase the number of samples, especially for the plasma AEA analysis, as that could help in confirming the predictive value of this eCB in the risk of PTL.

b. Examine all the available variables together for the assessment of the risk of PTL using multivariate analysis

c. Examine the possibility of predicting the timing of parturition from the time of obtaining the blood sample, i.e. a more accurate RTDI with a more stringent statistical method.

To do this a new method of analysis was applied in which all the factors that could result in PTB could be assessed. The tests used were univariate and multivariate analyses with logistic regression as a way of determining relationships between variables.

As a result, the aims of the (uni- and multi-variate) analyses were to establish:

a. A prediction of PTL from those women who deliver prematurely and at term.

b. An accurate prediction of RTDI calculated in days, rather than weeks (as was done in Chapter 2).

c. An accurate prediction of the gestational age at parturition, if possible.

A more robust statistical analysis of the data would be generated and a better conclusion on the predictability of PTL, with more accurate cut-off points of the plasma levels from a single plasma eCB measurement might be forthcoming.

6.2 Methods

This was an amalgamation of data reported in Chapter 2 with additional data generated by Dr. Vijaianitha Nallendran, my predecessor, in the ERG using data that had never been analysed. The processing of samples and the measurement of plasma AEA was conducted as described in Chapter 2, with the exception that measurement of OEA and PEA were not included. The number of additional data points are listed in Table 6-1.
Table 6-1: Numbers of samples for the multivariate analysis

<table>
<thead>
<tr>
<th>Samples for multivariate analysis</th>
<th>AEA</th>
<th>OEA</th>
<th>PEA</th>
<th>CL</th>
<th>IGFBP-1</th>
<th>FAAH</th>
<th>ofFN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current study</td>
<td>51</td>
<td>51</td>
<td>51</td>
<td>23</td>
<td>41</td>
<td>43</td>
<td>0</td>
</tr>
<tr>
<td>Additional data</td>
<td>166</td>
<td>0</td>
<td>0</td>
<td>42</td>
<td>0</td>
<td>0</td>
<td>79</td>
</tr>
<tr>
<td>*PTL</td>
<td>54</td>
<td>18</td>
<td>18</td>
<td>10</td>
<td>11</td>
<td>28</td>
<td>14</td>
</tr>
<tr>
<td>TL</td>
<td>112</td>
<td>33</td>
<td>33</td>
<td>55</td>
<td>28</td>
<td>15</td>
<td>65</td>
</tr>
<tr>
<td>Merged data</td>
<td>217</td>
<td>51</td>
<td>51</td>
<td>65</td>
<td>41</td>
<td>43</td>
<td>79</td>
</tr>
</tbody>
</table>

*For each variable, there were at least 10 cases of PTL, while for AEA there were 54 cases. For multivariate analysis, a minimum of 10 were required.

To ensure consistency throughout, only data obtained from patients who met the inclusion/exclusion criteria, detailed in Chapter 2, were included in this new set of analyses. The analysis are presented as a re-analysis of the data presented in Chapter 2 followed by an analysis of merged data.

6.2.1 Analyses

Data that were not normally distributed were normalised by logarithmic transformation and data for women who delivered at term were identified as zero (0) and those who delivered prematurely were identified as one (1). Each predictive variable was studied firstly by univariate linear regression and then as part of a multivariate analysis.

6.2.2 Multivariate analysis

Statistical analyses were performed at two levels: (1) only with the data from the current study and (2) on the merged data. The analysis was performed under the supervision of Dr. Maria Viskaduraki, the Biostatistics Manager and tutor at the Bioinformatics and Biology Hub. The data were analysed by using Stata Statistical Software version 14, Research Version (StataCorp LP, Texas, USA) and statistical significance accepted when p<0.05 and 95% CI recorded.
Chapter 6  Re-analysis of plasma anandamide for detection of preterm birth, the gestational age at delivery and the recruitment-to-delivery-interval and the possible contribution of other variables

6.3  Results

In order to assure that any data analysis of the merged data would be valid, the original data presented in Chapter 2 were re-analysed using the univariate and multivariate analyses. The outcomes, should thus be the same as those presented in Chapter 2.

6.3.1  Re-analysis and interpretation based on the data presented in Chapter 2

6.3.1.1  Logarithmic transformations

Plasma AEA and OEA data were not normally distributed (Chapter 2) and so logarithmic transformation was therefore applied. The use of logarithmic transformation was indicated for every variable with a skewed distribution (453). In addition, logarithmic transformation was used as it is more articulate when back-transformation is performed (454, 455). A first step was to determine what effect logarithmic transformations had on the use of plasma eCB concentrations in predicting PTL.

The histograms show the distribution of AEA in term (0) and preterm (1) birth groups before and after transformation (Figure 6-1) and for OEA (Figure 6-2) making them Gaussian distributed. The plasma PEA was already normally distributed (Figure 6-3).
Chapter 6   Re-analysis of plasma anandamide for detection of preterm birth, the gestational age at delivery and the recruitment-to-delivery-interval and the possible contribution of other variables

Figure 6-1: Logarithmic transformation of AEA data.
(a) the data not normally distributed (b) after logarithmic transformation the data are normally distributed. The data identified as (0) are for the term deliveries, whole (1) are for preterm deliveries.

Figure 6-2: Logarithmic transformation of OEA data.
(a) the data prior to transformation (b) the data after logarithmic transformation. The data identified as (0) are for the term deliveries, whole (1) are for preterm deliveries.

Figure 6-3: The PEA distribution.
Data were normally distributed hence not transformed.
Table 6-2 shows a comparison the analysis from the original data (already described in Chapter 2). AEA was statistically significant in both analyses. There was no significance for any other variable. All those variables that only contained data from the original study (logOEA, PEA and FAAH) of course produced the same outcomes with respect to the statistical coefficients. Multivariate analyses of these parameters (Table 6-3) showed that none of these variables were jointly linked to prematurity. This suggests that only AEA (whether log transformed or not) can be used to predict prematurity.

Table 6-2: Univariate logistic regression analyses for the prediction of preterm birth

<table>
<thead>
<tr>
<th>Variable</th>
<th>p-value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>logAEA</td>
<td>0.02</td>
<td>0.23 to 2.58</td>
</tr>
<tr>
<td>logOEA</td>
<td>0.09</td>
<td>-0.18 to 2.28</td>
</tr>
<tr>
<td>PEA</td>
<td>0.08</td>
<td>-0.01 to 0.19</td>
</tr>
<tr>
<td>Cervical length</td>
<td>0.76</td>
<td>-0.13 to 0.18</td>
</tr>
<tr>
<td>FAAH</td>
<td>0.29</td>
<td>-0.01 to 0.03</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>0.50</td>
<td>-11.53 to 23.07</td>
</tr>
</tbody>
</table>

*Only logAEA was significantly associated with prematurity.*
Table 6-3: Multi-variate logistic regression analysis for the prediction of PTL

<table>
<thead>
<tr>
<th>Variable*</th>
<th>p-value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>logAEA</td>
<td>0.74</td>
<td>-5.48 to 7.75</td>
</tr>
<tr>
<td>logOEA</td>
<td>0.47</td>
<td>-6.88 to 14.91</td>
</tr>
<tr>
<td>PEA</td>
<td>0.73</td>
<td>-0.74 to 0.52</td>
</tr>
<tr>
<td>Cervical length</td>
<td>0.91</td>
<td>-0.16 to 0.18</td>
</tr>
</tbody>
</table>

*None of the variables were statistically significant.

6.3.1.2 Regression analysis with gestational age (GA) at delivery as the continuous outcome variable

Once it was re-established that plasma AEA presented in Chapter 2 was indeed predictive of PTB, an important question was when is the best time to take the sample? To answer that query, a RDTI measurement using days instead of weeks was used. Figure 6-4 shows the relationships between gestational age (GA) (originally recorded in weeks and days, but converted to days) with the log transformed plasma AEA and OEA concentrations and non-transformed PEA concentrations. They also showed the relationship with lymphocytic FAAH activity. The data indicate an inverse relationship between gestational age at delivery with logAEA, logOEA, PEA, and FAAH, but a positive relationship with cervical length with the most accurate measurements appearing at ~260 days for the inverse relationship and ~270 days for the CL. The statistical data from these univariate analyses are presented in Table 6-2 and the results of the seven univariate regression analyses (one for each variable) show that there was no statistical significance in the prediction of gestational age at delivery by any of the different predictive factors.
Figure 6-4: Regression lines to the data used for the prediction of GA of delivery.
Graphs “a” to “e” represent the regression curves of logAEA, logOEA, PEA, cervical length (Cx Length), and FAAH, versus the GA of delivery.
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Table 6-4: Univariate linear regression analysis for the prediction of the GA of delivery

<table>
<thead>
<tr>
<th>GA at delivery</th>
<th>p</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>logAEA</td>
<td>0.14</td>
<td>-20.33 to 2.96</td>
</tr>
<tr>
<td>logOEA</td>
<td>0.77</td>
<td>-17.57 to 13.15</td>
</tr>
<tr>
<td>PEA</td>
<td>0.52</td>
<td>-1.63 to 0.84</td>
</tr>
<tr>
<td>Cervical length</td>
<td>0.38</td>
<td>-0.26 to 0.65</td>
</tr>
<tr>
<td>FAAH</td>
<td>0.41</td>
<td>-0.31 to 0.13</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>0.13</td>
<td>-3.45 to 25.46</td>
</tr>
</tbody>
</table>

Multivariate linear regression was performed to explore possible associations between gestational age at delivery and all of the possible predictive variables. No significant associations were identified (Table 6-3).

Table 6-5: Multi-variate linear regression analysis for the prediction of the GA of delivery

<table>
<thead>
<tr>
<th>GA at delivery</th>
<th>p</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>logAEA</td>
<td>0.13</td>
<td>-26.00 to 3.55</td>
</tr>
<tr>
<td>logOEA</td>
<td>0.26</td>
<td>-12.51 to 42.46</td>
</tr>
<tr>
<td>PEA</td>
<td>0.10</td>
<td>-2.72 to 0.29</td>
</tr>
<tr>
<td>Cervical length</td>
<td>0.21</td>
<td>-0.21 to 0.86</td>
</tr>
<tr>
<td>FAAH</td>
<td>0.37</td>
<td>-0.05 to 0.13</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>0.08</td>
<td>-24.50 to 1.37</td>
</tr>
</tbody>
</table>
Even though the regression lines (Figure 6-4) showed changes in GA with the different variables, both univariate and multivariate analyses showed no significant relationships.

6.3.1.3 Regression analysis with RTDI as the continuous outcome variable

Figure 6-5 shows regression lines fitted into the data using RTDI (days) as the outcome variable. Log transformed data were used when applicable.

The relationship of the variable RTDI in days and logAEA, logOEA, PEA, cervical length and FAAH was explored further using linear regression. In all cases, the most accurate RTDI was ~60 days. Table 6-4 shows the results from the seven separate univariate regression analyses and indicate that logAEA plasma concentrations was the only variable to be significantly associated with the prediction of RTDI, with number of RTDI days decreasing as logAEA increased (see Figure 6-5a). LogOEA did not reach statistical significance (p = 0.06), although a p-value close to the accepted value of p<0.05 suggests that an increase in sample numbers may have changed the relationship such that would become statistically significant. This was tested later (Section 6.4.2).
Figure 6-5: Regression lines to the data used for the prediction of RTDI in days.
Graphs “a” to “e” represent the lines of logAEA, logOEA, PEA, cervical (Cx) length, and FAAH versus days to delivery.
Table 6-6: Univariate linear regression analysis for the prediction of the RTDI in days

<table>
<thead>
<tr>
<th>Days</th>
<th>p</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>logAEA</td>
<td>0.04</td>
<td>-26.91 to -0.66</td>
</tr>
<tr>
<td>logOEA</td>
<td>0.06</td>
<td>-33.27 to 0.88</td>
</tr>
<tr>
<td>PEA</td>
<td>0.15</td>
<td>-2.43 to 0.37</td>
</tr>
<tr>
<td>Cervical length</td>
<td>0.53</td>
<td>-0.53 to 0.99</td>
</tr>
<tr>
<td>FAAH</td>
<td>0.53</td>
<td>-0.32 to 0.16</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>0.50</td>
<td>-11.53 to 23.07</td>
</tr>
</tbody>
</table>

*Only the AEA data had a predictive significance (p = 0.04).

Multivariate linear regression was performed to explore the possible association between the number of days (RTDI) and all of the exploratory variables. The cervical length was the only variable significantly associated with the RTDI (p = 0.04 and 95% CI 0.04 to 1.70) (Table 6-5).
6.3.1.4 Logistic Regression with “premature birth” as the binary outcome variable

To identify risk factors for preterm birth, the data were grouped into term and pre-term and logistic regression analysis applied. Table 6-6 shows the results of seven univariate logistic regression analyses (one for each variable). Only AEA was found to be a significant predictor ($p = 0.02$). The p-values for logOEA ($p = 0.09$) and PEA ($p = 0.08$) were close to being statistically significant, again suggesting that higher numbers might have resulted in statistical significance (Table 6-6).

Conversely, in the multivariate logistic regression of the association of prematurity with logAEA it was no longer detectable (Table 6-7) ($p = 0.74$), possibly due to the scarcity of the data.

Table 6-7: Multi-variate linear regression analysis for the prediction of the RTDI

<table>
<thead>
<tr>
<th>Multi-variate linear regression analysis</th>
<th>Days</th>
<th>p</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>logAEA</td>
<td>0.10</td>
<td>-41.60 to 4.08</td>
</tr>
<tr>
<td></td>
<td>logOEA</td>
<td>0.52</td>
<td>-29.37 to 55.61</td>
</tr>
<tr>
<td></td>
<td>PEA</td>
<td>0.82</td>
<td>-2.58 to 2.07</td>
</tr>
<tr>
<td></td>
<td>Cervical length</td>
<td>0.04</td>
<td>0.04 to 1.70</td>
</tr>
<tr>
<td></td>
<td>FAAH</td>
<td>0.14</td>
<td>-0.04 to 0.24</td>
</tr>
<tr>
<td></td>
<td>IGFBP-1</td>
<td>0.11</td>
<td>-36.03 to 3.96</td>
</tr>
</tbody>
</table>
Table 6-8: Univariate logistic regression analyses for the prediction of the risk of preterm birth

<table>
<thead>
<tr>
<th>Variable</th>
<th>p</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>logAEA</td>
<td>0.001</td>
<td>0.34 to 1.41</td>
</tr>
<tr>
<td>logOEA</td>
<td>0.09</td>
<td>-0.18 to 2.28</td>
</tr>
<tr>
<td>PEA</td>
<td>0.08</td>
<td>-0.01 to 0.19</td>
</tr>
<tr>
<td>Cervical length</td>
<td>0.46</td>
<td>-0.08 to 0.04</td>
</tr>
<tr>
<td>FAAH</td>
<td>0.29</td>
<td>-0.01 to 0.03</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>0.37</td>
<td>-0.82 to 2.18</td>
</tr>
</tbody>
</table>

6.3.2 Analysis of merged data

166 more samples were obtained for plasma AEA concentrations, an extra 42 data points for cervical length measurements and 79 extra data points for ofFN (full details about the number of samples are shown in Table 6-1).

Logarithmic transformation and identification (0 for term and 1 for PTL) were again used, as previously. AEA, OEA, and FAAH activity data were not normally distributed and so they were logarithmically transformed (Figure 6-6). By contrast, the data for plasma PEA, cervical lengths were normally distributed (Figure 6-7) and so were not transformed. The distributions of the remaining variables remained unchanged, because no additional data were included (Table 6-1).
Figure 6-6: Histogram of the merged AEA data normalisation.
(a) Data for 217 patients are not equally distributed. (b) Data transformed.

Figure 6-7: Merged data for cervical length distribution.
The data were normally distributed, hence not transformed.

6.3.2.1 Regression analysis of gestational age at delivery as a continuous outcome variable

Figure 6-8 shows the regression line fitted to the merged data for GA at delivery. This shows that GA at delivery was only significantly inversely associated with AEA (p = 0.004).
Figure 6-8: Regression line to the logAEA data used for the prediction of GA at delivery.

Table 6-9: Univariate linear regression analysis for the prediction of GA at delivery

<table>
<thead>
<tr>
<th>Univariate linear regression analysis</th>
<th>GA at delivery</th>
<th>p</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>logAEA</td>
<td>0.004</td>
<td>-2.27 to -0.43</td>
<td></td>
</tr>
<tr>
<td>logOEA</td>
<td>0.93</td>
<td>-2.45 to 2.23</td>
<td></td>
</tr>
<tr>
<td>PEA</td>
<td>0.28</td>
<td>-0.29 to 0.08</td>
<td></td>
</tr>
<tr>
<td>Cervical length</td>
<td>0.23</td>
<td>-0.03 to 0.11</td>
<td></td>
</tr>
<tr>
<td>FAAH</td>
<td>0.37</td>
<td>-0.05 to 0.02</td>
<td></td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>0.14</td>
<td>-3.52 to 0.53</td>
<td></td>
</tr>
</tbody>
</table>

No significant associations between GA at delivery and any of the other variables were found from the multivariate analysis although IGFBP-1 (Table 6-9).
Table 6-10: Multivariate linear regression analysis for the prediction of GA at birth

<table>
<thead>
<tr>
<th>Multi-variate linear regression analysis</th>
<th>GA at delivery</th>
<th>p</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>logAEA</td>
<td>0.19</td>
<td>-3.61 to 0.77</td>
<td></td>
</tr>
<tr>
<td>logOEA</td>
<td>0.38</td>
<td>-2.35 to 5.81</td>
<td></td>
</tr>
<tr>
<td>PEA</td>
<td>0.19</td>
<td>-0.36 to 0.08</td>
<td></td>
</tr>
<tr>
<td>Cervical length</td>
<td>0.19</td>
<td>-0.03 to 0.13</td>
<td></td>
</tr>
<tr>
<td>FAAH</td>
<td>0.49</td>
<td>-0.01 to 0.02</td>
<td></td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>0.08</td>
<td>-0.21 to 3.63</td>
<td></td>
</tr>
</tbody>
</table>

6.3.2.2 Regression analysis with RTDI as the continuous outcome variable

Figure 6-9 show regression lines fitted to the merged data for logAEA and cervical lengths. There was an inverse relationship between logAEA and RTDI. As the cervical length decreased, the number of days to delivery also significantly decreased. These significant associations are shown in Table 6-10.
Chapter 6  Re-analysis of plasma anandamide for detection of preterm birth, the gestational age at delivery and the recruitment-to-delivery-interval and the possible contribution of other variables

Figure 6-9: Regression lines for the merged data for logAEA and cervical lengths in relationship to RTDI.

Table 6-11: Univariate linear regression analysis for the prediction of RTDI in days

<table>
<thead>
<tr>
<th>Days</th>
<th>p</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>logAEA</td>
<td>0.02</td>
<td>-20.19 to -2.13</td>
</tr>
<tr>
<td>logOEA</td>
<td>0.06</td>
<td>-33.27 to 0.88</td>
</tr>
<tr>
<td>PEA</td>
<td>0.15</td>
<td>-2.43 to 0.37</td>
</tr>
<tr>
<td>Cervical length</td>
<td>0.002</td>
<td>0.50 to 2.03</td>
</tr>
<tr>
<td>FAAH</td>
<td>0.53</td>
<td>-0.32 to 0.16</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>0.50</td>
<td>-23.07 to 11.53</td>
</tr>
</tbody>
</table>

In multivariate analysis, the only significantly associated parameter was cervical length (p = 0.04, 95% CI 0.04 to 1.70) and logAEA lost its significant difference (p = 0.1, 95% CI -41.60 to 4.08).
Table 6-12: Multivariate linear regression analysis for the prediction of RTDI in days

<table>
<thead>
<tr>
<th>Days</th>
<th>p</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>logAEA</td>
<td>0.10</td>
<td>-41.60 to 4.08</td>
</tr>
<tr>
<td>logOEA</td>
<td>0.52</td>
<td>-29.37 to 55.61</td>
</tr>
<tr>
<td>PEA</td>
<td>0.82</td>
<td>-2.58 to 2.07</td>
</tr>
<tr>
<td>Cervical length</td>
<td>0.04</td>
<td>0.04 to 1.70</td>
</tr>
<tr>
<td>FAAH</td>
<td>0.14</td>
<td>-0.04 to 0.24</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>0.11</td>
<td>-3.96 to 36.03</td>
</tr>
</tbody>
</table>

### 6.3.2.3 Logistic Regression with “prematurity” as the binary outcome variable

Examination of the predictive value of each variable for the calculation of the risk of preterm birth showed a strong significance for AEA in a univariate analysis. None of the other variables showed significant association (Table 6-6). Multivariate logistic regression indicated that no predictor was statistically associated with prematurity (Table 6-13).

### 6.3.2.4 Analysis of oncofetal fibronectin

The predictive value of oncofetal fibronectin (ofFN) was examined by univariate analyses for the prediction of PTL and the RTDI (Tables 6-12 and 6-13, respectively). The results showed significance in predicting PTL ($p = 0.03$) but not for RTDI ($p = 0.09$).
Table 6-13: Univariate analysis of ofFN results with term/pre-term as the outcome variable and RTDI outcome

<table>
<thead>
<tr>
<th></th>
<th>p value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTL</td>
<td>0.03</td>
<td>0.13 to 2.90</td>
</tr>
<tr>
<td>RTDI (days)</td>
<td>0.09</td>
<td>-26.74 to 1.81</td>
</tr>
</tbody>
</table>

6.3.2.5 Analysis of fibronectin and IGFBP-1 combined

Since IGFBP-1 and ofFN are supposed to be useful predictors of prematurity in this cohort, the data from IGFBP-1 and ofFN measurements were combined into a single binary variable and logistic regression performed to test the association of prematurity with the combined variable; this was significant ($p = 0.02$) (Table 6-14).

Table 6-14: Univariate analysis of IGFBP-1 and ofFN combined results with term/preterm as the outcome variable

<table>
<thead>
<tr>
<th></th>
<th>p value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP-1 + ofFN</td>
<td>0.02</td>
<td>0.17 to 2.18</td>
</tr>
</tbody>
</table>

6.4 Discussion

In order to perform robust multivariate analysis, the variables to be examined are only of value if a minimum of 10 cases per variable develop the clinical complication of interest (456, 457). Therefore, in order to perform multivariate analysis on the value of the ECS in predicting PTL, it was necessary to choose variables where at least 10 women progressed to PTL. That was applicable on all variables available for analysis (Table 6-1); therefore, there was no restriction in data analysis.

Another fundamental principle in multivariate analysis is that even if some variables have not shown significant difference in univariate analyses, so long as one variable shows significance, then multivariate analysis should proceed, and such predictors should not be excluded from the analysis (169, 458-462). As a result, all potential predictors were used in this analysis.
Chapter 6  Re-analysis of plasma anandamide for detection of preterm birth, the gestational age at delivery and the recruitment-to-delivery-interval and the possible contribution of other variables

Analyses of the data presented in Chapter 2, and then with the merged data, confirmed that plasma AEA, as an individual marker, is a significant predictor of PTL ($p = 0.02$), but not in the multivariate analysis ($p = 0.74$).

An examination of the RTDI in days showed promising findings for log plasma AEA in the univariate analysis ($p = 0.04$), whereas in the multivariate study, only increased cervical length showed significance ($p = 0.04$). These data suggest that plasma AEA could provide a reasonable predictive test for PTL and of the RTDI. The data also suggest that a relationship might exist between AEA concentrations and CL in prematurity.

The observations that cervical length and IGFBP-1 and oFNF combined are significantly associated with prematurity indicates that the cohort under investigation were appropriate, and although, as mentioned in Chapters 1 and 2, cervical length results should always be interpreted with caution; the data are supportive of these factors being useful in the prediction of prematurity. The reason that cervical length should be considered with caution is that once the cervix starts to shorten, the type of management becomes secondary rather than primary (as PTL would be imminent), which means as a test transvaginal sonography (TVS) is not very useful for the prediction of PTL in asymptomatic, stable patients. A second reason is that cervical length measurements between clinicians and even by the same clinician when performing multiple measurements show significant variabilities. The predictive value of such measurements depends upon a few millimetres of difference and is thus really dependent upon an individual sonographers’ experience.

Among the non-significant results ($p = 0.06$), OEA showed a hint that it might be a future predictor of PTL. Because I have shown that increasing the number plasma AEA samples from 51 to 217 resulted in a p-value changing from 0.02 to 0.00, then it is possible that if a larger number of data points had been made available (or patients had been recruited) then significant results for plasma OEA may have been produced, providing an additional reliable predictor of PTL.

Unfortunately, the assay was unable to measure all three CBs at that time. If it had, then those data could have been merged with the data I collected and so could have produced more conclusive results for the entire study. Nevertheless, these observations provide the basis for future studies that may show that plasma AEA and OEA are good predictors of PTB.
Chapter 6  Re-analysis of plasma anandamide for detection of preterm birth, the gestational age at delivery and the recruitment-to-delivery-interval and the possible contribution of other variables

The merged data provided useful indicators for robustness of the analysis. The first was the increase in the number of patients who delivered prematurely from 33 to 55. The second sign was that univariate analysis showed plasma AEA as the only reliable predictor of the PTL risk of \( p = 0.001 \), the gestational age at delivery \( p = 0.004 \) and the RTDI in days \( p = 0.02 \). Cervical length was shown to be a predictor of RTDI: in both univariate \( p = 0.002 \) and multivariate \( p = 0.04 \) analyses, it was associated with PTL prediction in conformity with the literature (170-176). It also showed that the cohort under study (both as individual and as merged cohorts) were suitable for these studies.

The importance of significant results in a multivariate analysis is the possibility of deriving a predictive equation that can be used in clinical practice. In such an equation, the risk is estimated by using the calculated constant and the values of each predictive variable multiplied by their corresponding coefficient. The formula will thus be composed of the value of the constant with the addition or subtraction of the different variables, depending on coefficient of positivity or negativity.

Using the analysis in the table of the multivariate analyses of RTDI (Table 6-11) the following equation was derived:

\[
\text{Days} = 1.20 - (18.76 \times \log\text{AEA}) + (13.12 \times \log\text{OEA}) - (0.25 \times \text{PEA}) + (0.87 \times \text{CL}) + (0.10 \times \text{FAAH}) + (16.03 \times \text{IGFBP-1}) = \text{RTDI}
\]

Any of the non-significant variables can be removed from the model, however, if it is considered appropriate for clinical reasons, non-significant variables can be included in the equation. For instance, in the same example used, AEA does not show statistical significance, however, AEA findings were statistically significant in the univariate analysis of the RTDI \( p = 0.02 \) and in every other univariate predictive test. Consequently, one can argue that logAEA can be used in the equation. Taking into consideration significance of CL in the multivariate analysis \( p = 0.04 \), Table 6-17) and its predictability (RTDI) one can suggest that a larger study needs to be conducted where a comparison of CL results can be made with those of the CL plus logAEA of the plasma concentration.

The additional univariate analysis of ofFN showed a significant predictive value for PTL \( p = 0.03 \) but not for RTDI \( 0.09 \). In an “experimental” univariate analysis, where ofFN and IGFBP-1 swab results combined to produce a new single variable, improved their combined predictive value \( p = 0.02 \). One consequence of this significant finding is that it demonstrates that the correct
cohort was used in this study. It is important to clarify here that the data for ofFN was used as an individual predictor in the analysis, but not in the multivariate analysis, as it was not part of the original proposal. However, the additional “experimental” analysis of the combined predictive value of both IGFBP-1 and ofFN was performed in an “as realistic as possible” approach to what happens in daily clinical practice: in a pragmatic clinical scenario, clinicians would manage patients with a positive IGFBP-1 or ofFN measurement alike.

Neither ofFN nor IGFBP-1 showed any significant predictability when it came to RTDI. This is not surprising when one considers that ofFN is known to have high specificity when it comes to the diagnosis of PTL before 34 weeks (133), but it is also known that such risk counts on the concentration of the molecule (134), whilst the reliability of the test is not very good, primarily due to conflicting data presented in the literature where sensitivity values range between 37 and 90%. More importantly, the value of ofFN’s ‘predictability for PTL’ lies vaguely between 7 and 21 days (463), which is not useful when the RTDI values exceed that timeframe.

As for IGFBP-1, and despite its accuracy being similar to and if not superior to ofFN (155, 156) (accuracy: sensitivity: 74-100% and specificity: 77-98.2%), it cannot predict when labour might be initiated beyond 7 days of testing (145) and here again, the value of that prediction is vague. Even in cases of pPROM where 40% of the patients are expected to proceed to PTL (464-466) and where IGFBP-1 is used as a diagnostic test (416), there is no accurate prediction as to when such women will go into labour.

Due to the strong association between pPROM and PTL, other molecules have gained prominence in the prediction of PTL. For example, while the measurement of placental α-microglobulin-1 (PAMG-1) in the cervico-vaginal fluid, for the detection of pPROM, with a sensitivity 99% and a specificity 88-100% (467-475), the possible time of ‘predictability’ of labour does not exceed the 28 days (476) with a range between 7 and 28 days. This is interesting, but not as compelling as the data presented in this Chapter, where a much longer RTDI was predictable with less variability. The results from this present study and those in the worldwide literature indicate that predicting RTDI through the use of cervical swabs alone is insufficient. However, it is possible to conclude that plasma eCBs are more promising in detecting a RTDI than vagino-cervical or amniotic fluid tests. This was shown in Chapter 2 and confirmed here using previously collected data from a different patient cohort enabling more specific and powerful statistical analysis to be performed and presented.
6.5 Conclusion

It seems that the original premise for the studies presented in this thesis is correct and that PTL can be predicted by a single blood test! Plasma eCB concentrations (AEA and OEA) seem to offer a reliable promise in the prediction of PTL. There is no doubt that plasma AEA can achieve accurate predictive significance covering the risk of PTL, the GA at delivery and the RTDI in days. Such a test could revolutionise the management of PTL, as it will be the first biochemical test involved in a primary prevention of the condition. Plasma OEA, on the other hand, and possibly PEA (although the larger p-value suggest less so), could provide similar predictabilities if the number of recruits was increased. It would be interesting to see if increasing the numbers of subjects to a critical threshold might actually result in plasma PEA being included in the logistic equation.
Chapter 7 General discussion and future directions
7.1 Introduction

This thesis provides evidence that a single measurement of one of the eCBs can be used to predict (with some accuracy) the timing of birth. It also examines the possible mechanisms behind the derangement of that system so that one of the most common obstetric complications that trouble perinatologists and neonatologists’, i.e. preterm labour, can be better understood. The data presented herein suggest that a quick, cheap, reliable, and predictive test for PTL is within easy grasp. Such a test would prevent unnecessary admissions and allow clinicians to manage the pregnant women promptly and appropriately. The current tests used in clinical practice have a predictive value for up to two weeks and are used as part of secondary or even tertiary prevention, whereas the test presented in this thesis appears to have a predictive value of up to 8 to 10 weeks, depending upon whether plasma AEA measurements are used alone or in combination with plasma OEA measurements.

Before the inception of the studies that comprise this thesis, the ECS was shown to alter in pregnancy and parturition, with plasma concentrations of AEA increasing during miscarriage (180) and in the third trimester of pregnancy (355) with a further very large increase during term labour (310), potentially making it a promising tool for the prediction of PTB, especially in those women at risk for PTL. A pilot study showed that high-risk women for PTL tend to have higher plasma AEA concentrations (355), although these data needed confirmation and a mechanistic explanation.

As a result, this thesis focused primarily in examining the role of three eCBs in the prediction of PTL and examined changes in FAAH activity and serum sex hormones levels in women at risk of PTL. Finally, the placenta and FM s were examined for their expression of cannabinoid and sex steroid hormone receptors as well as the endocannabinoid enzymes, in an aim to elucidate the possible mechanism by which the ECS is involved in preterm parturition.

7.2 Plasma eCBs in the prediction of preterm labour

The marked increase in plasma AEA concentrations (Chapters 2 and 6) supported the original hypothesis set out in Chapter 1 that it is possible to predict PTL from a single blood test. High-risk women for PTL had higher plasma AEA concentrations, even when asymptomatic. Although the same analysis conducted for plasma OEA and PEA concentrations (the eCBs involved in the
“entourage effect” and promote the AEA concentrations) showed they increased in high-risk women, that increase was not statistically significant. The lack of statistical significance was most likely due to the limited number of samples (a theme that will recur throughout this discussion). The calculated sensitivity, specificity, NPV, and PPV for all three eCBs showed superior values when compared to those for CL, ofFN and IGFB-1 (Tables 2-5 and 6-14) suggesting that these observations offer hope for a more reliable predictive test for PTL, although a larger number of samples could have offered a more accurate cut-off point. Nevertheless, the ability to predict the gestational age at the point of labour as well as the RTDI from the same “single blood sample”, was an exciting finding because it will probably allow for more accurate management of such patients in the future. For example, the initial RTDI calculation using plasma AEA concentrations for high-risk women suggested a possible “safety window” of up to 10 weeks, but more importantly, the same outcome was noted for samples collected before 30 gestational weeks in asymptomatic patients who were recruited from the PPC. Certainly, these results warrant further research (as described in section 7.6), but they do raise the hypothesis that as a future test, this would prevent repeat visits to the clinic allowing for effective use of resources for those actually in need (something that can also be predicted from the plasma AEA test).

This observation could have a revolutionary effect in clinical practice because it suggests that the measurement of the plasma eCBs can offer a primary prevention months in advance of birth, something that none of the available tests can claim, which are reliable for only two weeks from the time of the examination and have poor sensitivities and PPVs. Furthermore, the opportunity to make the appropriate arrangements for patients who are at risk of imminent PTL, whether by the administration of steroids, inserting a cervical cerclage, or arranging an in-utero transfer can be predicted with the eCB test.

The initial RTDI assessment was performed by a relatively crude method where intervals were calculated in weeks. As a result, some bias may have been introduced (e.g. patients who delivered in exactly five weeks would be in the same group with patients who delivered in five weeks and 6 days). This potential inaccuracy was accounted for in Chapter 6, where the RTDI was calculated in days rather than in weeks and consequently the results were more reliable, but provided the same general conclusion that a single plasma AEA measurement can predict the timing of delivery.

Although both ofFN and IGFBP-1 measurements provide good NPVs, they are poor predictors of PTL (133, 136, 143, 144, 146, 160, 161) with IGFBP-1 being of no use in asymptomatic women (155, 157, 161, 165-169). Therefore, one third of pregnant women are admitted with ‘threatened
PTL’ and receive unnecessary treatment, even though most of their pregnancies go to term (17-19). Since starting this project, PAMG-1 assessment has been introduced into clinical practice and appears to produce better predictive values than other ‘more conventional’ tests (373, 374), even though its comparison with those tests has been limited. This suggests that in a best-case scenario, it too only gives a “safe window” of two weeks, as a part of any secondary prevention strategy. Even in my patient cohort, ofFN only confirmed that this safety window cannot be reached, as ofFN can predict PTL (p = 0.03; Table 6-14) but not the RTDI (p = 0.09; Table 6-15). As a result, in everyday clinical practice a lot of unnecessary admissions, managements, and parental anxiety seem to prevail, regardless of the type of cervico-vaginal test being used (17, 18, 20).

The only additional variable that could be of predictive value was CL assessment in my patient cohort. Nonetheless, it has been repeatedly advocated that cervical changes are not a good predictor per se rather than a contradictory assessment or an initial finding to the inevitable outcome of PTL because of the inherent intra- and inter-observer differences in cervical length measurements (477, 478). The optimal cut-off point of CL is also controversial in that CL < 30 mm (rather than the generally accepted < 25 mm) leads to PTL (479). Furthermore, in low-risk women with a mid-trimester short CL, only 25% proceed to PTL, while in high-risk women with a similar finding the rate is tripled (170, 480).

An extended assessment of plasma AEA measurements (Chapter 6), confirmed its ability to significantly predict PTL (p = 0.001), the RTDI in days (p = 0.02), and the gestational age at delivery (p = 0.004), especially in those women who were asymptomatic, providing three crucial management conclusions from a single blood test, thereby providing extra reassurance for the clinician and patient.

One more advantage of the assessment of the plasma eCBs is its cost effectiveness. In comparison to the other tests, it is significantly cheaper. The IGFBP-1 kit costs the NHS £20-30, while the ofFN kits are universally four times more expensive (155). The IGFBP-1 kit (Actim® PROM) that predicts pPROM is even more expensive. In addition, despite the fact that pPROM increases significantly the risk of PTL, a meta-analysis comparing the IGFBP-1 kit (Actim® PROM) to PAMG-1 (AmniSure®), expecting to favour the latter, did not show significant superiority of one test over the other (415). By contrast, the mass spectrometric eCB analysis of a single sample would cost the NHS less than £20-30, because of the higher sample throughput available. Adding to that cost, the presence of false positive results (and subsequent hospitalisation and medication),
the price of an inadequate diagnosis rises far higher than what seems to be simply the price of a kit.

Correlation analyses were used for all parameters measured in this thesis. This was done so as to start to understand any potential relationships and mechanisms of action. The three plasma eCBs are all substrates for FAAH and so positive correlations among them due to the “entourage effect” was expected and found. Furthermore, when comparisons were performed among patients with positive or negative IGFBP-1 tests and in women with CL > 25mm the results were equally reliable. These data support the “entourage effect” hypothesis, even though OEA and PEA plasma levels provided a suggested predictive value for PTL (but without statistical significance), the positive correlations with plasma AEA concentrations suggest that similar changes would occur in a larger patient cohort. Consequently, with a higher number of samples the hypothesis that OEA and PEA can have predictive importance in PTB could be tested and a reliable cut-off point generated from the subsequent ROC curves, as was achieved with plasma AEA (Chapter 2).

The possible question here is: ‘How can we identify a reliable cut-off point above which women at risk can be identified?’

There are different methods that can be used, but in our analyses ROC was the method of choice, as it includes specificity/sensitivity or NPV/PPV and offers valuable diagnostic material (481). From the available data, it appears that plasma AEA offers a novel diagnostic predictor of PTB, with a concentration > 1.095 nM (from Chapter 6) having a specificity of 87.14 %, a sensitivity of 25.93 %, a NPV of 70.24 % and a PPV of 61.2 %, and thus providing a better test than currently available anywhere.

### 7.3 Putative mechanisms

Once I had established that plasma eCBs could be used to predict PTB, it was considered important to assess the possible mechanisms that regulate those concentrations, where the eCBs could be synthesised and where they are catabolised. It was known before I started these studies that both progesterone and oestradiol inhibit lymphocytic and uterine FAAH activities (303, 317, 320, 390), and so changes in the levels of the sex steroid hormones could be the cause of the observed increases in plasma AEA. This hypothesis was tested, but the data were disappointing because most measurements and correlations were not statistically significant, possibly due to the small number of samples or because of the different possible mechanisms that result in emergency
admission might have affected ECS activity differently, resulting in the observed variability within the patient groups. There were, however, negative correlations between plasma E2 and AEA concentrations and E2 and PEA concentrations in this cohort suggesting that either NAPE-PLD expression was repressed or FAAH activity was elevated. But unfortunately, none of the FAAH comparisons, correlations or even RTDI analyses showed statistical significance, although FAAH activity in blood lymphocytes was unexpectedly found to be slightly higher in the group that delivered prematurely. Although, I planned to assess blood NAPE-PLD activities too, these samples were sent to collaborators in Italy, but were not assayed (see later in section 7.3).

The activity of FAAH was not only found to be higher in the blood of women who went through PTL, but also in all labour-affected placental transcripts, regardless of gestational age, suggesting that during labour, mechanisms exist to ‘switch on’ FAAH expression. At first sight, this may be counter-intuitive because plasma eCB concentrations are increasing at this point, but on second viewing it makes some sense, because the tissue samples are collected after the 3rd stage of labour has completed. In essence, the tissue is no longer viable. Similarly, in the fetal membranes the same increase in FAAH protein expression was noted in the PTL group in the chorion and decidua, while at the transcript level the reverse occurred supporting my ‘tissue death’ hypothesis. An alternative explanation for the discrepancies between protein and transcript expression could be attributed to the fact that IHC may have targeted one of the two FAAH isoforms, whilst the PCR primers were designed to detect both FAAH isoforms. Regardless of the statistical analysis outcomes, there was a reliable pattern of change of FAAH from the PTNL to PTL group, suggesting not only the involvement of FAAH in the process of labour, but more specifically in premature labour. By contrast, NAPE-PLD protein expression was visually greater in the PTNL group than in PTL group, in both the placenta and FM. The same pattern was seen in the placental transcripts, while in FM the NAPE-PLD transcripts were significantly lower at PTL. This reflects the higher consumption of the NAPE-PLD enzymes that led to higher plasma AEA concentrations. Collectively, this can be explained by the idea that as AEA is not needed in non-labouring states, its enzyme concentrations would be higher.

Having established that plasma AEA concentrations change in women at risk of PTL, it was necessary to examine the two CBs in the feto-maternal interface, because of course, the ligand cannot do anything unless the receptor it binds to is present. Several studies have previously shown the presence of CB1 in the placenta and FM, but not if its expression is modulated at term or by labour (322). By contrast, studies had shown the presence of CB2 in the placenta, and its
expression at term, but not if its expression is modulated by labour (422). CB2 expression in the FM had not been studied before, so any data generated here would be novel.

CB1 expression was lower in the placenta in all women in labour, indicating either a role for the activated receptor in the quiescence of pregnancy or more likely, allowing for the plasma eCBs to act/concentrate elsewhere, such as in the amnion, where CB1 expression was significantly higher in the women with PTL. Similarly, placental transcripts were significantly lower in the preterm stages, whilst in the FM the opposite was observed, suggesting that if AEA has an impact on the CB1 in the feto-maternal interface, it is more likely to be in the FM and more specifically in the amniotic layer. Hence, the CB1 correlations to PR and its isoforms were all positive in the FM.

Nevertheless, these observations on the actions of CB1 in labour were nothing when compared to that of CB2 in labour, where CB2 expression was higher in the placental tissues of the PTL group, mirrored by a marked rise in transcript levels. In the amniotic layer, changes were more evident, as CB2 expression was lowest in PTNL patients and highest in PTL patients (Figure 5-7). These data are similar to that of CB1, suggesting that CB2 has a critical role in PTL as its placental transcripts levels are higher at this stage and amniotic expression is notably higher too. At this point it should be pointed out that CB2 protein was found to be present in all FM layers at term and during prematurity. This should be noted because this was a novel finding and that the levels were modulated indicative a role for this receptor in the process of labour-induced FM rupture. Whether these changes are also present in pPROM, remains to be tested.

From all these data, it is not difficult to conclude that the ECS is involved in parturition, as the plasma results indicated, but when it comes to prematurity the entire system acts through a different mechanism from that shown at full term, with the CB1 activity during labour being focused on the FM, while CB2 is the principal cannabinoid receptor in placenta, especially in the PTL group.

Because the plasma E2 and P4 data were inconclusive, I thought that changes in the expression of their receptor isoforms might provide clues towards understanding how the components of the ECS might be regulated in the placenta and FM. The use of an antibody shown to identify all PR isoforms in IHC (PR-PAN) allowed a baseline from which to study the other PR isoforms and how they might link to the observed levels of the ECS. The significant decrease of all PR isoforms
from PTNL to PTL in all of the placental, chorionic and decidual tissues was not noticed in term tissue samples, suggesting that different patterns of PR isoform expression are associated with prematurity. This is not a new idea, but previously identified and discussed by others (335, 337), but what my study highlighted was that parturition in prematurity does not count solely on the functional withdrawal of the PR found in the myometrium at term (482), but also in the reduced expression of the entire receptor repertoire in the placenta and FM.

This notion was supported by the observation that levels of the PR-C transcript in the placenta was very similar to that of PR-PAN expression, while in the FM, PR-C transcripts tended to increase from a low level in the PTNL group to a higher level in the PTL group. The placental transcripts showed a decrease in all labour affected tissues, while in the FM, PTL transcripts were notably higher, suggesting a possible role of PR-C in PTL. As a result, the previously suggested role of PR-C in term parturition (328) seems to be applicable also in prematurity. The pattern in the changes observed in ER-alpha transcript levels in the FM was similar to that of PR-C, suggesting that the expression of these two receptors are related. Unfortunately, although I planned to examine ER-alpha and PR-C expression using IHC, I ran out of time and could not complete those studies. Consequently, I had to rely on the PR-C transcript levels through mathematical manipulations, in which PR-C transcript levels were calculated rather than measured, by subtracting PR-PAN from PR-AB and then relating that to the levels found in the control group (the TNL group). This method confidently identifies the levels of PR-C transcripts because the primers used were designed to detect PR isoforms A, B and C, and not any of the other isoforms (e.g. M, S, or T) or the recently identified membrane PR isoforms that are thought to be aquaporins (483, 484).

An equally important observation was confirmation of the presence of ER-alpha receptor transcript in the fetal membranes, especially after considering the difficulty others have had in detecting the two main ER isoforms in the FM (431-433). The only disappointment was that there has been no enough time to analyse the expression of the beta isoform in the FM samples, since this could have revealed a possible new avenue of research into the mechanisms of parturition.

### 7.4 The involvement of the ECS in the mechanism of preterm labour

In modern biology, the types of mechanisms available can be divided into three main categories: causal, explanatory and strategic (485, 486) that respectively, cover the processes in the science
according to the depth of the interpretation of every mechanism. The initial approach into the study of the role of the ECS in PTB (181) covered the “causal” mechanism level, because those observations suggest the involvement of the plasma AEA and other eCBs in parturition. The data presented in this thesis were to confirm that initial ‘mechanism’ and then to expand it to the “explanatory” level. The key observation was that although the ECS is involved in the mechanism of parturition, especially in prematurity, it did not provide that detailed explanation of what the ECS actually does, but did what all scientific studies should do and raised additional question for the future (see section 7.6), such as ‘how does the ECS integrate with the actions of the sex steroid hormones in parturition?’ The pivotal observations that the patterns of protein expression and transcript levels in the placenta and FM in the preterm (PTL and PTNL) samples differ from those at term (TL and TNL), suggest that the ECS has a role to play in PTB within the placenta and FM. However, although the patterns of expression and subsequent correlations were very similar, the actual differences were in most cases not statistically significant, and regardless of this, the patterns in prematurity samples did not always follow the corresponding analysis in term samples. This was noticed in almost all comparisons and analyses. Not surprisingly, all the significant differences between labour affected biopsies and CS biopsies noticed in the PTNL and PTL groups, had no corresponding alterations in the TNL and TL groups. Only in some cases could a similar pattern be noted in both term and preterm comparisons (e.g. in the CB1 placental protein expression (Figure 4-3) or in the FAAH placental transcript levels (Figure 4-7)). The PR-C transcripts in the FM (Figure 5-27), despite the lack of statistical significance, showed an increase in all labour affected tissues but with a very obvious change in the PTL group when compared to the PTNL group. These data suggest that both the ECS and the receptors for some PR isoforms are intimately involved in premature parturition and may be linked. Further experimentation of this hypothesis is therefore justified. Furthermore, there were several other examples where receptor or enzyme expression were different in PTNL and PTL samples when compared to TNL and TL samples. For example, NAPE-PLD placental expression in PTL was much lower than in PTNL (Figure 4-12), while a similar comparison at term offers neither significance nor a similar pattern. The same applies to PR expression in both placentae (Figure 4-15) and the chorio-decidual tissues (Figures 5-15 and 5-20), and CB2 transcript levels in the placenta (Figure 4-4) and FM (Figure 5-22). These data suggest that the changes from the non-labouring to a labouring state noticed in prematurity seem to be different from those occurring at term. These patterns suggest that parturition in prematurity does not necessarily follow the same mechanism of that at term. If such a hypothesis is shown to be correct, then preterm labour probably follows more intricate mechanistic pathways than those used by the same tissues at term.
7.5 Strengths and weaknesses

The main strength of this study was the way it was conducted, with a single operator responsible for everything from initial patient recruitment through to analysis and dissemination of the data, with one minor exception, the running of the UHPLC-MS/MS. The reason that I did not perform the actual UHPLC-MS/MS analyses, was that the ‘machine’ is a very complex piece of equipment that required specialists to run it. That should have been a strength, but actually turned out to be a weakness and created a major limitation. There were also several other limitations in this study, some of them major and some minor, even though what may seem to be a relatively minor and irrelevant incident at the time can destroy an entire study. These incidents are outlined here.

7.5.1 Major limitations

The first major limitation occurred when recruiting patients in the PPC, who having been through a PTB previously, were apprehensive about participating in the study. The main issue of concern was the speculum examination to obtain a cervical swab and so only 25% of those available for recruitment were actually recruited. Consequently, the inclusion criteria were amended and genital swab examination was not rendered compulsory. This resulted in a lower than expected number of IGFBP-1 and oFNF samples being made available for later analysis. Recruitment of patients for biopsy (placenta and FM) was not a problem and more than 200 samples were collected.

7.5.2 Minor limitations

The power calculations for the number of plasma samples required were performed based on the pilot study and showed that 122 samples were necessary. A similar power calculation was performed during the actual study with the new data obtained and it showed a similar result. The final AEA analysis (Chapter 6) was performed with the necessary number of samples, but still some analyses (particularly the PEA and OEA plasma measurements and serum E2 and P4 measurements) suggested the need for a larger number of samples. A similar challenge was faced with the placental and FM samples (Chapters 4 and 5), where the original number of samples required was based on validated methods. Indeed, some data were sufficiently powered to obtain statistically significant results, but other results indicated a larger sample size. Given the previously mentioned limitations with laboratory accessibility, staffing, and funding it was difficult to process and assess more specimens, even though I wanted to.
One more point that may be considered a ‘limitation’ was the use of real time, qRT-PCR for the measurement of transcript levels, especially for the PR isoform analyses might have been improved if I could have used northern blotting for the identification of the mRNA obtained from the placenta and FM biopsies. Using northern blotting allows the direct measurement of the different isoforms on the same nylon blot and so the data are directly comparable, whereas in the qRT-PCR method, some mathematical manipulation was required. The problems associated with the two commonly used northern blot methods [the use of radioactive probes, something that makes the experiment technically difficult and potentially dangerous, or non-radioactive colourimetric or fluorimetric probes, [although safer, but really expensive and technically more difficult (487, 488)] precluded their use in these studies.

7.6 Unanswered questions and future research

The clear observation that plasma AEA concentrations are predictive of PTB and can be used in predicting the actual time of delivery is of utmost importance with respect to this thesis, although the value of the other eCBs is less clear. The reason for this may be because all the blood samples were obtained from women at different gestational ages, starting from the age of fetal viability. A similar, future project, where the gestational age of recruitment is unified, would probably offer a more accurate cut-off point and have more universal acceptance in a routine clinical setting. Ideally, instead of limiting the study in high-risk women, a bigger cohort should be included and these women should be from different medical and obstetric backgrounds. By doing that, the resultant observations may offer a better understanding of the effect of eCB on parturition as well as on the patient’s underlying and presenting conditions. In this case, all 3 of the eCBs measured in this thesis and the untested eCB, 2-arachidonoylglycerol (2-AG) should be assessed, as well as the activity of both the enzymes that degrade and synthesise the eCBs (FAAH and NAPE-PLD) and those that synthesise and degrade 2-AG (diacylglycerol (DAGL) and monoacylglycerol (MAGL), respectively), and at different stages of pregnancy. With this in mind, the following would be proposed:

1). Plasma assessment for AEA, OEA, PEA, 2-AG, NAPE-PLD, FAAH, DAGL and MAGL would be made on blood and plasma samples collected at 20 gestational weeks (the age of viability in the USA), 24 weeks (the conventional international age of viability), 28 weeks (the age under which fetuses are considered very early preterm and the morbidities are at a maximum) and 32-34 weeks (the gestational age after which fewer interventions and complications are expected for the fetus). I would envisage that these studies would be successful and a clear RTDI would be identified.
2). The next step would be to standardise the test. To do that, the index biomarkers for PTB prediction would be examined in pregnant women at a time point equal to the RTDI before the time of viability. For example, since 24 weeks is the time of abortion limits and fetal viability, then I think the correct time to do this would be a standardised 24 weeks. To do these two studies effectively and efficiently, a multi-centre study would be suitable. This not only will reduce the length of the study, by making patient recruitment faster and more prompt, but it will also offer more subjective results through reproducibility of the experiments by different research groups, laboratories and equipment.

3). The ultimate aim of the plasma eCB test is to reach a stage where a prick test, like those performed by the diabetic patients, will be able to measure the plasma eCB concentrations within seconds would be used. For such a test to become available, the antibody technology to detect the eCBs on immobilised substrates would need to be created and validated, as has been achieved for the prostaglandins. The implementation of such a test would not only reduce the number of unnecessary admissions, but also reduce the number of referrals to the PPC, as such tests could be performed in a primary care setting.

4). One of the major limitations of this thesis was that the placental and FM studies lacked statistical power to reach real significance, even though the data tended towards significance. Consequently, these experiments will need to be repeated so that IHC covers every isoform of every protein and should be complemented with equivalent transcript data. This would require the collection of a new set of samples, ideally matched to the plasma eCB and enzyme activities. The ultimate ECS examination in placental and FM tissues will be concluded if the samples are obtained from patients whose plasma eCBs and enzymes have been already assessed in the antenatal period. Similar studies on the sex steroid hormone receptors and the serum levels of their ligands would provide additional information.

5). Similar analyses on the myometrium is suggested as a future direction, but with resilient reservation. As discussed in Chapter 4 (section 4.6), such a study can possibly offer answers to the involvement of the ECS and sex steroid hormones in parturition, especially if the results are combined with the findings from blood, plasma, serum, placenta, and fetal membranes. However, obtaining myometrial samples is technically very difficult, with ethical approval difficult to obtain, because procuring such samples from pregnant uteri carries several future risks for such
patients (398). Tissue banks can provide the samples required, both in quality and quantity, but those samples will not be serving the purpose of the suggested study, as, preferably all samples (plasma, myometrium, placenta, and FM) would be best obtained from the sample patient within the cohort.

A caveat to all of the proposed studies is that the number of patients recruited in every group needs to be sufficient (discussed in Chapters 4 and 5) so as to be of universal value in the management of patients at risk of PTB. This thesis provides the material to aid in those power analyses.

Finally, if we accept the validity of the hypothesis that term and preterm labours are based on mechanisms that are not entirely similar, then future studies should work on a ‘strategic’ mechanism that tests the hypothesis that the ECS and sex steroid hormones combine to not only regulate PTB, but also the entire process of parturition. The aim of such a study would be to search for not just a causal mechanism rather than a new explanatory mechanism for premature parturition.

### 7.7 Conclusion

This thesis has shown that changes in components of ECS can predict preterm labour, the length of pregnancy, and the timing of parturition. It has shown that the ECS is involved in the process of labour and more specifically in PTL, supporting the original hypothesis that many answers to questions related to preterm parturition can be obtained from a single blood test measuring the concentrations of plasma AEA. The changes in the ECS in the feto-placental interface, as well as the alterations in the sex steroid hormone receptors, in particular PR-C, indicate that parturition relies on the changes in receptor isoforms during labour, as predicted by Csapo’s functional progesterone withdrawal mode (489). That CB2 is the predominant player in both placental and FM tissues, while amniotic CB1 and PR-C are the suggested key players in PTL, was a key observation, has potential therapeutic potential, in that these might provide targeted therapy for the prevention of pPROM. Last, but not least, it seems that the mechanism of labour during term does not necessarily follow the same pathway as labour in prematurity.
Chapter 8   Appendices
8.1 Ethics committee letter of approval

Leicestershire, Northamptonshire & Rutland Research Ethics Committee 1
1 Standard Court
Park Row
Nottingham
NG1 6NN

Telephone: 01159123344
Fax: 01159123300

13 June 2006

Professor J Konje
Professor of Obstetrics & Gynaecology
University of Leicester
Robert Kipatrick Clinical Sciences Building,
Leicester Royal Infirmary
Leicester, LE2 7LX

Dear Professor Konje,

Full title of study: Endocannabinoids in Reproduction Tissue Bank
REG reference number: 08/Q2501/48

Thank you for your letter of 01 June 2006, responding to the Committee’s request for further
information on the above research and submitting revised documentation.

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

Confirmation of ethical opinion

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

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the
attached document. You are advised to study the conditions carefully.

Approved documents

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

<table>
<thead>
<tr>
<th>Document</th>
<th>Version</th>
<th>Date</th>
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<td>1</td>
<td>24 February 2006</td>
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<tr>
<td>Investigator CV</td>
<td>1</td>
<td>23 February 2006</td>
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<tr>
<td>Protocol</td>
<td>2</td>
<td>01 June 2006</td>
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<tr>
<td>Peer Review</td>
<td>2</td>
<td>13 February 2006</td>
</tr>
<tr>
<td>Letter of invitation to participants</td>
<td>2</td>
<td>30 May 2006</td>
</tr>
<tr>
<td>Participant Information Sheet</td>
<td>3</td>
<td>30 May 2006</td>
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<tr>
<td>Participant Consent Form</td>
<td>2</td>
<td>01 June 2006</td>
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<tr>
<td>Response to Request for Further Information</td>
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</tr>
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</table>

An advisory committee to Leicestershire, Northamptonshire and Rutland Strategic Health Authority.
**Leicestershire, Northamptonshire & Rutland Research Ethics Committee 1**

**LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION**

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

<table>
<thead>
<tr>
<th>REC reference number:</th>
<th>Issue number:</th>
<th>Date of issue:</th>
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</tr>
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<tbody>
<tr>
<td>09/Q250148</td>
<td>1</td>
<td>13 June 2006</td>
<td></td>
</tr>
</tbody>
</table>

**Chief Investigator:** Professor J.C. Konje

**Full title of study:** Endocannabinoids in Reproduction Tissue Bank

This study was given a favourable ethical opinion by Leicestershire, Northamptonshire & Rutland Research Ethics Committee 1 on 12 June 2006. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS Care organisation has been confirmed.

<table>
<thead>
<tr>
<th>Principal Investigator</th>
<th>Post</th>
<th>Research site</th>
<th>Site assessor</th>
<th>Date of favourable opinion for this site</th>
<th>Notes (if any)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Professor J.C. Konje</td>
<td></td>
<td>University Hospitals, Leicester Royal Infirmary, Leicestershire, Northamptonshire &amp; Rutland Research Ethics Committee 1</td>
<td>Professor of Obstetrics and Gynaecology and Honorary Consultant</td>
<td>13/06/2006</td>
<td></td>
</tr>
</tbody>
</table>

Approved by the Chair on behalf of the REC:

(Signature of Chair Administrator)

(Date)

(Name)

(1) The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.
8.2 Patient information leaflet for plasma eCB study

PATIENT INFORMATION LEAFLET

(Pregnant Volunteer in Preterm Labour)

Title of Study:

The role of endogenous cannabinoids (chemicals similar to cannabis but produced naturally in the human body) in reproduction

Principal Investigator: Professor Justin Konje
Professor of Obstetrics and Gynaecology
Reproductive Sciences Section
University Hospitals of Leicester NHS Trust
Leicester Royal Infirmary
Leicester, LE2 7LX
Phone: 0116 252 5826
Pager through switchboard.

Invitation paragraph

You are being invited to take part in a research study. Before you decide it is important for you to understand, why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.
What is the purpose of the study?

Endogenous cannabinoids are naturally occurring substances in the body, which are similar to cannabis. Studies in animals have shown that these substances are vital for pregnancy to succeed. High levels are associated with miscarriages but a critical level in the blood is essential for a successful pregnancy. The precise way in which these substances influence human reproduction is not known. While there have been lots of studies on the role of these substances in animal reproduction, very little research has been undertaken in human beings. The main reason for this has been the inability of researchers to measure these substances in the blood. We very recently developed a means of measuring these substances and successfully used the method in a small group of pregnant women and non-pregnant women. Our results were very encouraging and supported our belief that these substances play a part in successful pregnancies. Despite our results, there are several unanswered questions about the exact role of these substances in reproduction. We believe that by defining these questions and looking for answers, we will provide opportunities for improving the outcome of pregnancies especially in women who undergo repeated miscarriages.

We would therefore like to study further the different ways in which these substances may be involved in:

- women during normal pregnancy and
- women with different complications of pregnancy

In addition, we would like to investigate:

the way in which these substances work in different parts of the body (for example the womb, fallopian tubes, ovaries, placenta) in pregnant women and those who are not pregnant.

We hope to do this by:

- measuring the levels of these cannabis-like substances in the blood, and the tissues which we collect
- studying the way these substances work on the tissues we collect and
- investigating the genes that control how the body produces them and how they work in the various tissues we collect.
The tissue(s) collected will be used for the research but any left over will be kept in a special bank called the “Female Reproduction Research Tissue Bank” at the University of Leicester. We will ask for your permission to put your tissue(s) into this Bank but before we do this, you will be given an information leaflet about the bank to read and to give us permission by signing a consent form. If you do not want your tissue(s) to be kept in the Tissue Bank, it will be used only for the studies outlined above. Any future research will only be undertaken on the tissues after the Ethics committee has given its approval.

**Why have I been chosen?**

You were chosen because you are presenting with symptoms of preterm labour. We are inviting as many women as possible with preterm labour to partake in the study.

**Do I have to take part?**

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw from the study at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

**What will happen to me if I take part?**

If you agree to take part, a small amount of blood (one tablespoonful) will be taken from your arm. When you have your swab test to check whether you are actually in preterm labour, we will use part of the test result for this study.

If then go on have an emergency Caesarean section, we would again take a blood sample whilst you are having your operation. Once the baby has been delivered, we would like to take a very tiny tissue from your womb (less than one tenth of an inch from the edge of the hole through which we deliver the baby). Taking this sample will not affect your pregnancy or any future pregnancies you may have.

After your baby has been delivered and separated from the placenta, we would like to take some blood (one tablespoon) from the cord attached to the placenta and from the placenta itself.

The tissue obtained from you will not be used to undertake any genetic tests whose results may have adverse consequences on either you or your families insurance or employment.
What do I have to do?

If you would like to take part in the study, the only thing you need to do is to sign the consent form and allow us to take the necessary samples. There are no restrictions on what you may or may not do.

Will I receive payment for the tissue that I donate for this research study?

You will not receive any payment for the tissue. The tissue is a gift - neither yourself nor your relatives will benefit from any inventions that result from the use of the tissue.

What are the side effects of any treatment received when taking part?

None, since this research does not require you to take any treatment.

What are the possible disadvantages and risks of taking part?

The only disadvantage to you of this study is the slight discomfort (scratch) you may feel when your blood samples are taken. However, we will keep this to a minimum by taking the blood when you will normally have a pregnancy blood test.

What are the possible benefits of taking part?

You will not benefit directly from taking part in this study. However, with your help we may be able to help patients in future by understanding the way in which these substances are involved in the process of pregnancy.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the substances being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw, your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

What happens when the research study stops?

This will not affect you or your baby as you are not receiving any medication for this study.
What if something goes wrong?

If you are harmed by taking part in this research project there are no special compensation arrangements. If you are harmed due to someone’s negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Will my taking part in this study be kept confidential?

If you consent to take part in the research any of your medical records may be inspected by a member of the research team for the purposes of analysing the results.

All information, which is collected about you during the course of the research will be kept strictly confidential. Any information about you, which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?

Please be reassured that personal information will not be published or presented. However, the findings of the whole study will be presented at various national and international meetings and published in medical journals. These presentations and publications will not probably happen until at least 2 years from the start of the study to allow us to gather information. If you would like to see the findings, a copy of this could be obtained from your research doctor or from the medical library.

Who is organising and funding the research?

This is a huge research programme being funded by the University of Leicester, University Hospitals of Leicester NHS Trust and BUPA Foundation (a UK Charity). We plan to seek for additional funding from various UK charities and the Medical Research Council to support this programme.

Who has reviewed the study?

All research that involves NHS patients or staff, information from NHS medical records or uses NHS premises or facilities must be approved by an NHS Research Ethics Committee before it
goes ahead. Approval does not guarantee that you will not come to any harm if you take part. However, approval means that the committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits and that you have been given sufficient information on which to make an informed decision.

For more information, who do I contact?

Professor Justin Konje
Reproductive Sciences Section, CSMM
University of Leicester
Robert Kilpatrick Clinical Sciences Building
Leicester Royal Infirmary
Leicester LE2 7LX

Telephone: 0116 252 5826 or via Pager through the Leicester Royal Infirmary switchboard on 0116 2541414.

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

You will be given a copy of the information sheet and a signed consent form for you to keep.
8.3 Patient information leaflet for placental and fetal membrane study

(Version 1c – March 2005)

“Novel progesterone receptor expression control at the fetal-maternal interface and its involvement in human parturition (the birth process)” – a study into the hormonal control of premature birth.

Principal Investigator: Dr Anthony Taylor

This study is sponsored by: University of Leicester

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.
What is the purpose of the study?

This is a 3-year study into the action of the pregnancy hormone, progesterone, in the control of the birth process. This is being performed in order to help us understand why some babies are born prematurely, and to help us develop treatments to prevent this.

In order to do this, we need to look at a number of types of tissue, including placenta and attached membranes women who have delivered normally at full term. The tissue samples will be collected anonymously (we will not take any of your personal details), and will be used for molecular and cellular studies on the control of progesterone action. Most of the sample we take will be analysed immediately, however some may be stored for analysis at a later date. Once the study has been completed your sample will be destroyed. The research study will be performed at the University of Leicester.

Why have I been chosen?

You have been chosen because your baby was born normally and at full term. Approximately 10 women who have had normal births at full term will be included in the research study.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

After your baby and placenta have been safely delivered, the research team will take a sample of the placenta (2 cm x 2 cm) and attached membranes (1 cm x 3 cm), and the remainder disposed of by the hospital in the routine way.

What do I have to do?

Nothing. We do not require any further action from you after your placenta and attached membranes has been collected and processed.
Will I receive payment for the tissue that I donate for this research study?

No. You will not receive any payment for the tissue. The tissue is a gift - neither yourself nor your relatives will benefit from any inventions that result from the use of the tissue.

What are the possible disadvantages and risks of taking part?

There are no risks to taking part, nor any disadvantages to you. The tissue samples are being removed as part of your planned clinical treatment, and we are asking your permission for us to use a small part of this for our research study. As we are not taking any of your personal details, you will not be contacted by us in the future regarding the tissue sample that you donate to our research project.

What are the possible benefits of taking part?

There is no clinical benefit to you from taking part in this study, however, the information we obtain from using your sample in the research may lead to ways of preventing premature birth.

What if something goes wrong?

There is little chance of you being harmed by taking part in this research project, and so there are no special compensation arrangements. If you are harmed due to someone’s negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms would be available to you.

Will my taking part in this study be kept confidential?

Yes. We will not be taking any of your personal details when we collect the tissue sample, therefore your participation in this study is entirely confidential.

What will happen to the results of the research study?

The research study will last for 3 years, and after analysis of the data we aim to publish our findings in scientific journals. If wished, a copy of the results may be obtained from the principal investigator.
Who is organising and funding the research?

The research is organised and funded by the Preterm Birth Research Group at the University of Leicester. The researchers are committed to finding ways to prevent premature birth and so the doctors and scientists involved in the research do not receive any payment for including you in the study.

Who has reviewed the study?

All research that involves NHS patients or staff, information from NHS medical records or uses NHS patients or staff, information from NHS medical records or uses NHS premises or facilities must be approved by an NHS Research Ethics Committee before it goes ahead. Approval does not guarantee that you will not come to any harm if you take part. However, approval means that the Committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits and that you have been given sufficient information on which to make an informed decision to take part or not.

Contact for Further Information
Dr Anthony Taylor
University of Leicester
Tel: 0116 252 5822

Thank you for reading this information leaflet.

You will be given a copy of the information sheet and a signed consent form to keep
### 8.4 Normal values of oestradiol

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<tbody>
<tr>
<td>Males</td>
<td>10 – 45</td>
</tr>
<tr>
<td>Postmenopausal females</td>
<td>10 – 45</td>
</tr>
<tr>
<td>Ovulating females:</td>
<td></td>
</tr>
<tr>
<td>(day 0 = LH peak)</td>
<td></td>
</tr>
<tr>
<td>Day:</td>
<td></td>
</tr>
<tr>
<td>-10</td>
<td>13 – 80</td>
</tr>
<tr>
<td>-4</td>
<td>20 – 165</td>
</tr>
<tr>
<td>-1</td>
<td>73 – 410</td>
</tr>
<tr>
<td>0</td>
<td>119 – 417</td>
</tr>
<tr>
<td>+2</td>
<td>22 – 154</td>
</tr>
<tr>
<td>+5</td>
<td>44 – 174</td>
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<tr>
<td>+10</td>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>3rd trimester</td>
<td>4200 – 32000</td>
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1 Table obtained from the E2-EASIA protocol (DIAsource ImmunoAssays S.A (Belgium))
### 8.5 Normal values of progesterone

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2 Table obtained from the PROG-EASIA protocol (DIAsource ImmunoAssays S.A (Belgium))
highlighted boxes in green are the significant values mentioned in the thesis. The highlighted in grey are the values that were close to significance.

In the upper left part of the figure are the "r" values, while in the lower left are the "p" values. The highlighted boxes in green are the significant values mentioned in the thesis. The highlighted in grey are the values that were close to significance.
### 8.7 Correlations of all IHC results from placental samples

In the upper left part of the figure are the “r” values, while in the lower left are the “p” values. The highlighted boxes in yellow are the significant values mentioned in the thesis.

<table>
<thead>
<tr>
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### 8.8 Correlations of all PCR results from FM samples

In the upper left part of the figure are the “r” values, while in the lower left are the “p” values. The highlighted boxes in green are the significant values mentioned in the thesis. The highlighted in grey are the values that were close to significance.
### 8.9 Correlations of all IHC results from FM samples

In the upper left part of the figure are the “r” values, while in the lower left are the “p” values. The highlighted boxes in yellow are the significant values mentioned in the thesis.

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8.10 Presentations arising from this thesis: posters

Predicting the risk of preterm birth, the gestational age and timing of delivery from a single Anandamide measurement.

P. Bachkangi, V. Nallendran, A.H. Taylor, J.C. Konje
Poster presentation at the SRI 63rd Annual Scientific Meeting (March 2016), Montreal, Canada

In search for a predictive model for the risk of preterm birth.

P. Bachkangi, A.H. Taylor, J.C. Konje
Poster presentation at the SRI 63rd Annual Scientific Meeting (March 2016), Montreal, Canada

The prediction of preterm labour by plasma Anandamide in asymptomatic high-risk women.

P. Bachkangi, V. Nallendran, A.H. Taylor, J.C. Konje
Poster presentation at the SRI 63rd Annual Scientific Meeting (March 2016), Montreal, Canada

The role of plasma and placental fatty acid amide hydrolase in parturition in high-risk women for preterm labour.

P. Bachkangi, A. H. Taylor, M. Maccarrone, J. C. Konje
Poster presentation at the October 2015 BMOGS meeting, Birmingham, UK

Towards revealing the mechanisms by which the endocannabinoid system and female sex hormones interact in term and preterm birth.

P. Bachkangi, A. H. Taylor, M. Maccarrone, M. Bari, J. C. Konje
Poster presentation at the SRI 62nd Annual Scientific Meeting (March 2015), San Francisco, USA

Differential expression of CB1 and CB2, and the enzyme NAPE-PLD in the placenta of preterm and term pregnancies.

P. Bachkangi, A. H. Taylor, J. C. Konje
Poster presentation at the SRI 62nd Annual Scientific Meeting (March 2015), San Francisco, USA

Is the expression of the endocannabinoid system in fetal membranes different at term and in preterm?

P. Bachkangi, A. H. Taylor, J. C. Konje
Poster presentation at the SRI 62nd Annual Scientific Meeting (March 2015), San Francisco, USA
Are plasma endocannabinoid concentrations good predictors of preterm birth risk?

**Panayoti Bachkangi**, Anthony H. Taylor and Justin C. Konje
Poster presentation at the BMOGS meeting, October 2014, Leicester, UK

**Prediction of preterm birth risk using a single blood test.**

Poster presentation 2014 SGI 61st Annual Scientific Meeting (March 2014), Florence, Italy

The use of plasma endocannabinoids to predict the time of delivery in asymptomatic women at ‘high-risk’ of preterm birth.

Poster presentation 2014 SGI 61st Annual Scientific Meeting (March 2014), Florence, Italy
8.11 Published Abstracts


8.12 Oral presentations

Can plasma endocannabinoids predict the time of labour early in asymptomatic women at high risk of pre-term labour

Presented in the International BMOGS meeting in Athens. 2015

(Available to watch on https://www.youtube.com/watch?v=N8-gjO-oURQ)

Endocannabinoids and preterm birth

Presented at the University of Leicester as part of the “end of the first year” progress. 2014

8.13 Public presentations and Press interviews

Prediction of preterm birth risk using a single blood test

Presented to the general public at the Leicester Adult Education College. 2013

University Press Office Interview

An invitational interview with the Press Office of the UoL to discuss our project and findings. 2014
Chapter 9

References


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261
References


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